# On the enzymatic mechanism of 2-hydroxyisocaproyl-CoA dehydratase from *Clostridium difficile*

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Buckel, W., Hetzel, M. & Kim, J. (2004) ATP-driven electron transfer in enzymatic radical reactions. *Curr. Opin. Chem. Biol. 8*, 462-467

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# Contents

## Abbreviations

| | |
|---|---|
| DTT | Dithiothreitol |
| EPR | Electron Paramagnetic Resonance |
| FPLC | Fast Protein Liquid Chromatography |
| FMN | Riboflavin-5'-phosphate |
| FAD | Flavin Adenine Dinucleotide |
| Maldi-TOF MS | Matrix-assisted laser desorption ionisation - time of flight mass spectrometry |
| Mops | 4-Morpholinepropanesulfonic acid |
| OD | Optical Density |
| SDS | Sodium dodecylsulfate |
| TEMED | *N*,*N*,*N'*,*N'*-Tetraethylethylenediamine |
| TCA | Trichloroacetic acid |
| Tris | 2-Amino-2-(hydroxymethyl)-1,3-propanediol |
| UV-vis | Ultraviolet visible |

## Zusammenfassung

Die Gene *ldhA* und *hadA* aus *Clostridium difficile* (DSMZ 1296$^T$) wurden kloniert und in *Escherichia coli* exprimiert. Die erhaltenen Proteine wurden gereinigt und als D-2-Hydroxyisocaproat-Dehydrogenase (LdhA) und 2-Hydroxyisocaproat-CoA-Transferase (HadA) identifiziert. Die Enzyme katalysieren zwei Schritte in der Fermentation von Leucin zu Ammonium, $CO_2$, Isovalerat und Isocaproat. Die nächsten im Genom von *C. difficile* liegenden Gene *hadBC* und *hadI* wurden ebenfalls aktiv exprimiert und als 2-Hydroxyisocaproyl-CoA-Dehydratase (HadBC) und ihrem Aktivator (HadI) identifiziert. Die Dehydratase katalysiert die Eliminierung von Wasser aus (*R*)-2-Hydroxyisocaproyl-CoA zu Isocaprenoyl-CoA, die eine chemisch schwierige Reaktion darstellt, da das Proton in der β-Position nicht aktiviert ist (p*K* ca. 40). Wir postulieren, dass erst die Reduktion des Substrats mit einem Elektron die Eliminierung ermöglicht, wobei der p*K* mindestens bis 14 gesenkt wird. Anschließend wird das Elektron wieder ans Enzym zurückgegeben.

Die heterodimere Dehydratase und der homodimere Aktivator sind Eisen-Schwefel-Proteine, in denen keine weiteren prosthetischen Gruppen (oder Metalle wie Molybdän) detektiert werden konnten. Der durch Ferredoxin reduzierte Aktivator überträgt unter ATP-Hydrolyse ein Elektron auf die Dehydratase, die dadurch in den katalytisch aktiven Zustand überführt wird. Dieser ATP-getriebene Elektronen-Transfer ähnelt dem der Nitrogenase. Die aktivierte und vom Aktivator abgetrennte Dehydratase katalysiert ca. 10000 Umsätze bis das Elektron durch Oxidation verloren geht. Durch anschließende Zugabe von ATP und Aktivator kann die inaktivierte Dehydratase wieder voll aktiviert werden. Die Bildung eines stabilen aktiven $AlF_4^-$-induzierten Komplexes aus Dehydratase und Aktivator stützt den postulierten Elektronentransport vom Aktivator zur Dehydratase und zeigt ebenfalls die Rückgewinnung des benötigten Elektrons nach jedem Turnover. In Übereinstimmung damit werden zur maximalen Aktivität nur substöchiometrische Mengen an Aktivator (Aktivator/Dehydratase = 1:10) benötigt. Mit Hilfe der EPR-Spektroskopie wurde zum ersten Mal während der Dehydratisierung eines 2-Hydroxyacyl-CoA-Derivats ein organisches Radikalsignal detektiert. Mit Hilfe von isotopmarkierten Substraten veränderten sich die EPR-Spektren in einer für das Ketylradikalanion des Isocaprenoyl-CoA charakteristischen Weise.

## Summary

The genes *ldhA* and *hadA*, from *Clostridium difficile* (DSMZ 1296$^T$) were cloned and expressed in *Escherichia coli*. The obtained proteins were purified and characterised as D-2-hydroxyisocaproate dehydrogenase (LdhA) and 2-hydroxyisocaproate CoA-transferase (HadA) involved in two consecutive steps in the pathway of leucine fermentation to ammonia, $CO_2$, isovalerate and isocaproate. The downstream genes *hadBC* and *hadI* were also functionally expressed and shown to encode the novel 2-hydroxyisocaproyl-CoA dehydratase (HadBC) and its activator (HadI). The activated dehydratase catalyses the dehydration of (*R*)-2-hydroxyisocaproyl-CoA to isocaprenoyl-CoA, which is a chemically difficult step since the proton in the β-position is not activated (p*K* ca. 40). We postulated that the reduction of the substrate by one electron enables the elimination, whereby the p*K* is lowered to at least 14. After the reaction the electron is returned to the dehydratase, which may catalyse many turnovers.

The extremely oxygen-sensitive homodimeric activator as well as the heterodimeric dehydratase contain iron-sulfur cluster(s); other prosthetic groups specifically molybdenum were not detected. The reduced activator transfers one electron to the dehydratase concomitant with hydrolysis of ATP, a process similar to that observed with the unrelated nitrogenase. The reduced dehydratase separated from the activator and ATP catalysed almost $10^4$ dehydration turnovers until the electron was lost by oxidation. By adding activator and ATP the enzyme could be fully reactivated. The active tight complex of the two protein components induced by $AlF_4^-$ and ATP underpins the postulated electron transfer from the activator to the dehydratase and demonstrates again that the electron is recycled after each turnover. In agreement with this observation, only substoichiometric amounts of activator (activator/dehydratase = 1:10) were required to generate full activity. An organic radical proposed to mediate the dehydration was detected by EPR spectroscopy for the first time in a 2-hydroxyacyl-CoA dehydratase. The changes of the EPR spectra induced by the use of labelled substrates showed that the radical was substrate-derived. These results are the first clear evidence for a radical involved in a dehydration mechanism and suggest a new way to form a radical in enzymatic reactions.

# Introduction

## 1. Fermentation of amino acids by clostridia

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 (6, 8, 50). These organisms are able to ferment amino acids to ammonia, $CO_2$, short chain fatty acids and molecular hydrogen. 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 the other amino acid is reduced to a fatty acid with the same carbon skeleton. An example is the pairwise 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. Thus *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 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 or the conversions of single amino acids to intermediates, which are able to perform redox reactions, are unique in most cases. Most α-amino acids have to be deaminated by β-elimination to unsaturated fatty acids, in order to be reduced to the saturated carboxylates.

In general α-amino acids are resistant towards β-elimination, since the p$K$ of the non-activated β-proton (p$K \approx 40$) 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 (73). On the other hand, β-amino acids are easy to deaminate, since the p$K$ of the α-hydrogen is about 30 and can be lowered to 21 by CoA-thiol ester formation (1) 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 (34).

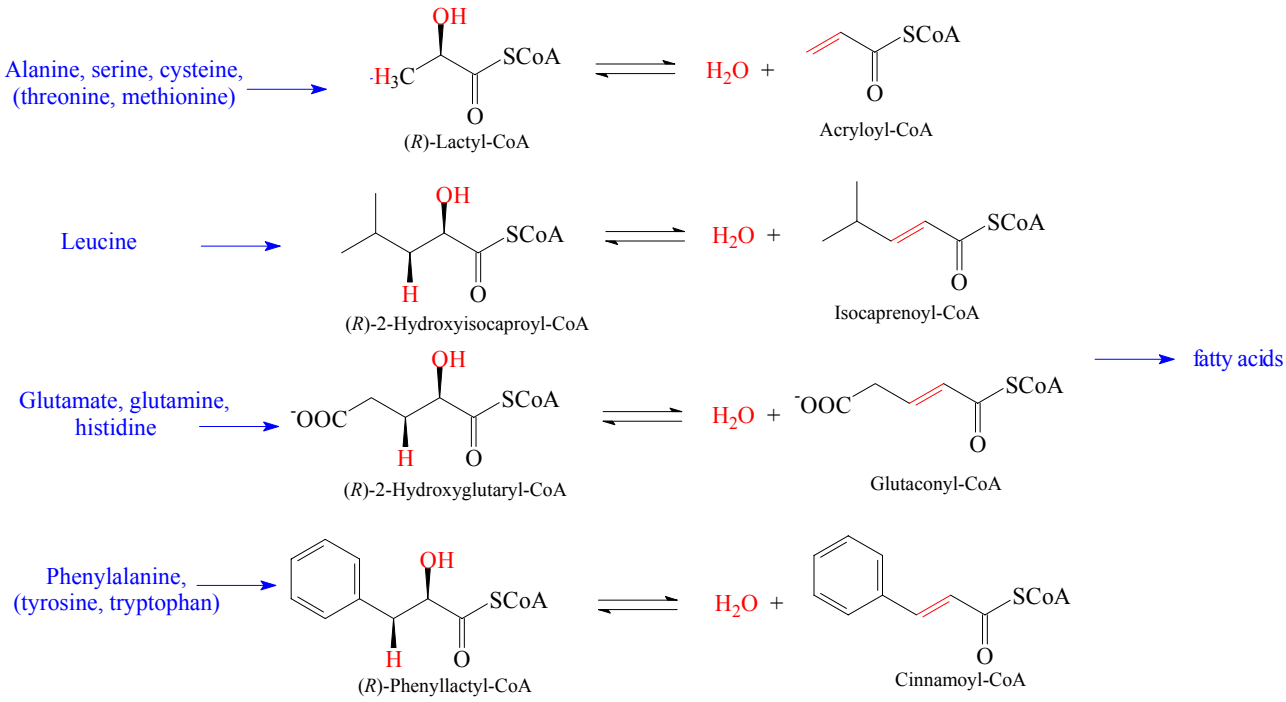



**Fig. 1. Conversion of amino acids to fatty acids via dehydration of their corresponding (*R*)-2-hydroxyacyl-CoAs to enoyl-CoAs.**

Of the twenty proteinogenous amino acids, twelve can be dehydrated by the mechanism outlined above (Fig. 1). 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 $NAD^+$ 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 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 dehydration of 2-hydroxy acid is not involved. Thus the α-amino 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 the α-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.

## 2. Radicals in enzymatic processes

Until recently, many enzymes have been reported to catalyse by radical mechanisms. These enzymes utilize the high reactivity of radicals to perform catalysis. Catalytic radicals are either the radicals derived from cofactors, such as AdoCbl (adenosylcobalamin) (92) and *S*-adenosylmethionine (38), or protein radicals (85). Radical-catalysed reactions have a common feature: substrates of these enzymes can not be activated by an acid-base mechanism. It is worth noting that all of these reactions are chemically difficult under mild, physiological conditions without enzymes. Enzymatic radical catalysis can therefore be defined as the mechanism of catalysis by which enzymes catalyse chemically difficult reactions by utilizing the high reactivity of free radicals.

Enzymes produce radicals by three different ways: homolysis of a weak covalent bond, one-electron oxidation, and one-electron reduction. The first way is applied by coenzyme $B_{12}$-dependent enzymes, such as glutamate mutase, in which the homolysis of the weak carbon–cobalt bond of adenosylcobalamin (130 kJ/mol) affords cob(II)alamin and the 5'-deoxyadenosine radical necessary to initiate the rearrangement of (*S*)-glutamate to (2*S*,3*S*)-3-methylaspartate by abstraction of the 4*Si*-hydrogen (17). The aerobic ribonucleotide reductase is an example, in which the tyrosine radical is formed by one-electron oxidation with molecular oxygen activated by the dinuclear iron centre, although the primary event might be homolysis of the O–O-bond of the peroxo intermediate (5). A one electron oxidation has been postulated for the catalysis of 4-hydroxybutyryl-CoA dehydratase, in which the enolate anion of the substrate is oxidised by the prosthetic group FAD to an enoxy radical (17). The formation of this radical lowers the p*K* of the β-proton of 4-hydroxybutyryl-CoA from about 40 to 14, enabling the dehydration to crotonyl-CoA via a ketyl radical anion (82). The emerging large family of *S*-adenosylmethionine (SAM) radical enzymes uses one-electron reduction of the 'high-energy' compound SAM to generate the 5'-deoxyadenosine radical (38). Another example of radical formation by one-electron reduction is the *syn*-dehydration of (*R*)-2-hydroxyacyl- CoA to (*E*)-2-enoyl-CoA. Apparently a one-electron transfer to the thiol ester carbonyl affording the ketyl radical anion initiates this reaction. The ketyl acts as a nucleophile and expels the adjacent hydroxyl group. To meet the low redox potential of the thiol ester carbonyl, the reducing power of ferredoxin is enhanced by hydrolysis of ATP (55).

### 3. Dehydration of (*R*)-2-hydroxy acids: 2-hydroxyacyl-CoA dehydratase

It has been proposed that the dehydration of (*R*)-2-hydroxyacyl-CoA to enoyl-CoA can only be achieved by conversion of the electrophilic thiol ester carbonyl into a nucleophile, a process called 'Umpolung' (charge reversal). The thiol ester carbonyl has properties of an electrophile ketone, which can be reduced by one electron to a nucleophilic ketyl radical anion. Hence, reduction of 2-hydroxyacyl-CoA to its ketyl radical anion would facilitate the elimination of the hydroxyl group to yield an enoxy radical, which can be deprotonated to the ketyl radical anion of the product enoyl-CoA. Oxidation of the latter to the unsaturated product by the next incoming substrate would complete the catalytic cycle (15, 68) (Fig. 2). It has been calculated that the p*K* of the enoxy radical has been lowered to 14, about 26 units less than the p*K* of the β-proton of the 2-hydroxy acid (82). Similar to β-hydroxyacyl-CoA, the p*K* of the enoxy radical may further be lowered by hydrogen bonding.

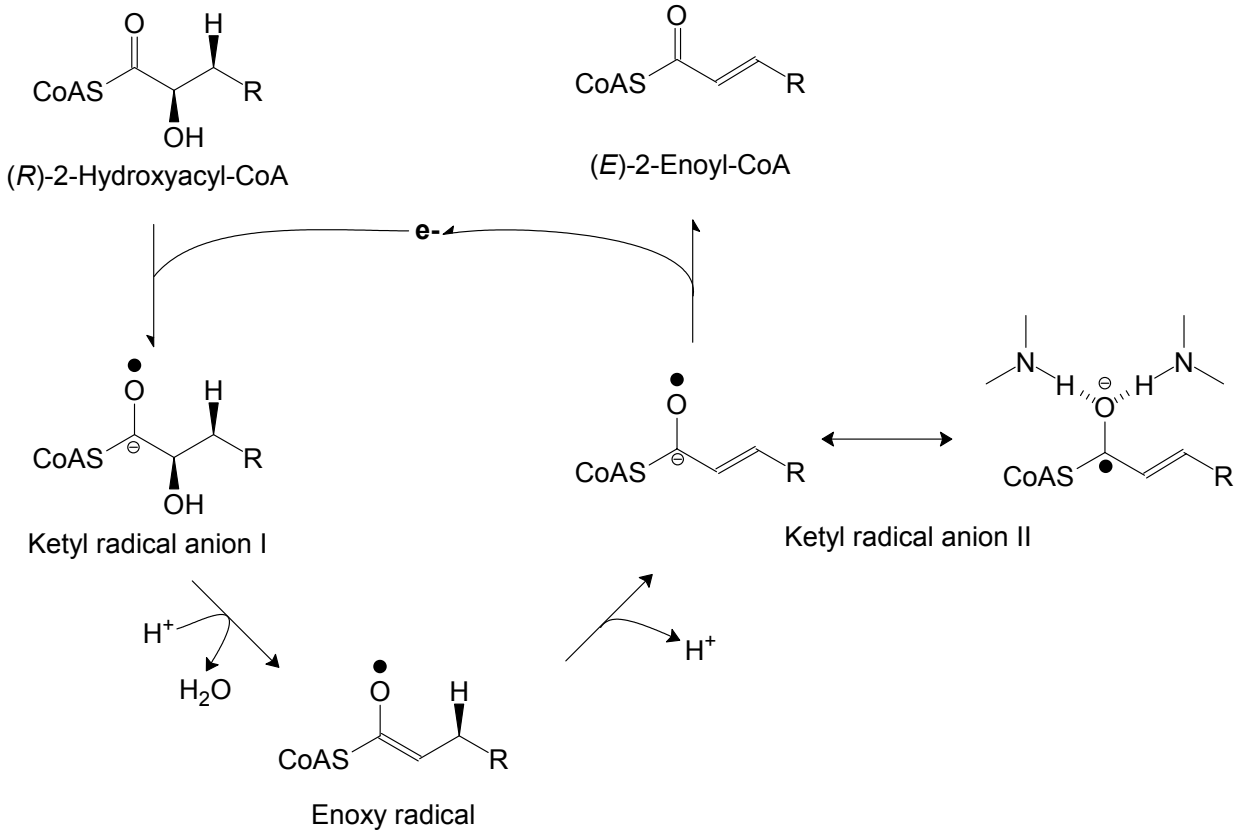



**Fig. 2. Proposed mechanism for the dehydration of (*R*)-2-hydroxyacyl-CoA to (*E*)-2-enoyl-CoA using an electron as cofactor catalysed by dehydratase.** The function of activator is only to initiate the catalytic cycle by ATP-induced electron transfer. Although calculations of the ketyl radical anion have indicated that the negative charge sits mainly at the oxygen, the resonance structure depicted here contributes to the stabilisation of the radical and shows how the ketyl acting as nucleophile expels the hydroxyl group (11).

Until now six different 2-hydroxyacyl-CoA dehydratases have been purified and characterised (Table. 1), lactyl-CoA dehydratase from *C. propionicum* (48, 78), 2-hydroxyglutaryl-CoA dehydratase from *A. fermentans* (43, 79), *C. symbiosum* (45) and *F. nucleatum* (56), phenyllactate dehydratase from *C. sporogenes* (27, 28) as well as 2-hydroxyisocaproyl-CoA dehydratase from *C. difficile* (54). All dehydratases are enzyme systems composed of two separable components, an extremely oxygen-sensitive activator or initiator (component A) and a moderately oxygen-sensitive component D, the actual dehydratase. The activator, initiator or component A from *A. fermentans* produced in *E. coli* (44) is a homodimeric enzyme with one [4Fe-4S] cluster between the two subunits (2 × 27 kDa) (66). The most remarkable feature of component A are two helices, each from one subunit pointing with their N-termini towards the [4Fe-4S] cluster forming a helix-cluster-helix angle of 105°.

**Table 1. Characteristics of 2-hydroxyacyl-CoA dehydratases**

| Organism | Substrate | Component A | Component D | Specific activity ($s^{-1}$) |
|---|---|---|---|---|
| *Acidaminococcus fermentans* | (*R*)-2-Hydroxy-glutaryl-CoA | HgdC, $\gamma_2$, $[4Fe\text{-}4S]^{1+/2+}$ | HgdAB, $\alpha\beta$, $[4Fe\text{-}4S]^{2+}$, FMN, riboflavin | 6 |
| *Clostridium symbiosum* | (*R*)-2-Hydroxy-glutaryl-CoA | Not purified[1] | HgdAB, $\alpha\beta$, 2 $[4Fe\text{-}4S]^{2+}$, FMN | 30<br>50* |
| *Fusobacterium nucleatum* | (*R*)-2-Hydroxy-glutaryl-CoA | HgdC[1], $\gamma_2$, $[4Fe\text{-}4S]^{1+/2+}$ | HgdABD, $\alpha\beta\delta$, $[4Fe\text{-}4S]^{2+}$, riboflavin | <30 |
| *Clostridium sporogenes* | (*R*)-Phenyl-lactate | FldI[1], $\eta_2$, $[4Fe\text{-}4S]^{1+/2+}$ | FldABC, $\alpha\beta\gamma$, $[4Fe\text{-}4S]^{2+}$ | 1* |
| *Clostridium difficile* | (*R*)-2-Hydroxy-isocaproyl-CoA | HadI[1], $\eta_2$, $[4Fe\text{-}4S]^{1+/2+}$ | HadBC, $\alpha\beta$, 1-2 $[4Fe\text{-}4S]^{2+}$, FMN? | 100* |
| *Clostridium propionicum* | (*R*)-Lactyl-CoA | Partially purified | LcdAB, $\alpha\beta$, 2 $[4Fe\text{-}4S]^{2+}$, FMN, riboflavin | low |
| *Megasphera elsdenii* | (*R*)-Lactyl-CoA | Not purified | LcdAB, $\alpha\beta$, 2 $[4Fe\text{-}4S]^{2+}$?, FMN? | low |

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

A similar architecture is found in the phylogenetically unrelated iron protein of nitrogenase from *Azotobacter vinelandii* with a helix-cluster-helix angle of 150°. Upon binding to component D, probably the angle opens to 180° as observed in the complex of nitrogenase iron protein with molybdenum-iron protein in the presence of ADP-$AlF_4^-$ (76). Component A has a low ATPase activity (ca. 0.1 $s^{-1}$) but only in the reduced $[4Fe\text{-}4S]^+$ state. 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. 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) (88) indicating a potential of about –350 mV or even higher. The closely related components A from *C. sporogenes* and *F. nucleatum* (86) have also been purified in the same way and revealed almost identical properties. Each known genome of an anaerobic bacterium (including *E. coli*) or archaeon contains at least one deduced homologue of this exciting protein. There are even four homologous genes of component A in the genome of *Clostridium acetobutylicum* (69).

Component D of *A. fermentans* has been characterised as a heterodimeric enzyme (54 + 42 kDa) containing one [4Fe-4S] cluster, one riboflavin-5′-phosphate (FMN) and about 0.1 riboflavin. Molybdenum has also been found in this protein, but the content of 0.1 mol/heterodimer appears to be too low to be significant. Furthermore, the same amount of Mo has been detected in component D from *C. symbiosum*, but this metal is absent in 2-hydroxyglutaryl-CoA dehydratase from *F. nucleatum*. Interestingly, component D from *C. symbiosum* contains two [4Fe-4S] clusters. The smaller β-subunit of this protein could be crystallised and its crystal structure has been determined. It revealed one [4Fe-4S] cluster and one FMN at a distance of 17 Å. Unexpectedly, only three iron atoms of the cluster are coordinated by cysteines, whereas the fourth has a not conserved tyrosine as ligand (Holger Dobbek and Berta Martins, unpublished). The 2-hydroxyglutaryl-CoA dehydratase from *F. nucleatum* is unique, since it is composed of three different subunits (53, 56, 86). The third subunit does not seem to be related to any other protein. Component D of phenyllactate dehydratase from *C. sporogenes* is also a trimeric protein. The third and largest subunit (46 kDa), however, has been characterised as a cinnamoyl-CoA:phenyllactate CoA-transferase, which catalyses the formation of (*R*)-phenyllactyl-CoA. The other two subunits are homologues of the α and β-subunits (45 and 37 kDa) of components D from *C. symbiosum* and *A. fermentans*. Hence, activity of phenyllactate dehydratase requires in addition to (*R*)-phenyllactate, ATP, $MgCl_2$, a reducing agent (see below) and component A also catalytic amounts of cinnamoyl-CoA. The mechanism comprises a combination of the mechanisms of

citrate lyase and 2-hydroxyglutaryl-CoA dehydratase. Initially (*R*)-phenyllactyl-CoA and the final product (*E*)-cinnamate are generated from cinnamoyl-CoA and (*R*)-phenyllactate. In the next step cinnamoyl-CoA is regenerated by dehydration of (*R*)-phenyllactyl-CoA. This enzyme complex clearly shows that formation of the thiol ester substrate is a prerequisite for the dehydration of 2-hydroxy acids. In the fermentation of glutamate via 2-hydroxyglutarate, the dehydration at the thiol ester level could be due to the participation of the CoA-ester in the consecutive decarboxylation step (27, 28).

The reductive activation of component D of the dehydratases requires component A, ATP, $MgCl_2$, and a reducing agent. In vitro dithionite or Ti(III)citrate are suitable one-electron donors, whereas in vivo a clostridial-type, two [4Fe-4S] cluster-containing ferredoxin (88) or flavodoxin (43) serve for this purpose. The further fate of the electron in the activation process remains unclear. Whereas the Mössbauer spectrum clearly revealed oxidation of component A during activation, the concomitant reduction of the [4Fe-4S] cluster(s) of component D could not be observed by this method. The active component D, however, exhibited an EPR-signal ($g < 2.0$), which has been interpreted as that of Mo(V) (43). The recently detected tyrosine-coordination of the [4Fe-4S] cluster by *X*-ray crystallography may lead to another speculation. This non-innocent ligand could be reduced to a radical anion stabilised by the cluster.

