

**Substrates and mechanism of
2-hydroxyglutaryl-CoA-dehydratase from
*Clostridium symbiosum***

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Dedicated to all students, teachers
and practitioners of Science.

**We are what we think.
All that we are, arises with our thoughts.
With our thoughts, we make the world.
The Buddha.**

Ein Teil der im Rahmen dieser Dissertation erzielten Ergebnisse werden in folgender Publikation veröffentlicht:

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Abbreviations

AHT	Anhydrotetracycline
DMSO	Dimethyl sulfoxide
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EPR	Electron Paramagnetic Resonance
ESI-MS	Electro Spray Ionisation mass spectrometry
FAD	Flavin Adenine Dinucleotide
FMN	Riboflavin-5'-phosphate
FPLC	Fast Protein Liquid Chromatography
β-IPTG	beta-Isopropyl thiogalactoside
MALDI-TOF	Matrix-assisted laser desorption ionisation – time of flight (mass spectrometry)
Mops	4-Morpholinepropanesulfonic acid
NMR	Nuclear Magnetic Resonance
OD	Optical Density
SDS	Sodium dodecylsulfate
TEMED	N, N, N', N'- Tetraethylethylenediamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Tris	2-Amino-2-(hydroxymethyl)-1, 3-propanediol
UV-vis	Ultraviolet visible

Zusammenfassung

Muconyl-CoA, 2-Hydroxyadipoyl-CoA, Oxalocrotonyl-CoA und Butynedioyl-CoA wurden als neue Substrate für die (*R*)-2-Hydroxyglutaryl-CoA-Dehydratase aus *Clostridium symbiosum* synthetisiert und charakterisiert. Die Spezifität des Enzyms für diese Substrate wurde bestimmt und die Reaktionsprodukte mit MALDI-TOF-Massenspektrometrie nachgewiesen. Als Inhibitor der Dehydrataseaktivität ($K_i = 0.069$ mM) wurde 2,2-Difluoroglutaryl-CoA synthetisiert und charakterisiert.

Die in früheren Untersuchungen gezeigte Inaktivierung der Dehydratase durch Metronidazol ist wahrscheinlich auf die Zerstörung des Eisen-Schwefel-Clusters des Aktivators zurückzuführen. Der Aktivator wird als akzesorisches Enzym für den Start der Dehydratasereaktion benötigt.

Weiterhin wurde für die Lactyl-CoA-Dehydratase aus *Clostridium propionicum* ein Aktivitätstest entwickelt und das Enzym bis zur Homogenität gereinigt. Eine Kombination aus kinetischen Experimenten mit (*R*)-2-Hydroxyglutaryl-CoA-Dehydratase und Lactyl-CoA-Dehydratase in Gegenwart ihrer natürlichen Substrate und theoretischer Kalkulationen zeigt, dass die chemische Struktur der 2-Hydroxyacyl-CoA-Esters einen großen Einfluss auf das Gleichgewicht der Umwandlung zum 2-Enoyl-CoA hat.

In dieser Arbeit gelang es, zwei neue Substrate (Oxalocrotonat und 2-Hydroxyadipat) und einen kompetitiven Inhibitor (2,2-Difluoroglutarat, $K_i = 0.62$ mM) der (*R*)-2-Hydroxyglutarat-Dehydrogenase aus *Acidaminococcus fermentans* zu identifizieren. Die biochemischen Beobachtungen konnten durch die Modellierung dieser Substanzen in das aktive Zentrum des Enzyms unterstützt werden.

Summary

Muconyl-CoA, 2-hydroxyadipoyl-CoA, oxalocrotonyl-CoA and butynedioyl-CoA were synthesised and characterised as substrates of the (*R*)-2-hydroxyglutaryl-CoA dehydratase from *Clostridium symbiosum*. The specificity of the enzyme for these substrates were determined and the reaction products identified by MALDI-TOF mass spectrometry. 2,2-Difluoroglutaryl-CoA was synthesised and characterised as an inhibitor of the dehydratase activity ($K_i = 0.069$ mM).

It could be shown that the inhibition of the dehydratase by metronidazole observed by earlier investigators was most likely due to the destruction of the iron-sulfur cluster of the activator (which is an accessory enzyme required to start the dehydratase catalysis).

Further, lactyl-CoA dehydratase from *Clostridium propionicum* was assayed spectrophotometrically and purified to apparent homogeneity. A combination of kinetic experiments performed with (*R*)-2-hydroxyglutaryl-CoA dehydratase and lactyl-CoA dehydratase and their respective substrates, and theoretical calculations showed that the chemical structure of the 2-hydroxyacyl-CoA had a large effect on the equilibrium constant of its conversion to 2-enoyl-CoA.

Finally, two new substrates (oxalocrotonate and 2-hydroxyadipate), and a competitive inhibitor (2,2-difluoroglutarate, $K_i = 0.62$ mM) of the (*R*)-2-hydroxyglutarate dehydrogenase from *Acidaminococcus fermentans* were characterised. Modelling of these compounds into the active site of this enzyme supported the biochemical observations.

