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Dehydration of (*R*)-2-hydroxyacyl-CoA to enoyl-CoA in the fermentation of α-amino acids by anaerobic bacteria

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Abstract

Several clostridia and fusobacteria ferment α-amino acids via (*R*)-2-hydroxyacyl-CoA, which is dehydrated to enoyl-CoA by *syn*-elimination. This reaction is of great mechanistic interest, since the β-hydrogen, to be eliminated as proton, is not activated (p*K* 40–50). A mechanism has been proposed, in which one high-energy electron acts as cofactor and transiently reduces the electrophilic thiol ester carbonyl to a nucleophilic ketyl radical anion. The 2-hydroxyacyl-CoA dehydratases are two-component systems composed of an extremely oxygen-sensitive component A, an activator, and component D, the actual dehydratase. Component A, a homodimer with one [4Fe–4S]cluster, transfers an electron to component D, a heterodimer with 1-2 [4Fe–4S]clusters and FMN, concomitant with hydrolysis of two ATP. From component D the electron is further transferred to the substrate, where it facilitates elimination of the hydroxyl group. In the resulting enoxyradical the β-hydrogen is activated (p*K*14). After elimination the electron is handed-over to the next incoming substrate without further hydrolysis of ATP. The helix–cluster–helix architecture of component A forms an angle of 105°, which probably opens to 180° upon binding of ATP resembling an archer shooting arrows. Therefore we designated component A as 'Archerase'. Here, we describe 2-hydroxyglutaryl-CoA dehydratase from *Acidaminococcus fermentans*, *Clostridium symbiosum* and *Fusobacterium nucleatum*, 2-phenyllactate dehydratase from *Clostridium sporogenes*, 2-hydroxyisocaproyl-CoA dehydratase from *Clostridium difficile*, and lactyl-CoA dehydratase from *Clostridium propionicum*. A relative of the 2-hydroxyacyl-CoA dehydratases is benzoyl-CoA reductase from *Thauera aromatica*. Analogous but unrelated archerases are the iron proteins of nitrogenase and bacterial protochlorophyllide reductase. In anaerobic organisms, which do not oxidize 2-oxo acids, a second energy-driven electron transfer from NADH to ferredoxin, the electron donor of component A, has been established. The transfer is catalysed by a membrane-bound NADH–ferredoxin oxidoreductase driven by an electrochemical Na^+-gradient. This enzyme is related to the Rnf proteins involved in *Rhodobacter capsulatus* nitrogen fixation.
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Keywords: Amino acid fermentation; Dehydration of 2-hydroxyacyl-CoA; ATP hydrolysis; Nitrogenase; Iron-sulfur clusters; Flavins; NADH–ferredoxin oxidoreductase

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1. Fermentation of amino acids

Many chemotrophic organisms are able to thrive from proteinogenous α-amino acids. Aerobes and respiring anaerobes usually convert these valuable nutrients to the corresponding α-oxo acids and oxidise them further via the Krebs cycle to CO_2. In the absence of electron acceptors, such as oxygen, nitrate or sulfate, only "Clostridia", "Fusobacteria" and a few other anaerobes can use amino acids as energy substrates [1–3]. These organisms are able to ferment amino acids to ammonia, CO_2, short chain fatty acids and molecular hydrogen. Minor products are hydrogen sulfide, methylmercaptane, phenols and alcohols, which form together with fatty acids the typical putrefying odour. In the famous Stickland reaction one amino acid is oxidised to ammonia, CO_2 and a fatty acid, whose chain has been shortened by one carbon as compared to that of the parent substrate, whereas another amino acid is reduced to a fatty acid with the same carbon skeleton. An example is the pair wise fermentation of isoleucine and leucine by *Clostridium difficile* to isobutyrate and 4-methylpentanoate (isocaproate), respectively. On the other hand, various Clostridia use fermentation pathways, in which single amino acids act as electron donors as well as acceptors. For instance *Acidaminococcus fermentans* and *Fusobacterium nucleatum* convert glutamate to crotonyl-CoA, which is oxidised to acetate and reduced to butyrate. In many fermentations also hydrogen is produced, whereby protons rather than a part of the substrate act as electron acceptors. The oxidative pathways usually do not differ from those of respiring organisms, whereas the reductive branches of Stickland reactions and the conversions of single amino acids to intermediates, which are able to perform redox reactions, are unique in most cases. Ideal intermediates are α, β-unsaturated acyl-CoAs (enoyl-CoAs), which are subject to β-oxidation and reduction. An easy way to enoyl-CoA would be a direct β-elimination of α-amino acids.

Most α-amino acids, however, are resistant towards β-elimination, since the $pK = 40–50$ of the non-activated β-hydrogen is too high for a basic residue of an enzyme. This may be one reason, why Nature has chosen α-amino acids as building blocks of proteins. The only exceptions are aspartate, which can be considered as α- as well as β-amino acid, as well as histidine and phenylalanine. Enzymes with the electrophilic prosthetic group MIO (methylidene imidazolone) catalyse the β-elimination of ammonia from both aromatic amino acids. The electrophilic MIO adds to the aromatic ring and thus lowers the p*K* of the β-hydrogen [4]. On the other hand, β-amino acids are easy to deaminate, since the p*K* of the β-hydrogen is about 30. It can be lowered to 21 by CoA-thiol ester formation [5] and further to about 7 by hydrogen bonding from two backbone amides of the enzyme to the carbonyl group, as shown for octanoyl-CoA in medium chain acyl-CoA dehydrogenase [6]. Nevertheless several Clostridia and *F. nucleatum* are able to deaminate α-amino acids, but use a complex mechanism via conversion to the corresponding (*R*)-2-hydroxyacyl-CoA derivatives, which are dehydrated to enoyl-CoAs. These dehydrations are also difficult, since the exchange of the amino group to a hydroxy group and the formation of the thiol ester has not lowered the high p*K* of the β-hydrogen (Fig. 1). It has been proposed that this dehydration is achieved by one-electron reduction of the electrophilic thiol ester carbonyl to a nucleophilic ketyl radical anion, a process called 'Umpolung' (charge reversal) [7]. The elimination of the hydroxyl group from the ketyl radical anion yields an enoxy radical, which is deprotonated to the product-derived ketyl radical anion and oxidised by the next incoming substrate to enoyl-CoA, in order to complete the catalytic cycle [8,9]. It has been calculated that the enoxy radical has the low $pK = 14$, about 26 units lower than the p*K* of the β-proton of the 2-hydroxy acid [10]. The p*K* of the enoxy radical may further be lowered to around 7 by hydrogen bonding. In summary, just one electron, the smallest possible cofactor, is involved in the catalytic cycle.