The putative mechanism of activation and dehydration can now be described in the following way (Fig. 3): The cluster of component A, to which two ADP are bound, is reduced by ferredoxin or flavodoxin with one electron to $[4Fe\text{-}4S]^{+}$. Then ADP is exchanged by ATP, which causes the helix-cluster-helix angle to open from 105° to 180°. This conformational change enables component A to dock on 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 returns to its ‘ground state’ with two ADP and oxidised $[4Fe\text{-}4S]^{2+}$. Upon addition of (*R*)-2-hydroxyacyl-CoA to the reduced component D the electron is further transferred to the substrate to form the ketyl radical anion, which initiates the dehydration as proposed above. Afterwards the electron is 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 from glutamate, since the fermentation only yields 0.6 mol ATP/mol glutamate.

There are still several weak 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 the ketyl radical anion and enoxy radical intermediates. The electron transfer from component A to D driven by ATP hydrolysis is comparable to an archer shooting arrows. Furthermore the conformational changes of the string of his bow during shooting are similar to the proposed opening of the helix-cluster-helix angle from 105° to 180°. Hence, all enzymes related to component A have been called Archerases (55).

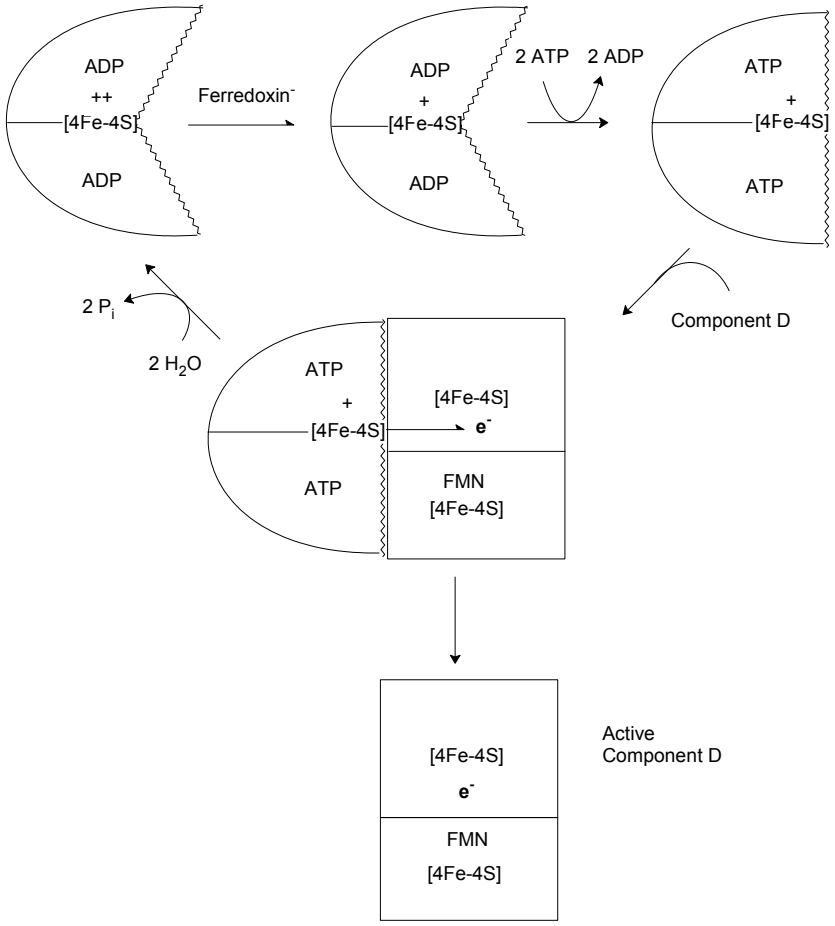



**Fig. 3. Proposed mechanistic scheme of the activation of component D.** At the left, component A is in the resting state with the oxidized $[4Fe\text{-}4S]^{2+}$ cluster and one molecule of ADP bound to each subunit. The helix-cluster-helix motif forms an angle of 105°. Then one electron from reduced ferredoxin is transferred spontaneously to component A to yield reduced $[4Fe\text{-}4S]^{1+}$ and oxidized ferredoxin. Addition of ATP leads to an exchange with ADP and causing a large conformational change, whereby the helix-cluster-helix angle is opened to 180°. After docking to component D, the electron is transferred concomitant with ATP hydrolysis: Component D is activated by reduction with one electron and component A returns to the ground state.

## 4. Fermentation of leucine by *Clostridium difficile*

*Clostridium difficile* is a strictly anaerobic, gram-positive and spore-forming human pathogen belonging the genus *Clostridium*, family *Clostridiaceae*, order *Clostridiales*, class *Clostridia*, phylum *Firmicutes* (39), another scheme classified *C. difficile* as a member of clostridium clusters I (23).

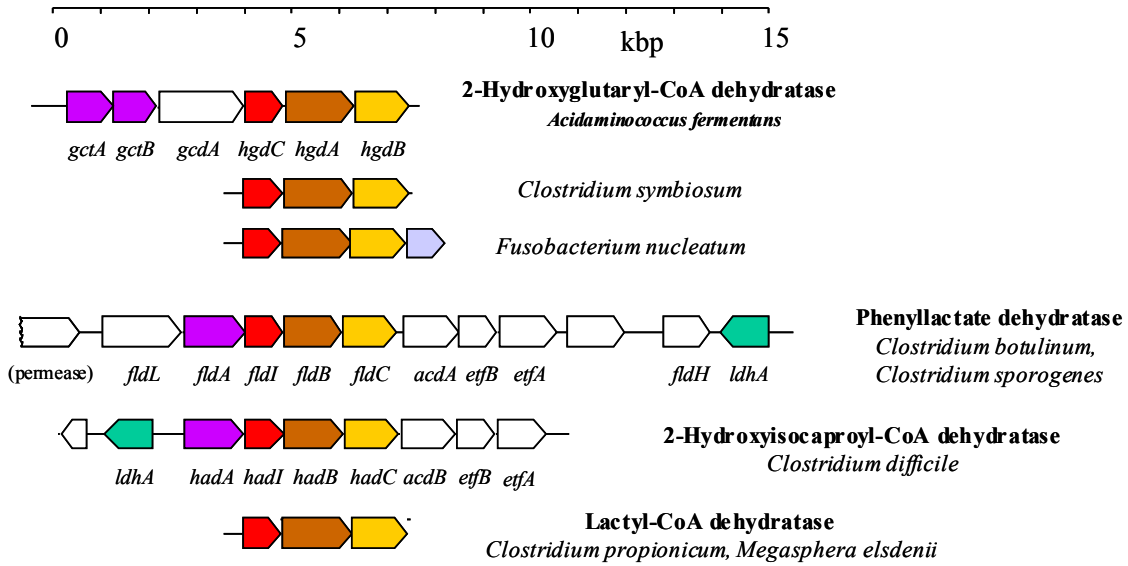



**Fig. 4. Gene cluster encoding 2-hydroxyacyl-CoA dehydratases and accessory enzymes.**

The previous study on phenyllactate dehydratase of *C. sporogenes* revealed a similar arrangement of homologous genes in the genome of *C. difficile*, designated as *hadA*, *hadI*, *hadB* and *hadC*, had for **h**ydroxy**a**cyl-CoA **d**ehydratase (27). Upstream of *hadA* an open reading frame in the opposite direction (*ldhA*) was detected encoding a putative D-2-hydroxy acid dehydrogenase (Fig. 4). We speculated that these genes could be involved in the fermentation of leucine (3), the preferred substrate of *C. difficile*. Leucine had been considered only as an electron donor in the Stickland reaction but it was found that *C. difficile* ferments leucine to isovalerate and isocaproate. Therefore leucine should act also as an electron acceptor. Three mol leucine are fermented by this organism to a mixture of fatty acids (eqn 1); two mol are reduced to isocaproate, whereas one mol is oxidised to isovalerate and $CO_2$ (10, 31), eqn. 1, for structures see Fig. 5.

3 L-Leucine + 2 $H_2O$ = 3 $NH_4^+$ + $CO_2$ + Isovalerate$^-$ + 2 Isocaproate$^-$;

$\Delta G^{\circ\prime}$ = –146 kJ/reaction (89); (eqn 1).

A proposed pathway is shown in Fig. 5. The reductive branch of the pathway might be carried out by enzymes deduced from the genes. The formation of isocaproate should proceed via the dehydration of (*R*)-2-hydroxyisocaproyl-CoA to 2-isocaprenoyl-CoA.

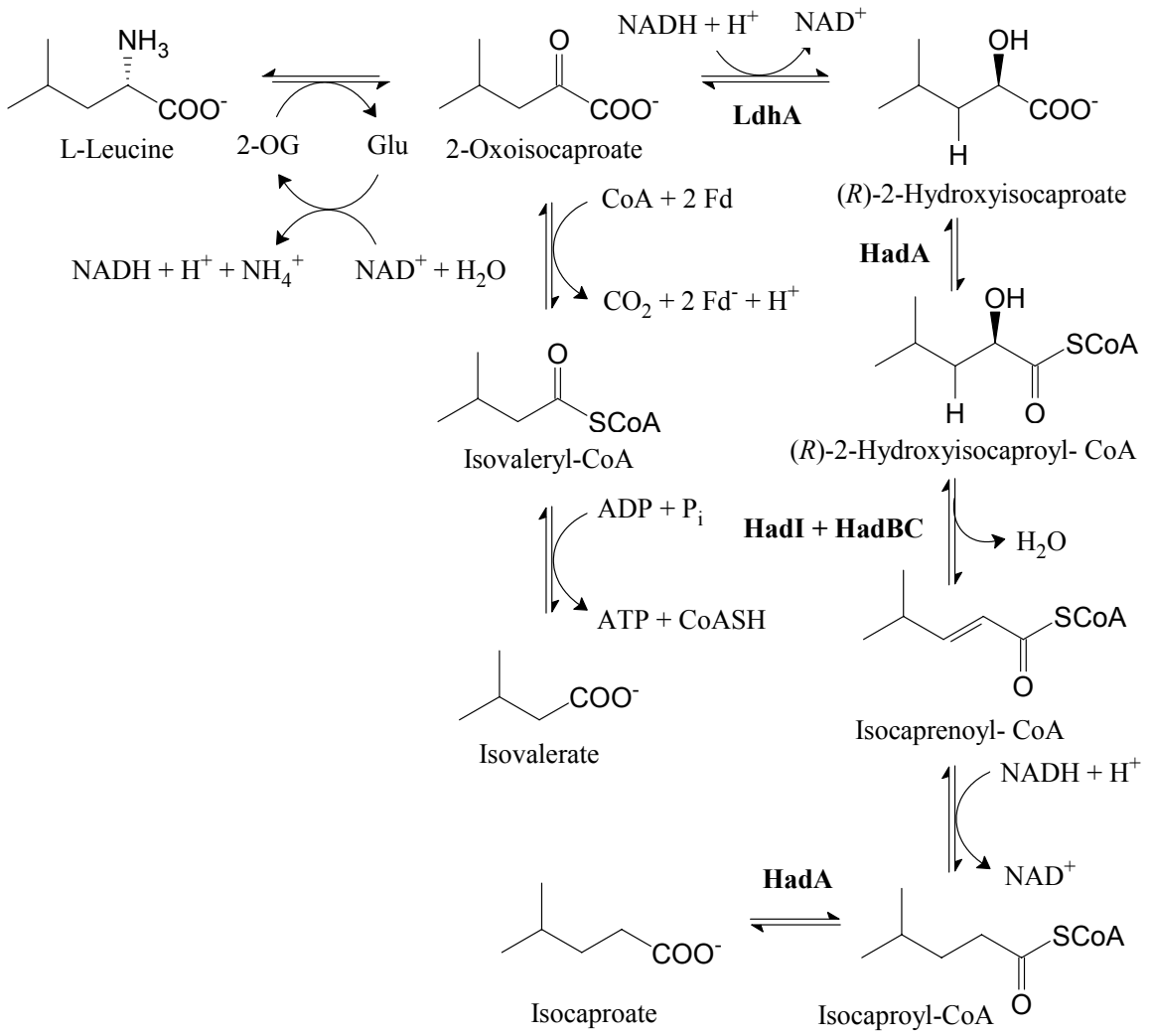



**Fig. 5. Proposed L-leucine fermentation pathway of *C. difficile.*** **LdhA**, (*R*)-2-hydroxyisocaproate dehydrogenase; **HadA**, Isocaprenoyl-CoA: 2-hydroxyisocaproate CoA-transferase; **HadI**, activator of dehydratase; **HadBC**, 2-hydroxyisocaproyl-CoA dehydratase; $Fd^-$, reduced ferredoxin

## 5. Goals of the work

Before the study on 2-hydroxyisocaproyl-CoA dehydratase, 2-hydroxyacyl-CoA dehydratases from 5 different organisms were purified and characterised in our group. However several weak points in the proposed mechanism were not clarified as mentioned. One of the major problems is to localise the electron in dehydratase (component D). Most of the characterised dehydratases contain FMN and iron-sulfur cluster for a possible place of electron, however, none of them seemed to change their redox state. As another target the metal molybdenum was detected in 2-hydroxyglutaryl-CoA dehydratases, but is unlikely to be the place for the electron. The other point for the verification of the mechanism are the proposed ketyl radical anion and enoxy radical intermediates, which have not been observed. Therefore, this thesis focuses on clarification of weak points in the proposed mechanism of 2-hydroxyacyl-CoA dehydratase through the study of 2-hydroxyisocaproyl-CoA dehydratase of *C. difficile*.

# Materials and Methods

## 1. Materials

### 1.1. Chemicals and reagents

All used chemical compounds and reagents were purchased, if not mentioned separately in the text, from the companies, Sigma (Steinheim), Merck (Darmstadt), Roth (Karlsruhe), Fluka (Neu-Ulm), Bio-Rad-Laboratories (München) or Serva (Heidelberg). The materials for molecular biology were obtained from New England Biolabs (Frankfurt am Main), Abgene (Hamburg), Roche (Mannheim) and Amersham Biosciences (Freiburg). The primers were synthesised by MWG (Ebersberg).

#### 1.1.1. (*R*)- and (*S*)-2-Hydroxyisocaproate

(*R*)- and (*S*)-2-Hydroxyisocaproate were synthesized by Dr. Daniel Darley from D- and L-leucine respectively by treatment of the corresponding amino acids with sodium nitrite in dilute sulfuric acid (24).

#### 1.1.2. (*E*)-2-Isocaprenoate (4-methyl-*trans*-2-pentenoate)

(*E*)-2-Isocaprenoate was synthesized by condensation of isobutyraldehyde and malonic acid in pyridine-piperidine (40).

#### 1.1.3. 2-Hydroxyisocaproyl-CoA

2-Hydroxyisocaproyl-CoA was synthesized from the mixed acid anhydride of 2-hydroxyisocaproate by a modified method of the thiol ester synthesis described by Kawaguchi. 2-Hydroxyisocaproate (110 µmol) and 1,1'-carbonyldiimidazole (100 µmol) were dissolved in 500 µl tetrahydrofuran (> 99% HPLC grade). To form the CoA-ester, 200 µl of mixed acid anhydride was added to 10 µmol CoASH in 500 µl 100 mM $NaHCO_3$ and incubated at room temperature for 1 hour. The reaction was stopped by acidifying of the reaction with 1 M HCl (< pH 4.0) and loaded on a Sep-pak® $C_{18}$ cartridge (Waters, USA) which was equilibrated with 0.1 % trifluoroacetic acid. The column was washed with 5 column volume of 0.1% (v/v) trifluoroacetic acid and CoA ester was eluted in 5 ml 1% (v/v) trifuloroacetic acid and 50% (v/v) acetonitrile. For the molecular mass confirmation by MALDI-TOF mass spectroscopy, the purified CoA ester was applied on thin layer of indole-2-carboxylic acid prepared from a solution of 300 mM indole-2-carboxylic acid in acetone. MALDI-TOF mass spectra were collected at an accelerating voltage of 15,000 V, 58% grid

voltage, and a delay time of 50 ns in the reflector mode of the instrument at a mirror ratio of 1.07 with indole-2-carboxylic acid as matrix [56].

### 1.1.4. (*R*)-2-Hydroxy[2-$^{2}H_{1}$]isocaproate

(*R*)-2-Hydroxy[2-$^{2}H_{1}$]isocaproate was synthesised by reduction of 2-oxoisocaproate by (*R*)-2-hydroxyisocaprate dehydrogenase (LdhA) with [2-$^{2}$H]formate, formate dehydrogenase and $NAD^{+}$;

| | |
|---|---|
| 50 mM | Photassium phosphate buffer pH 7.6 |
| 0.1 mM | $NAD^{+}$ |
| 75 mM | 2-Oxoisocaproate |
| 300 mM | [2-$^{2}$H]formic acid (neutralized with NaOH) |
| 0.1 U | (*R*)-2-Hydroxyisocaprate dehydrogenase |
| 0.4 U | Formate dehydrogenase |

The reaction mixture was made up to total volume 1 ml $H_2O$ and incubated overnight at room temperature. After acidification (pH ≈ 1) with 1 M HCl, the excess formic acid was removed by either extractions (3 x 3 ml) and the product dissolved in $D_2O$ was confirmed by NMR.

### 1.1.5. (*R*)-2-Hydroxy[3-$^{2}H_{2}$]isocaproate

2-Oxoisocaproate (150 µmol) was dissolved in 1 ml $D_2O$ and incubated at 80 °C for 3 hours yielding 2-oxo[3-$^{2}H_{2}$]isocaproate (≈ 100 % exchange confirmed by NMR). Subsequently, reduction by (*R*)-2-hydroxyisocaprate dehydrogenase with formic acid (HCOOH), formate dehydrogenase and $NAD^{+}$ was prepared as described in 1.1.4.

### 1.1.6. (*R*)-2-Hydroxy[2,3-$^{2}H_{3}$]isocaproate

2-Oxo[3-$^{2}H_{2}$]isocaproate was synthesised as described in 1.1.5. and reduced by (*R*)-2-hydroxyisocaprate dehydrogenase with [2-$^{2}$H]formate, formate dehydrogenase and $NAD^{+}$ as described in 1.1.4.

### 1.1.7. (*R*)-2-Hydroxy[1-$^{13}$C]isocaproate

2-Oxo[1-$^{13}$C]isocaproate was purchased from Cambridge Isotope Laboratories, Inc. and reduced by (*R*)-2-hydroxyisocaprate dehydrogenase with formic acid (HCOOH), formate dehydrogenase and $NAD^{+}$ as described in 1.1.4.

### 1.2. Instruments and columns

Beckman (Munich) supplied the ultra centrifuge, Sorvall (München) the cooling centrifuges. Anaerobic experiments have been done in an anaerobic glove box supplied by Coy Laboratories, Ann Arbor MI, (USA). The FPLC system and the UV-vis photometer, Ultrospec 400, installed in glove box were obtained from Amersham Biosciences (Freiburg). HP 8453 UV-visible diode array spectrophotometer (USA) was used for measuring UV-vis spectra and Amersham Biosciences Kontron spectrophotometer was used for aerobic activity assays. The columns DEAE Sepharose HR 26/10, Phenyl Sepharose FF HR 26/10, Superdex 200 HR 26/10, Q-Sepharose High performance HiLoadTM 26/10, Superdex-G25 (5 ml) were obtained from Amersham Biosciences (Freiburg). HPLC columns were from Merck (Darmstadt). *Strep*-Tactin MacroPrep column was purchased from IBA GmbH (Göttingen).

### 1.3. Anaerobic work

Purification of the activator and 2-hydroxyisocaproyl-CoA dehydratase were performed at 15 – 20 °C in an anaerobic glove box under a nitrogen atmosphere containing 5 % $H_2$. Buffers for enzyme purification were prepared by boiling and cooling under vacuum. Afterwards the buffers were flushed with nitrogen and transferred to the anaerobic chamber where dithiothreitol (2 - 5 mM) was added and stirred overnight. Enzyme activity was determined inside the anaerobic chamber with an Ultrospec 4000 spectrophotometer.

### 1.4. Bacteria and culture media

#### 1.4.1. *Clostridium difficile*

*Clostridium difficile* (DSMZ $1296^T$) was cultivated under anaerobic conditions in 100 ml serum bottles. For the 100 L fermenter culture of the organism, a 10 L overnight pre-culture was used to inoculate the fermenter. The culture medium had the following composition:

Per liter

| | | |
|---|---|---|
| $NaHCO_3$ | 5.0 | g |
| Glucose | 2.0 | g |
| Leucine | 1.0 | g |
| Thioglycolic acid | 0.5 | g |
| Resazurine | 1 | mg |
| 100-fold Phosphates | 10.0 | ml |
| 100-fold Chlorides | 10.0 | ml |

| | | |
|---|---|---|
| 100-fold Sulfates | 10.0 | ml |
| 100-fold Amino acids and Vitamins | 10.0 | ml |

100-fold Phosphates per liter

| | | |
|---|---|---|
| $KH_2PO_4$ | 30.0 | g |
| $Na_2HPO_4$ | 150.0 | g |

100-fold Chlorides per liter

| | | |
|---|---|---|
| NaCl | 90.0 | g |
| $CaCl_2 \times 2H_2O$ | 2.6 | g |
| $MgCl_2 \times 6H_2O$ | 2.0 | g |
| $MnCl_2 \times 4H_2O$ | 1.0 | g |
| $CoCl_2 \times 6H_2O$ | 0.1 | g |

100-fold Sulfates per liter

| | | |
|---|---|---|
| $(NH_4)_2SO_4$ | 4.0 | g |
| $FeSO_4 \times 7H_2O$ | 0.4 | g |

100-fold Amino acids and Vitamins per liter

| | | |
|---|---|---|
| Proline | 80.0 | g |
| Cysteine | 50.0 | g |
| Each: Arginine, Glycine, Histidine, Isoleucine, Methionine, Threonine, Tryptophan, Valine | 10.0 | g |
| Ca-Pantothenate | 100 | mg |
| Pyridoxine-HCl | 10 | mg |
| Biotin | 1 | mg |

The bottle of the prepared medium was tightly closed with a rubber stopper and a hypodermic needle was introduced for pressure released. The medium was boiled until the blue-red colour of resazurine disappeared then the air above the medium was replaced by nitrogen. The autoclaved medium could be stored at room temperature in a dark place.

### 1.4.2. *Escherichia coli*

*Escherichia coli* was normally grown at 37 °C in Standard I medium (Merck: 1.5% Pepton, 0.3 % yeast extract, 100 mM NaCl, 6 mM D-Glucose) containing antibiotic(s) depending on the harboured plasmid. The strain DH5α {$F^-$ *ϕ80 Δ lacZΔM15Δ(lacZYA-argF)U169, deoR, recA1, end A1,hsdR17(*$rk^-$*,* $mk^+$*), phoA, supE44,*$\lambda^-$*, thi-1, gyrA96, relA1*} was used for gene cloning and BL21-CodonPlus(DE3)-RIL {*E. coli* B $F^-$, *ompT*, *hsdS*($r_B^-$ $m_B^-$), *dcm*$^+$, $Tet^r$, *gal* λ(DE3), *endA*, Hte [*argU ileY leuW* $Cam^r$]} for the gene expressions.

## 1.5. Plasmids

pASK-IBA7 (IBA GmbH) (tet promoter/operator, N-terminal *Strep*-tag II, cytosolic localization of the recombinant protein, $Amp^r$) was used for the *ldhA* expression and pASK-IBA3 (IBA GmbH) (tet promoter/operator, C-terminal *Strep*-tag II, cytosolic localization of the recombinant protein, $Amp^r$) used for *hadA*, *hadI* and *hadBC* expression.

## 1.6. Antibiotics

The stock of antibiotics was prepared and used as described below.

| Antibiotic | Stock | Final concentration |
|---|---|---|
| Ampicillin | 100 mg/ml $H_2O$ | 100 µg/ml |
| | sterilised by filtration (0.2 µm) | |
| Chloramphenicol | 50 mg/ml 70 % ethanol | 50 µg/ml |

## 2. Methods for DNA work

### 2.1. Plasmid DNA isolation

Plasmid DNA isolation was done by alkaline lysis methods using solutions described below.

Solution I
50 mM Glucose
10 mM EDTA
25 mM Tris/HCl pH 8.0

Solution II
0.2 M NaOH, 1% SDS (made fresh)

Solution III
3 M Potassium acetate / glacial acetic acid pH 4.8

Standard I medium 5 ml containing antibiotic(s) was inoculated with a bacterial colony and incubated with gyration overnight at 37 °C. The culture was transferred into an Eppendorf tube and harvested at 13000 x *g* in microfuge for 2 minutes. The bacterial pellet was suspended in 100 µl Solution I then lysised by adding 200 µl Solution II, and neutralized with 150 µl Solution III. The soluble supernatant was separated from cell debris by centrifugation for 5 minutes and transferred new Eppendorf tube. The plasmid DNA was extracted with 2 volumes of isopropanol and obtained by centrifugation as a white pellet. DNA pellet was washed with 1 ml 70 % ethanol, dried and dissolved in TE buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA).