Introduction

1. Anaerobic food chain and phylogeny of clostridia

Bacteria of the orders Clostridiales (Firmicutes, phylum Bacteria XIII) and Fusobacteriales (Fusobacteria, phylum Bacteria XXI; Garrity 2001) are able to ferment amino acids (Barker, 1961). The phylogenetic position of these bacteria is indicated in fig 1. A large, heterogeneous group of Gram positive, anaerobic, spore-forming bacteria are presently grouped together in the genus *Clostridium* (Johnson and Francis, 1975; Cato and Stackebrandt, 1989; Lawson, et al., 1993, Summanen, 1993; Collins, et al., 1994) More than 100 clostridial 16S rRNA gene sequences have now been determined and the resulting data have provided the basis for a taxonomic revision of the clostridia (Collins, et al., 1994).

These organisms occur in soil, sewage sludge, marine and freshwater sediments and in the gastrointestinal tract of animals. In these anoxic environments, the bacteria participate in the anaerobic food chain by which polymers such as proteins are finally degraded to methane and carbon dioxide. Anaerobic degradation of carbohydrates and proteins starts with the hydrolysis of these macromolecules by the action of exogenous enzymes to sugars and small peptides and single amino acids respectively. These are then usually fermented by bacteria in the orders *Clostridiales* and *Fusogenes*. Among these, the solvent producing and saccharolytic species are described elsewhere (Stern and Bambers, 1966; Stern, et al., 1966; Dorner and Schink, 1990; Keis, et al., 1995, Valentin, et al., 2000; Seedorf, et al., 2008). Clostridia also ferment other types of molecules like purines (Barker, 1942; Rabinowitz and Barker, 1956) or organic acids (van der Wielen, 2002) which will not be discussed further. This work pertains to the organisms fermenting amino acids only. Methanogenesis from acetate, molecular hydrogen and carbon dioxide is carried by different archaea. The whole food chain is depicted in fig 2.

Acetogenic organisms use CO₂ and H₂ for the synthesis of acetate, whereas syntrophic bacteria oxidise the short chain fatty acids to acetate, CO₂ and H₂. The latter process is thermodynamically possible only if methanogenic archaea keep the partial pressure of H₂ at very low values, by reducing CO₂ to methane.

Extensive literature is available on various aspects of *Clostridium symbiosum* (Buckel and Semmler, 1983; Brunhuber and Blanchard, 1994; Hans, et al., 1999; Elsayed and Zhang, 2004; Decousser, et al., 2007; Hamza and Engel, 2008), *Acidaminococcus fermentans* (Rogosa, 1969; Buckel, et al., 1981; Schweiger, et al., 1987; Rohde, et al., 1988; Dutscho, et al., 1989; Bendrat, et al., 1993, Müller and Buckel, 1995; Härtl and Buckel, 1996; Selmer and Buckel, 1999; Hans, et al., 2000; Hans and Buckel, 2000; Locher, et al., 2001; Thamer, et al., 2003; Hans, et al., 2008), *Clostridium propionicum* (Cardon and Barker, 1946; Barker

and Wiken, 1948; Johns, 1952; Leaver, et al., 1955; Schweiger and Buckel, 1984, 1985; Kuchta and Abeles, 1985; Chartrain and Zeikus, 1986; Janssen, 1991; Hofmeister and Buckel, 1992; Hofmeister, et al., 1993; Jossek and Steinbuchel, 1998; Zinecker, et al., 1998; Valentin, et al., 2000, Seeliger, et al., 2002; Selmer, et al., 2002; Hetzel, et al., 2003; Herrmann, et al., 2005), enzymes from which are the subject of this work (fig. 3) and the related *Fusobacterium nucleatum* (Loesche and Gibbons, 1968; Dzink and Socransky, 1990; Klees, et al., 1992; Ramezani, et al., 1999; Karpathy, et al., 2007). However this work only discusses certain amino acid fermentations.