Of the 20 proteinogenous amino acids, 12 can be dehydrated by the mechanism outlined above (Fig. 2). Most of the (*R*)-2-hydroxy acids are formed from these amino acids by amino transfer to 2-oxoglutarate followed by an NADH-dependent reduction, whereby alanine yields (*R*)-lactate, phenylalanine (*R*)-3-phenyllactate, leucine (*R*)-2-hydroxyisocaproate, tyrosine (*R*)-3-(4-hydroxyphenyl)-lactate and tryptophan (*R*)-3-(3-indolyl)-lactate. Direct oxidation of glutamate by

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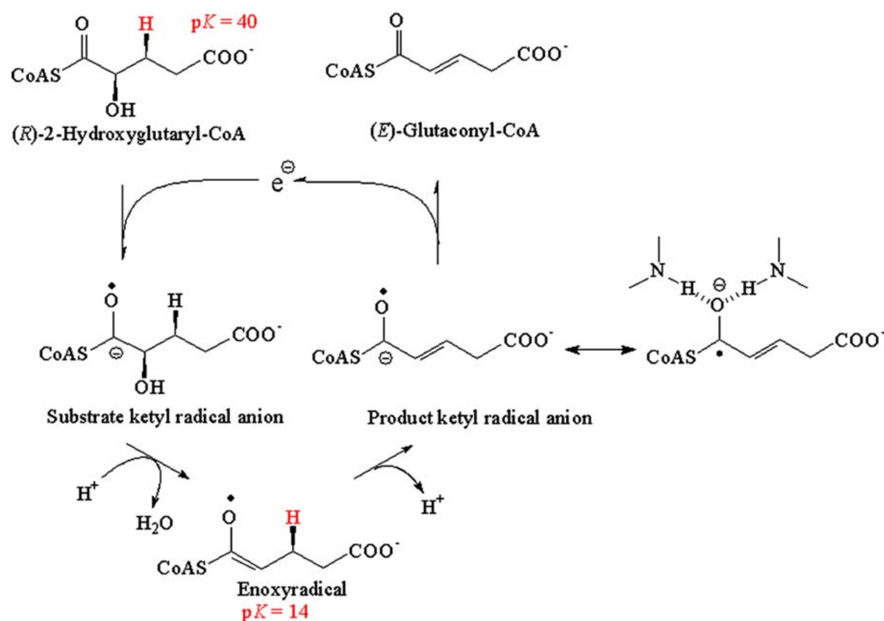

Fig. 1. Proposed mechanism for the dehydration of (*R*)-2-hydroxyglutaryl-CoA to (*E*)-glutaconyl-CoA. The possible stabilisation by hydrogen bonding from two backbone amides is only shown for the 'product ketyl radical anion', but it is also applicable for the other intermediates.

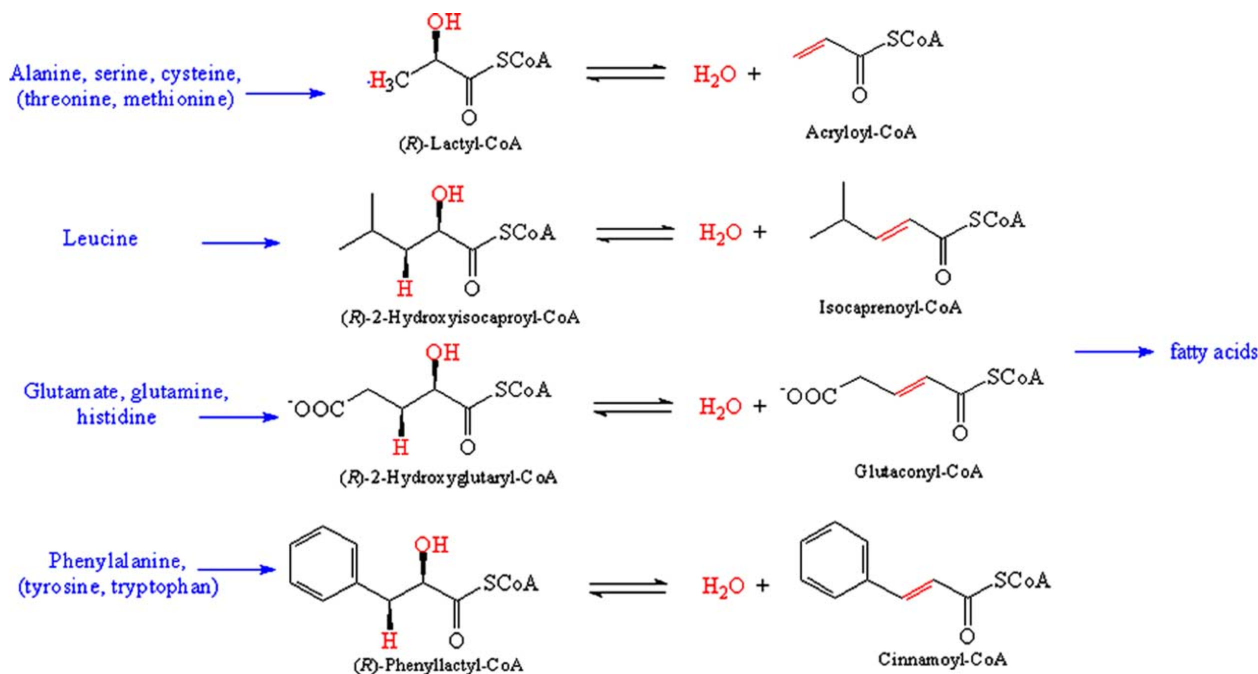

Fig. 2. Conversion of amino acids to fatty acids via dehydration of their corresponding (*R*)-2-hydroxyacyl-CoAs to enoyl-CoAs.

NAD^+ followed by an NADH-dependent reduction leads to (*R*)-2-hydroxyglutarate. Degradation of histidine and glutamine via glutamate also gives rise to (*R*)-2-hydroxyglutarate. Elimination of water from serine and threonine yields pyruvate and 2-oxobutyrate, which are reduced to (*R*)-lactate and (*R*)-2-hydroxybutyrate, respectively. (*R*)-Lactate can also be derived from cysteine by β-elimination of H_2S followed by reduction of the pyruvate. Similarly, methionine gives rise to (*R*)-2-hydroxybutyrate via γ-elimination of methylmercaptane and reduction. Prior to dehydration, all these (*R*)-2-hydroxy acids are converted to the (*R*)-2-hydroxyacyl-CoA derivatives using specific CoA-transferases.

The remaining eight proteinogenous amino acids are fermented by pathways, in which the dehydration of a 2-hydroxy acid is not involved. Thus the α-amino

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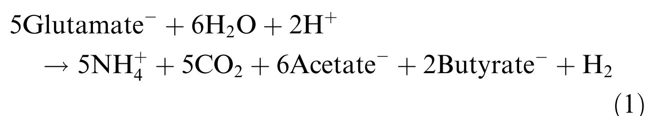
groups of glycine and proline are directly substituted by hydrogen, the amino group of the α, β-amino acid aspartate (and asparagine) can be easily eliminated and amino groups of lysine and ornithine (from arginine) are shifted to the β-position. Isoleucine and valine are only oxidised, probably because branching at the β-carbon may be not accepted by the dehydratases.

Until now six different 2-hydroxyacyl-CoA dehydratases have been purified and characterised, 2-hydroxyglutaryl-CoA dehydratase from *A. fermentans* [9,11–14], *Clostridium symbiosum* [15] and *F. nucleatum* [16], phenyllactate dehydratase from *Clostridium sporogenes* [17,18], 2-hydroxyisocaproyl-CoA dehydratase from *C. difficile* (J. Kim, T. Selmer & W. Buckel, unpublished), and lactyl-CoA dehydratase from *Clostridium propionicum* [19–23]. All these dehydratases are enzyme systems composed of two separable components, an extremely oxygen-sensitive activator or initiator (component A or archerase, see below) and a moderate oxygen-sensitive component D, the actual dehydratase. The 2-hydroxyacyl-CoA dehydratases have to be assayed in the presence of ATP, Mg^{2+}, and a one-electron reducing agent, either Ti(III)citrate or dithionite under strict anoxic conditions [11,24]. The reactions have been followed by various methods, such as the determination of the product or substrate by HPLC [21], discontinuous or continuous enzymatic assays [16,17,25], or liqud scintillation counting of ^{3}HOH released from 2-hydroxy-[3-^{3}H]acyl-CoA [25]. The continuous direct assay, by which the highest activities have been obtained, is based on the absorbance difference between enoyl-CoA and 2-hydroxyacyl-CoA at 290 nm ($\Delta\varepsilon = 2.2$ mM^{-1} cm^{-1}). In the case of phenyllactate dehydratase the absorbance difference between phenyllactate and cinnamate at 290 nm is much higher ($\Delta\varepsilon = 9.0$ mM^{-1} cm^{-1}) [18].