### 2.2. Genomic DNA isolation from *C. difficile*

For the genomic DNA isolation, 2 g of *C. difficile* cells were suspended in 3 ml Tris-sucrose buffer (10 mM Tris/HCl pH 8.0, 25 % sucrose). The suspended cells were incubated at 37 °C for 90 minutes with gentle shaking after adding 100 mg lysozyme. Then, 4 ml of 10 mM Tris/HCl pH 8.0, 25 mM EDTA was added and incubated on ice for 15 minutes. After adding 20 mg proteinase K and 100 mg RNase, the mixture was incubated at 37 °C for 3 hours. The protein by extraction with 3 x saturated phenol and 1 x chloroform/isoamylalcohol (24 : 1). The aqueous phase was transferred to a dialysis bag for overnight dialysis in TE (10 mM Tris/HCl, 1 mM EDTA pH 8.0) buffer.

### 2.3. Agarose gel electrophoresis

Agarose powder was mixed with electrophoresis TAE-buffer (2 M Tris, 1 ml acetic acid, 50 mM EDTA (50x)) to the desired concentration, then heated in a microwave oven until completely melted. After cooling the solution to about 60°C, it was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. After the gel had solidified, the comb was removed and the gel was inserted horizontally into the electrophoresis chamber just covered with buffer. DNA samples mixed with loading buffer (0.21% Bromophenol Blue, 0.21% Xylene Cyanol FF, 0.2 M EDTA, pH 8.0, and 50% Glycerol) were then pipetted into the sample wells, and a voltage was applied. Bromophenol blue and xylene cyanol dyes migrate through agarose gels at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively. When adequate migration had occurred, DNA fragments were stained with ethidium bromide and placed on a ultraviolet transilluminator.

### 2.4. Elution of DNA fragments from agarose gel

DNA bands were exposed on an UV-illuminator (using short wavelength) and rapidly cut out from the agarose gel. Extraction was performed following the manual of the QIAquick Gel Extraction Kit (QIAGEN GmbH).

### 2.5. DNA restriction and ligation

Restriction reactions were usually performed following the enzyme insert manual. For ligations of double stranded DNA, T4-DNA ligase (Amersham Biosciences) were used following the enzyme insert manual.

### 2.6. Dialysis of ligation mixtures

The ligation mixture was dialysed before electrotransformation. The ligation mixture was pipetted on Millipore-Membrane (#VSWP 02500) which was floating on the water or TE buffer. After 30 minutes of dialysis, the ligation mixture was carefully recovered from the membrane and used for electrotransformation.

### 2.7. Preparation of competent *E. coli* cells for electrotransformation

An overnight 5 ml standard I medium culture inoculated with a fresh *E. coli* single colony from a plate was used to inoculate a 500 ml main culture grown till the exponential phase ($OD_{578}$ = 0.5 – 0.8). The cells were harvested by a pre-cooled (4 °C) high-speed centrifuge

with 6000 x *g* for 20 minutes. The harvested cell was washed two times with 500 ml ice-cold sterile $H_2O$ and one time with 20 ml 10 % glycerol. The washed cells were resuspended with 1 ml 10 % glycerol and 40 µl aliquots in thin-wall 500 µl tubes were stored at –80 °C.

## 2.8. Electrotransformation

The dialysed ligation mixture was added to 40 µl competent cells and transferred to a Gene-Pulser cuvette (Bio-Rad cat# 165-2086). A pulse was given to the cuvette using the following settings: 25 µF, 1.8 kV and 200 Ohm. The cuvette was washed with 500 µl Standard I medium and transferred to a sterile 1.5 ml Eppendorf tube. The transformation mixture was incubated for 30 minutes at 37°C before plating on a Standard I agar plate containing antibiotic(s). The agar plate was incubated overnight at 37 °C to get the colonies.

## 2.9. DNA concentration and purity determination

The DNA concentration and purity were determined measuring $OD_{260}$ and $OD_{280}$.

$OD_{260}$ = 1 corresponds to 50 µg/ml of dsDNA

$OD_{260}/OD_{280} < 1.8$ indicates contamination with protein or phenol

$OD_{260}/OD_{280} > 1.8$ indicates contamination with RNA

$OD_{260}/OD_{280} \approx 1.8$ indicates pure dsDNA

## 2.10. PCR reactions

PCR reactions were performed using a proofreading DNA polymerase, Extensor Hi-Fidelity PCR Enzyme Mix (ABgene®) and the reaction mixtures were made with following concentration of the ingredients and cycling program:

Concentration of ingredients

| | Final concentration |
|---|---|
| dNTP | 200 µM |
| Forward primer | 500 nM |
| Reverse primer | 500 nM |
| Template DNA | 20 to 200 pg/µl (plasmid DNA) |
| | 1 to 2 ng/µl (genomic DNA) |
| Proofreading DNA polymerase | 1 U |

Cycling program

| | |
|---|---|
| 1. 94 °C | 3 min |
| 2. 94 °C | 30 sec |
| 3. 55 °C (depending on primer) | 30 sec |
| 4. 68 °C | 1 min 30 sec (depending on the length of target gene) |
| 5. 68 °C | 10 min |

29 x from 2. to 4.

## 2.11. PCR primers

PCR primers were designed using free software, Primer D'Signer (IBA GmbH) introducing *Bsa*I restriction site (underlined) on the primers. The primers are described below:

For *ldhA*

Forward, 5'-ATGGTAGGTCTCAGCGCAAAATACTAGTATTTGGAGCACGCG-3'

Reverse, 5'-ATGGTAGGTCTCATATCAATTTACTCTATTAGTAGCAGTTCCTG-3'

For *hadA*

Forward, 5'-ATGGTAGGTCTCAAATGCTTTTAGAAGGAGTTAAAGTAGTAGA-3'

Reverse, 5'-ATGGTAGGTCTCAGCGCTATATCTTACAACTTTACTATCTTTAAAG-3'

For *hadI*

Forward, 5'-ATGGTAGGTCTCAAATGTACACAATGGGATTAGATATAGGTTC-3'

Reverse, 5'-ATGGTAGGTCTCAGCGCTTATATTTTTCACTTCTTTTTGTGATTCT-3'

For *hadBC*

Forward, 5'-ATGGTAGGTCTCAAATGTCTGAAAAAAAAGAAGCTAGAGTAGT-3'

Reverse, 5'-ATGGTAGGTCTCAGCGCTCGCTAAACTCATCATCTCAGCAAA-3'

## 2.12. Cloning of the genes

The amplified fragments of the genes, *ldhA* (999 bp), *hadA* (1200 bp), *hadI* (801 bp) and *hadBC* (2354 bp) were restricted and ligated into *Bsa*I restriction site of pASK-IBA3 (provides C-terminal *Strep-tag* II peptide, Trp-Ser-His-Pro-Gln-Phe-Glu-Lys, fused proteins, HadA, HadI, HadBC) or IBA7 (provides N-terminal *Strep-tag* II peptide, Trp-Ser-His-Pro-Gln-Phe-Glu-Lys, fused protein, LdhA)

## 2.13. Sequencing of the cloned genes

IRD (Infra-Red-Dye) labelled primers (5' IRD 700 forward and 5' IRD 800 reverse primers) were synthesised for sequencing:

Standard primers,

pASK-IBA forward: 5'-AGA GTT ATT TTA CCA CTC CCT-3'

pASK-IBA reverse: 5'-GCT CCA TCC TTC ATT ATA GC-3'

Internal primers,

*ldhA* forward internal: 5'-TGA TTA CCG TTG GAT AGC TG-3'

*ldhA* reverse internal: 5'-GAC GCA GTA GCG GTA AAC G-3'

*hadA* forward internal: 5'-ATC TCC AGC AAA TAC AGC AG-3'

*hadA* reverse internal: 5'-GAC GCA GTA GCG GTA AAC G-3'

*hadBC* forward internal: 5'-GAA ATT ATA CAT GCA GCT GG-3'

*hadBC* reverse internal: 5'-GAC GCA GTA GCG GTA AAC G-3'

*hadBC* forward internal II: 5'-TTC TCC TTC TCC AAT GAA TG-3'

*hadBC* reverse internal II: 5'-TCA AGT TCT CTT TTT ACG CC-3'

The standard primers were used for all sequencings and the internal primers were used to complete sequences, which were too long to be determined by the standard primers. In order to exclude possible errors by DNA polymerase, three different clones from three different PCRs were sequenced and mutations (nucleotides in one clone different from the same nucleotide in the other two colnes) were removed by recombination of the clones.

## 3. Methods for protein work

### 3.1. Gene expressions and protein purification

For the expression of genes, plasmid constructs were transformed into *E. coli* BL21-CodonPlus(DE3)-RIL harbouring extra rare codon (*arg*, *ileY* and *leuW*) tRNA genes. An overnight pre-culture (100 ml) inoculated with a fresh single colony from a Standard I agar plate was grown in the Standard I medium with ampicillin (100µg/ml) and chloramphenicol (50µg/ml) was used to inoculate 2 L Standard I medium containing the same antibiotics at 37 °C, 30 °C, or room temperature under aerobic or anaerobic conditions. When the culture reached the mid-exponential phase ($A_{590}$ = 0.5 - 0.7) gene expression was induced with anhydrotetracycline (200µg/L). After another 3h growth, the cells were harvested and re-suspended in equilibration buffer. The cells were broken using a French Press operating at 140 MPa or sonication and cell debris were removed by ultra-centrifugation at 100,000 x *g* for 1 h. The supernatant was loaded on a 5 mL *Strep*-Tactin MacroPrep column, which was equilibrated with equilibration buffer. After loading the cell free extract, the column was washed with at least 10 column volumes of equilibration buffer. The pure protein was eluted with equilibration buffer + 3 mM D-desthiobiotin. Buffers for recombinant protein purification;

| **Protein** | **Equilibration buffer** | **Elution buffer** |
|---|---|---|
| LdhA | 100 mM Tris/HCl pH 8.0<br>300 mM NaCl<br>1 mM EDTA | Equilibration buffer<br>+ 3 mM D-desthiobiotin |
| HadA | 100 mM photassium phosphate pH 7.5<br>300 mM NaCl<br>1 mM EDTA | Equilibration buffer<br>+ 3 mM D-desthiobiotin |
| HadI | 50 mM Mops pH 7.2<br>300 mM NaCl<br>10 mM $MgCl_2$<br>2 mM DTT | Equilibration buffer<br>+ 1 mM ADP<br>+ 3 mM D-desthiobiotin |
| HadBC | 50 mM Mops pH 7.0<br>300 mM NaCl<br>2 mM DTT | Equilibration buffer<br>+ 3 mM D-desthiobiotin |

### 3.2. Purification of (*R*)-2-hydroxyisocaproyl-CoA dehydratase

*C. difficile* cells were cultivated as described in Materials 1.4.1. in 2 L tightly closed bottles containing anoxic defined medium supplemented with L-leucine (1g/L; 7.6 mM). Cells were harvested, washed and resuspended in buffer A containing 50 mM Mops pH 7.0 and 2 mM dithiothreitol, yield 3 g wet cell paste. The preparation of the cell free extract was performed as that described in the activator purification. The cell free extract was filtered (0.45 µm pore size) and loaded a DEAE-Sepharose fast-flow column (3 × 10 cm) equilibrated with buffer A. The column was washed with 70 mL buffer A and the proteins were eluted at a rate of 3 mL $min^{-1}$ with a linear gradient of 0 – 1.0 M NaCl in buffer A. The active brown fractions were eluted around 0.4 M NaCl. An equal volume of 2.0 M $(NH_4)_2SO_4$ in buffer A was added to the pooled fractions from the first column, which were then loaded on a phenyl-Sepharose column (3 x 10 cm) equilibrated with buffer B (50 mM Mops pH 7.0, 1.0 M $(NH_4)_2SO_4$, 2 mM dithiothreitol). After washing the column with 70 mL buffer B, the active brown dehydratase eluted around 0.1 M $(NH_4)_2SO_4$ with a linear gradient of 1.0 – 0 M $(NH_4)_2SO_4$ in buffer B at a rate of 3 mL $min^{-1}$. The dehydratase fractions were concentrated on an Amicon PM 30 cell and desalted against buffer A, then loaded on a Q-Sepharose column (1.8 × 10 cm) equilibrated with buffer A. After a washing step with 60 ml buffer A, the dehydratase was eluted around 0.5 M NaCl with a linear gradient of 0 – 1.0 M NaCl in buffer A at a rate of 3 mL $min^{-1}$. The dehydratase was finally concentrated with an Amicon Ultra-4 PLTK Ultracel-Pl (30 kDa cut off).

The recombinant 2-hydroxyisocaproyl-CoA dehydratase from *E. coli* was purified by the same method, since the enzyme was not absorbed at the *Strep*-Tactin MacroPrep column. After the phenyl-Sepharose column the enzyme was already pure and therefore the Q-Sepharose column could be omitted.

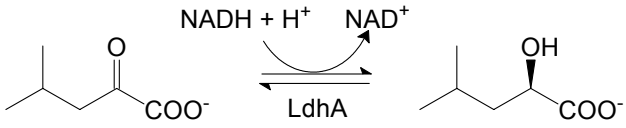
### 3.3. Preparation of soluble membrane protein

The *C. difficile* cell debris and membranes separated from cell-free extract as described above were washed three times with 50 mM Mops pH 7.0, 2 mM DTT. The washed membrane fraction was dissolved in 10 ml 50 mM Mops pH 7.0, 2 mM DTT 10 % n-dodecyl-β-D-matoside. The soluble part of membrane fraction was separated from the insoluble part by ultra-centrifugation at 100,000 x *g* for 30 minutes.

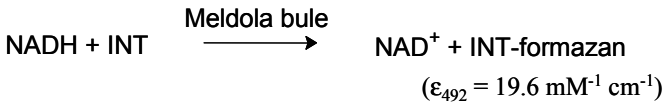
## 3.4. Enzyme activity assays

### 3.4.1. (*R*)-2-Hydroxyisocaproate dehydrogenase

(*R*)-2-Hydroxyisocaproate dehydrogenase activity was measured aerobically in 50 mM Tris/HCl pH 8.0, 0.2 mM NADH and 0.1 mM 2-oxoisocaproate in total volume of 1.0 mL at room temperature. After addition of enzyme, the decrease of NADH ($\Delta\varepsilon_{340}$ = 6.2 $mM^{-1}$ $cm^{-1}$) absorbance was followed at 340 nm.




The reverse reaction could also be measured by the formation of formazane from iodonitrosotetrazolium chloride in presence of meldola blue. Contents in total volume of the assay (1 mL ) was as shown below. The assay was started by addition of the enzyme and followed by the increase of the absorbance of formazane at 492 nm ($\varepsilon_{492}$ = 19.6 $mM^{-1}$ $cm^{-1}$).



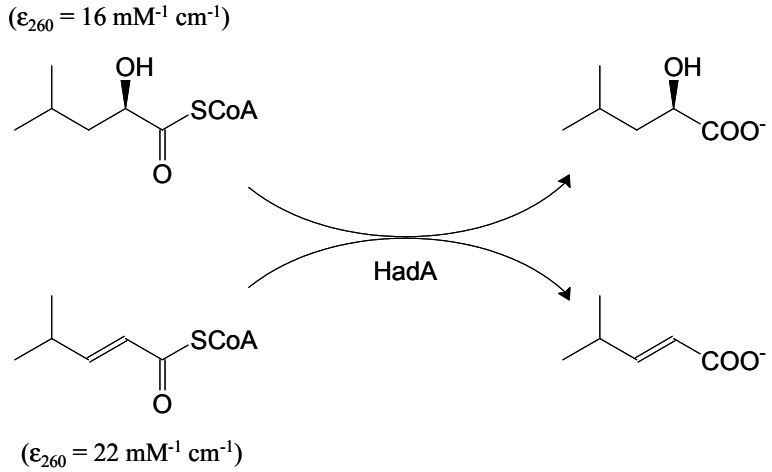

In 1 mL test

| | |
|---|---|
| 65 mM | TEA (Triethanolamine) |
| 8 mM | Potassium phosphate pH 7.6 |
| 4.3 mg | Triton –X100 |
| 77 µM | INT (Iodonitrosotetrazolium chloride) |
| 165 µg | Meldola blue |
| 2 mM | $NAD^+$ |

### 3.4.2. (*E*)-2-Isocaprenoyl-CoA:2-hydroxyisocaproate CoA transferase

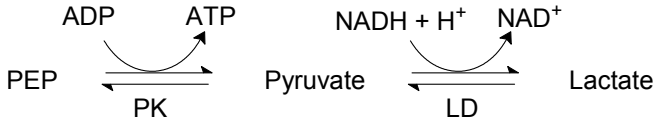
(*E*)-2-Isocaprenoyl-CoA:(*R*)-2-hydroxyisocaproate CoA-transferase was measured aerobically in 50 mM potassium phosphate pH 7.0 and 100 µM (*R*)-2-hydroxyisocaproyl-CoA ($\varepsilon_{260}$ = 16 $mM^{-1}$ $cm^{-1}$) in total volume of 1.0 mL at room temperature. After addition of the enzyme, the reaction was initiated by addition of (*E*)-2-isocaprenoate (1 mM final

concentration) and the absorbance increase by formation of (*E*)-2-isocaprenoyl-CoA ($\varepsilon_{260}$ = 22mM$^{-1}$ cM$^{-1}$) was followed at 260 nm ($\Delta\varepsilon_{260}$ = 6 mM$^{-1}$ cm$^{-1}$).



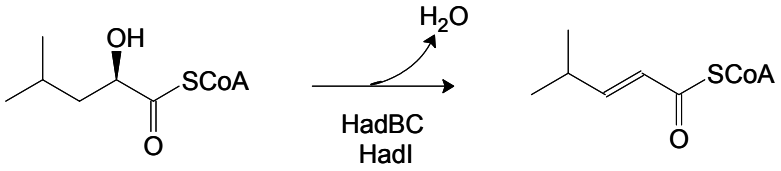

### 3.4.3. ATPase activity of activator

ATPase activity of activator was measured by determination of ADP-formation using a coupled assay with PK (pyruvate kinase) and LD (lactate dehydrogenase) (26, 42). The total volume of 1.0 ml contained the activator in 50 mM Tris-HCl pH 8.0, 1 mM PEP (phosphoenolpyruvate), 10 mM $MgCl_2$, 1 mM ATP, 0.2 mM NADH, 2 U pyruvate kinase and 2 U lactate dehydrogenase by following the absorbance decrease of NADH at 340 nm ($\varepsilon_{340}$ = 6.2 mM$^{-1}$ cm$^{-1}$) after addition of the dehydratase.




### 3.4.4. (*R*)-2-Hydroxyisocaproyl-CoA dehydratase

2-Hydroxyisocaproyl-CoA dehydratase activity was measured by a continuous direct assay and relying on the difference of the extinction coefficient between 2-hydroxyisocaproyl-CoA and 2-isocaprenoyl-CoA at 290 nm ($\Delta\varepsilon$ = 2.2 mM$^{-1}$ cm$^{-1}$). Dehydratase was incubated for 5 minutes in 0.5 ml total volume with an equal molar amount of recombinant activator in the presence of 5 mM $MgCl_2$, 0.4 mM ATP, 0.1 mM dithionite or Ti(III) citrate and 5 mM DTT in 0.5 ml total volume. The assay was started by addition of (*R*)-2-hydroxyisocaproyl-CoA (0.2 mM final concentration), and followed by the absorbance increase of 2-isocaprenoyl-CoA at 290 nm.




($\Delta\varepsilon_{290}$ = 2.2 $mM^{-1}$ $cm^{-1}$)

### 3.4.5. NADH:ferredoxin oxidoreductase

The membrane NADH:ferredoxin oxidoreductase activity was measured in 100 mM Tris/HCl pH 8.0, 200 µM NADH, ferricyanide and addition of the enzyme probe started the reaction. The reaction was followed by the absorbance decrease of ferrocyanide reduced from ferricyanide at 420 nm ($\varepsilon_{420}$ = 1.02 $mM^{-1}$ $cm^{-1}$).

## 3.5. Determination of protein concentration

Protein concentration was determined by the Bradford method (9). The assay is based on the shift of the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 from 465 nm to 595 nm upon binding of protein. A standard 0 – 7 µg of BSA was made up to an 800 µl volume with water and 200 µl Coomassie Brilliant Blue G-250 reagent. The reactions were incubated in the dark at room temperature for 30 minutes and the absorbance was measured at 595 nm.

## 3.6. Non-heme iron determination

The iron complexed by the protein is liberated by treatment with hydrochloric acid. Excess acid is neutralized with ammonium acetate, $Fe^{3+}$ is converted to $Fe^{2+}$ by reduction with ascorbic acid. Precipitated protein is complexed with sodium dodecylsulfate. Finally the iron chelator is added to form a blue $Fe^{2+}$-chelator complex (35). Used compounds are described below:

| | | |
|---|---|---|
| 1 % | (m/v) | HCl |
| 7.5 % | (m/v) | Ammoniumacetate |
| 2.5 % | (m/v) | Sodium dodecylsulfate (SDS) |
| 4 % | (m/v) | Ascorbic acid, (freshly prepared) |
| 1.5 % | (m/v) | Iron chelator, (3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazine, disodium salt |
| 0.2 mM | | $(NH_4)_2Fe(SO_4)_2$ x $6H_2O$ (freshly prepared) |

Three samples of the unknown, two blanks and six samples of iron standard (2,4,8,12,16 and 20 µM final concentrations) were diluted to 100 µl with water in Eppendorf tubes, subsequently, 100 µl 1 % HCl was added. The samples were mixed by gentle shaking, incubated at 80 °C for 10 minutes and cooled down to room temperature. 500 µl ammoniumacetate, 100 µl 4 % ascorbic acid, 100 µl sodium dodecylsulfate and 100 µl iron chelator were added sequentially with vortex and short centrifugation. The reaction mixtures were centrifuged at 9000 x *g* for 10 minutes and the absorbance at 593 nm was measured against water.

### 3.7. Acid-labile sulfur determination

The iron-sulfur protein is denatured in an alkaline medium containing zinc hydroxide. Released sulfide is co-precipitated with $Zn(OH)_2$ as ZnS. After acidification, $H_2S$ condenses with two molecules of N,N'-dimethyl-p-phenylenediamine to form methylene blue (22). The reagents are described below:

| | | |
|---|---|---|
| 1 % | (m/v) | Zinc acetetate (freshly prepared from 10 %) |
| 7 % | (m/v) | Sodium hydroxide |
| 0.1 % | (m/v) | N,N'-dimethyl-p-phenylenediamine (DMPD) in 5 M HCl |
| 10 mM | | $FeCl_3$ in 1 M HCl |
| ≈ 2 mM | | Sulfide standard ($Na_2S$ x $9H_2O$); |

A crystal of appropriate size (≈ 0.5 g) is blotted on filter paper, rapidly weighed and added to a 1 L volumetric flask containing 10 mM NaOH which has been purged of air with nitrogen. The flask is closed immediately and the solution is stirred magnetically. The solution was independently standardized iodometrically.

Three protein samples, two blanks, five sulfide standards (5 – 50 µM) and two protein samples with sulfide standard additions were put in Eppendorf tubes and made up to 200 µl with distilled water, 0.6 ml 1 % zinc acetate and 0.05 ml of 7 % NaOH were added, mixed and incubated for 15 minutes at room temperature. After adding 150 µl DMPD and $FeCl_3$, the tubes were closed immediately and vortexed vigorously for 30 seconds and incubated for 20 minutes at room temperature. The reaction tubes were centrifuged at 9,000 x *g* for 5 minutes and the absorbance were measured at 670 nm against water.

### 3.8. Iodometric determination of the sulfide standard

The gravimetric preparation of a sulfide standard using $Na_2S$ x $9H_2O$ is inaccurate because of the hygroscopic nature of the compound leading to overestimation of the sulfide in the protein sample. An accurate amount of iodine ($I_2$) is partly reduced with a known volume of sulfide standard solution. Remaining iodine is then determined by titration with sodium thiosulfate, and the sulfide concentration of the $Na_2S$ solution can be calculated by substraction of this volume from titration of the same amount of idodine without added $S^{2-}$.

| | |
|---|---|
| 45 mM (should be accurate) | Sodium thiosulfate ($Na_2S_2O_3$) |
| 40 mM in 50 ml 300 mM KI | $I_2$ |
| 0.35 g/70 ml $H_2O$ | Soluble starch (indicator) boiled and cooled down under continuous stirring |

In a 100 ml Erlenmeyer flask, 25 ml water was mixed with exactly 5 ml iodine solution and 1 ml 2 N sulfuric acid. The mixture was titrated with sodium thiosulfate until the solution turned almost colourless. Then 0.5 ml indicator solution was added for further titration with sodium thiosulfate. After the accurate amount of iodine was determined, the same amount of iodine was partly reduced with 25 ml sulfide standard and the remaining iodine was titrated with sodium thiosulfate. The stoichiometry ($I_2 + 2S_2O_3 = 2I^- + S_4O_6$, $I_2 + S^{2-} = S + 2I^-$) was used for calculation.