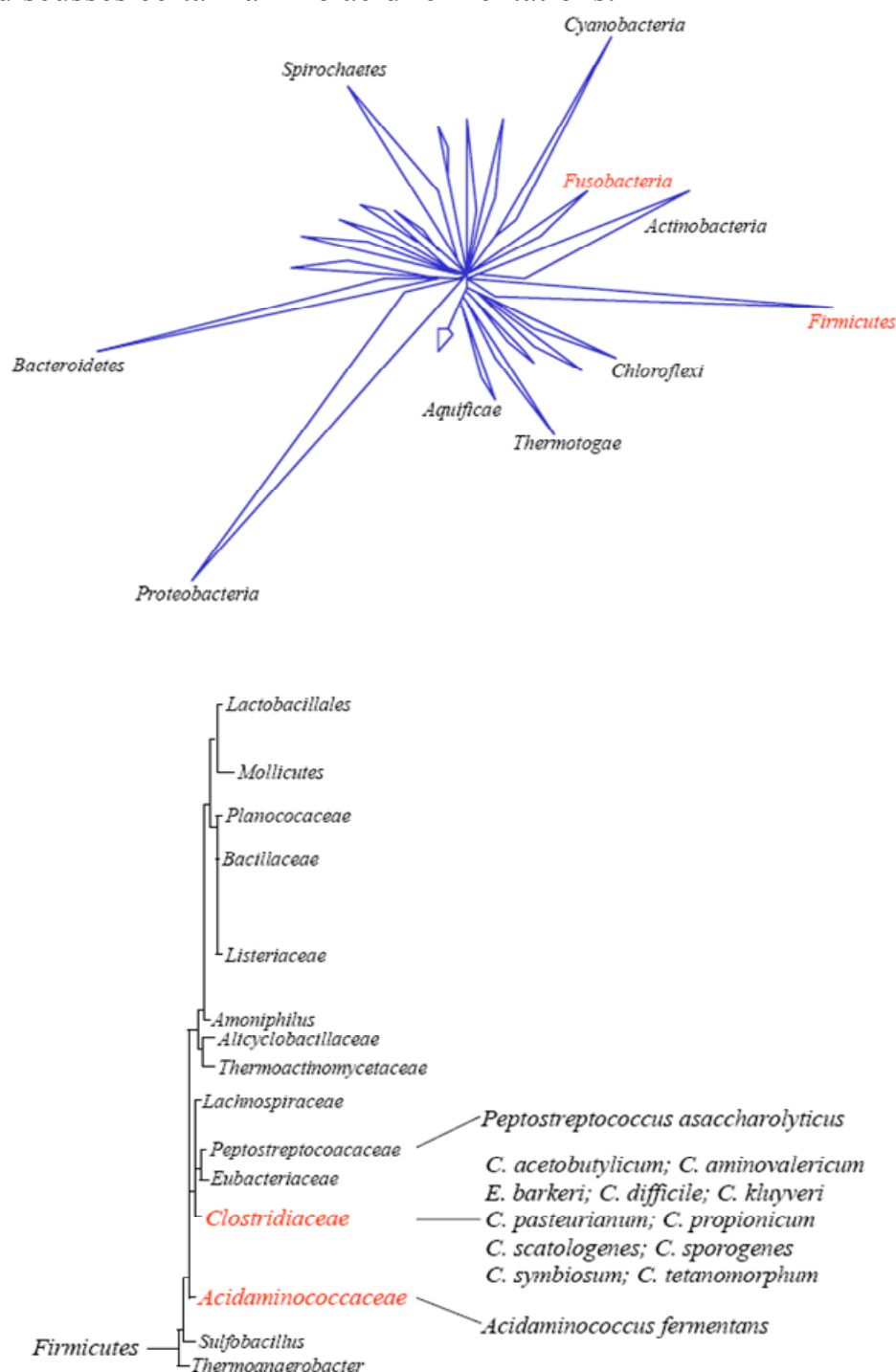


Fig.1. Phylogenetic position of clostridia and related organisms

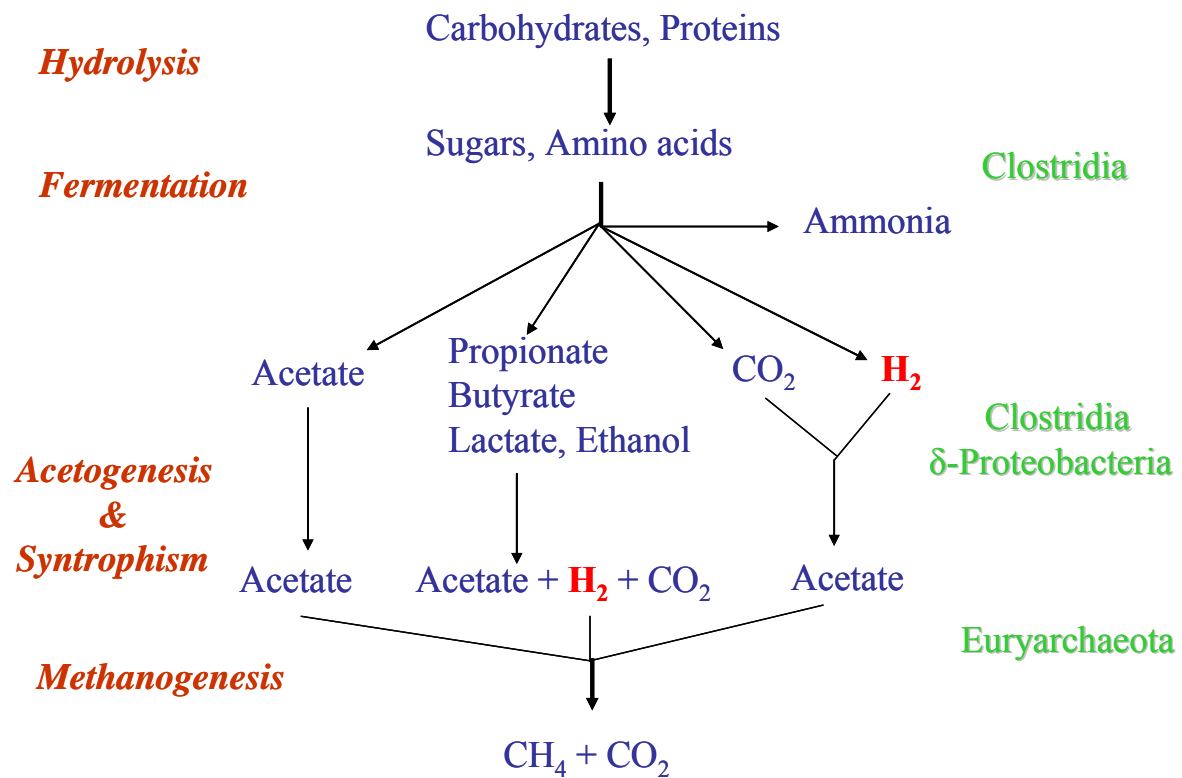


Fig. 2. Anaerobic food chain – fermentation of sugars and aminoacids

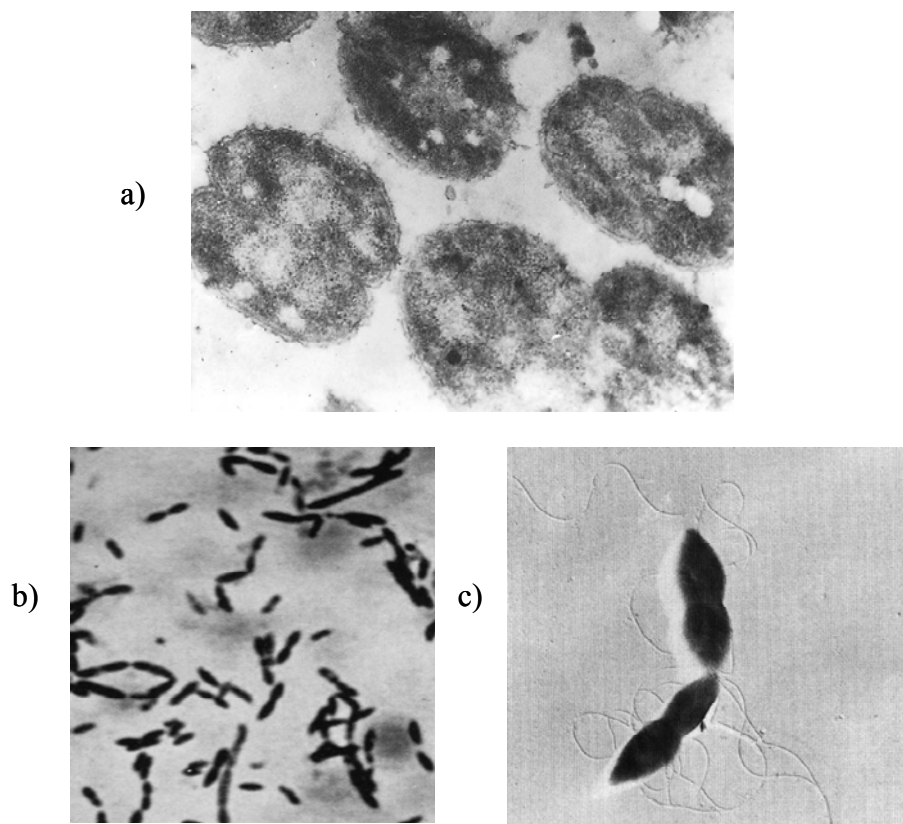


Fig 3. a) and c) electron-micrographs of *A. fermentans* and *C. symbiosum* , respectively; b) light microscope picture of *C. propionicum*.

2. Fermentation of amino acids

The biochemically most versatile organisms of the anaerobic food chain described above are the fermentative bacteria since, for every one of the 20 proteinogenic amino acids, at least one pathway is known (Barker, 1961, 1981). Twelve of the twenty proteinogenous amino acids can be fermented by different clostridia and related organisms, via their 2-hydroxyacids (fig 4; Buckel, 1999; Buckel, et al., 2004) to various products like acetate, propionate, butyrate, other short chain fatty acids, arylacetates, arylpropionate, ammonia, carbon dioxide and molecular hydrogen.

The oxidative branches of the 2-hydroxyacid pathways are similar to those of aerobic organisms, but the reductive branches contain a unique class of dehydratases. These are the key enzymes in their respective pathways and all of them consist of two components – component D or dehydratase; and component A or “activator”. A summary of the gene arrangements of the dehydratases and their accessory enzymes in various organisms is shown in fig 5.