2. 2-Hydroxyglutaryl-CoA dehydratase from *A. fermentans*

The strict anaerobic Gram-negative coccus has been classified as a member of the order *Clostridiales*, family *Acidaminococcaceae* [1]. It ferments glutamate via the 'hydroxyglutarate pathway' [26] to ammonia, CO_2, hydrogen, acetate and butyrate, approximately according to equation (1)



$$5\text{Glutamate}^- + 6H_2O + 2H^+ \rightarrow 5NH_4^+ + 5CO_2 + 6\text{Acetate}^- + 2\text{Butyrate}^- + H_2 \quad (1)$$

$\Delta G^{\circ\prime} = -62.6$ kJ/mol glutamate, whereby 0.6 ATP/glutamate can be obtained by substrate level phosphorylation [27].

The hydroxyglutarate pathway involves the NAD^+-dependent deamination of (*S*)-glutamate to 2-oxoglutarate followed by the NADH-dependent reduction to (*R*)-2-hydroxyglutarate. The activation to (*R*)-2-hydroxyglutaryl-CoA by acetyl-CoA is catalysed by glutaconate CoA-transferase [1], an octameric enzyme composed of two different subunits $(\alpha\beta)_4$. The mechanism of this class I CoA-transferase [28] proceeds via a specific glutamate residue, which forms a CoA-thiol ester intermediate [29,30]. For the *syn*-elimination of water from (*R*)-2-hydroxyglutaryl-CoA to (*E*)-glutaconyl-CoA [25] (Fig. 2), two enzymes are required, component A, the activator or initiator, and component D, the actual dehydratase (Table 1) [11]. The subsequent decarboxylation of glutaconyl-CoA to crotonyl-CoA is mediated by an integral membrane enzyme, which converts the free reaction enthalpy into an electrochemical Na^+-gradient [31]. The enzyme is composed of four different subunits, the carboxyltransferase (α), the biotin containing carrier protein (γ), the carboxybiotin decarboxylase with 12 transmembrane helices (β), and a membrane anchor (δ) [32]. The α-subunit catalyses the transfer of CO_2 from glutaconyl-CoA to biotin [33] attached to a flexible arm of the γ-subunit, which swings over to the β-subunit, where the decarboxylation of carboxybiotin drives the Na^+-pump. In the following steps of the hydroxyglutarate pathway, crotonyl-CoA disproportionates to acetate and butyrate and molecular hydrogen according to equation (1); for individual steps, see [7].

Component A of 2-hydroxygluatryl-CoA dehydratase is encoded by the gene *hgdC* located on *A. fermentans* DNA upstream of two genes (*hgdA* and *hgdB*) encoding component D (Fig. 3). The genes *gctA* and *gctB* for glutaconate CoA-transferase and *gcdA* for the carboxytransferase subunit of glutaconyl-CoA decarboxylase precede the three *hgd*-genes. Hence the genes *gctA*, *gctB*, *gcdA*, *hgdA*, *hgdB*, *hgdC* are clustered in this order and form the 'hydroxyglutarate operon' as shown by northern blot analysis [34]. Interestingly, the genes encoding the other three subunits of glutaconyl-CoA decarboxylase are located in another cluster apparently not adjacent to the hydroxyglutarate operon [32].

The gene *hgdC* has been expressed in *Escherichia coli* as a protein with a C-terminal fused Streptag® and purified by streptavidin affinity chromatography [13,35]. Component A is very sensitive to oxygen with a half-life of about 10 s under air. Under anoxic conditions and in the presence of 1 mM ATP + 10 mM $MgCl_2$ the protein is stable for a few days. Probably, the actual stabiliser is Mg–ADP, which is formed by hydrolysis of Mg–ATP, see below. Crystallisation of component A occurred

[1] The enzyme was called glutaconate CoA-transferase, since glutaconate was shown to be the best substrate (highest k_{cat}/K_m) [29]. At this time it was not recognised that the dehydration of 2-hydroxyglutarate occurs at the CoA-level, whereas glutaconyl-CoA was identified as the substrate for the subsequent decarboxylation to crotonyl-CoA.

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Table 1
Characteristics of 2-hydroxyacyl-CoA dehydratases

Organism	Substrate	Component A	Component D	Specific activity (s^{-1})
Acidaminococcus fermentans	(*R*)-2-Hydroxyglutaryl-CoA	HgdC, γ_2, 2×27 kDa; $[4Fe–4S]^{1+/2+}$	HgdAB, αβ, 54 + 42 kDa; $[4Fe–4S]^{2+}$, FMN, riboflavin	10
Clostridium symbiosum	(*R*)-2-Hydroxyglutaryl-CoA	Not purified[1] 2×27 kDa	HgdAB, αβ, 54 + 42 kDa; 2 $[4Fe–4S]^{2+}$, FMN	50 80*
Fusobacterium nucleatum	(*R*)-2-Hydroxyglutaryl-CoA	HgdC[1], γ_2, 2×28 kDa; $[4Fe–4S]^{1+/2+}$	HgdABD, αβδ, 49 + 39 + 24 kDa $[4Fe–4S]^{2+}$, riboflavin	<50
Clostridium sporogenes	(*R*)-Phenyllactate	FldI[1], η_2, 2×28 kDa; $[4Fe–4S]^{1+/2+}$	FldABC, αβγ, 46 + 43 + 40 kDa; $[4Fe–4S]^{2+}$, flavin not detected	1*
Clostridium difficile	(*R*)-2-Hydroxyisocaproyl-CoA	HadI[1], η_2, 2×29 kDa; $[4Fe–4S]^{1+/2+}$	HadBC, βγ, 46 + 43 kDa 1-2 $[4Fe–4S]^{2+}$, FMN?	150* (V_{max})
Clostridium propionicum	(*R*)-Lactyl-CoA	Partially purified	LcdAB, αβ, 48 + 41 kDa 2 $[4Fe–4S]^{2+}$, FMN, riboflavin	Low
Megasphaera elsdenii	(*R*)-Lactyl-CoA	Not purified	LcdAB, αβ, 2 $[4Fe–4S]^{2+}$?, FMN?	Low

*Activities measured with the direct assay at 290 nm; the other activities were obtained with the coupled enzymatic assay [16].
[1] Component A (HgdC) from *A. fermentans* was (also) used.

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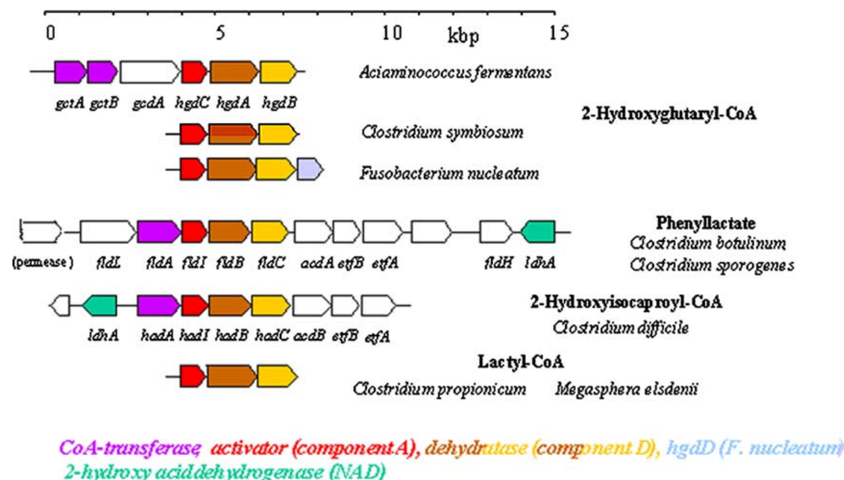

Fig. 3. Genes encoding 2-hydroxyacyl-CoA dehydratases and accessory enzymes. Explanations of gene abbreviations are given in the text.