### 3.9. Flavin determination

The flavin bound to the protein was characterized with HPLC. The enzyme solution was denatured with 3 % TCA and centrifuged to remove the denatured protein. The yellow supernatant was analysed using a hydrophobic reverse phase column, RP-18 column (5 µm). The sample was eluted with 25 % methanol in 50 mM ammonium formate. Riboflavin, FMN and FAD (10 µM) were used as standards, which were treated in the same way as the enzyme solution. A flow rate of 1 ml/min was used and the absorbance at 266 nm was used for detection of flavin.

### 3.10. Separation of activated dehydratase from activator

Dehydratase (4.4 mg) was activated with 1.0 mg activator in the presence of 50 mM Mops pH 7.0, 0.4 mM ATP, 5 mM $MgCl_2$, 5 mM dithiothreitol, and 0.1 mM dithionite (total volume 2.0 ml) as described in activity assay but in the absence of bovine serum albumin. After 30

min incubation at room temperature, 1 µL was assayed for activity without further activation and the reaction mixture was loaded on a 5 mL *Strep*-Tactin MacroPrep column, previously reduced with 50 mM Mops pH 7.0, 5 mM dithiothreitol and 0.1 mM dithionite, and equilibrated with 50 mM Mops pH 7.0, 300 mM NaCl, 10 mM $MgCl_2$ and 5 mM dithiothreitol. The tagged activator was bound to the column while the dehydratase-containing flow-through was collected in 1 mL fractions. An UV/visible spectrum was taken from the peak fraction (1.2 mg dehydratase/mL), which was also analysed for activity. Therefore a 2 µL aliquot was added to 50 mM Tris/HCl pH 8.0 and the reaction was started with 0.2 µmol (*R*)-2-hydroxyisocaproyl-CoA, total volume 0.5 ml, d = 1 cm. After the reaction had ceased, two additional 0.2 µmol (*R*)-2-hydroxyisocaproyl-CoA aliquots were added. Finally the enzyme was completely re-activated by 0.1 mM dithionite, 0.4 mM ATP, 5 mM $MgCl_2$ and 5 mM dithiothreitol and 30 µg activator (added last). On an SDS/polyacrylamide gel, to which 20 µL of the separated dehydratase was applied, the double band of the dehydratase (40 kDa) but no trace of the activator (30 kDa) was visible upon Coomassie staining.

### 3.11. Complex of dehydratase and activator; formation and purification

The complex formation was performed as described for the nitrogenase complex using the transition state ATP analogue ADP-$AlF_4$ (76). Dehydratase and activator (1.5 : 1 molar ratio) were incubated for 30 minutes at room temperature in the anoxic globe box with the mixture described below:

| | |
|---|---|
| 50 mM | Tris/HCl pH 8.0 |
| 5 mM | $MgCl_2$ |
| 5 mM | Dithiothreitol |
| 1 mM | ATP |
| 1 mM | Dithionite |
| 1 mM | $AlF_3$ |
| 10 mM | KF |

The complex could be purified using Strep-tag affinity, DEAE Sepharose Fast flow and gel-filtration columns. However, the fastest and most stable way was concentration and buffer (50 mM Tris/HCl pH8.0) exchange on Amicon Ultra-4 PLTK Ultracel-Pl (100 kDa cut off). After purification two protein bands of dehydratase and activator were shown on SDS-

polyacrylamide gel. The purified complex was used for crystallisation and the dehydratase activity measurement.

### 3.12. Protein molecular mass determination

Apparent molecular mass of enzymes were determined by gel filtration on a Superose 6 column in 150 mM NaCl with 50 mM Tris/HCl, pH 8.0 at a flow rate of 0.5 mL/min. Amylase, aldolase, bovine serum albumin, catalase and cytochrome *c* were used for calibration. The molecular mass standards were obtained from Roche Molecular Biochemicals (Mannheim, Germany).

# Results

## 1. Putative gene cluster for the reduction of leucine by *C. difficile*

Fig. 6 shows the gene arrangement found on the genomic DNA sequence of *C. difficile* strain 630 (http://www.sanger.ac.uk/Projects/C_difficile/). The putative amino acid sequence of the first ORF *hadA* showed 45% identity to FldA [(*E*)-cinnamoyl-CoA: (*R*)-phenyllactate CoA-transferase] of *Clostridium sporogenes* (27), 24% to BbsE (succinyl- CoA: (*R*)-benzylsuccinate CoA-transferase) of *Thauera aromatica* (61, 62), 24% to Frc (formyl-CoA: oxalate CoA-transferase) of *Oxalobacter formigenes* (4, 81) and 25% to CaiB (butyrobetainyl-CoA: (*R*)-carnitine CoA-transferase) of *Escherichia coli* (32, 33), which belongs to the highly substrate specific CoA-transferase family III (46). The second ORF *hadI* showed 50%, 47%, 46% and 48% amino acid sequence identities to the activator of phenyllactate dehydratase of *C. sporogenes*, 2-hydroxyglutaryl-CoA dehydratase from *A. fermentans* (44), *F. nucleatum* (86) and putative activator of *C. symbiosum*, respectively. Two ORFs (*hadBC*) of 2-hydroxyacyl-CoA dehydratase were found showing 37 to 50% identities on the amino acid level to the subunits of 2-hydroxyacyl-CoA dehydratase of *C. sporogenes*, *C. symbiosum*, *F. nucleatum* and *A. fermentans*. Furthermore, downstream, three ORFs, one for an acyl-CoA dehydrogenase and two for subunits of electron transfer flavoprotein were located. And in the upstream of the ORFs, *ldhA* was found in an opposite direction of transcription showing 42% identities to D-lactate dehydrogenase of *Lactobacillus bulgaricus* (57, 58), 35% to D-2-hydroxyisocaproate dehydrogenase of *Lactobacillus casei* (60) and 31% to phenyllactate dehydrogenase of *C. sporogenes* (27).

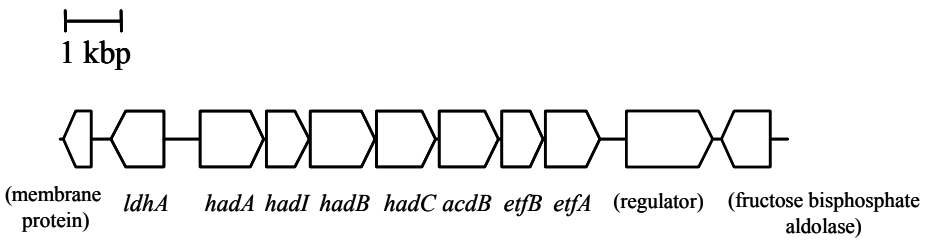



**Fig. 6. Gene arrangement for the 2-hydroxyisocaproyl-CoA dehydratase system of *C. difficile*.** *ldhA*, 2-hydroxyisocaproate dehydrogenase; *hadA*, 2-isocaprenoyl-CoA: 2-hydroxyisocaproate CoA-transferase; *hadI*, activator of dehydratase; *hadBC*, dehydratase; *acdB*, acyl-CoA dehydrogenase; *etfBA*, electron transferring flavoprotein.

## 2. (*R*)-2-Hydroxyisocaproate dehydrogenase

### 2.1. Analysis of *ldhA*

The gene *ldhA* was composed of 999 base pairs (A and T 677, 67.8% and C and G 322, 32.2%) and coding for 332 amino acids with a calculated molecular mass of 36519.84 Da and isoelectric point of 4.9. The codon usage of the gene showed a pattern, different from the codon usage of *E. coli*, which could be anticipated from the low GC content of *C. difficile*. The direction of the transcription was reversed from the other genes for the dehydratase system and no additional gene was found between *ldhA* and *hadA*. The deduced amino acid sequence showed identities to the D-lactate and D-2-hydroxyisocaproate dehydrogenase family.

### 2.2. Cloning and expression of *ldhA* and protein purification

PCR primers were designed as described in Materials and Methods. The restriction enzyme *Bsa*I site was introduced in the primers for in-frame cloning into the expression vector pASK-IBA7. The amplified DNA fragment (≈ 1 kb) was cut with *Bsa*I and ligated into the expression vector, which supports an N-terminal fused *Strep-tag* II peptide protein for one-step purification. Three clones from three different PCR reactions were sequenced to exclude possible errors of the DNA polymerase. The cloned *ldhA* was composed of 999 nucleotide base pairs with three nucleotide bases substitutions (C555 → T, T894 → C and T948 → C) (Fig. 7) compared to the known sequence from Sanger Center, but the same substitutions were found in all three different clones. Therefore, it was concluded that these three substituted nucleotides were due to a difference in the strain used, however, the deduced 332 amino acids were 100% identical to the known sequence.

The plasmid construct named p7ldhA was transformed into *E. coli* strain BL21-CodonPlus(DE3)-RIL to get more efficient protein production. Cultures were grown under aerobic conditions at 37 °C with 5% inoculation and induced with anhydrotetracycline (200 µg/L) in the exponential phase ($A_{590}$ = 0.5 – 0.7). After another 3 h of growth, cells were harvested. The cell free extract was obtained after centrifugation of cells opened by sonication. Finally, pure protein was purified by purification using *Strep*-Tactin affinity chromatography (Fig. 8).

```
   1 ATGAAAATAC TAGTATTTGG AGCACGCGAT TATGAAGAAC CAGTAATAAA AAAATGGTCT
  61 GAAGAACATA AGGATGTTCA AGTGGATATT TATCCTGAAA ACATGACTGA AGAAAATGTA
 121 GTTAAAGCTA AAGGGTATGA TGGTATATCT ATACAACAAA CTAACTATAT AGATAATCCT
 181 TATATTTATG AAACTTTAAA AGATGCTGGG GTTAAAGTTA TAGCTTCAAG AACTGCAGGG
 241 GTTGACATGA TACATTTTGA TTTAGTTAAT GAAAATGGAC TTATCGTTAC AAACGTTCCT
 301 TCTTATTCAC CTAATGCAAT AGCTGAATTA GCTGTTACTC AAGCTATGAA CCTTTTAAGA
 361 AAGACTCCTC TAGTAAAGAA AAAAGTCTGT GAAGGTGATT ACCGTTGGAT AGCTGAACTT
 421 CTTGGAACAG AAGTTAGATC TATTACAGTT GGTGTTATAG GTACAGGAAA AATAGGTGCT
 481 ACTTCTGCAA AATTATTCAA AGGCCTAGGA GCTAATGTAA TTGCATTTGA CCAATATCCA
 541 AATAGTGATT TAAATGATAT ATTAACTTAC AAAGATTCTT TAGAAGACCT TCTAAAAGAA
 601 GCTGACCTTA TAACATTACA TACTCCTTTA CTTGAAGGAA CAAAACATAT GATAAATAAA
 661 GATACTCTAG CTATAATGAA GGATGGAGCT TACATAGTAA ATACTGGCCG TGGTGGTTTA
 721 ATTAATACAG GGGATTTAAT AGAAGCACTA GAGTCAGGAA AAATTAGAGC TGCTGCCCTT
 781 GATACATTTG AAACTGAAGG ATTGTTCTTA AACAAAAAAA TGAATCCTGG AGAATTAACT
 841 GACCCAGAAA TAAATAAACT TCTTTCTATG GAACAAGTTA TATTCACTCA TCACCTTGGT
 901 TTCTTCACTA GTACAGCGAT TGAAAATATA GTTTATTCTA GTTTAAGTAG TGCTGTAGAA
 961 GTTATAAAAA CAGGAACTGC TACTAATAGA GTAAATTAG
```

**Fig. 7. The nucleotide sequence of *ldhA* of *C. difficile* strain DSMZ 1296$^T$ (GeneBank accession number AY772817).** Start and stop codons derived from the vector are underlined and three nucleotides differing from strain 630 (http://www.sanger.ac.uk/Projects/C_difficile/) are depicted as bold letters in grey boxes.

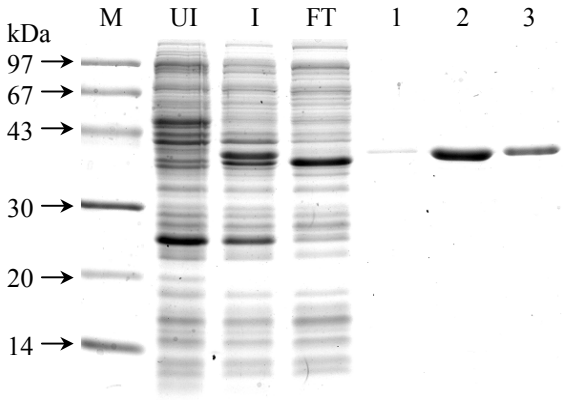



**Fig. 8. SDS-PAGE gel for purified recombinant LdhA, (*R*)-2-hydroxyisocaproate dehydrogenase.** The 15% gel was stained with Coomassie Brilliant Blue. M; molecular mass marker; UI; cell free extract of uninduced cells, I; cell free extract of cells after induction with anhydrotetracycline (200µg/L); FT; flow through of the *Strep*-Tactin column; 1-3; fractions containing purified enzyme upon elution with D-desthiobiotin.

### 2.3. Substrate specificity

The purified LdhA showed a molecular mass of approximately 38 kDa on SDS-PAGE, which agreed well with the calculated mass of the deduced amino acids (36.5 kDa + 1kDa *Strep-tac* II peptide). The protein behaved as a monomer on gel filtration, which is uncommon for D-specific hydroxy acid dehydrogenases (25). The enzyme activity of the forward reaction was measured under aerobic conditions following the decrease in absorbance of NADH at 340 nm. The reverse reaction was followed by the formation of formazane as described in Materials and Methods.

The purified LdhA exhibited highest activity at pH 10 ± 0.2 (forward reaction) and in the early stationary phase of *C. difficile* growth. $K_m$ and $V_{max}$ of 2-oxoisocaproate were determined as 68 µM and 49 U/mg, respectively. The activity showed inhibition by excess substrate (over 10-fold $K_m$). Other several possible 2-oxo acids were tested to access the substrate specificity. The $K_m$, $V_{max}$ values and the catalytic parameter $V_{max}/K_m$ of each substrate are shown in Table 2. LdhA accepted 2-oxoisocaproate, 2-oxopentanoate, 2-oxohexanoate and phenylpyruvate as the substrates with different $V_{max}/K_m$ values. On the other hand, pyruvate, 3-methyl-2-oxopentanoate (2-oxoisovalerate) and 2-oxobutyrate were not accepted as substrates. 2-Oxopentanoate, 2-oxohexanoate and phenylpyruvate showed 2-, 70- and 70-fold lower $V_{max}/K_m$ values, respectively, as compared to 2-oxoisocaproate. Comparing the $V_{max}/K_m$ data clearly showed that 2-oxocaproate was the most efficient and possible native substrate. In the reverse reaction, LdhA stereospecifically accepted (*R*)-2-hydroxyisocaproate with $K_m$ = 2.8 mM and $V_{max}$ = 81 U/mg but not (*S*)-2-hydroxyisocaproate. This stereospecificity is in agreement with that expected from amino acid sequence comparisons. Therefore, LdhA was named as (*R*)-2-hydroxyisocaproate dehydrogenase.

**Table 2. Substrate specificity of LdhA, (*R*)-2-hydroxyisocaproate dehydrogenase.**

| Substrate | $K_m$ (mM) | $V_{max}$ (U/mg) | $V_{max}/K_m$ |
|---|---|---|---|
| 2-Oxoisocaproate | 0.068 | 49 | 721 |
| (*R*)-2-Hydroxyisocaproate | 2.8 | 81 | 29 |
| 2-Oxopentanoate | 0.084 | 35 | 417 |
| 2-Oxohexanoate | 5 | 53 | 11 |
| Phenylpyruvate | 10 | 100 | 10 |

## 3. (*E*)-2-Isocaprenoyl-CoA:2-hydroxyisocaproate CoA-transferase

### 3.1. Analysis of *hadA*

The gene *hadA* was composed of 1200 base pairs (A and T 809, 67.6% and C and G 391, 32.62%) for 399 amino acids with a calculated molecular mass of 44236.94 Da and an isoelectric point of 5.0. The deduced amino acid sequence showed identities to family III CoA transferases. Recently, the structures of two formate CoA-transferases (Frc of *O. formigenes* (74, 75) and YfdW of *E. coli* (41)) were reported. They suggested a possible reaction mechanism, in which two conserved amino acid residues in the formate CoA-transferases ($Tyr^{59}$ and $Asp^{169}$, the numbering is for Frc) play an important role. However only one amino acid residue corresponding to $Asp^{169}$ is conserved in FldA (cinnamoyl-CoA:phenyllactate CoA-transferase) of *C. sporogenes* and HadA (2-isocaprenoyl-CoA:2-hydroxyisocaproate CoA-transferase) of *C. difficile* (Fig. 9).

### 3.2. Cloning and expression of *hadA* and protein purification

The ORF, *hadA* was cloned into pASK-IBA3 (named p3hadA) to produce a C-terminal *Strep*-tag II-fused protein, because the N-terminal *Strep-tag* II fused HadA from pASK-IBA7 clone showed no activity, presumably, due to disturbance of the active site by the *Strep-tag* II peptide. The DNA sequence, obtained from three clones of three different PCR reactions, was composed of 1200 bp for 399 amino acids. There were two nucleotides (Fig. 10) different from the strain 680 sequence at G459 → A and T816 → C causing no change to the encoded amino acids. The production and purification of HadA was performed in the same way with LdhA except that *E. coli* cells were grown at 30 °C to increase the yield of the protein.

### 3.3. Properties

On SDS/PAGE purified HadA appeared just below the 43 kDa protein marker (Fig. 11) (The calculated molecular mass of HadA = 44.2 kDa + 1kDa of *Strep-tac* II peptide). On Superose 6 gel filtration the protein behaved as homodimer. The enzyme activity of HadA was measured at 260 nm using the difference of the extinction coefficients ($\Delta\varepsilon_{260}$ = 6 $mM^{-1}$ $cm^{-1}$) of (*R*)-2-hydroxyisocaproyl-CoA ($\varepsilon_{260}$ = 16 $mM^{-1}$ $cm^{-1}$) and 2-isocaprenoyl-CoA ($\varepsilon_{260}$ = 22 $mM^{-1}$ $cm^{-1}$) as described in Materials and Methods. The measurement was performed by following the absorbance increase of (*E*)-2-isocaprenoyl-CoA in the presence of (*E*)-2-isocaprenoate started by adding (*R*)-2-hydroxyisocaproyl-CoA.

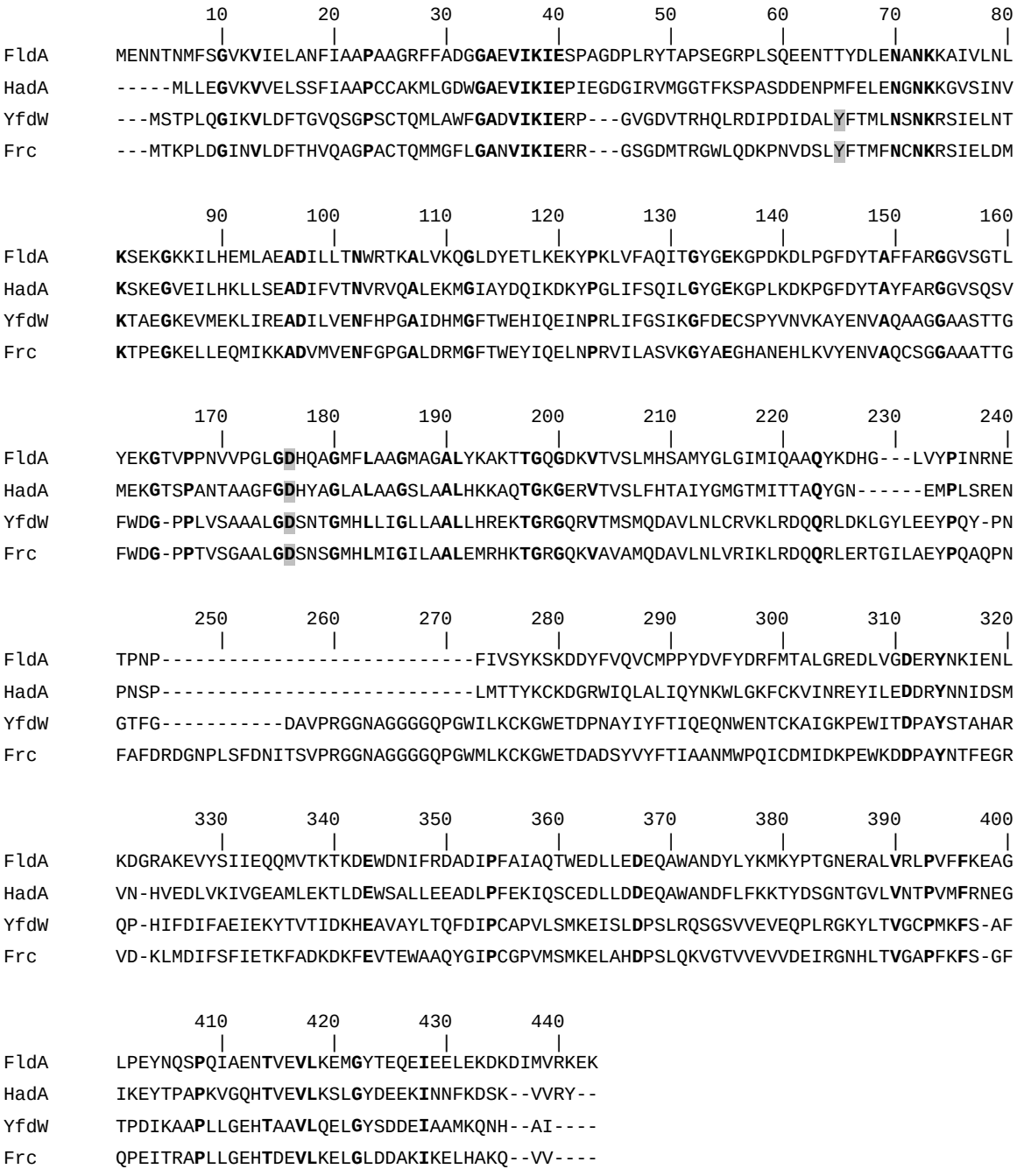



**Fig. 9. Homology analysis of CoA-transferases.** HadA, 2-hydroxyisocaproate CoA-transferase of *C. difficile*; FldA, cinnamoyl-CoA:phenyllactate CoA-transferase of *C. sporogenes*; YfdW, formate CoA-transferases of *E. coli*; Frc, formate CoA-transferases of *O. formigenes*. Identical amino acid residues are in bold letters and the putative active site Tyr and Asp are in grey boxes.

```
   1 ATGCTTTTAG AAGGAGTTAA AGTAGTAGAA CTTTCAAGTT TCATCGCAGC ACCATGTTGT
  61 GCAAAAATGT TAGGTGACTG GGGTGCAGAG GTTATTAAGA TTGAACCTAT AGAAGGTGAT
 121 GGAATAAGAG TTATGGGTGG AACATTTAAA TCTCCAGCAT CAGATGATGA AAACCCTATG
 181 TTTGAATTAG AAAATGGAAA TAAAAAGGGT GTAAGTATTA ATGTAAAATC AAAAGAAGGA
 241 GTAGAAATAT TACATAAATT ATTATCAGAA GCAGACATAT TTGTAACTAA TGTTAGAGTT
 301 CAAGCATTAG AAAAAATGGG TATAGCTTAT GACCAAATAA AAGATAAGTA TCCAGGATTA
 361 ATATTCTCTC AAATATTAGG ATATGGTGAA AAAGGACCTT TAAAAGATAA ACCAGGATTT
 421 GACTATACTG CATACTTCGC AAGAGGAGGA GTTAGCCAAT CTGTTATGGA AAAAGGAACA
 481 TCTCCAGCAA ATACAGCAGC AGGATTTGGT GACCACTATG CAGGTCTAGC ACTAGCAGCA
 541 GGAAGTTTAG CAGCATTACA TAAAAAAGCT CAAACTGGTA AAGGTGAGAG AGTAACAGTA
 601 AGTCTTTTCC ATACAGCTAT ATATGGAATG GGAACAATGA TAACAACAGC ACAATACGGA
 661 AATGAAATGC CTTTATCAAG AGAAAATCCA AACAGCCCAT TAATGACTAC ATATAAATGT
 721 AAAGATGGAA GATGGATTCA ATTAGCTTTA ATACAATACA ACAAGTGGTT AGGCAAATTC
 781 TGTAAGGTTA TAAATAGAGA ATATATATTA GAAGACGATA GATATAATAA CATAGATTCA
 841 ATGGTTAATC ATGTTGAAGA TTTAGTTAAG ATAGTTGGAG AAGCTATGTT AGAAAAAACA
 901 TTAGACGAGT GGTCAGCTTT ATTAGAAGAA GCAGACTTAC CATTTGAAAA AATTCAAAGC
 961 TGTGAAGATT TATTAGATGA CGAACAAGCT TGGGCAAATG ACTTCTTATT TAAGAAAACA
1021 TACGATAGCG GAAATACAGG TGTCTTAGTT AATACTCCAG TTATGTTTAG AAATGAAGGA
1081 ATTAAAGAAT ATACACCAGC ACCAAAAGTA GGTCAACATA CTGTAGAAGT ATTAAAATCT
1141 TTAGGCTACG ATGAAGAGAA AATAAATAAC TTTAAAGATA GTAAAGTTGT AAGATATTAA
```

**Fig 10. The nucleotide sequence of *hadA* of *C. difficile* strain DSMZ 1296$^T$ (GeneBank accession number AY772818).** Start and stop codons derived from the vector are underlined and two nucleotides differing from strain 630 (http://www.sanger.ac.uk/Projects/C_difficile/) are depicted as bold letters in grey boxes.