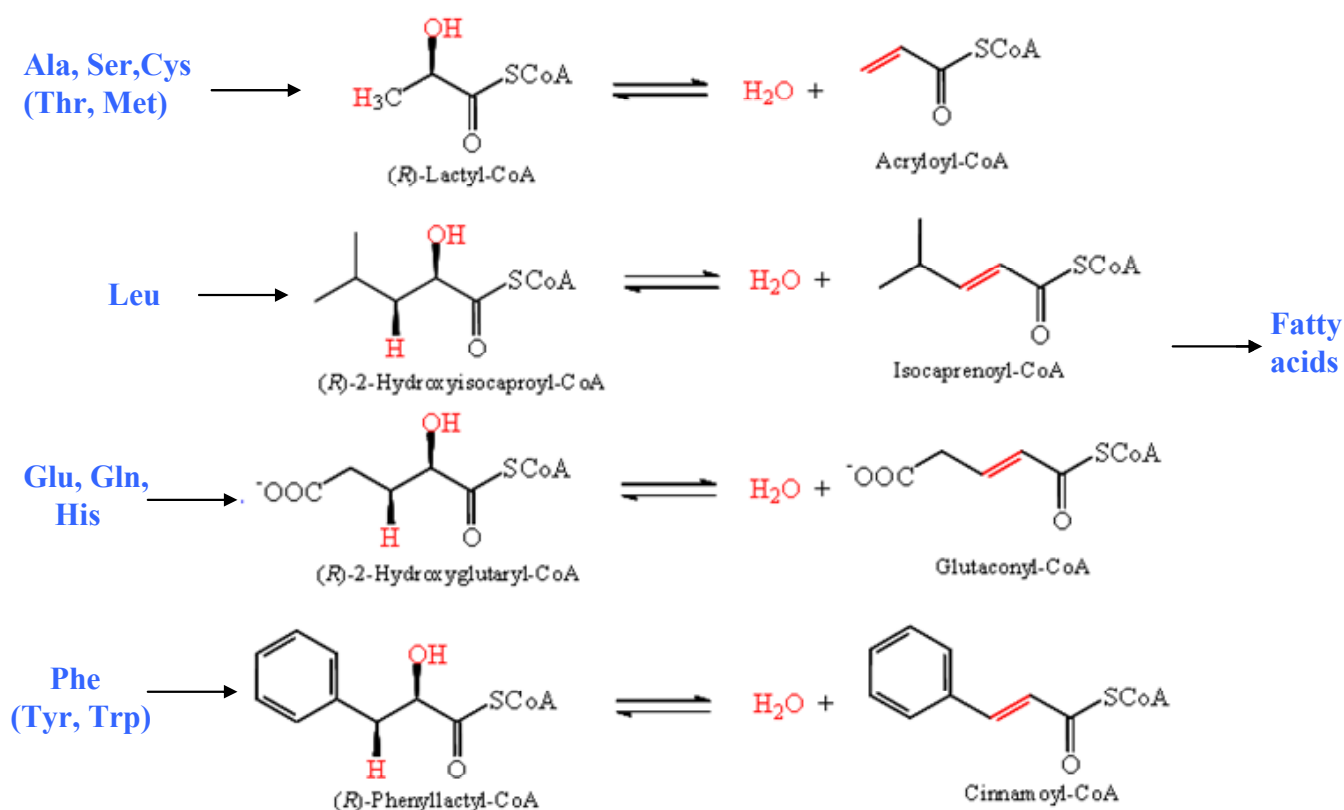


Fig. 4. Conversion of amino acids to fatty acids via dehydration of their corresponding (R)-2-hydroxyacyl-CoAs to enoyl-CoAs.

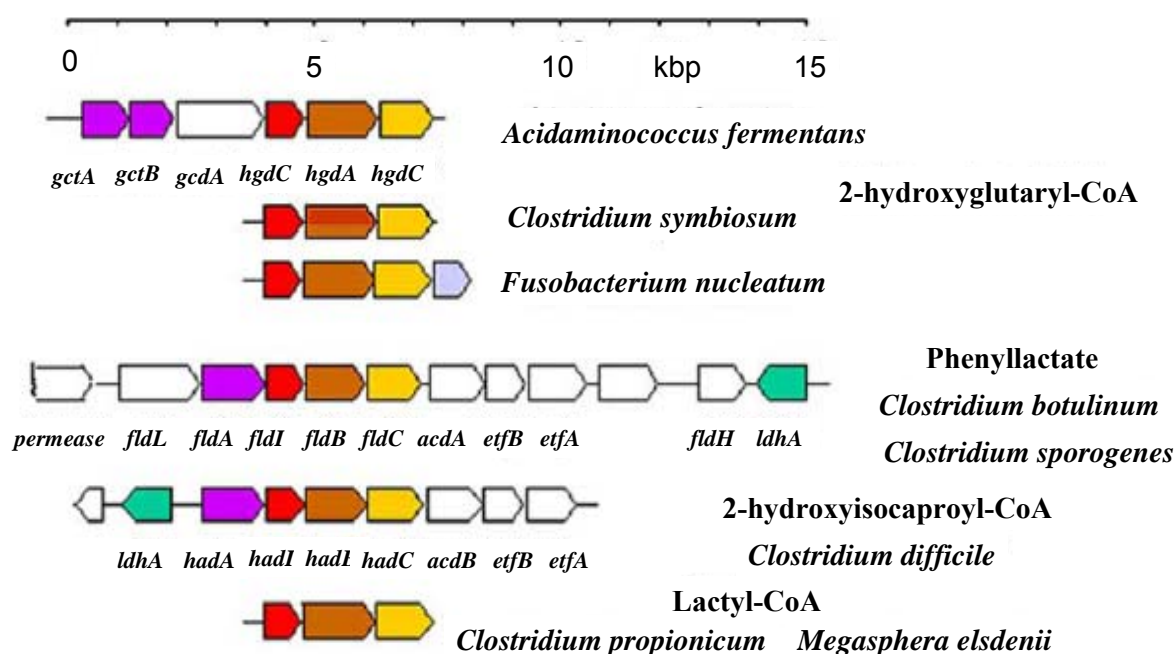


Fig 5. Genes encoding 2-hydroxyacyl-CoA dehydratases and accessory enzymes in different organisms; Colour codes: Magenta = CoA transferase; Red = Activator of dehydratase; Yellow, Brown and Blue = Dehydratase; Green = 2-Hydroxyacid dehydrogenase (NAD-dependent).