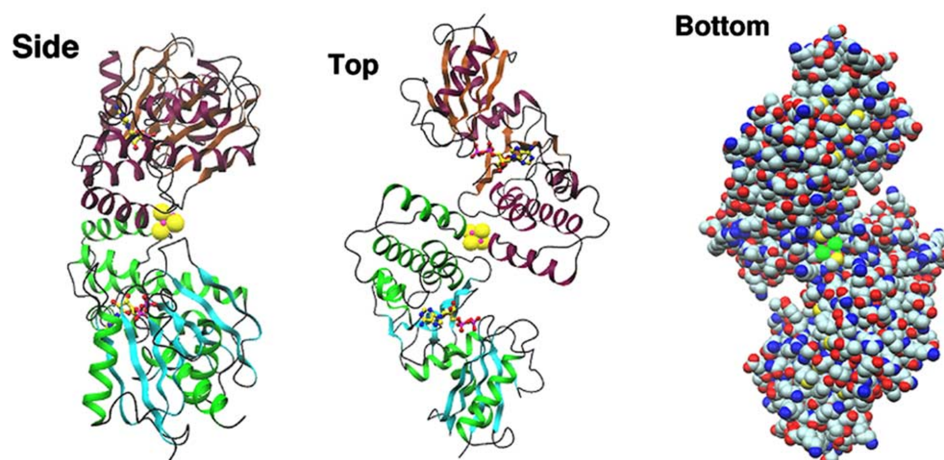

Fig. 4. Different views of component A from *A. fermentans* at 3 Å resolution. The bottom view represents a space-filling model. The yellow (S) and red (Fe) balls are the atoms of the [4Fe–4S] cluster. Below and above ADP molecules are seen ('side' and 'top'). The green balls in 'bottom' are the iron atoms.

over night and the structure has been solved by the MAD technique using a data set obtained at the Stanford synchrotron [36] (Fig. 4). The structure revealed a fold related to that of acetate kinase, sugar kinases, HSP70, and actin family of ATP-binding proteins, called ASKHA [37]. Each of the two subunits of component A contained one non-covalently bound ADP molecule, but Mg^{2+} ion could not be seen at the low resolution of 3 Å. The most remarkable feature of component A are two helices, each from one subunit pointing with their N-termini towards a $[4Fe–4S]^{1+/2+}$ cluster forming a helix–cluster–helix angle of 105°. A similar architecture, absent in other ASKHA proteins, is found in the iron protein of nitrogenase from *Azotobacter vinelandii* with a helix–cluster–helix angle of 150° [38]. The nitrogenase iron-protein, however, is related to the G-proteins rather than to the ASKHA-proteins.

In contrast to an earlier report [14] component A has a negligible ATPase activity, either in the reduced or oxidised state. In the presence of equal amounts of components A and D, the ATPase activity becomes measurable (ca. 1 s^{-1}). Reduction of component A increases the activity 2-fold (J. Kim, M. Hetzel and W. Buckel, unpublished). The structure of component A also revealed that the [4Fe–4S] cluster is easily accessible from the solvent. This may be the reason for the extreme oxygen-sensitivity and the destruction of the cluster by the iron-chelating agent *o*-phenanthroline. In the presence of ATP the chelating rate is about 10-fold increased indicating the induction of a large conformational change by the nucleoside triphosphate. Probably the helix–cluster–helix angle opens to 180° as observed in the complex of nitrogenase iron protein with molybdenum–iron protein in the presence of ADP–AlF_4^- [39]. The redox potential of component A could not be measured, but the cluster becomes almost completely reduced by flavodoxin (E'_0 ca. − 420 mV) or ferredoxin ($E'_0 = -405$ mV) [40] indicating a potential of about −350 mV or even higher. Remarkably, in the presence of an excess of Ti(III)citrate the $[4Fe–4S]^+$ cluster of component A could be partially further reduced to the inactive 'all-ferrous' state, $[4Fe–4S]^0$ (Hans, Bill & Buckel, unpublished), similar to the iron protein of nitrogenase [41].

Component D of *A. fermentans* has been purified from the original cells, where about 12% of the soluble protein comprises this enzyme. It has been characterised as a heterodimeric enzyme (Table 1) containing 1.0 mol [4Fe–4S] cluster, 1.0 mol riboflavin-5′-phosphate (FMN) and about 0.3 mol riboflavin [9]. Molybdenum has also been found in this protein, but the content of 0.1 mol/heterodimer appears to be too low to be significant [14]. Furthermore, the same amount of Mo has been detected in component D from *C. symbiosum*, but this metal is absent in the enzymes from *F. nucleatum* and *C. difficile* (J. Kim, A. Tamannaei & W. Buckel, unpublished).

The activation of component D of 2-hydroxyglutaryl-CoA dehydratases requires component A, ATP, $MgCl_2$, and a reducing agent. In vitro dithionite or Ti(III)citrate are suitable one-electron donors; in vivo a clostridial-type two $[4Fe–4S]^{1+/2+}$ cluster-containing ferredoxin or a flavodoxin serve for this purpose. It has been shown that with 45 μM Fe in the growth medium *A. fermentans* produces almost exclusively this ferredoxin, whereas under iron-limiting conditions (7 μM Fe) a flavodoxin is the dominant species [40]. The reduction of component A by flavodoxin has been observed by UV/visible, EPR and Mössbauer spectroscopy. The reduced $[4Fe–4S]^+$ cluster exhibited a spin 3/2 system [13]. The further fate of the electron in the activation process remains unclear. Whereas the Mössbauer spectrum revealed oxidation of component A during activation, the concomitant reduction of the cluster of component D could not be observed by this method. The activated component D, however, exhibited an EPR-signal ($g < 2.0$), which has been interpreted as that of Mo(V) [14]. More recent experiments, however, question the presence of Mo in component D.

3. 2-Hydroxyglutaryl-CoA dehydratase from *C. symbiosum*

C. symbiosum ferments glutamate by the same pathway as *A. fermentans* [26] (Fig. 2). The three genes encoding components A and D from *C. symbiosum* are clustered together in the order *hgdC*, *hgdA*, *hgdB*; the same arrangement was found in *A. fermentans* (Fig. 3). The sequence identities between the deduced proteins of the corresponding genes from both organisms are 76%, 73% and 63%, respectively. Upstream of the three-gene cluster, however, no genes of enzymes of the hydroxyglutarate pathway have been detected [15]. The only other characterised gene from the glutamate fermentation pathway of *C. symbiosum* encodes the initial enzyme, glutamate dehydrogenase [42]. The structure of this enzyme, the first one of a glutamate dehydrogenase, has been determined by X-ray crystallography [43]. Component D was purified from *C. symbiosum*, where it comprises 4% of the total soluble protein. The specific activity was determined as 50 U/mg protein (80 s^{-1}), about 8 times higher than that of component D from *A. fermentans*. Notably, component A from *A. fermentans* was used as activator in both cases, whereas component A from *C. symbiosum* was lost during purification. Component D from *C. symbiosum* is composed of two different subunits and contains 1.0 FMN, almost no riboflavin and surprisingly two $[4Fe–4S]^{2+}$ clusters, whereas only one has been detected in component D from *A. fermentans* (Table 1). Also in component D

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from *C. symbiosum* 0.07 Mo/heterodimer has been found and Mo is eluted from chromatography columns parallel with dehydratase activity.