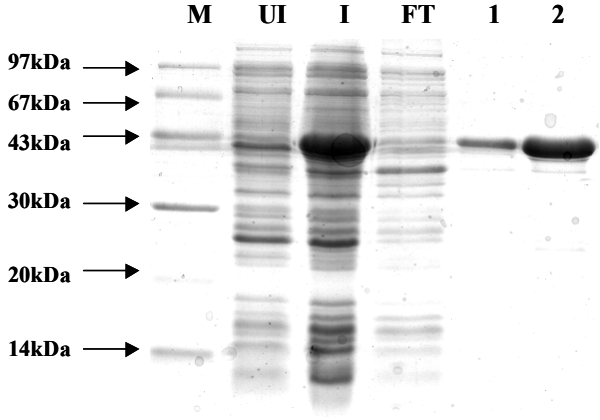



**Fig. 11. SDS-PAGE gel for purified recombinant HadA, 2-hydroxyisocaproate CoA-transferase.** The SDS-polyacrylamide gel (15%) was stained with Coomassie Brilliant Blue. M, molecular mass marker; UI, cell free extract of uninduced cells; I, cell free extract of cells after induction with anhydrotetracycline (200µg/L); FT, flow through from the *Strep*-Tactin column; 1-2, purified fractions obtained by elution with D-desthiobiotin.

The measurement could be performed under aerobic conditions even though other enzymes of the leucine fermentation pathway (dehydratase and activator) were oxygen sensitive. The purified enzyme could be kept at –80 °C for at least one month without any significant activity loss. Table 3 compares specific activities of *C. difficile*, induced *E. coli* and purified HadA. The activities were not high, but both cell free extracts showed the same range of activity. Purified HadA accepted (*R*)- and (*S*)-2-hydroxyisocaproyl-CoA as CoA donor but acetyl-CoA could not be used as CoA donor to (*E*)-2-isocaprenoate. The measurement of the reverse reaction of CoA transfer from (*E*)-2-isocaprenoyl-CoA to (*R*)-2-hydroxyisocaproate was tried but no absorbance decrease rather than a slow spontaneous degradation of (*E*)-2-isocaprenoyl-CoA at pH 7 was observed.

**Table 3. Activities of 2-isocaprenoyl-CoA:2-hydroxyisocaproate CoA-transferase.**

| | Specific Activity (U/mg) |
|---|---|
| *C. difficile* cell free extract | 0.54 |
| *E. coli* (p3hadA) cell free extract | 0.62 |
| Purified HadA | 2.70 |

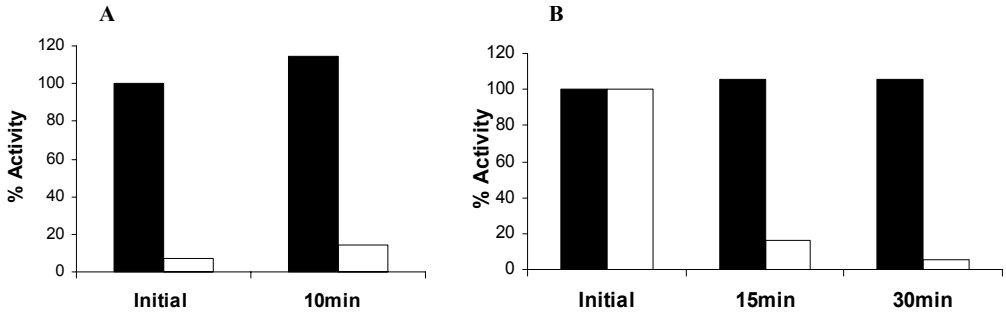



**Fig. 12. Inactivation of 2-hydroxyisocaproate CoA-transferase by $NaBH_4$ (A) or hydroxylamine (B) in the presence or absence of (*R*)-2-hydroxyisocaproyl-CoA.** Purified HadA (320 µg) was incubated with 10 mM $NaBH_4$ or 200 mM hydroxylamine in 200 µM (*R*)-2-hydroxyisocaproyl-CoA at pH 7 and room temperature. The enzyme activities were measured at indicated time points using the enzyme assay described in Materials and Methods. Black bars are the incubation of the enzyme in the absence of (*R*)-2-hydroxyisocaproyl-CoA as controls and white bars are in the presence of (*R*)-2-hydroxyisocaproyl-CoA.

### 3.4. Inactivation by $NaBH_4$ or hydroxylamine

In case of family I CoA transferases, the reaction proceeds via a ping-pong mechanism, forming a CoA-thioester intermediate on the glutamate residue of the enzyme (46, 80). The glutamyl-CoA-thioester was reduced by $NaBH_4$ or cleaved by hydroxylamine yielding irreversibly inactive enzyme (16). The reaction mechanism of family III CoA transferases has not been elucidated, but kinetic analysis suggested the reaction might be proceeded via a ternary complex formation of the substrates (46, 62). Nevertheless the partial inactivation of two family III CoA transferases (BbsEF of *T. aromatica* and FldA of *C. sporogenes*) was reported by the reduction with $NaBH_4$ or cleavage with hydroxylamine in the presence of corresponding CoA ester. A similar inactivation of HadA was obtained as shown in Fig. 12. HadA exhibited 90% inactivation by $NaBH_4$ reduction, while in the absence of a CoA ester substrate no inactivation was observed. Hydroxylamine also inactivated the enzyme showing 20% residual activity after 15 minutes and only 5 % after 30 minutes incubation.

## 4. Activator of (*R*)-2-hydroxyisocaproyl-CoA dehydratase

### 4.1. Analysis of *hadI*

The gene *hadI* was identified by homology analysis with known activators of *C. sporogenes* (FldI) and *A. fermentans* (HgdC) showing 55 and 51 % amino acid sequence identities, respectively. The identified gene was composed of 810 bp (A and T 538, 66.4% and C and G 72, 33.6%) coding for 269 amino acids. Amino acid sequence alignment with the other activators revealed conserved ATP binding motifs G(I/V/L)D(I/V)G and (V/I)IDIG and the 2 cysteine residues known to coordinate the [4Fe-4S] cluster (20, 52). The conserved cluster helix known from HgdC of *A. fermentans* plays a role in binding to [4Fe-4S] cluster located at the interface and bridging the monomers (66). The deduced amino acid sequence of the 810-bp *hadI*-gene contained three in-frame methionines (MLKMYTM) in the N-terminal region. Furthermore the lack of a nucleotide space between the ribosome binding site (A/GGAGG/A) and the start codon (ATG) (Fig. 13) indicated a possible error in the assignment of the start codon. It was assumed, therefore, that the next in-frame ATG could be the real start codon of *hadI*, consequently expanding the nucleotide space from 0 to 9 nucleotides and revising the ORF, *hadI*, from 810 to 801 bp.

| Gene | Nucleotide sequence | Number of Nucleotides between G/AGAAG/A and ATG |
|---|---|---|
| *ldhA* | CA**GGAGG**GTATTGAT**ATG**AAAATACTA | 8 |
| *hadA* | AA**GGAGG**AAAACGTT**ATG**CTTTTAGAA | 8 |
| *hadI* | AA**GGAGA**ATGCTAAAG**ATG**TACACAAT | 9 |
| *hadB* | AA**AGAGG**GATGAAA**ATG**TCTGAAAAA | 7 |
| *hadC* | AT**AGAGG**TGAGATATA**ATG**GAAGCTATT | 9 |

**Fig. 13. Nucleotide sequences around the ribosome binding site and start codons of the genes.** The ribosome binding sites and the start codons are shown in bold letters. Abbreviations of the genes are as described in Fig. 6. The number of nucleotides shows the nucleotide space between ribosome binding site and start codon. By using the start codons shown in grey no active proteins could be obtained.

```
  1 ATGTACACAA TGGGATTAGA TATAGGTTCA ACTGCATCAA AGGGAGTAAT CTTAAAGAAT
 61 GGGGAAGATA TTGTAGCTTC TGAAACAATA TCCTCTGGTA CTGGGACTAC TGGACCATCA
121 AGAGTTTTAG AAAAATTATA TGGCAAGACA GGTCTTGCAA GAGAAGATAT TAAAAAAGTT
181 GTAGTTACAG GATATGGAAG AATGAACTAT TCAGATGCTG ATAAGCAAAT AAGTGAATTA
241 AGCTGTCATG CTAGAGGGGT AAATTTCATA ATTCCAGAGA CAAGAACCAT TATTGACATA
301 GGTGGTCAAG ATGCAAAGGT ATTAAAATTA GATAATAATG GAAGACTATT AAACTTTCTT
361 ATGAATGACA AATGTGCTGC AGGTACAGGA AGATTTTTAG ATGTAATGGC AAAAATAATA
421 GAGGTTGATG TATCTGAACT CGGAAGTATA TCTATGAATT CTCAAAATGA AGTATCAATA
481 AGCAGTACAT GTACAGTATT TGCAGAGTCT GAGGTTATAT CACATTTATC TGAAAATGCA
541 AAAATTGAAG ATATAGTGGC AGGTATTCAT ACTTCAGTAG CAAAGAGAGT TTCTAGCCTA
601 GTAAAAAGAA TAGGAGTACA AAGAAATGTA GTTATGGTTG GTGGGGTTGC TAGAAATAGT
661 GGTATTGTAA GAGCTATGGC AAGAGAAATC AACACAGAAA TTATTGTACC TGATATACCT
721 CAATTAACTG GTGCTTTAGG AGCAGCGTTA TATGCTTTTG ATGAAGCAAA AGAATCACAA
781 AAAGAAGTGA AAAATATATA A
```

**Fig 14. The nucleotide sequence of modified and cloned *hadI* of *C. difficile* strain DSMZ 1296$^T$ (GeneBank accession number AY772815).** Start and stop codons derived from vector are underlined and two nucleotide substitutions compared to the strain 680 are depicted as the thick letters in grey box.

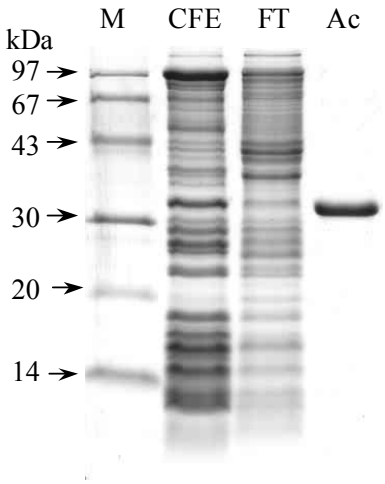



**Fig. 15. Purified recombinant HadI, activator of dehydratase.** SDS-PAGE (15%) stained with Coomassie Brilliant Blue. M, molecular mass marker; CFE, cell free extract of the cells induced with anhydrotetracycline 200µg/L; FT, flow-through from the column; Ac, purified activator after elution with D-desthiobiotin.

## 4.2 Cloning and expression of *hadI* and protein purification

Since the activators of 2-hydroxyacyl-CoA dehydratases are known to be extremely sensitive against oxygen and difficult to purify in sufficient amount from the native organism (67), No effort was made to obtain pure HadI from *C. difficile* cell free extracts. Therefore expression of the *hadI* gene in *E. coli* seemed to be the preferred method. The produced activator from 810 bp *hadI* as *Strep-tag* II peptides fused to the N- or C-terminus was obtained as insoluble or non-functional, which neither did exhibit ATPase activity nor activate dehydratase. We decided to modify the ORF choosing the second methionine as start codon and these shortened *hadI* from 810 to 801 bp. The modified *hadI* was amplified using the PCR primers, which are described in Materials and Methods, and cloned into pASK-IBA3 named Np3I. The nucleotide sequence (Fig. 14) showed two nucleotide substitutions at C303 → T and A645 → G comparing to the strain 680, but the encoded amino acids were 100% identical to those of the sequence from the Sanger Center. The cloned gene was coding for 266 amino acids starting with (MYTM), instead of 269 amino acids.

In order to produce the activator of 2-hydroxyisocaproyl-CoA dehydratase, *E. coli* cells harbouring the Np3I were grown and induced under anaerobic conditions. The harvested cells were opened by a French Press to avoid heating the sensitive enzyme and the produced protein fused with a *Strep*-tag II peptide on the C-terminus was purified using a *Strep*-Tactin affinity column. The pure activator eluted in Mops pH 7.0 containing, in addition to 3 mM D-desthiobiotin, 1 mM ADP and 10 mM $MgCl_2$ to maintain stability (Fig. 15).

## 4.3. UV-vis spectra and ATPase activity

The UV-visible spectra of the purified activator as isolated showed a shoulder around 370 nm, which was shifted to 420 nm after reduction by a 10 fold excess of dithionite with concomitant formation of an absorbance band at 570 nm visible. After oxidation of the isolated form with a 10 fold excess of thionine, the 370 nm shoulder was shifted to 400 nm with 10% decrease of absorbance (Fig. 16). By chemical analysis, 4 ± 0.5 non-heme iron and 2 ± 0.1 acid labile sulfur were detected indicating one [4Fe-4S] cluster in the isolated protein. The low observed sulfur content presumably resulted from loss of $H_2S$ during storage of the extremely labile [4Fe-4S] protein. The other known activators from *C. sporogenes* and *A. fermentans* showed ATP hydrolysis activities when the proteins were oxidized (>2.0 $s^{-1}$ and 4-6 $s^{-1}$, respectively) (27, 43). However, both reduced and oxidized HadI showed low ATP hydrolysis activity (< 1.5 U/mg), but the reduced HadI in presence of dehydratase showed

very efficient ATP hydrolysis (up to 50 U/mg) which did not change significantly by addition of substrate (Fig. 17).

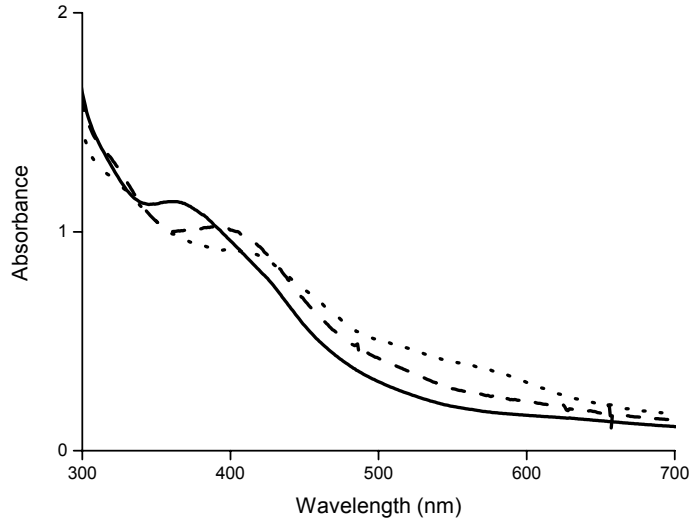



**Fig. 16. UV-vis spectra of purified HadI, activator of dehydratase.** Solid line, 4.2 mg/ml as isolated; dotted line, 0.5 mg/ml (8 fold amplified) reduced with 10 fold excess dithionite; dashed line, 0.5 mg/ml (8 fold amplified) oxidized with 10 fold excess thionine. The excess dithionite or thionine was removed by desalting through a Sephadex G-25 column.

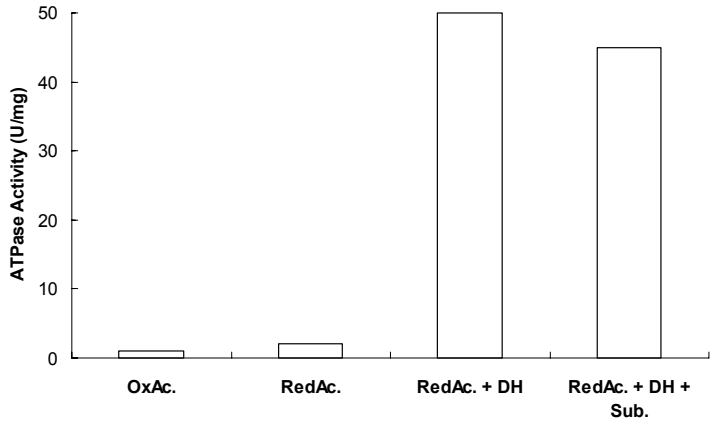



**Fig. 17. ATPase activity of the activator.** OxAc., oxidised activator; RedAc., reduced activator; RedAc. + DH, reduced activator in the presence of the dehydratase; RedAc. + DH + Sub., reduced activator in the presence of the dehydratase and (*R*)-2-hydroxyisocaproyl-CoA. The oxidation and reduction were performed as described in Fig. 16. The total volume (1.0 ml) of assay contained the activator (1 µg) in 50 mM Tris/HCl pH 8.0, 1 mM phosphoenolpyruvate, 10 mM $MgCl_2$, 1 mM ATP, 0.2 mM NADH, 2 U pyruvate kinase and 2 U lactate dehydrogenase. The reaction was started by the addition of the dehydratase (20 µg) or the dehydratase (20 µg) and (*R*)-2-hydroxyisocaproyl-CoA (0.2 mM), then, the absorbance decrease of NADH at 340 nm ($\varepsilon_{340}$ = 6.2 $mM^{-1}$ $cm^{-1}$) was followed.

## 5. (*R*)-2-Hydroxyisocaproyl-CoA dehydratase

### 5.1. Analysis of *hadBC*

The deduced amino acid sequence of *hadBC* of the two subunits of (*R*)-2-hydroxyisocaproyl-CoA dehydratase showed 37 – 50% identity to related 2-hydroxyacyl-CoA dehydratases of *C. sporogenes* (FldBC), *A. fermentans* (HgdAB), *C. symbiosum* (HgdAB) and *F. nucleatum* (HgdAB). The ORF *hadB* for the B-subunit was composed of 1233 bp (A and T 883 67.7% and C and G 397 32.3%) encoding 410 amino acids. The next ORF *hadC* for the C-subunit was 1128 bp (A and T 768 68.1% and C and G 360 31.9%) encoding 375 amino acids. The start codon of *hadC* had an overlap of 1 nucleotide with the stop codon of *hadB*. A presumable error in the assignment of the start codon was found on *hadB* (Fig. 13). Two in-frame methionines (MKM) were found in N-terminus of the HadB. When the second methionine was assigned as the start, the nucleotide space between ribosome binding site and the start codon could be extended to 7 instead of 1. A possible ribosome binding site for subunit C was found in the structural gene *hadB* 9 nucleotides apart from the start codon of *hadC*, which was expressed.

### 5.2. Cloning and expression of *hadBC* and protein purification

Finally *hadBC* was modified from 2360 bp to 2354 extending the space between the ribosome binding site of *hadB* and its start codon to 7 bp. The modified *hadBC* for the two subunits of dehydratase was amplified as one fragment with the primers described in Materials and Methods. The PCR amplified ≈ 2.4 kb fragment was cut and ligated into the *Bsa*I restriction site of pASK-IBA3 giving Np3BC. The nucleotide sequences from three different PCRs showed 5 – 10 mutations, which were removed by recombination of the clones. The final nucleotide sequence of recombined *hadBC* (Np3BC) was composed of 2354 bp coding for 783 amino acids and having three silent nucleotide substitutions at G285 → A, T870 → C and T2003 → C (Fig. 18).

*E. coli* cell harbouring the plasmid construct Np3BC was grown anaerobically at room temperature to increase the solubility of the recombinant protein. The *E. coli* cell free extract after induction showed thick protein bands by SDS/PAGE around the 43-kDa molecular mass marker, which were not seen in non-induced *E. coli*. After addition of activator, a dehydratase activity of 9 U/mg equal to that in the *C. difficile* cell-free extract was obtained in *E. coli* cell-free extract while no activity could be measured when the non-revised *hadBC* genes were expressed. Harvesting and opening the cells were done as described in Materials and Methods. The protein could not be purified using the affinity column as while the other

recombinant protein purifications. The recombinant HadBC did not bind to the column and was found in flow-through, probably the *Strep*-tag II peptide on C-terminus of the HadC subunit was buried inside the protein structure.

```
   1 ATGTCTGAAA AAAAAGAAGC TAGAGTAGTA ATTAATGATT TATTAGCTGA ACAATATGCA
  61 AATGCATTTA AAGCTAAAGA AGAAGGAAGA CCTGTAGGTT GGTCAACATC AGTATTTCCT
 121 CAAGAGTTAG CAGAAGTATT TGACTTAAAC GTATTATATC CAGAAAACCA AGCAGCTGGA
 181 GTAGCAGCTA AAAAAGGTTC TTTAGAATTA TGTGAAATAG CTGAATCTAA AGGATATTCT
 241 ATTGACCTAT GTGCATATGC AAGAACAAAT TTTGGTCTTT TAGAAAATGG TGGATGTGAA
 301 GCTTTGGATA TGCCAGCTCC AGATTTCCTA CTTTGCTGTA ACAATATATG TAACCAAGTT
 361 ATAAAATGGT ATGAAAATAT TTCAAGAGAA TTAGATATAC CTTTAATAAT GATTGATACA
 421 ACTTTCAATA ATGAAGACGA AGTTACTCAA TCAAGAATAG ATTATATTAA AGCTCAATTT
 481 GAAGAAGCTA TAAAACAACT AGAAATTATA TCAGGAAAGA AATTTGACCC TAAGAAGTTT
 541 GAAGAAGTAA TGAAAATATC AGCTGAAAAC GGAAGACTAT GGAAGTATTC TATGAGTTTA
 601 CCAGCAGATT CTTCTCCTTC TCCAATGAAT GGATTTGACT TATTTACTTA CATGGCTGTA
 661 ATAGTTTGTG CTAGAGGTAA AAAAGAAACT ACAGAAGCAT TTAAGTTACT TATAGAAGAA
 721 TTAGAGGACA ACATGAAAAC TGGTAAATCT TCTTTCAGAG GGGAAGAAAA ATACAGAATA
 781 ATGATGGAAG GTATACCTTG TTGGCCATAT ATAGGATACA AGATGAAAAC ATTAGCTAAA
 841 TTTGGAGTTA ACATGACAGG TAGTGTTTAC CCACATGCTT GGGCATTACA ATATGAAGTT
 901 AATGATTTAG ATGGAATGGC AGTAGCATAT AGTACTATGT TTAACAATGT AAACCTAGAC
 961 CGTATGACAA AATATAGAGT TGATTCTTTA GTAGAGGGTA AATGTGATGG AGCATTCTAT
1021 CATATGAACA GAAGCTGTAA ACTTATGAGT TTAATACAAT ATGAAATGCA AAGAAGAGCA
1081 GCTGAAGAAA CTGGATTACC ATATGCTGGA TTTGATGGTG ACCAAGCAGA CCCTAGAGCT
1141 TTCACTAATG CTCAATTTGA AACAAGAATT CAAGGTTTAG TTGAAGTAAT GGAAGAAAGA
1201 AAAAAACTTA ATAGAGGTGA GATATAATGG AAGCTATTTT ATCTAAAATG AAAGAAGTAG
1261 TTGAAAATCC AAATGCGGCT GTAAAAAAAT ATAAAAGTGA AACTGGTAAA AAAGCTATAG
1321 GTTGTTTCCC AGTTTATTGC CCAGAAGAAA TTATACATGC AGCTGGAATG CTTCCAGTTG
1381 GTATATGGGG AGGACAAACA GAATTAGATT TAGCTAAACA ATATTTCCCT GCATTTGCAT
1441 GTTCAATAAT GCAATCATGT TTAGAATATG GATTAAAAGG TGCTTATGAT GAATTATCTG
1501 GAGTTATTAT ACCAGGTATG TGTGATACAC TAATTTGTTT AGGACAAAAC TGGAAATCAG
1561 CAGTACCTCA TATAAAATAT ATATCATTAG TACACCCACA AAATAGAAAA CTTGAAGCTG
1621 GTGTAAAATA CTTAATCAGT GAGTACAAAG GCGTAAAAAG AGAACTTGAA GAAATTTGTG
1681 GATATGAAAT AGAAGAAGCA AAAATTCATG AAAGTATAGA AGTTTACAAT GAACATAGAA
1741 AAACTATGAG AGACTTTGTT GAAGTAGCTT ATAAACATTC TAATACTATA AAACCATCAA
1801 TAAGAAGCTT AGTAATTAAG AGTGGGTTCT TTATGAGAAA AGAAGAACAT ACTGAGCTAG
1861 TGAAAGATTT AATAGCAAAA TTAAATGCTA TGCCAGAAGA AGTCTGTTCT GGAAAGAAAG
1921 TTTTATTAAC AGGTATATTA GCTGATTCTA AAGATATATT AGACATTTTA GAAGACAACA
1981 ATATATCAGT TGTAGCTGAC GACTTAGCAC AAGAAACAAG ACAATTCAGA ACAGATGTAC
2041 CAGCAGGTGA TGATGCGTTA GAGAGATTAG CAAGACAATG GTCAAACATA GAAGGATGTT
2101 CATTAGCTTA TGACCCTAAG AAAAAACGTG GGTCACTTAT AGTAGATGAA GTTAAAAAGA
2161 AAGATATAGA TGGTGTTATC TTCTGTATGA TGAAATTCTG TGACCCAGAA GAATACGATT
2221 ATCCTTTAGT TAGAAAAGAT ATAGAAGATA GTGGAATACC TACTTTATAT GTTGAAATCG
2281 ACCAACAAAC TCAGAATAAT GAACAAGCCA GAACTCGTAT TCAAACTTTT GCTGAGATGA
2341 TGAGTTTAGC GTAA
```

**Fig. 18. The nucleotide sequence of the revised and cloned *hadBC* of *C. difficile* strain DSMZ 1296$^T$ (GeneBank accession number AY772816).** Start and stop codons derived from vector and structural genes are underlined. The grey box is for ribosome binding site for *hadC* and three nucleotide substitutions are depicted in bold letters in grey box. The 5 underlined bases in the middle are the overlap of the *hadB* stop codon and the *hadC* start codon.