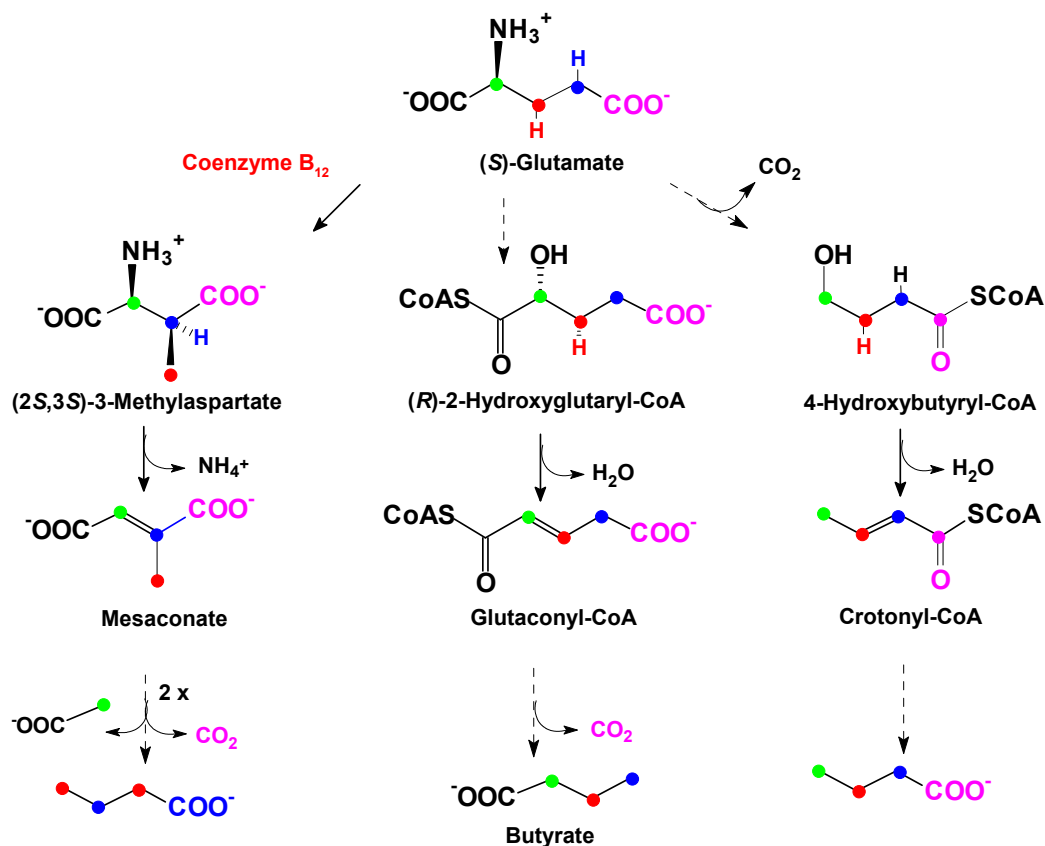


Fig 6. Three different clostridial fermentations leading from (S)-glutamate to butyrate as elucidated by the use of isotopically labelled glutamate.

2.1 Fermentation of glutamate

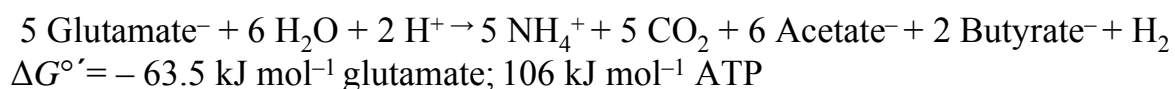
(*S*)-glutamate may not only be degraded via (*R*)-2-hydroxyglutarate, but also 4-hydroxybutyrate and 5-aminovalerate, as well as via coenzyme B₁₂ dependent rearrangement (Buckel, 2001). Figure 6 shows a schematic of the three pathways leading from glutamate to butyrate (Barker, 1961, 1978 and 1981).

2.1.1 The B₁₂ dependent mutase pathways

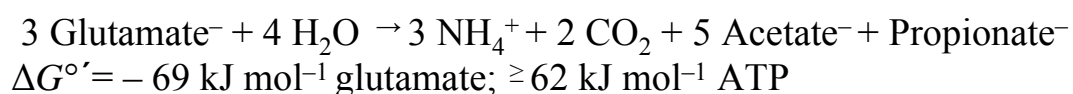
Two pathways proceed to ammonia, acetate and pyruvate via the coenzyme B₁₂-dependent glutamate mutase, which catalyses the re-arrangement of the linear carbon skeleton to that of the branched-chain amino acid (2*S*, 3*S*)-3-methylaspartate. They have been found in Clostridiales isolated from soil or sewage sludge and diverge at the level of pyruvate in their reductive branches.

In the pathway, (*S*)-glutamate is converted via (2*S*, 3*S*)-3-methylaspartate to ammonia, acetate and pyruvate. One butyrate and two CO₂ are formed from two molecules of the 2-oxoacid via acetyl-CoA, with concomitant production of some H₂ and additional acetate, whereby 0.6 ATP per glutamate is conserved via substrate-level phosphorylation (Thauer, et al., 1977; Buckel and Miller 1987; Härtel and Buckel 1996).