Full activity of component D was already achieved by using only substoichiometric amounts of component A (1:10). As reported for nitrogenase [39] the addition of ATP and AlF_4^- induces the formation of a complex of component A from *A. fermentans* and component D from *C. symbiosum*, which was characterised by size exclusion and ion exchange chromatography. (M. Hetzel, J. Kim & W. Buckel, unpublished). The structure of this complex could reveal, whether the angle helix–cluster–helix of component A indeed is opened from 105° to 180°.

4. 2-Hydroxyglutaryl-CoA dehydratase from *Fusobacterium nucleatum*

Fusobacterium nucleatum, occurring in dental plaques, ferments glutamate also via the 2-hydroxyglutarate pathway [26] (Fig. 2). 2-Hydroxyglutaryl-CoA dehydratase was purified from this organism as an extremely oxygen-sensitive, ATP-requiring protein, which surprisingly was active without a component A. The composition of three different subunits suggested the presence of a stable complex of components A and D (Table 1). The heterotrimer contained 0.5 riboflavin/112 kDa and a [4Fe–4S] cluster [16]. The recent elucidation of the whole fusobacterial genome [44], however, changed the interpretation of the subunit functions. The N-termini of the three subunits and the cloned gene coding for the α-subunit perfectly matched a three-gene cluster, which did not comprise the homologous gene for component A (*hgdC*) located upstream of *hgdA* (Fig. 3). Hence, only the heterotrimeric fusobacterial component D has been isolated. Apparently it contained also component A, but in too small amounts to be visible on the gel. Treatment of component D with *o*-phenanthroline or chromatography on the size exclusion column Superdex-200 removed the traces of component A and resulted in an inactive but still trimeric enzyme. Its reactivation required in addition to dithionite and ATP/Mg^{2+} component A from *A. fermentans*. In order to obtain component A from *F. nucleatum*, its *hgdC* gene was cloned with a C-terminal Streptag® and expressed in *E. coli*. The affinity-purified component A could replace component A from *A. fermentans* very efficiently (A. Tamannaei & W. Buckel, unpublished).

5. Phenyllactate dehydratase from *Clostridium sporogenes*

Clostridium sporogenes ferments phenylalanine to ammonia, CO_2, phenylacetate and 3-phenylpropionate. Initially (*S*)-phenylalanine is oxidised to phenylpyruvate by amino-transfer to 2-oxoglutarate. Further oxidation of phenylpyruvate mediated by phenylpyruvate–ferredoxin oxidoreductase yields phenylacetyl-CoA, from which phenylacetate and ATP are generated. The reductive branch involves the NADH-dependent reduction of phenylpyruvate to (*R*)-3-phenyllactate, which is dehydrated to (*E*)-cinnamate with a *syn*-geometry [45] and further reduced to phenylpropionate. All three enzymes of this reductive branch have been purified and characterised [17]. The NAD^+-dependent phenyllactate dehydrogenase (FldH; F for phenylalanine) is related to D-lactate dehydrogenases and the reduction of cinnamate to phenylpropionate is catalysed by an NADH-dependent enoate reductase (FldZ). Phenyllactate dehydratase (FldABC) and its activator or initiator (FldI), which together mediate the formation of cinnamate, are the topic of this chapter (Fig. 2). Thereby the question arises, why *C. sporogenes* needs three enzymes and the activator for the conversion of (*S*)-phenylalanine to (*E*)-cinnamate rather than using the plant enzyme phenylalanine ammonia lyase, which catalyses the deamination of the amino acid in one step [4]. The answer may be the intermediate phenylpyruvate, which also has to be oxidised to phenylacetyl-CoA, in order to generate ATP and reduced ferredoxin for the reduction of cinnamate to the final product phenylpropionate.

In the presence of the activator FldI, ATP, Mg^{2+}, dithionite, and catalytic amounts of cinnamoyl-CoA, the dehydratase (FldABC; $m = 130$ kDa) catalyses the overall conversion of (*R*)-phenyllactate to (*E*)-cinnamate without stoichiometric participation of a CoA-thiol ester. Therefore the enzyme FldABC was called phenyllactate dehydratase. The requirement of cinnamoyl-CoA established a new mechanism, in which FldA acts as a CoA-transferase mediating the formation of (*R*)-phenyllactayl-CoA and (*E*)-cinnamate from (*R*)-phenyllactate and cinnamoyl-CoA. The subsequent dehydration of (*R*)-phenyllactyl-CoA catalysed by FldBC regenerates cinnamoyl-CoA (Fig. 5). In all other 2-hydroxyacyl-CoA dehydratase systems a separate CoA-transferase is present.

This mechanism demonstrates that the dehydration of 2-hydroxy acids must proceed at the thiol ester level (Fig. 5), as proposed in the introduction. In the other systems this finding is less apparent, since the formed enoyl-CoA undergoes subsequent reactions, which also require CoA-thiol esters such as decarboxylation of glutaconyl-CoA to crotonyl-CoA or reduction of acryloyl-CoA to propionyl-CoA. Upon a second chromatography of the heterotrimeric phenyllactate dehydratase complex (FldABC) on Q-Sepharose, the oxygen-sensitive phenyllactyl-CoA dehydratase (FldBC) could be separated from the oxygen-insensitive CoA-transferase (FldA), which was characterised as class III enzyme [17,28]. The FldBC heterodimer contained

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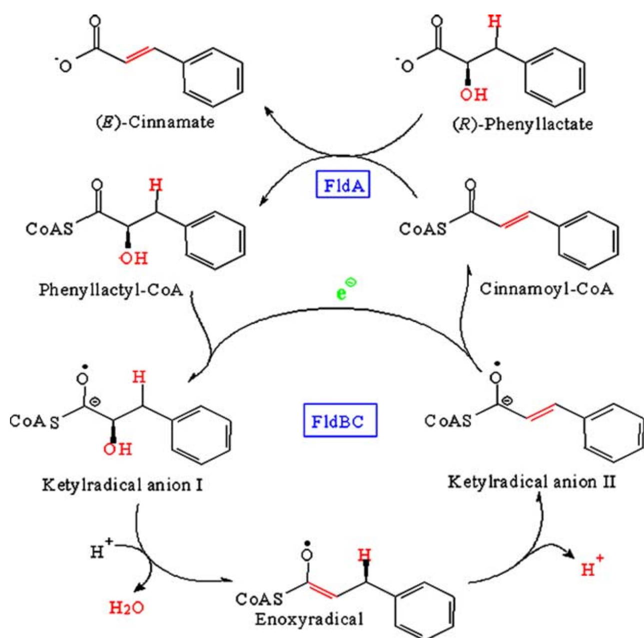

Fig. 5. Proposed mechanism of the dehydration of (*R*)-phenyllactate to (*E*)-cinnamate in *C. sporogenes*.

about one [4Fe–4S] cluster; but a flavin was not detected and FAD was not required for activity.

The genes from *C. sporogenes* encoding the three subunits of phenyllactate dehydratase have been cloned and sequenced [18]. This task has been facilitated by the availability of the almost finished genome of *Clostridium botulinum* type A (Hall strain, Sanger Centre). Both organisms are closely related, since the nucleotide sequences of their 16*S* rRNAs differ only by 0.2% [46]; therefore it is no surprise that the deduced amino acid sequences of the three subunits of phenyllactate dehydratase from *C. sporogenes* show about 95% identity with those from *C. botulinum*. The genes are arranged in the order *fldL–fldA–fldI–fldB–fldC* (Fig. 3). From these genes *fldI*, coding for the initiatior or component A, has been overexpressed in *E. coli* as an active C-terminal Streptag fusion protein. Upstream of *fldA* there is an open reading frame *fldL*, which probably encodes a cinnamoyl-CoA synthetase. The enzyme might be required for the synthesis of the small amount of cinnamoyl-CoA necessary for starting the highly specific CoA-transferase cycle. Cinnamoyl-CoA cannot be replaced by other CoA-thiol esters, such as acetyl-CoA or phenylacetyl-CoA.