## 6. Dehydratase purification from *C. difficile*

The dehydratase was therefore purified from *C. difficile* cell-free extracts by three chromatography columns (Table 4). SDS-PAGE of the purified enzyme showed two protein bands (calculated masses of two subunits, HadB = 46578 Da and HadC = 42350 Da) just below the 43 kDa protein molecular mass marker (Fig. 19). On a gel filtration column, the enzyme eluted at a size (≈ 90 kDa) corresponding to a heterodimer (89.93 kDa). The N-terminal amino acid sequences of the two subunits determined by the Edman degradation method revealed that the upper band was the slightly smaller HadC (MEAILSKMKE) and the lower band the somewhat larger HadB (SEKKEARVVI) confirming the correct start codon. The UV-visible spectrum of purified 2-hydroxyisocaproyl-CoA dehydratase showed a spectrum typical for iron-sulfur cluster(s) containing proteins (Fig. 23). Chemical analysis revealed 5.7 ± 0.1 non-heme iron and 6.1 ± 0.5 acid labile sulfur. Metal contents were also estimated by inductively coupled plasma atomic emission spectroscopy (ICP-AES, model Optima 3000, Perkin–Elmer) (See Results 12).

Using the same method as applied for the purification of the 2-hydroxyisocaproyl-CoA dehydratase from cell-free extracts of *C. difficile*, the recombinant enzyme with a non-functional *Strep*-tag at the C-terminus of the C-subunit could be also obtained in pure form from *E. coli*. The properties of the recombinant dehydratase ($V_{max}$ and $K_m$, see the next section) were identical to those of the enzyme from *C. difficile*.

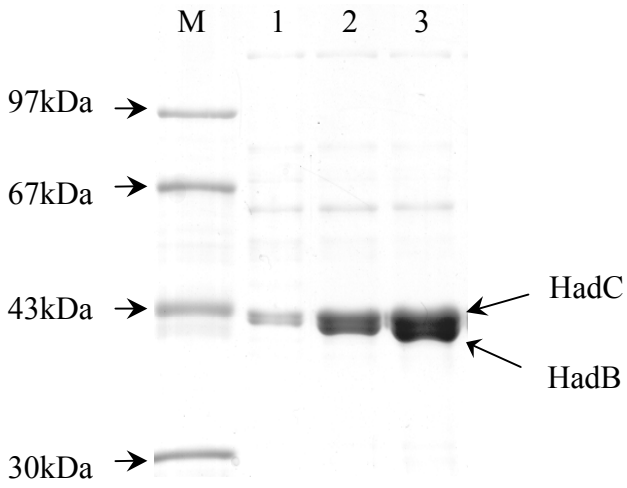



**Fig. 19. Purified 2-hydroxyisocaproyl-CoA dehydratase HadBC from *C. difficile*.** SDS-PAGE (8%) stained with Coomassie Brilliant Blue. M, molecular mass marker; 1 – 3, purified protein

**Table. 4. Purification of 2-hydroxyisocaproyl-CoA dehydratase from *C. difficile* and from *E. coli* (Np3BC).**

| Step | Protein (mg) | Activity (U) | Specific Activity (U/mg) | Enrichment (fold) | Yield (%) |
|---|---|---|---|---|---|
| *C. difficile* cell-free extract | 700 | 6300 | 9 | 1 | 100 |
| DEAE Sepharose | 140 | 3220 | 23 | 3 | 51 |
| Phenyl Sepharose | 50 | 2250 | 45 | 5 | 36 |
| Q-Sepharose | 17 | 2210 | 130 | 15 | 35 |
| *E. coli* cell-free extract | 153 | 1363 | 9 | 1 | 100 |
| DEAE Sepharose | 43 | 887 | 21 | 2 | 65 |
| Phenyl Sepharose | 6 | 593 | 99 | 11 | 44 |

## 7. Direct continuous activity assay

The activity of 2-hydroxyglutaryl-CoA dehydratase has been measured in a coupled assay following the absorbance increase of NADH produced from β-oxidation of crotonyl-CoA. However, the 2-hydroxyisocaproyl-CoA dehydratase activity could not be measured using an analogous coupled assay, because of the lack of auxiliary enzymes in this system. Therefore, another assay was developed using the absorbance difference between 2-hydroxyisocaproyl-CoA and isocaprenoyl-CoA at 290 nm ($\Delta\varepsilon$ = + 2.2 $mM^{-1}cm^{-1}$). 2-Hydroxyisocaproyl-CoA dehydratase activity was measured in the presence of ATP, $MgCl_2$, dithionite, dithiothreitol, serum albumin and activator. Addition of (*R*)-2-hydroxyisocaproyl-CoA started this assay and the formation of isocaprenoyl-CoA was followed at 290 nm. Due to the high absorbance of the adenine moiety of CoA and ATP, the absorbance maximum at 263 nm could not be used. The product isocaprenoyl-CoA was identified by MALDI-TOF mass spectrometry (Fig. 20, molecular mass = 865) and by comparison with the chemically synthesized compound. The apparent $K_m$ value for (*R*)-2-hydroxyisocaproyl-CoA was 50-80 μM and $V_{max}$ was determined as 110-150 U/mg (160-220 $s^{-1}$) using different purifications of dehydratase. In assays using (*E*)-isocaprenoyl-CoA as substrate, no activity could be observed suggesting that the dehydration is irreversible under these conditions (see Discussion). Isocaprenoyl-CoA (400 μM) was shown to decompose slowly (5 nmol/min) under the assay conditions regardless whether the dehydratase was present. The product could not be identified by MALDI-TOF spectrometry.

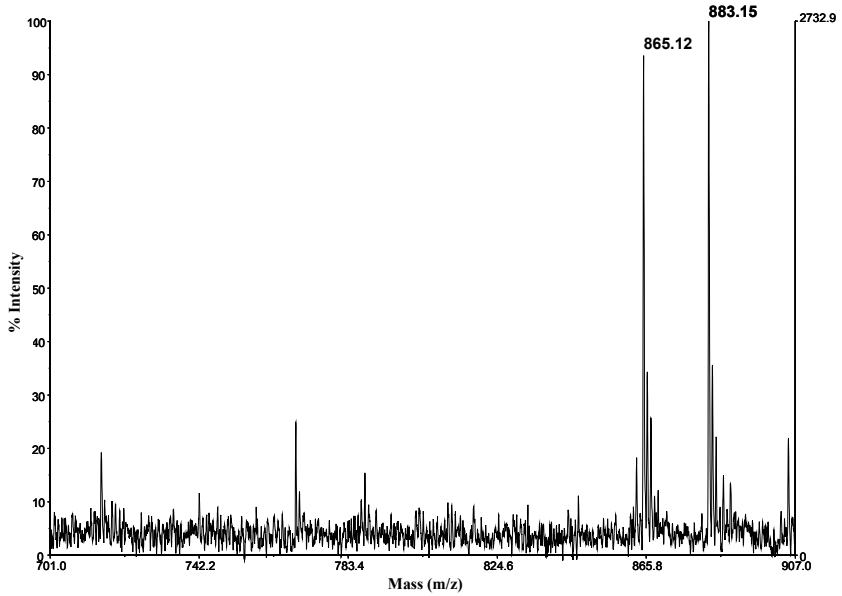



**Fig. 20. MALDI-TOF mass spectrometry of CoA esters isolated from dehydratase reaction.** CoA esters were purified using Sep-pak® $C_{18}$ cartridge from the standard activity assay mix incubated for 5 minutes and acidified with 1 M HCl (< pH 4.0). The calculated masses of 2-hydroxyisocaproyl-CoA and 2-isocaprenoyl-CoA are 883 and 865 Da, respectively.

## 8. Catalytic activation of the dehydratase by its activator

Recombinant HadI activated the dehydratase in the presence of ATP, $MgCl_2$ and a one-electron reducing agent Ti(III)citrate or dithionite, but the initial experiments revealed a dependence of the activity on the applied amount of activator (Fig. 21). Hence, it appeared that each dehydratase molecule required one activator molecule and ATP is hydrolysed during every turnover. A true activator, however, should act catalytically; it should be able to serve many dehydratase molecules, each of which catalyses many turnovers without further hydrolysis of ATP. Subsequent experiments indicated that the low dehydratase/activator ratio ≤ 1 was due to the instability of the activator in the assay mixture. The activator HadI could be stabilized with 5 mM dithiothreitol and 1 μM bovine serum albumin, probably by removing trace amounts of oxygen and preventing dissociation into subunits. Under these conditions a dehydratase/activator ratio of 10 gave an even higher dehydratase activity than a ratio of 0.2 in the absence of the stabilisators (Fig. 21). The experiments indicate, however, that at a dehydratase/activator ratio of 10 at least a pre-incubation time of 40 min is required to reach full activity. Immediate activation was only obtained by using dehydratase/activator ratios ≤ 0.1 (see also Fig. 21).

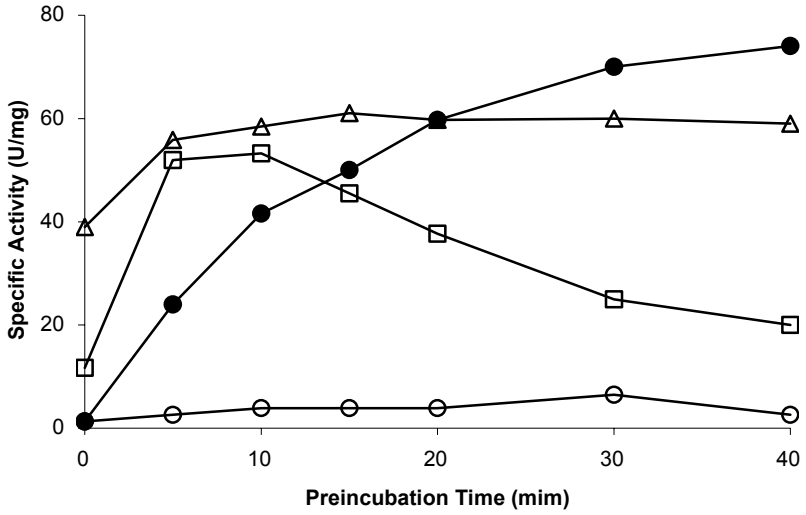



**Fig. 21. Activation of dehydratase by activator.** Dehydratase activities were measured in presence of 5 mM $MgCl_2$, 0.1 mM dithionite and 0.4 mM ATP, and the reactions were started by adding 200µM 2-hydroxyisocaproyl-CoA at the indicated pre-incubation times. Molar ratios of dehydratase/activator, open triangles, 0.2; open squares, 1; open circles, 10; closed circles, 10, in presence of 5 mM dithiothreitol and 1 µM BSA.

## 9. Electron recycling: separation of the activated dehydratase from its activator

In a critical experiment 4.4 mg dehydratase was activated for 30 min in the presence of 1.0 mg activator (dehydratase/activator = 3), 0.4 mM ATP, 10 mM $MgCl_2$, 5 mM dithiothreitol and 0.1 mM dithionite in 50 mM Mops pH 7.0 (total volume 2 ml) and was assayed by diluting a 1.0 µL sample into 0.5 ml 0.4 mM (*R*)-2-hydroxyisocaproyl-CoA in 50 mM Tris/HCl pH 8.0 (139 $s^{-1}$). The active dehydratase was separated from its activator through a *Strep*-Tactin column. The tagged activator bound to the column, while the active dehydratase passed through. A 2.0 µL sample of the flow-through was assayed in the same manner as above (69 $s^{-1}$); after two successive substrate additions the activity was almost completely lost (Fig. 22). SDS/PAGE revealed the double band of the dehydratase around 43 kDa but no band at 30 kDa indicating that > 95 % of activator had been removed. Activation by 0.4 mM ATP, 0.1 mM dithionite and a >10-fold molar excess of activator immediately restored the complete activity (68 $s^{-1}$). Hence the activated dehydratase irreversibly lost 50 % of its activity during passage through the *Strep*-Tactin column; the remaining 13.5 pmol active enzyme dehydrated 103 nmol (*R*)-2-hydroxyisocaproyl-CoA (7630 turnovers) until activity ceased.

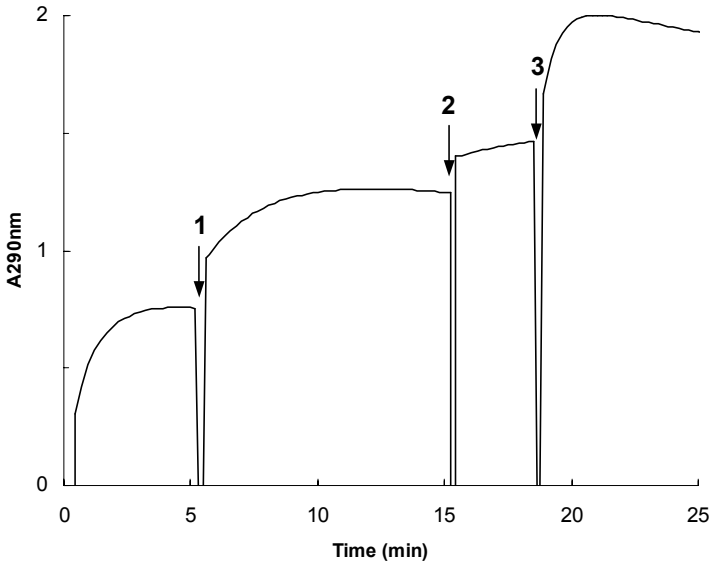



**Fig. 22. The activity assay of activated dehydratase separated from the activator by passage through a *Strep*-Tactin column.** The assay (total volume 500 µl) contained 27 pmol active dehydratase in 50 mM Tris/HCl pH 8.0 in absence of ATP, $MgCl_2$, dithionite and dithiothreitol. The reaction was started by adding 0.2 µmol of the substrate (*R*)-2-hydroxyisocaproyl-CoA (arrow 1). After the substrate was consumed ($\Delta A_{290nm}$ = 0.455), further 2 × 0.2 µmol substrate was added at arrows 1 and 2. The activity was recovered by addition of an excess amount of activator (>10 fold), 0.4 mM ATP, 5 mM $MgCl_2$, 5 mM dithiothreitol and 0.1 mM dithionite (arrow 3). The decrease in absorbance after 20 min was due to the instability of the product isocaprenoyl-CoA.

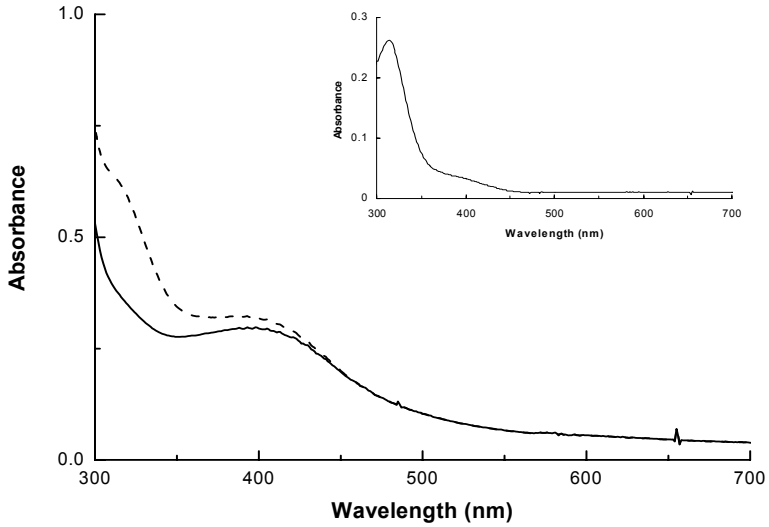



**Fig. 23. UV-visible spectra of 2-hydroxyisocaproyl-CoA dehydratase.** Solid line, as isolated (1.2 mg/ml); dashed line, activated dehydratase separated from activator (1.2 mg/ml). The insert shows the difference spectrum of activated dehydratase (dashed line) minus isolated dehydratase (solid line). The peak at 320 nm stems from dithionite.

This experiment shows that the activated dehydratase retains its activity (i) in the absence of 0.4 mM ATP, which was diluted in the assay prior to the affinity chromatography to 0.8 µM; (ii) after affinity chromatography at < 0.8 µM ATP and in the absence of at least 95 % of the activator (dehydratase/activator > 160 and absence of stabilisators); (iii) turnover causes rapid inactivation. The UV-visible spectra between 300 and 700 nm of the dehydratase as isolated and after activation + affinity chromatography are shown in Fig. 23, which reveal the absorbance of a $[4Fe\text{-}4S]^{2+}$ cluster around 400 nm. In the difference spectrum (insert of Fig. 23) the peak at 320 nm stems from dithionite, whereas the increase in absorbance around 400 nm may be caused by the irreversible inactivation of 50 % of the dehydratase during affinity chromatography. In another experiment, in which the *Strep*-Tactin column was not treated with dithionite prior to the affinity chromatography (see Materials and Methods 3.10), the yield of active dehydratase was only 10 %, but the absorbance increase around 400 nm was higher. In contrast to that expected for a reduction of a $[4Fe\text{-}4S]^{2+}$ cluster, no decrease in absorbance was observed.

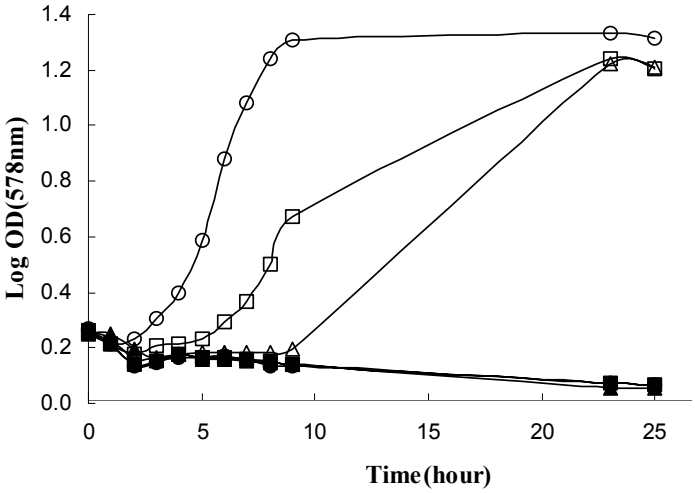



**Fig. 24. The effect of metronidazole on *C. difficile* growth.** Open circles, no metronidazole; open squares, 5 µM; open triangles, 10 µM; closed circles, 25 µM; closed squares, 50 µM; closed triangles, 100 µM.

## 10. Metronidazole effect

It was suggested that 2-hydroxyisocaproyl-CoA dehydratase or the activator could be a very sensitive target of metronidazole (26), which has been used as an antibiotic for *C. difficile* infections in human body (83). Indeed, metronidazole and the other nitro-compound such as 4-nitrophenol and chloramphenicol inhibited 2-hydroxylglutaryl-CoA dehydratases at 10 µM

concentration, probably, by oxidizing the enzymes (45, 68). The effect of metronidazole on *C. difficile* growth was tested and showed complete inhibition of growth at 25 µM (Fig. 24). The activity of purified dehydratase was completely abolished at 20 µM probably by the oxidation of the activated enzyme with the nitro group of the inactivator.

## 11. UV-vis spectrum of cofactor supernatant

The supernatant of the enzyme purified from *C. difficile* after treatment with anoxic 0.2 M trichloroacetic acid showed a characteristic UV-visible spectrum of oxidised flavin (peaks at 370 nm and 450 nm), but no significant flavin content (< 5 % of the dehydratase) was detected by HPLC comparing with FMN, FAD and riboflavin standards. After oxidation with air the UV-visible spectrum of the supernatant showed a new peak at 300 nm and a shoulder around 325 nm, which could not be assigned to any known cofactor. Probably this absorption was due to oxidised iron sulfide (Fig. 25).

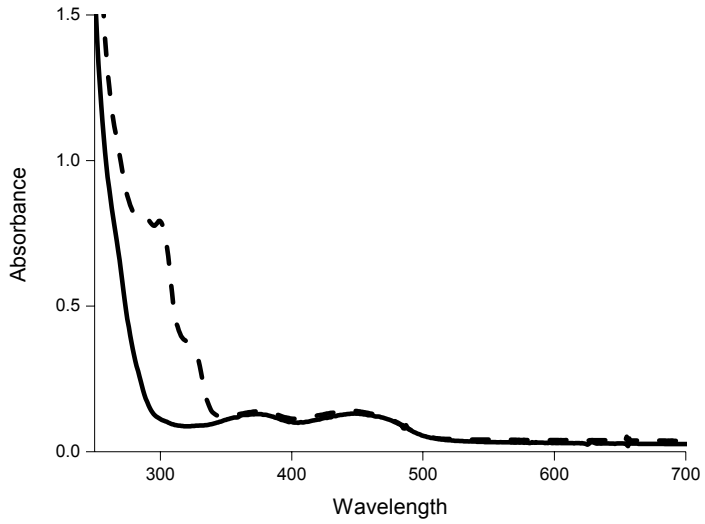



**Fig. 25. UV-vis spectrum of cofactor(s) released from dehydratase.** The purified dehydratase (10mg/ml) was treated with anaerobically prepared trichloroacetic acid (final concentration of TCA 3 % (m/v)) and precipitated protein was removed by centrifugation (solid line) and after oxidation of released cofactors against air for 6 hours (dash line).

## 12. Metal analysis

It was suggested that the $d^1$-metal Mo(V) could be involved in 2-hydroxyacyl-CoA dehydratases because the EPR spectrum of the 2-hydroxyglutaryl-CoA dehydratase from *A. fermentans* reduced by component A showed a signal similar to that of a $d^1$-metal ion and the enzyme activities were proportional to the Mo contents (0.07 – 0.2 per mol enzyme) during the purification (43). However, no molybdenum could be detected by atomic absorption spectroscopy in 2-hydroxyisocaproyl-CoA dehydratase, which exhibited the highest activity

($V_{max}$ = 150 U/mg) of all known 2-hydroxyacyl-CoA dehydratases. Interestingly, crude preparations of 2-hydroxyisocaproyl-CoA dehydratase indeed contained Mo; but upon further purification the Mo peak could be separated without loss of activity (Fig. 26). The other method, ICP-AES, was used for metal analysis with two different preparation of dehydratase from *C. difficile* and a recombinant dehydratase from *E. coli*. As shown in Table 5, the iron content of 3.8, 4.1 and 5.3 mol/mol homodimer were estimated in two native and a recombinant dehydratase, respectively. Other metals Co, Ni and Mo were absent in native probes. A small amount of Co and Ni were detected in recombinant probe, which might be due to a contamination. But surprisingly stoichiometric amounts of Zn were found 1.1, 1.9 mol/mol protein in two separate preparations of the dehydratase from *C. difficile* and 3.3 mol/mol in a recombinant dehydratase from *E. coli*.