Since in most fermentative and respiratory pathways only about 70–80 kJ are required to synthesise 1 mol ATP (Thauer et al. 1977), the additional energy of about 30 kJ mol⁻¹ may be conserved via electrochemical H⁺ or Na⁺ gradients (ΔμH⁺/Na⁺) generated during butyrate synthesis. An additional 0.3 ATP per glutamate may be conserved via electron bifurcation involving the butyryl-CoA dehydrogenase-electron transfer flavoprotein (BCD-ETF) complex and ferredoxin (Herrmann, et al., 2008).



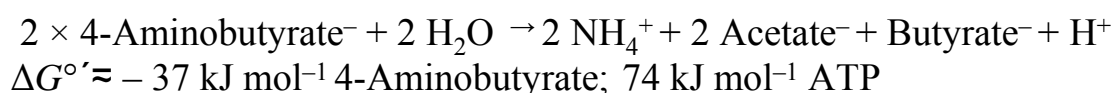
In a variant to this pathway, pyruvate disproportionates to acetate, CO₂ and propionate whereby 2 ATP are conserved via acetyl-CoA and up to 2 × (2/3) ATP are generated via ΔμH⁺ by fumarate respiration in the succinate pathway (Lancaster, et al., 1999) leading from pyruvate to propionate involving the B₁₂ dependent methylmalonyl-CoA mutase.



In addition to *Clostridium tetanomorphum* (Barker 1978), the pathway leading to butyrate has been detected in other members of the Clostridiaceae, like *C. cochlearium*, *C. lentoputrescens*, *C. limosum*, *C. malenominatum* and *C. tetani* (Buckel and Barker, 1974; Buckel 1980). The pathway leading to propionate has been detected in *Selenomonas acidaminophila* (Nanninga, et al., 1987) and more recently in *Barkera propionica* (Janssen, Sievers, Liesack, and Buckel, unpublished), both of which belong to the family Acidaminococcaceae, as well as in *C. pascui* (Elamparithi, J., unpublished).

2.1.2 The 4-aminobutyrate pathway

The 3-methylaspartate pathway and the 2-hydroxyglutarate pathway require only one organism each to perform the whole sequence of reactions. Many aerobic or anaerobic organisms transform the very abundant and versatile amino acid glutamate to other amino acids, which are then fermented by Clostridiales. In order to counterbalance acid stress, *E. coli* decarboxylates glutamate to 4-aminobutyrate, whereby one proton is consumed (Castanie-Cornet, et al., 1999). The γ -amino acid is then fermented by *C. aminobutyricum* via 4-hydroxybutyryl-CoA and crotonyl-CoA to acetate and butyrate (Hardman and Stadtman, 1960). The reversible dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA catalysed by the 4-hydroxybutyryl-CoA dehydratase in *C. aminobutyricum* (Scherf and Buckel, 1993, Müh, et al., 1996, Çinkaya, et al., 1997) is an unusual reaction, comparable to the dehydrations of the 2-hydroxyacyl-CoAs. It represents the key step in the fermentation of 4-aminobutyrate to ammonia, acetate and butyrate. The reaction also occurs in the pathway of succinate reduction to butyrate in *C. kluyveri*. The energy yield is about 37 kJ mol⁻¹ per cycle of fermentation (Buckel, 2001).



2.1.3 The 2-hydroxyglutarate pathway

Organisms living in anoxic niches within humans and animals ferment glutamate by the 2-hydroxyglutarate pathway via (*R*)-2-hydroxyglutaryl-CoA, glutaconyl-CoA and crotonyl-CoA. The latter disproportionates to acetate, butyrate and H₂, the same products as those formed by *C. tetanomorphum* via 3-methylaspartate. In the 2-hydroxyglutarate pathway (Fig. 3, Eq. 1), the extra energy is conserved via $\Delta\mu\text{Na}^+$ generated by decarboxylation of glutaconyl-CoA (Buckel and Semmler, 1983; Buckel, 2001).

The pathway has been found in *Acidaminococcus fermentans* (Acidaminococcaceae), *C. sporosphaeroides*, *C. symbiosum*, *Fusobacterium*

nucleatum (Fusobacteriaceae) and *Peptostreptococcus asaccharolyticus* (Peptostreptococcaceae, Buckel and Barker, 1974; Buckel, 1980). Glutamate is converted to ammonia, CO₂, acetate and butyrate and molecular hydrogen. A detailed view of this pathway is shown below (fig 7).