6. 2-Hydroxyisocaproyl-CoA dehydratase (Had) from *Clostridium difficile*

During the study of phenyllactate dehydratase, the *had* genes homologous to the *fld* genes were detected in *C. difficile* [17,18], which is able to ferment several amino acids and whose genome has also been sequenced by the Sanger Centre (Fig. 3). The assumption that these genes encode enzymes of the reductive branch of leucine fermentation (Fig. 2) has been recently verified in our laboratory (J. Kim, T. Selmer & W. Buckel, unpublished). The genes *ldhA* and *hadA* were cloned, overexpressed in *E. coli*, and characterised as NAD^+-dependent 2-hydroxyisocaproate dehydrogenase and isocaprenoyl-CoA-2-hydroxyisocaproate CoA-transferase, a class III enzyme [28], respectively. Hence, (*S*)-leucine is most likely oxidised by amino-transfer to 2-oxoglutatrate yielding 2-oxoisocaproate, which is reduced to (*R*)-2-hydroxyisocaproate (2-hydroxy-4-methylpentanoate) followed by activation to the CoA-thiol ester. The oxygen-sensitive 2-hydroxyisocaproyl-CoA dehydratase (HadBC) could be purified as a heterodimer from cell-free extracts of *C. difficile* (Table 1). There is no indication for a complex formation between the CoA-transferase (HadA) and the dehydratase (HadBC), which contains flavin, [4Fe–4S] clusters, but no molybdenum. Using component A from *A. fermentans* or the closely related HadI from *C. difficile*, both produced in *E. coli*, HadBC could be activated to a specific activity of about 100 U/mg, the highest obtained with a 2-hydroxyacyl-CoA dehydratase. Thereby HadI was present only in a concentration of 10% of that of HadBC. Hence, as postulated in the introduction, the activation is a catalytic process. In contrast to component A from *A. fermentans*, HadI from *C. difficile* exhibited no ATPase activity, neither in the reduced nor in the oxidised form. But in the presence of HadBC, HadI hydrolysed ATP very efficiently. Furthermore, the formation of a complex of HadBC and reduced HadI could be demonstrated, if ATP and AlF_4^- have been added. The complex could be isolated by streptavidin affinity chromatography using the C-terminal fused Streptag® of component A. Most likely the complex is stabilised by the transition state analogue $ADP \times AlF_4^-$ [39].

7. The dehydration of lactyl-CoA

Clostridium propionicum ferments alanine, serine and cysteine to CO_2, H_2, propionate and acetate, whereas the fatty acids derived from threonine are butyrate and propionate [2]. In a similar manner *Megasphaera elsdenii* converts lactate to acetate, propionate, butyrate, valerate, and caproate [47,48]. In all pathways either (*R*)-lactyl-CoA or (*R*)-2-oxobutyryl-CoA are dehydrated in a *syn*-fashion to acryloyl-CoA or crotonyl-CoA, respectively [23,49] (Fig. 2). Component D from *C. propionicum* has been purified and characterised as an FMN, riboflavin, and probably two [4Fe–4S] cluster-containing protein (Table 1), although an EPR-signal of a [3Fe–4S] cluster has been observed. Since such a signal was also found in component D of *A. fermentans* com-

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prising only 10% of the total cluster content, the [3Fe–4S] cluster may arise from inactive enzyme. The genes encoding component D from *C. propionicum* were partially cloned and show high sequence identities to those from *A. fermentans* (T. Selmer & W. Buckel, unpublished). The conversion of lactate (2-hydroxypropionate) via (*R*)-lactyl-CoA and acryloyl-CoA to 3-hydroxypropionyl-CoA catalysed by crotonase has been proposed as a biotechnological process to produce various valuable polymers [50].

8. On the mechanism of the *syn*-dehydration of (*R*)-2-hydroxyacyl-CoA to (*E*)-enoyl-CoA

The mechanism of activation and dehydration can now be described in the following way (Fig. 6): The $[4Fe–4S]^{2+}$ cluster of component A, to which two ADP are bound, is reduced by ferredoxin or flavodoxin with one-electron to $[4Fe–4S]^{+}$. Then ADP is exchanged by ATP, which probably causes the helix–cluster–helix angle to open from 105° to 180°. This conformational change might enable component A to dock at component D and the electron is transferred from A to D with concomitant hydrolysis of two ATP. Thereby the electron transfer becomes irreversible and component A

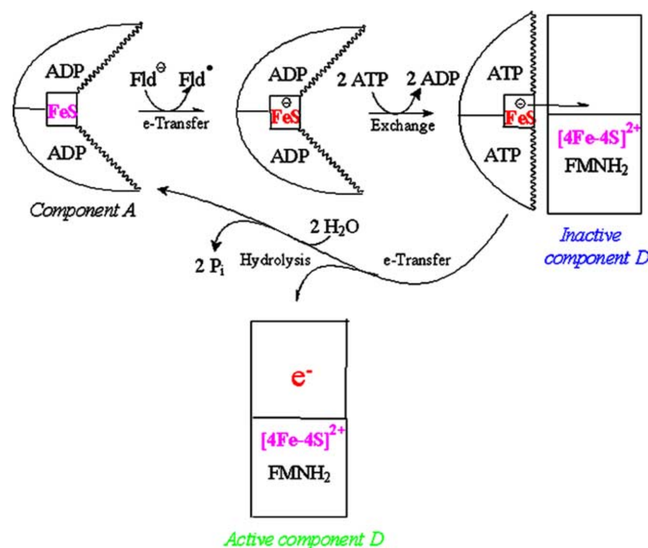

Fig. 6. Proposed mechanistic scheme of the activation of component D. At the left, component A is in the resting state with the oxidized $[4Fe–4S]^{2+}$ cluster and one molecule of ADP bound to each subunit. The helix–cluster–helix architecture forms an angle of 105°. Then one electron from flavodoxin hydroquinone is transferred spontaneously to component A to yield reduced $[4Fe–4S]^{1+}$ and blue flavodoxin semiquinone. Addition of ATP probably leads to an exchange with ADP and might cause a large conformational change, whereby the helix–cluster–helix angle is opened to 180°. After docking to component D, the electron could be transferred concomitant with ATP hydrolysis: Component D is activated by reduction with one electron and component A returns to the ground state.

returns to its 'ground state' with two ADP and oxidised $[4Fe–4S]^{2+}$. Upon addition of (*R*)-2-hydroxyacyl-CoA to component D, the electron could be further transferred to the substrate to form the ketyl radical anion, which initiates the dehydration as proposed above. Afterwards the electron should be returned to component D and transferred further to the next incoming substrate. Thus multiple turnovers are possible without additional consumption of ATP. Only if the electron is lost by oxidation, another activation with hydrolysis of two ATP becomes necessary. If each turnover would require hydrolysis of ATP, the organism would be unable to thrive, since the fermentation of one mol glutamate via the dehydration only yields 0.6 mol ATP [27]. There are still several uncertain steps in this proposed mechanism, which need to be clarified. The major problem comprises the localisation of the electron in component D and the verification of ketyl radical anion and enoxy radical intermediates. It has been suggested that molybdenum in component D is the site at which the electron is 'parked', before it is further transferred to the substrate [14]. But due to the discovery of the highly active 2-hydroxyisocaproyl-CoA dehydratase devoid of Mo, this hypothesis must be abandoned. We speculate therefore that the electron may be located as a ketyl radical anion at an amino acid side chain and is stabilised by the [4Fe–4S] cluster. A candidate could be the tyrosine residue 37 of the HgdB subunit from *C. symbiosum*, which coordinates one of the four iron atoms, whereas the other three Fe are coordinated by cysteines (B. Martins, M. Hetzel, W. Buckel & H. Dobbek, unpublished crystal structure of HgdB).