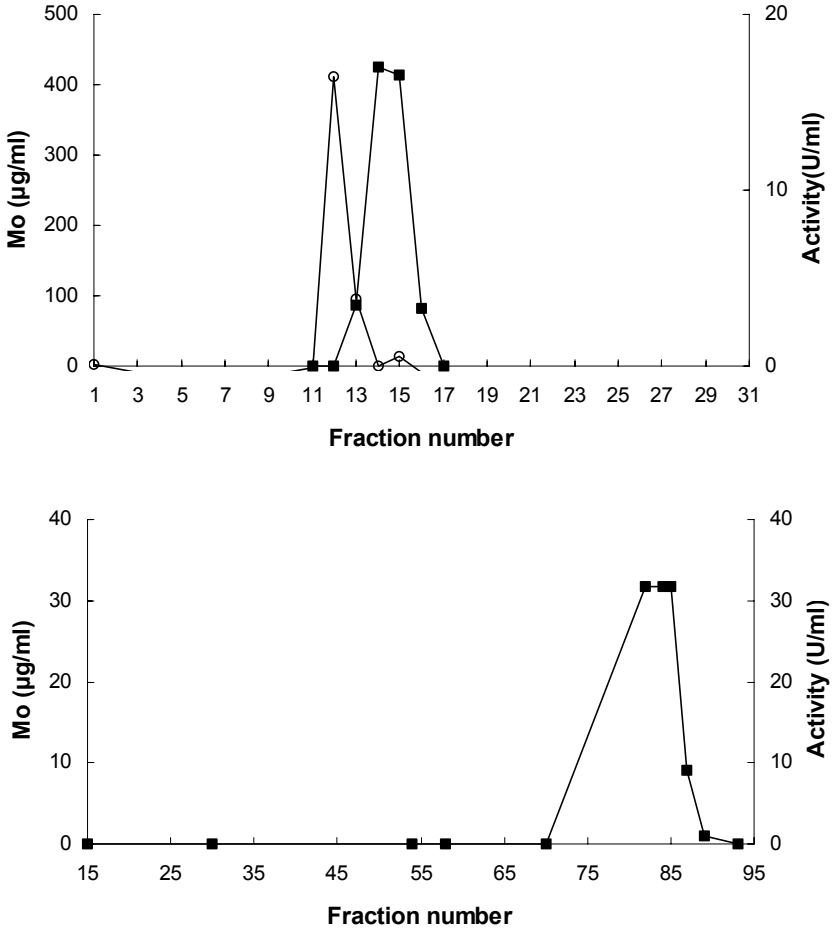



**Fig. 26. Separation of molybdenum from active dehydratase during the purification.** Molybdenum contents (open circles) and the enzyme activity (black squares) of the fractions after the first DEAE-Sepharose (**A**) and the second Phenyl Sepharose column (**B**).

**Table 5. Metal contents estimated by inductively coupled plasma atomic emission spectroscopy (ICP-AES).** 1 and 2, preparation 1 and 2; <, lower than the detection limit (9 x $10^{-3}$ mol/mol protein).

| | Metal contents / mol protein | | | | |
|---|---|---|---|---|---|
| Probe | Co | Fe | Ni | Zn | Mo |
| 1 Dehydratase from *C. difficile* | < | 3.77 | < | 1.15 | < |
| 2 Dehydratase from *C. difficile* | < | 4.11 | < | 1.86 | < |
| Recombinant dehydratase from *E. coli* | 0.08 | 5.32 | 0.20 | 3.22 | < |

## 13. Complex of activator and dehydratase

Prior to the complex formation of 2-hydroxyisocaproyl-CoA dehydratase with its activator, the dehydratase activity was measured in the presence of $AlF_4^-$ and ATP. However, no significant inhibition was observed (Fig. 27), which might be explained in two possible ways; i) as shown by the proposed mechanism above (see Results 9), the electron can be recycled for several turnovers, hence the complex formation can not cause a significant inhibition of the activity or ii) $AlF_4^-$ and ATP can not induce the tight complex formation.

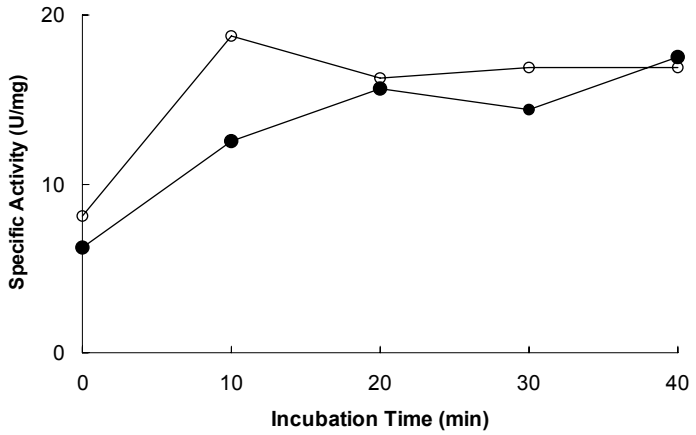



**Fig. 27. Inhibition of 2-hydroxyisocaproyl-CoA dehydratase activity in the presence of $AlF_4^-$.** Dehydratase and activator (molar ratio 1 : 1) were incubated in 50 mM Tris/HCl pH 8.0, 5 mM $MgCl_2$, 5 mM DTT, 0.4 mM ATP and 0.1 mM dithionite in the presence of 1 mM $AlF_3$ and 10 mM KF (closed circles) or in the absence of $AlF_3$ and KF (open circles). The measurement of dehydratase activity was started by adding 0.2 mM (*R*)-2-hydroxyisocaproyl-CoA in 50 mM Tris/HCl pH 8.0 at indicated incubation time points.

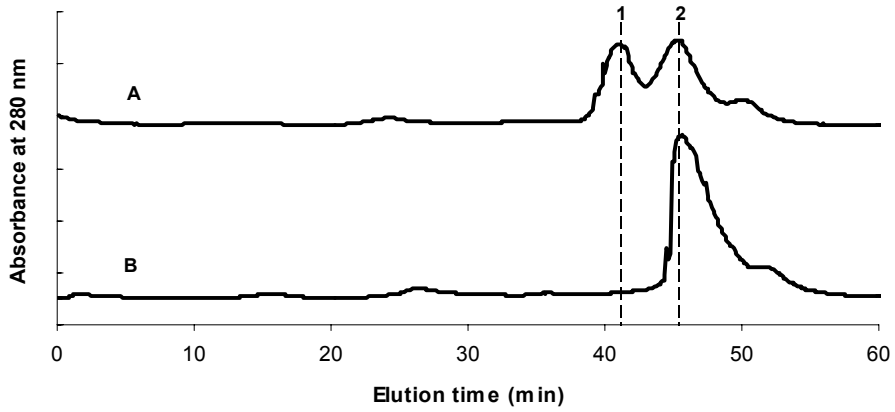



**Fig. 28. Separation of $AlF_4^-$ induced complex by gel filtration on Superdex 200. A**, dehydratase (120 µg) and activator (144 µg) were incubated as described in Fig. 27 for 30 minutes at room temperature in the presence of 1 mM $AlF_3$ and 10 mM KF; **B**, same as **A** in the absence of $AlF_3$ and KF. Dashed line 1, the elution peak for the complex; Dashed line 2, the elution peak for the overlap of dehydratase and activator.

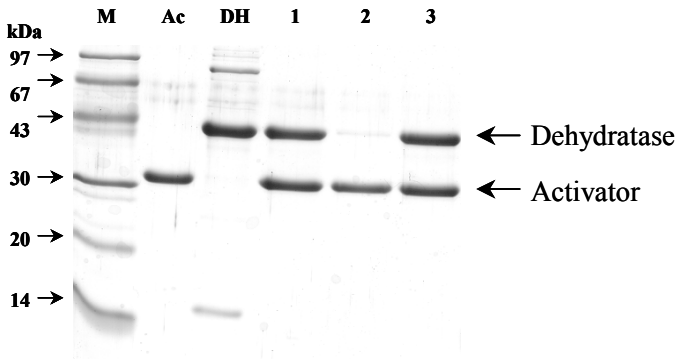



**Fig. 29. Coomassie stained SDS-PAGE (15 %) of tight complex purified using *Strep*-Tactin affinity column.** M, protein size marker; Ac, purified activator; DH, partially purified dehydratase; 1, purified complex after incubation of dehydratase/activator (1.8/1 molar ratio) as described in Fig. 27 in the presence of 1 mM $AlF_3$ and 10 mM KF; 2, in the absence of $AlF_3$ and KF; 3, in presence of 1 mM $AlF_3$, 10 mM KF and 1 mM (*R*)-2-hydorxyisocaproyl-CoA.

In order to get an answer, the purification of the complex of the activator and the dehydratase was performed by gel filtration chromatography (Fig. 28). The complex induced by $AlF_4^-$ and ATP could be seen by an earlier elution time but the separation was not good enough due to the overlap of non-complexed proteins. Another purification of the complex using the *Strep*-Tactin affinity column could be applied. When the activator forms the complex with the dehydratase, the complex could be bound to the column by using the *Strep-tag* II peptide on the C-terminus of the activator. The purified complex from the affinity column showed clearly two protein bands of the dehydratase and the activator (intensities of the dehydratase : activator = 1 : 1 were confirmed by Image quantitator) while one protein band of the activator

could be seen on SDS-PAGE of the non-induced reaction (Fig. 29). The complex formation was not affected by the addition of the substrate showing two protein bands on SDS-PAGE. However, the purified complex from the affinity column was not stable enough to be concentrated on 100 kDa cut off spin column, probably because of the high salt concentration (300 mM) in the elution buffer. Afterwards the complex was purified on Amicon Ultra-4 PLTK Ultracel-Pl (100 kDa cut off) by exchanging the buffer and removing non-complxed proteins. The purified complex exhibited about 20 % dehydratase activity of the non-purified complex, which might be loss of the electron by the oxidation of the sensitive complex as the activated dehydratase (See Results 9). The purified active $AlF_4^-$-induced complex was used for the crystallisation trial for the structure analysis. The obtained microcrystals exhibited bands of the dehydratase and activator on SDS/PAGE (performed by Dr. Berta M. Martin in Universität Bayreuth).

## 14. Detection of a substrate-derived organic radical by EPR spectroscopy

EPR spectra were obtained to probe the formation of an organic radical which might be involved in 2-hydroxyacyl-CoA dehydration mechanism. The activator of 2-hydroxyisocaproyl-CoA dehydratase from *C. difficile* was reduced with 10 fold excess dithionite and excess dithionite was removed by gel filtration on Sephadex G-25.

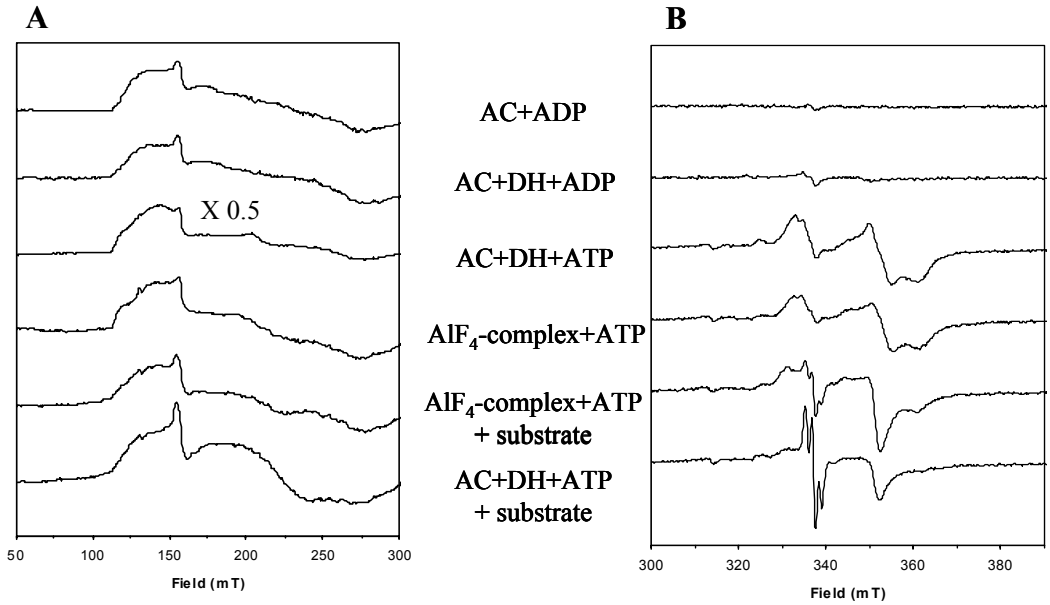



**Fig. 30. High spin (A) and low spin (B) EPR signals of (*R*)-2-hydroxyisocaproyl-CoA dehydratase.** Activator/dehydratase 50 µM, ATP/ADP 10 mM, $MgCl_2$ 5 mM, substrate 0.1 mM. EPR conditions: microwave frequency, 9.458 GHz; modulation frequency, 100 kHz; temperature, 10 K; modulation amplitude, 1.25 mT; microwave power, 20 mW (B), 0.1 mW (A).

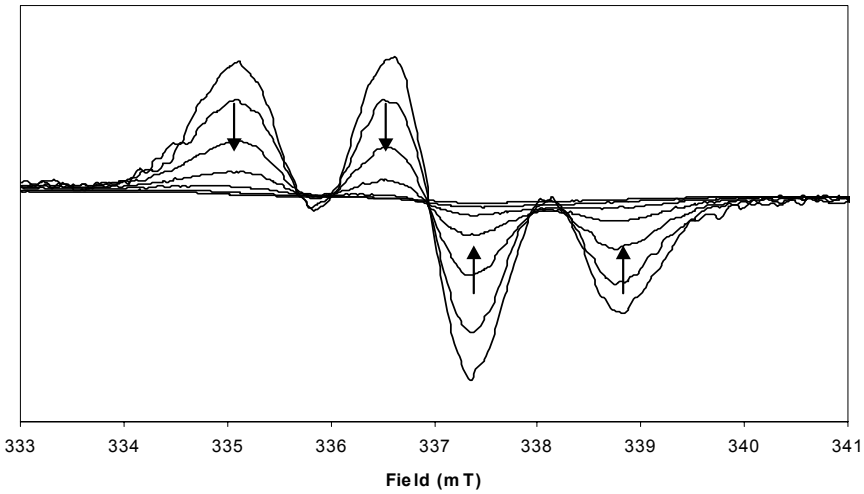



**Fig. 31. EPR power saturation of the (*R*)-2-hydroxyisocaproyl-CoA-induced radical signal in hydroxyisocaproyl-CoA dehydratase and activator in presence of ATP.** The decrease of the signal by increasing the power is shown by the arrows. EPR conditions: microwave frequency, 9.458 GHz; modulation frequency, 100 kHz; temperature, 10 K; modulation amplitude, 1.0 mT; microwave power, 32, 8, 2, 0.5, 0.13, 0.032, and 0.008 mW (signal intensities corrected to a microwave power of 0.008 mW)

Reduced activator in presence of ADP showed clear $S = 3/2$ signals ($g = 4\text{-}6$) which changed depending on presence of dehydratase, ATP/ADP and substrate (Fig. 30A). $S = 3/2$ signals were observed in the closely related activators of 2-hydroxyglutaryl-CoA dehydratase from *A. fermentans* and phenyllactate dehydratase from *C. sporogenes* with weak $S = 1/2$ signals ($g \approx 2$) (27, 44). But, interestingly not even a weak $S = 1/2$ signal was observed in the activator of 2-hydroxyisocaproyl-CoA dehydratase and the recent EPR spectra of the activator from *A. fermentans* also showed less $S = 1/2$ signal (47). 2-Hydroxyisocaproyl-CoA dehydratase alone was EPR silent but addition of reduced activator in the presence of ADP showed only $S = 3/2$ signals. In the presence of ATP, new $S = 1/2$ signals of $[4Fe\text{-}4S]^+$ clusters were detected possibly due to the reduction of the dehydratase or to change of the $S = 1/2$ and $S = 3/2$ signals of the activator upon the complex formation. The $S = 3/2$ signals showed small changes depending on the presence of $AlF_4^-$. Furthermore, an organic radical signal was generated by addition of substrate (*R*)-2-hydroxyisocaproyl-CoA. The radical signal ($g_{average} = 2.0038$) showed hyperfine couplings from two protons with coupling constants of 1.45 mT and 1.15 mT Fig. 30B and 31). The EPR spectra of the $AlF_4^-$ induced complex were also obtained showing almost no difference in the $S = 1/2$ signals and minor changes in the $S = 3/2$ signals from non-complex proteins. The organic radical signal was also observed in the $AlF_4^-$-induced complex by addition of substrate confirming that the complex was active. The 3-fold less intense radical signal of the active complex after $AlF_4^-$ induction might be due to spontaneous loss of the electron, which could not be activated again. Fig. 31 shows the microwave power

saturation of the radical signal. Though the radical signal saturates at 10 K (P ½ = 0.1 mW), the saturation is not typical for free radical species at this temperature, implicating the radical might be located near a paramagnetic metal-containing center leading to a more efficient relaxation. This center could be the iron-sulfur cluster in the dehydratase. In order to confirm that the observed radical signal is substrate-derived, 2-hydroxy[3-$^{2}H_2$]isocaproyl-CoA and 2-hydroxy[1-$^{13}$C]isocaproyl-CoA were synthesized and used for preparation of EPR samples. The EPR-spectra with the 3-$^{2}H_2$-labelled substrate showed hyperfine coupling from a single proton while the 1-$^{13}$C-labelled substrate showed hyperfine couplings from two protons and $^{13}$C (Fig.32).

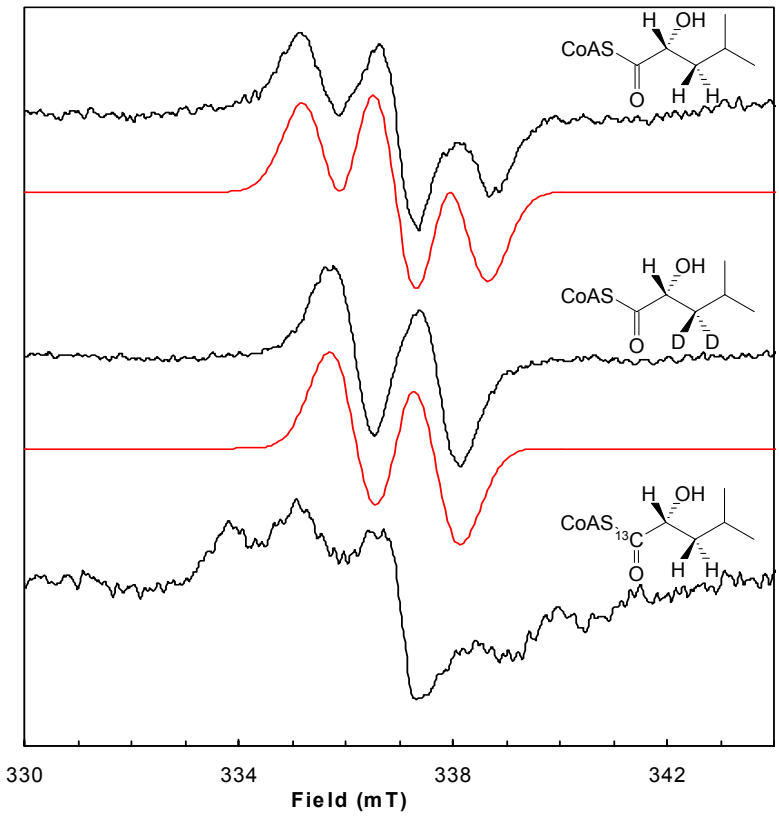



**Fig. 32. Experimental EPR spectra (black traces) and simulated (red traces) of the substrate-induced radical signal in the 2-hydroxyisocaproyl-CoA dehydratase and activator.** EPR conditions: microwave frequency, 9.460 GHz; modulation frequency, 100 kHz; temperature, 10 K; modulation amplitude, 0.6 mT; microwave power, 8.0 µW. Simulation parameters: Non-labelled substrate ($a$ = coupling constant), $a$(1H) = 1.45 mT and $a$ (1H) = 1.15 mT; 3-[$^{2}H_2$] labelled substrate, $a$(1H) = 1.45 mT, $a$(2H) = 0.177 mT, line width = 0.95 mT, g = 2.0038. The spectrum of the $^{13}$C-labelled substrate was not simulated due to the low-signal to noise.

**Table 6. Summary of the substrate deuterium kinetic isotope effects.**

| | $V_{max}$ (U/mg) | $K_m$ (μM) | $V_{max}/K_m$ | $k_H/k_D$ |
|---|---|---|---|---|
| (*R*)-2-Hydroxyisocaproyl-CoA | 110 | 78 | 1.4 | (=1) |
| (*R*)-2-Hydroxy[3-$^2H_2$]isocaproyl-CoA | 64 | 58 | 1.1 | 1.3 |
| (*R*)-2-Hydroxy[2-$^2H_1$]isocaproyl-CoA | 88 | 88 | 1.0 | 1.4 |
| (*R*)-2-Hydroxy[2,3-$^2H_3$]isocaproyl-CoA | 54 | 61 | 0.9 | 1.6 |

## 15. Deuterium kinetic isotope effects

The kinetic isotope effects ($k_H/k_D$) on the overall 2-hydroxyisocaproyl-CoA dehydratase reaction were obtained with deuterium labelled 2-hydroxyisocaproyl-CoA (Table 6) by measuring the $V_{max}/K_m$ values. Labelled substrates were synthesised and the activity was measured as described in Materials and Methods.

## 16. Preliminary stereochemistry

The dehydratase accepted the *R*-isomer more efficiently while only 10 % activity could be obtained with the (*S*)-2-hydroxyisocaproyl-CoA, which might be due to a contamination with the *R*-isomer. Since an equal mixture of (*R*)- and (*S*)-2-hydroxyisocaproyl-CoA gave only half of the enzymatic activity, we assume that that the *S*-isomer can also bind at the active site of the enzyme but can not be dehydrated. In assays using (*E*)-isocaprenoyl-CoA as substrate, no activity could be observed suggesting that the dehydration produces (*Z*)-2-isocaprenoyl-CoA or is irreversible under these conditions. Isocaprenoyl-CoA (400 μM) was shown to decompose slowly (5 nmol/min) under the assay conditions regardless whether the dehydratase was present.

# Discussion

## 1. (*R*)-2-Hydroxyisocaproate dehydrogenase

D-2-Hydroxyisocaproate dehydrogenase was discovered together with its L-specific counterpart, named L-2-hydroxyisocaproate dehydrogenase (77). The low specificity of the enzymes with respect to the substrate's side-chain makes them attractive for biotechnological applications for the synthesis of chiral compounds (49). D-2-Hydroxyisocaproate dehydrogenase belongs to the $NAD^+$ dependent 2-hydroxycarboxylate dehydrogenase family. 2-Hydroxycarboxylate dehydrogenases showing broad substrate specificity can be divided into two classes (7): the mandelate dehydrogenase family, which prefers substrate with branched C3-atoms and the 2-hydroxyisocaproate dehydrogenase family, for which a branch in the substrate side chain at the C3-position is unfavorable. (*R*)-2-Hydroxyisocaproate dehydrogenase from *C. difficile* (LdhA) is the third enzyme in the second class. The other known two D-2-hydroxyisocaproate dehydrogenases are from *Lactobacillus casei* (25), whose the crystal structure was solved, and from *Lactobacillus delbrueckii* (7), whose gene was cloned and the protein was produced in *E. coli*. HadA is a monomeric enzyme whereas the other two D-specific dehydrogenases are homodimers; despite this difference, all three enzymes show similar substrate specificities. The crystal structure of the enzyme from *L. casei* revealed that some amino acid residues (fairly conserved in HadA) are responsible for substrate specificity. They suggested that Leu51 (in the *L. casei* enzyme) is the key residue for the differences in substrate specificity between D-2-hydroxyisocaproate and D-lactate dehydrogenase. Recently, D-lactate dehydrogenase form *Lactobaciluus pentosus* was converted to D-2-hydroxyisocaproate dehydrogenase by replacing the corresponding Tyr by Leu (90). This key residue is not conserved but occupied by Glu in LdhA which might explain the complete absence of any activity with pyruvate, 3-methyl-2-oxopentonate and 2-oxobutyrate. Even though a lot is known about structure and substrate specificity so far, the role of the D-2-hydroxyisocaproate dehydrogenase from lactobacilli in their metabolism is unknown. However the gene arrangement (Fig. 6) in the genome of *C. difficile* shows that *ldhA* belongs to the gene cluster of the 2-hydroxyisocaproyl-CoA dehydratase system for leucine reduction. The substrate specificity of the enzyme reveals that 2-hydroxyisocaproate should be the natural substrate *in vivo*. Therefore HadA is clearly the first enzyme in the reductive branch of leucine fermentation.

## 2. (*E*)-2-Isocaprenoyl-CoA:2-hydroxyisocaproate CoA transferase

According to the proposed mechanism, the dehydration of 2-hydroxyalkanoates to enoates must proceed at the thiol ester level. The formation of 2-hydroxyacyl-CoA prior to dehydration was apparent in the phenyllactate dehydratase system from *C. sporogenes* showing a dehydratase complex with a CoA transferase (28). But there is no indication that (*R*)-2-hydroxyisocaproyl-CoA dehydratase forms a complex with the CoA transferase since both enzymes separate during purification of the dehydratase from *C. difficile*. It is not clear yet which metabolic intermediate is the CoA donor to (*R*)-2-hydroxyisocaprate, because HadA activity was measured in the reverse reaction (CoA transfer from (*R*)-2-hydroxyisocaproyl-CoA to (*E*)-2-isocaprenoate) due to the low stability of (*E*)-2-isocaprenoyl-CoA. Subsequently, isocaprenoyl-CoA seems to be reduced to isocaproyl-CoA by a putative enoyl-CoA reductase (3 U/mg of activity was measured in the cell free extract) which might be encoded by *acdB* downstream of *hadAIBC* (Fig. 6). Isocaproyl-CoA might be a natural CoA donor to 2-hydroxyisocaproate yielding the final product isocaproate mediated by the CoA transferase (Fig. 5).