Ladd and Walker discovered in 1959 that the fermentation of lactate by cell-free extracts of *M. elsdenii* could be inhibited by μM-concentrations of 2,4-dinitrophenol, hydroxylamine and azide, suggesting a mechanism similar to mitochondrial oxidative phosphorylation. The purification of 2-hydroxyglutaryl-CoA dehydratases, however, excluded any participation of membranes [24]. Repeating of the experiments with purified 2-hydroxyglutaryl-CoA dehydratase revealed a lag phase in the coupled assay system, when 3 μM 2-nitrophenol was added. This lag phase was increased at higher 2-nitrophenol concentrations and decreased, when more component A was added. Apparently 2-nitrophenol oxidised component A [9]. Since the antibiotic metronidazole (2-methyl-5-nitroimidazole-1-ethanol) has the same effect, it has been suggested recently that the 2-hydroxyisocapryloyl-CoA dehydratase of *C. difficile* might be a much more sensitive target than reduced ferredoxin [18]. Interestingly 5 μM metronidazole inhibits growth of *F. nucleatum* and *C. symbiosum* by about 50% (A. Tamannaei, L. Schlüter & W. Buckel, unpublished). The action of hydroxylamine was different. Upon titration of the dehydratase system with hydroxylamine (NH_2OH) or nitrite (NO_2) no inhibition

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or inactivation could be observed until a certain threshold was reached. Above 30 μM hydroxylamine or 6 μM nitrite, the dehydratase was irreversibly inactivated. It could be shown that the threshold was due to the non-enzymatic reduction of 30 μM hydroxylamine to ammonia by 60 μM Ti(III)citrate present in the assay. The reason for the subsequent irreversible inactivation was not established. The concentration of hydroxylamine certainly is too low to cleave the thiol ester bond of the substrate, for which at least 100 mM hydroxylamine are necessary to complete the reaction within about 30 min at 25 °C.

9. Sources of reduced ferredoxin and flavodoxin

For the reduction of component A reduced ferredoxins or flavodoxins ($E_0' = -400$ to -450 mV) are required. These highly negative redox potentials can be easily supplied by oxidation of pyruvate to acetyl-CoA ($E_0' = -500$ mV) as catalysed by pyruvate–ferredoxin oxidoreductase or pyruvate–flavodoxin oxidoreductase. Since such or related enzymes participate in the fermentations of alanine in *C. propionicum* (pyruvate–ferredoxin oxidoreductase), leucine in *C. difficile* (2-oxoisocaproate–ferredoxin oxidoreductase) or phenylalanine in *C. sporogenes* (phenylpyruvate–ferredoxin oxidoreductase), the reduction of the electron carriers in these organisms provides no problem. In *A. fermentans* and *F. nucleatum*, however, only NADH ($E_0' = -320$ mV) is formed in the fermentation pathway. It has been postulated, therefore, that *A. fermentans* contains a membrane-bound enzyme, which catalyses the endergonic reduction of ferredoxin by NADH driven by an electrochemical Na^+-gradient generated by the decarboxylation of glutaconyl-CoA [27]. A possible candidate is the highly active NADH-dehydrogenase present in membranes of *A. fermentans* using ferricyanide as electron acceptor. The enzyme could be solubilised with dodecylmaltoside and purified until only several bands were visible by SDS–PAGE. Although some N-terminal sequences could be determined, the lack of a genome sequence of *A. fermentans* impeded further characterisation (J. Bresser & W. Buckel, unpublished).

A similar NADH-dehydrogenase has been detected in membranes of the glutamate fermenting *C. tetanomorphum*, a close relative of *Clostridium tetani*, whose genome has recently been elucidated [51]. The pathway of glutamate fermentation in *C. tetanomorphum* differs from that of *A. fermentans*, although both pathways lead to the same products. In *C. tetanomorphum* the carbon skeleton of (2*S*)-glutamate is rearranged to that of (2*S*, 3*S*)-3-methylaspartate catalysed by the coenzyme B_{12}-dependent glutamate mutase [52]. 3-Methylaspartate is further converted to acetate and pyruvate, which is oxidised to acetyl-CoA. Thereby reduced ferredoxin is generated, 20% of which is used for hydrogen formation and 80% for the NADH-dependent butyrate synthesis [27]. Hence, the NADH-dehydrogenase could be involved in the reverse direction, the reduction of NAD^+ by ferredoxin, whereby an electrochemical Na^+-gradient is established useful for transport purposes. The enzyme was solubilised with dodecylmaltoside and purified by three chromatographic steps until a six-band pattern was visible on SDS-PAGE. The N-termini of two of the bands were sequenced and showed high sequence identities to the proteins RnfC and RnfG deduced from the *rnf*-like gene cluster of *C. tetani*. Three bands (RnfACD) revealed fluorescence upon irradiation at 365 nm, indicating the presence of covalently attached flavins. From sequence and chemical analysis it was deduced that the RnF-like complex (ABCDEF) contains 6 Fe-S clusters, 2 [4Fe–4S] in RnfC and 3 [4Fe–4S] + 1 [3Fe–4S] in RnfB; the latter was designated as polyferredoxin. Finally it could be shown that the NADH dehydrogenase indeed catalyses the reduction of NAD^+ in an atmosphere of hydrogen using ferredoxin and hydrogenase (both from *Methanosarcina barkeri*) as electron transmitters. Thus the enzyme can be regarded as a NADH–ferredoxin oxidoreductase (NFOR) probably generating a Na^+ motive force (C. D. Boiangiu and W. Buckel, unpublished).

The abbreviation Rnf is derived from Rhodobacter nitrogen fixation, because the *rnf*-genes have been detected first in *Rhodobacter capsulatus* [53]. There is genetic evidence that the Rnf-proteins may be involved in the electron transport to the nitrogenase iron protein. The discovery of NFOR in *C. tetanomorphum* strongly suggests that the Rnf proteins of *R. capsulatus* form a similar hexameric complex, which catalyses the reduction of ferredoxin by NADH driven by H^+ or Na^+-motive force.

In the genome of *F. nucleatum* [44] also an *rnf*-like gene cluster has been found concomitant with a strong NADH-dehydrogenase activity (30 U/mg). Solubilisation and partial purification of the membrane enzyme (ca. 2000 U/mg) revealed a pattern of bands by SDS-PAGE; the N-terminus of the strongest staining band (Coomassie) was identical to that of the deduced RnfC homologue. These data lead to the conclusion that this NFOR may be necessary to provide reduced ferredoxin for component A of 2-hydroxyglutaryl-CoA dehydratase (D. Brügel, C. D. Boiangiu and W. Buckel, unpublished).

10. Homologues of the 2-hydroxyacyl-CoA dehydratase system

Benzoyl-CoA reductase from *Thauera aromatica* catalyses the ATP dependent reduction of benzoyl-CoA to cyclohexadienecarboxyl-CoA [54,55], which is converted

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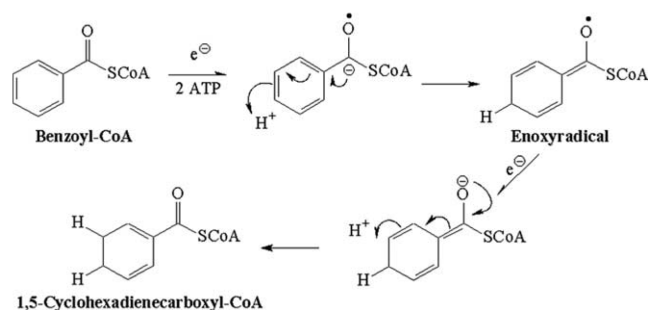

Fig. 7. Proposed mechanism for the reduction of benzoyl-CoA catalysed by benzoyl-CoA reductase from *Thauera aromatica*.

by a series of β-oxidation steps to CO_2 and 3 acetyl-CoA [56]. It has been proposed that in the Birch-like reduction one-electron energised by hydrolysis of two ATP converts the thiol ester carbonyl of benzoyl-CoA to a ketyl radical anion (Fig. 7). The highly reactive ketyl radical anion is trapped by protonation in the *p*-position of the aromatic ring yielding an enoxy radical, which is further reduced by a second electron and protonated in the *m*-position to the final diene [8,57]. Benzoyl-CoA reductase is a heterotetrameric protein complex, of which two different subunits share sequence similarities with component A of 2-hydroxyglutaryl-CoA dehydratase from *A. fermentans*, whereas the other two subunits seem to be related to the HgdA and B subunits of component D [15]. Although the dehydratase and the reductase catalyse different ATP-dependent reactions, the common origin of the enzymes may be due to the common mechanism. Like component A, benzoyl-CoA reductase requires reduced ferredoxin as electron donor, which is generated by oxidation of 2-oxoglutarate [58]. Naphthoyl-CoA-reductase, an enzyme possibly involved in anaerobic naphthalene oxidation, could be related to benzoyl-CoA reductase. By applying the mechanism of benzoyl-CoA reduction to naphthoyl-CoA reduction, 5,6,7,8-tetrahydro-2-naphthyl-CoA could be an intermediate (Fig. 8). In a sulfate-reducing enrichment culture using naphthalene as substrate indeed 5,6,7,8-tetrahydro-2-naphthalene carboxylic acid has been detected [59].

Other homologues of *hgdC* coding for component A from *A. fermentans* have been detected in the genomes of methanogenic archaea and in *E. coli*, but no function could be attributed to these genes. Deletion of the *yjiL*-gene from *E. coli*, a homologue of *hgdC* (43% sequence identity), gave no phenotype by growing the mutant on different substrates under aerobic and anaerobic conditions (S. Textor, Ph. D. thesis, Philipps-Universität Marburg, 1999). Remarkably, every sequenced genome of an anaerobic or facultative anaerobic bacterium revealed at least one homologue of *hgdC*; in *Clostridium acetobutylicum* even four different homologues have been described [60]. Currently over 50 entries are in the databases.

11. Convergent evolution of the Archerases

The 2-hydroxyacyl-CoA dehydratases from Clostridia and *F. nucleatum*, the benzoyl-CoA reductase from *T. aromatica*, as well as the deduced homologues of component A form a new family of enzymes, for which we coined the term 'Archerases' [61]. The ATP-driven electron transfer resembles an archer, who shoots arrows energised by ATP hydrolysis in his muscles (Fig. 9). Furthermore, just before shooting the string of the bow forms an angle of about 105°, the same angle is exhibited by the helix–cluster–helix architecture of component A. During shooting the angle is enlarged to 180° as proposed for the conformational change of component A in the presence of ATP. The helix–cluster–helix architecture of the nitrogenase iron protein exhibits an angle of 150°, which is also enlarged to 180° as shown in the complex with the iron–molybdenum protein in the presence of ADP$\times$AlF$_4^-$ [39]. Therefore we consider nitrogenase as a member of the 'Archerases', though of different phylogenetic origin. The bacterial ATP-dependent protochlorophyllide reductase from *R. capsulatus* is another 'Archerase'[62]. The enzyme catalyses the ferredoxin-dependent reduction of one double bond of an 18π-electron-containing aromatic system (Fig. 10). Apparently this difficult reduction requires

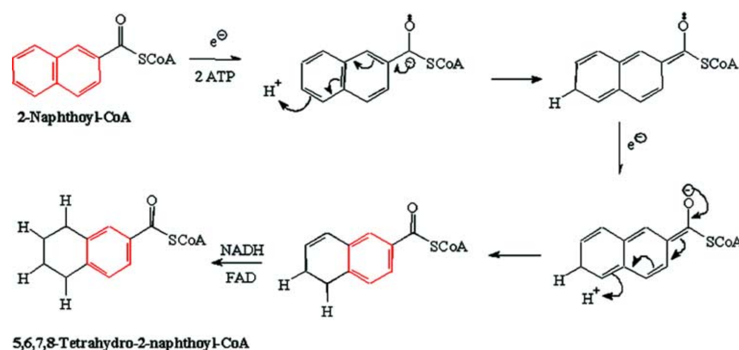

Fig. 8. Possible reductive pathway of naphthalene-2-carboxylate in a sulfate-reducing enrichment culture obtained from a contaminated aquifer.

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Fig. 9. The archer. The relief 'King Ashurbanipal hunting wild Asses' originated from the palace of Nineveh ca. 645 BC; now it is in the British Museum, London. The helix–cluster–helix architecture with two bound ADP from the 'Archerase' component A of *A. fermentans* has been superimposed on the string of the bow.

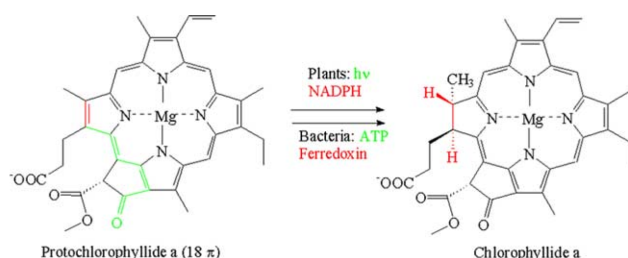

Fig. 10. Reactions catalysed by protochlorophyllide reductases from plants and bacteria.

ATP in addition to a strong reductant, similar to nitrogenase, with which it shares sequence similarities. As with benzoyl-CoA reductase the first electron could form a ketyl radical anion at the carbonyl group of protochlorophyllide. The ketyl is trapped by protonation at the double bond to be reduced. Notably the plant protochlorophyllide reductase uses NADPH and is energised by light [63].

Hence, the helix–cluster–helix architecture seems to be a versatile tool for certain enzymes. In evolution this tool probably has been combined with an ATP-hydrolysing enzyme, either with the ASKHA-proteins to form component A of 2-hydroxyacyl-CoA dehydratases and benzoyl-CoA reductase or with G-proteins to form nitrogenase iron protein and the corresponding iron protein of protochlorophyllide reductase. Thus the newly recognised family of Archerases may have evolved in a convergent manner.

The question arises, whether the dehydration of 2-hydroxy acids was an early invention of life or evolved later after the main pathways of amino acid metabolism had been established. The hydration of an enoyl-CoA at the α-carbon could have been a step on an early route to α-amino acids. On the other hand, scenarios of the primordial metabolism start with the synthesis of acetyl thiol esters from CO_2 and H_2S [64,65], followed by reductive carboxylation to pyruvate, which gives rise to alanine. Carboxylation of pyruvate yields oxaloacetate, the precursor of the aspartate family, and the reversed Krebs cycle leads to 2-oxoglutarate, the precursor of the glutamate family of amino acids [66]. Therefore it appears more likely that 2-hydroxyacyl-CoA dehydratases were only involved in amino acid degradation and perhaps in the biosynthesis of some β-amino acids, which are derived by the facile addition of ammonia to enoyl-CoA at the β-carbon [67,68].

Acknowledgements

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