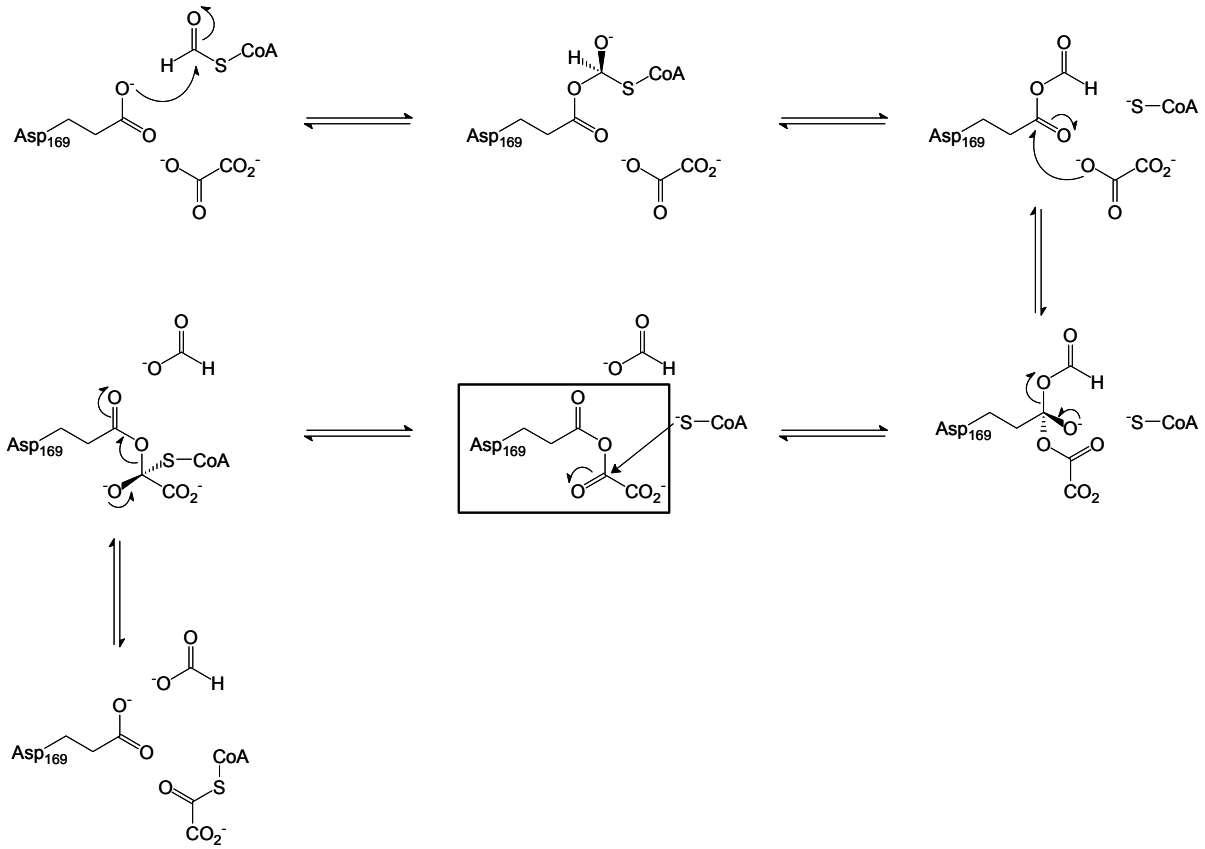



**Fig. 33. Proposed catalytic mechanism of *O. formigenes* formyl-CoA transferase Frc (51).** The anhydride intermediate in the box was observed in the structure.

2-Hydroxyisocaproate CoA-transferase is homologous only with class III enzymes supposing that the reaction is mediated by a ternary complex. Recently, a detailed mechanism (Fig. 33) was proposed on the basis of structural and kinetic analysis of formate CoA-transferase (41, 51, 75). Crystal structure of wild type and site-specific mutants revealed that $Asp^{169}$ as conserved residue in class III enzymes formed an anhydride intermediate with the substrate carboxylate. The authors also observed kinetics different from the ping-pong scheme of class I enzymes even though the same chemical strategy is suggested for the formation of anhydride intermediates. Double reciprocal plots of activity vs. oxalate concentration at three different formyl-CoA concentration showed the intersection of lines as observed in other class III enzymes. The result indicates a formation of the ternary complex of the enzyme with two substrates, which is different from a transient thiolester formation as shown for class I enzymes. The different kinetics were explained with the position of free CoA in the active site. In the class I enzymes, bound CoA reacts with the initial anhydride in the absence of the second substrate releasing the first product and forming a glutamyl-CoA-thiol ester which was irreversibly reduced or cleaved by sodium borohydride or hydroxylamine, respectively. On the other hand, the free CoA in formate CoA-transferase is positioned too far away from the relevant carbonyl group of $Asp^{169}$ to form an enzyme-linked thiol ester and the first product can be released only by the second substrate.

This proposed mechanism does not explain all results from the class III enzymes. A curious partial inactivation by sodium borohydride was reported for cinnamoyl-CoA:phenyllactate CoA transferase (FldA) of *C. sporogenes* (28) and succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase (BbsF) of *Thauera aromatica* (62) even though similar kinetic results with double-reciprocal plots were obtained. In case of 2-hydroxyisocaproyl-CoA transferase (HadA), complete inactivation by sodium borohydride was observed. However the kinetic results obtained were not precise enough due to the low sensitivity of the activity assay. $Asp^{169}$, suggested in formate CoA-transferase as the anhydride forming residue, is conserved in FldA and HadA (Fig. 9) but not in BbsF, furthermore, $Tyr^{59}$, which might stabilize the tetrahedral anhydride intermediates, is not conserved in FldA and HadA. Therefore the proposed mechanism for the class III enzyme might be incorrect, or at least does not apply to all class III enzymes. Further studies on HadA could help to define the mechanism of class III enzymes.

### 3. Activator

Previous work on 2-hydroxyglutaryl-CoA dehydratase showed that the activator alone has ATPase activity (4 – 6 $s^{-1}$) only in the oxidised state, which was not improved in presence of dehydratase and substrate (42). The results in this work, which revealed less ATPase activity (< 1.5 $s^{-1}$) of the activator HadI regardless of its oxidation state, question those data. Therefore the original data obtained with component A of 2-hydroxyglutaryl-CoA dehydratase have been re-calculated and found too high by at least a factor of ten. Furthermore, repetition of the ATPase measurements with the activator of *A. fermentans* by applying the conditions used in this work gave only insignificant activities (47). Therefore we conclude that the activators from *A. fermentans* and *C. difficile* both are devoid of significant ATPase activity. Addition of dehydratase to the corresponding reduced activator/component A, however, gave high ATPase activities; in case of HadI + HadBC up to 50 U/mg activator were achieved. This result fits much better to the proposed mechanism, since the electron should only be transferred in a complex of both proteins driven by ATP-hydrolysis.

It was known that the activator is extremely sensitive against oxygen and even under anoxic conditions, ADP or ATP and $MgCl_2$ had to be added in the buffers in order to maintain stability. In this work two additional stabilising factors have been discovered, DTT and high protein concentration, which was achieved by adding BSA. In previous work on the activator of 2-hydroxyglutaryl-CoA dehydratase, the coupled activity assay already contained DTT and sufficient protein in the form of helping enzymes. It is assumed that the activator of 2-hydroxyisocaproyl-CoA dehydratase is more stable than the other activators, since only $S$ = 3/2 signals were detected. Component A of 2-hydroxyglutaryl-CoA dehydratase revealed additional $S$ = 1/2 signals, which was interpreted as irreversibly inactivated $[2Fe\text{-}2S]^{2+}$ clusters (44). The less stable component A might be due to the presence of ATP instead of ADP in the elution buffer. The binding of ATP at the activator causes a conformational change which exposes the [4Fe-4S] cluster to solvent leading to a more rapid inactivation. The usual spin ground state of $[4Fe\text{-}4S]^{1+}$ found in biological systems is $S$ = 1/2, but several cases of mixtures of $S$ = 1/2 and $S$ = 3/2 were reported in the study of nitrogenase Fe-protein (63, 64), the *Pyrococcus furiosus* ferredoxin (94), the *Bacillus subtilis* glutamine phosphoribosylpyrophosphate amidotransferase (70) and the *Clostridium thermoaceticum* CO dehydrogenase (65). Pure $S$ = 3/2 signals of $[4Fe\text{-}4S]^{1+}$ cluster are not as common as spin mixtures, but only in the case of the A33Y mutant of *P. furiosus* ferredoxin a pure $S$ = 3/2 state was observed. The wild type *P. furiosus* ferredoxin has mixed spin states of $[4Fe\text{-}4S]^{1+}$ coordinated by four cysteins in the ferredoxin-like arrangement (–C-$X_2$-C-$X_2$-C- and a remote

–C-P-) (29). The pure $S$ = 3/2 signal in the reduced activator shown in this work is the first example for $[4Fe\text{-}4S]^{1+}$ clusters coordinated by non-ferredoxin-like arranged cysteines. Similar changes of $S$ = 3/2 and 1/2 signals were observed for the $[4Fe\text{-}4S]^{1+}$cluster of the nitrogenase Fe-protein depending on ATP or ADP binding. The nucleotide binding on the Fe-protein causes the conformational change, which could switch the electronic ground state of the $[4Fe\text{-}4S]^{1+}$cluster. Although the sequence homology does not show any relationship, the activator has a similar protein structure, probably changing the conformation by nucleotide binding. Indeed $S$ = 1/2 signals were induced in the presence of ATP and dehydratase, but EPR signals could not certainly be assigned to the $[4Fe\text{-}4S]^{1+}$cluster of the activator or of the dehydratase. Further studies by Mössbauer spectroscopy will have to clarify this aspect.

### 4. (*R*)-2-Hydroxyisocaproyl-CoA dehydratase

The results described in this work clearly show that the *hadIBC*-genes of *C. difficile* encode a novel 2-hydroxyacyl-CoA dehydratase (HadBC) and its activator (HadI), probably specific for the dehydration of (*R*)-2-hydroxyisocaproyl-CoA to isocaprenoyl-CoA, but besides the *S*-isomer no other substrate was tested yet. Since the known enzymatic eliminations of water from (*R*)-2-hydroxyacyl-CoA to (*E*)-2-enoyl-CoA, (*R*)-2-hydroxyglutaryl-CoA to (*E*)-glutaconyl-CoA (14), (*R*)-lactyl-CoA to acryloyl-CoA (12) and (*R*)-phenyllactyl-CoA to (*E*)-cinnamoyl-CoA (71) all occur in a *syn*-fashion, we assume that this will also be the case for (*R*)-2-hydroxyisocaproyl-CoA to (*E*)-2-isocaprenoyl-CoA, which, however, remains to be determined. The inability to measure the hydration of the chemically synthesised (*E*)-2-isocaprenoyl-CoA could be either due to the unfavourable equilibrium or due to the *Z*-isomer being the correct substrate. It has been shown that 2-hydroxyglutaryl-CoA dehydratase catalysed the reverse reaction. The conditions, however, were different; this experiment was performed in the cell-free extract using (*E*)-glutaconate in the presence of acetyl-CoA as substrate and the formed (*R*)-2-hydroxyglutarate was determined enzymatically (14).

The requirement of activator (HadI), dehydratase (HadBC), ATP, $Mg^{2+}$, and dithionite for activity of 2-hydroxyisocaproyl-CoA dehydratase indicates that this enzyme acts by the same mechanism as that proposed for 2-hydroxyacyl-CoA dehydratase (18, 55). The reduced activator transfers one electron to the dehydratase concomitant with hydrolysis of ATP. Although the stoichiometry of 1 or 2 ATP/electron remains to be determined, the homodimeric structure of the activator with one [4Fe-4S] cluster strongly suggests 2 ATP/electron as observed with nitrogenase (55). The reduced dehydratase transfers the electron further to the substrate to generate the ketyl radical anion I, which expels the adjacent

hydroxyl group. The formed enoxy radical can now be deprotonated at the β-position to the product-related ketyl radical anion II, which is oxidised to isocaprenoyl-CoA by the next incoming substrate 2-hydroxyisocaproyl-CoA, whereby the electron is recycled (Fig. 34). It has been calculated that the extremely high p*K* of the β-protons of 2-hydroxyisocaproyl-CoA (ca. 40), is lowered by 26 units to p*K* = 14 in the enoxy radical (82). This fairly low p*K* could be even further decreased to about 7 by hydrogen bonds from backbone amides of the enzyme to the carbonyl oxygen and thus gets into the range of the p*K* of carboxylates or imidazolyl residues of the enzyme (34).

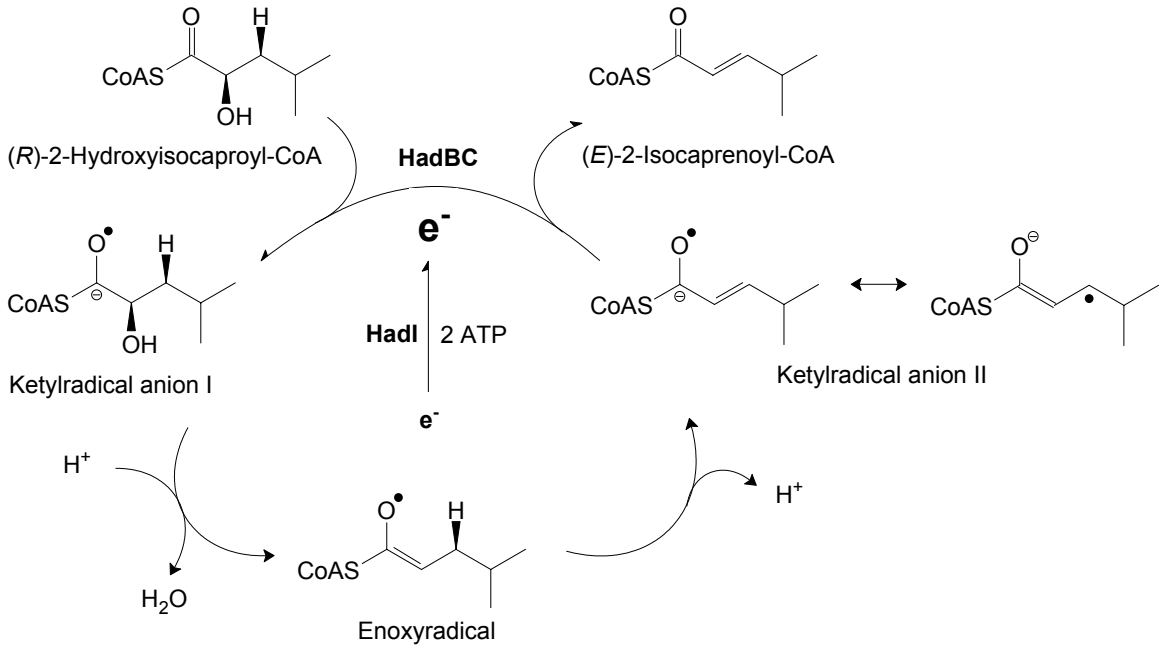



**Fig. 34. Proposed mechanism of dehydration from (*R*)-2-hydroxyisocaproyl-CoA to (*E*)-2-isocaprenoyl-CoA.** A mesomeric structure of the radical which could be that detected by EPR spectroscopy is shown in the box.

For the first time, three experiments probed that the full activation of the dehydratase needs only catalytic amounts of activator since the electron of the reduced dehydratase can be recycled. 1) The maximum dehydratase activity could be obtained by substoichiometric amounts of activator (HadI/HadBC = 1:10) and a pre-incubation time. This important finding was due to the development of a direct spectrophotometric assay of the dehydratase and to the improved stability of HadI by the addition of serum albumin and dithiothreitol. In previous work an assay with six auxiliary enzymes was used and hence gave only qualitative data (13, 56). 2) The activated reduced dehydratase could be separated from the activator and retained its activity for almost $10^4$ turnovers. These experiments clearly demonstrated that ATP and $Mg^{2+}$ are only required for activation and ATP is not used to phosphorylate the hydroxyl

group in order to facilitate the elimination as suggested in the early work on lactyl-CoA dehydratase (2). The authors R. L. Anderson and W. A. Wood already addressed the energetic enigma if each dehydration would require one ATP, this means that, in the case of *C. difficile*, generation of one ATP by substrate-level phosphorylation consumes two ATP (Fig. 5 and eqn 1). Therefore it was proposed that one ATP must be sufficient to activate the dehydratase for at least 100 turnovers (55), which has now been experimentally verified. The activated enzyme may become inactivated simply by one-electron oxidation with traces of oxygen or by a second electron transfer to a radical intermediate, which would result in isocaproyl-CoA rather than isocaprenoyl-CoA as product, but according to the measured turnover only one in $10^4$. The inactivation by substrate is reminiscent of coenzyme $B_{12}$-dependent mutases. The suicide inactivation of β-lysine 5,6-aminomutase is caused by the substrate-induced one electron transfer from cob(II)alamin to the 5′-deoxyadenosine radical resulting in the inactive pair of cob(III)alamin and 5′-deoxyadenosine (87). 3) Another experimental evidence was obtained from the active complex of the dehydratase and the activator. In case of nitrogenase, the inhibition of the activity was observed in presence of $AlF_4^-$ and MgATP (ADP-$AlF_4^-$ a transition state of ATP hydrolysis (21)) which induce the tight complex of the Fe-and MoFe-protein (30, 72). The Fe-protein plays a role in electron transfer to the main enzyme MoFe-protein which is concomitant with the hydrolysis of 2 ATP/electron. When the reduction of the MoFe-protein has occurred, the transient complex dissociates again for further electron transfers. The reduction of 1 mol dinitrogen to 2 mol ammonia needs 8 electrons and 16 MgATPs requiring 7 times dissociation. The tight complex can only transfer once an electron and then inhibits nitrogenase. In the dehydratase system a one-electron transfer is sufficient for catalysis explaining the lack of inhibition by $AlF^{4-}$, furthermore, the active complex could be purified. In previous publications on 2-hydroxyglutaryl-CoA dehydratase (43), the terms component A and component D were used for the activator and the dehydratase, respectively, implicating that only both components together are able to form an active enzyme. The important result that even in the complete absence of activator the reduced 2-hydroxyisocaproyl-CoA dehydratase is catalytically active has consequences for the nomenclature. From a recently submitted paper (54) onwards we decided to call component A just *activator* or archerase (55) and component D just *dehydratase*.

The result in this work most important for the mechanism is the detection of a substrate-derived organic radical by EPR spectroscopy. An organic radical has never been detected in any known 2-hydroxylacyl-CoA dehydratase system, although a radical mechanism was proposed already 20 years ago (79). The detected organic radical is substrate-

derived, which was proved with labelled substrates, however, a series of other labelled substrates (Fig.35) would be needed to define the ketyl radical anion II as the EPR active species (Fig. 34 in box). As described in the introduction a radical generation in enzyme reactions needs cofactors such as adenosylcobalamin (91, 92), *S*-adenosylmethionine (37, 38) or diferric-tyrosyl radical (84, 85).

The 2-hydroxyisocaproyl-CoA dehydratase might suggest a new way of an organic radical generation since no known cofactor was detected and needed for the dehydration. The only possible cofactor is the putative [4Fe-4S] cluster as shown by the induction of EPR $S = 1/2$ signals during the reduction of the dehydratase, which has to be confirmed by Mössbauer spectroscopy after separation of the activated dehydratase separated from the activator. Furthermore the substrate-derived radical signal was shown to be relaxed by increasing the microwave power implicating a possible role of the [4Fe-4S] cluster in radical generation.

Another important result of this work is the finding that 2-hydroxyisocaproyl-CoA dehydratase, the 2-hydroxyacyl-CoA dehydratase with highest ever-observed activity (up to 220 $s^{-1}$), contains no molybdenum and hardly any flavin. Therefore these two cofactors seem not to play any role in the other 2-hydroxyacyl-CoA dehydratases. Molybdenum may be an impurity that could not be separated from 2-hydroxyglutaryl-CoA dehydratase and flavin (FMN and/or riboflavin) could bind fortuitously. Interestingly, crude preparations of 2-hydroxyisocaproyl-CoA dehydratase obtained from *C. difficile* do contain Mo, which is lost during further purification. The only prosthetic group of the dehydratase, which after activation could carry the catalytic electron, is a putative iron-sulfur cluster, whose structure remains to be determined by spectroscopic and crystallographic methods. This cluster must have a very negative redox potential ($E_0' < -600$ mV), since no activity could be observed after treatment of the inactive dehydratase with excess dithionite or Ti(III)citrate in the absence of the activator HadI and ATP. On the other hand this cluster cannot be very unusual, since it is synthesised by enzymes not only present in *C. difficile* but also in *E. coli* (36) as shown by the functional heterologous expression of the *hadBC* genes. Hence, isocaproyl-CoA dehydratase and its activator appear as simple iron-sulfur proteins without any special cofactors or rare elements. Owing to this simplicity, one may conclude that 2-hydroxyacyl-CoA dehydratases have evolved very early during the emergence of life (93), probably with an unknown biosynthetic anabolic rather than catabolic function. Even though no cofactor for radical formation is needed in 2-hydroxyacyl-CoA dehydration, one could suggest an alternative dehydration mechanism as known from AdoCbl-dependent diol dehydratase catalysing the conversion of 1,2-diols to corresponding aldehyde (91). The very low primary

deuterium isotope effects ($k_H/k_D$ = 1.3 – 1.6) with labelled substrates excluded this possibility, while a significant primary isotope effect ($k_H/k_D$ > 3) was observed in the mechanism of diol dehydratase because the breaking C-H bond is the rate-limiting step.

## 5. Outlook

The crystal structure of the dehydratase and the tight complex will reveal and confirm a lot of points which should be verified. The crystallisation of proteins for the structural analysis is an ongoing project in cooperation with Dr. Holger Dobbek and Dr. Berta M. Martin in the Universität Bayreuth. Another key experiment, Mössbauer spectroscopy will be done to clarify electron transfer from activator to dehydratase and further to the substrate. At the same time EPR spectroscopy with other labelled substrates (Fig. 35) are needed to define the structure of the ketyl radical anion. I would like to close discussion with the scheme for the preparation of labelled substrates applying the enzymes involved in the leucine biosynthesis (discussion with Dr. Antonio J. Pierik and Dr. Daniel Darley) (19, 59) (Fig. 35). Using commercially available compounds (2-keto[3-$^2$H]isovalerate for **1**, [2-$^{13}$C]acetate for **2** or 2-keto[2-$^{13}$C]isovalerate for **3** in Fig. 35), labelled 2-ketoisocaproates can be synthesised and then converted to (*R*)-2-hydroxyisocaproate by (*R*)-2-hydroxyisocaproate dehydrogenase (LdhA) as described in Materials and Methods.

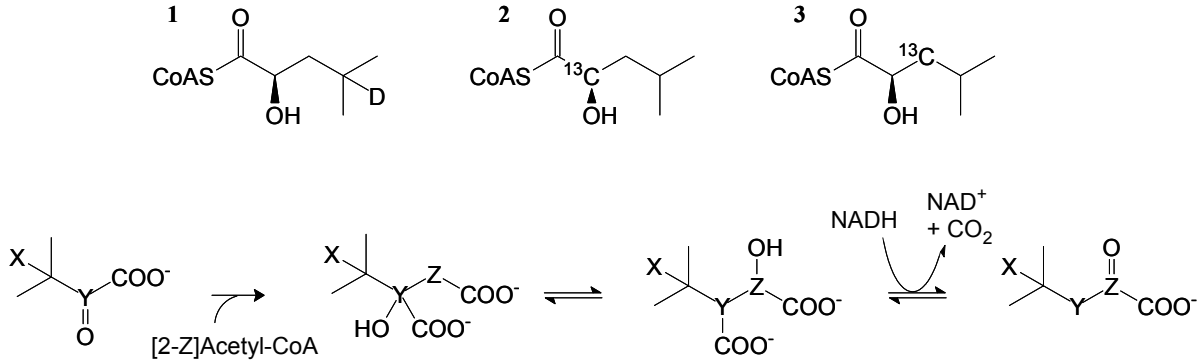



**Fig. 35. Labelled substrates for the determination of ketyl radical anion and the scheme of the synthesis.** α-Isopropylmalate synthase, isomerase and β-isopropylmalate dehydrogenase (decarboxylase) will be involved in the synthesis. X = H or $^2$H, Y and Z = C or $^{13}$C.

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