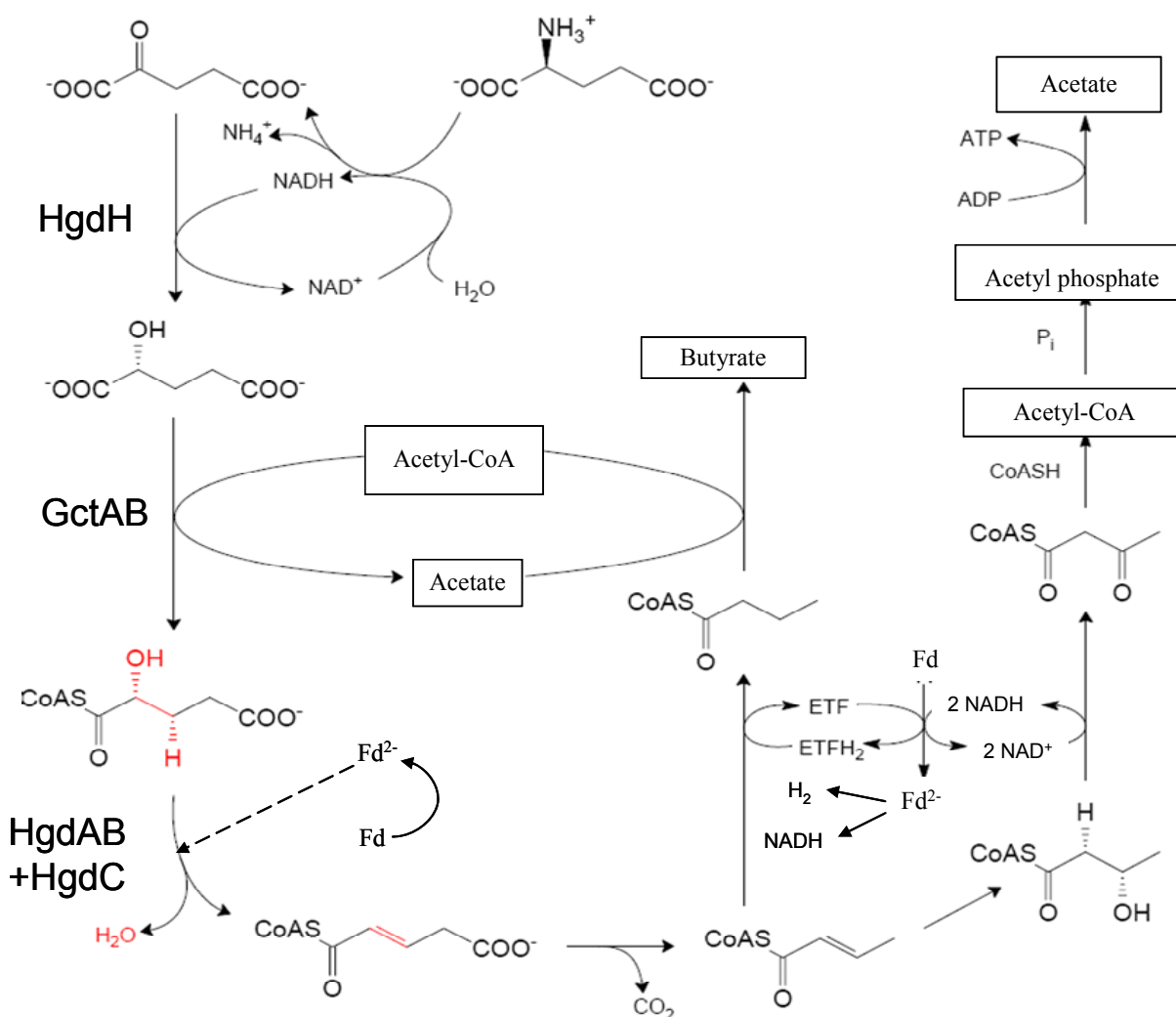
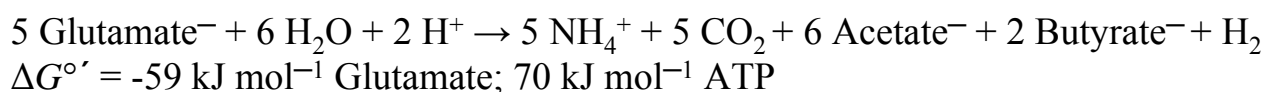


Fig 7. Glutamate fermentation via the 2-hydroxyglutarate pathway, the enzymes HgdAB (2-hydroxyglutaryl-CoA dehydratase), HgdC (activator of the dehydratase), HgdH (2-hydroxyglutarate dehydrogenase) and GctAB (glutaconate-CoA transferase) were used in this work.

The key intermediate is (R)-2-hydroxyglutaryl-CoA, which is dehydrated to glutaconyl-CoA, followed by decarboxylation to crotonyl-CoA (Buckel and Semmler, 1983). The hydroxyglutarate pathway contains an unusual biochemical transformation, the reversible dehydration of (R)-2-hydroxyglutaryl-CoA to (E)-glutaconyl-CoA (Buckel, 1980; Schweiger, et al.,

1987; Hans, et al., 1999; Hans et al., 2000). This transformation is carried out by the (R)-2-hydroxyglutaryl-CoA dehydratase, in the presence of its activator.

Variations of this remarkable enzyme system have been found in *C. symbiosum*, *A. fermentans* and *F. nucleatum*. Component D from *C. symbiosum* contains two $[4\text{Fe-4S}]^{2+}$ clusters instead of the one found in component D of *A. fermentans*, although both components D share 70% sequence identity.

Furthermore, component A from *A. fermentans* is active with component D from *C. symbiosum*, reaching a specific activity of 60 s^{-1} , as compared to 10 s^{-1} with component D from *A. fermentans*. Component A of *C. symbiosum* has a deduced amino acid sequence that shares 73% identity with that from *A. fermentans* (Hans, et al., 1999). The 2-hydroxyglutaryl-CoA dehydratase from *F. nucleatum* contains only one $[4\text{Fe-4S}]^{2+}$ cluster, but is composed of three different subunits (α , β , γ) in equal amounts (Tamannaei, A., Diploma thesis, Philipps University, Marburg); the function of the γ -subunit is not known. Figure 8 represents the gene arrangement of the *C. symbiosum* dehydratase system and some of its properties.

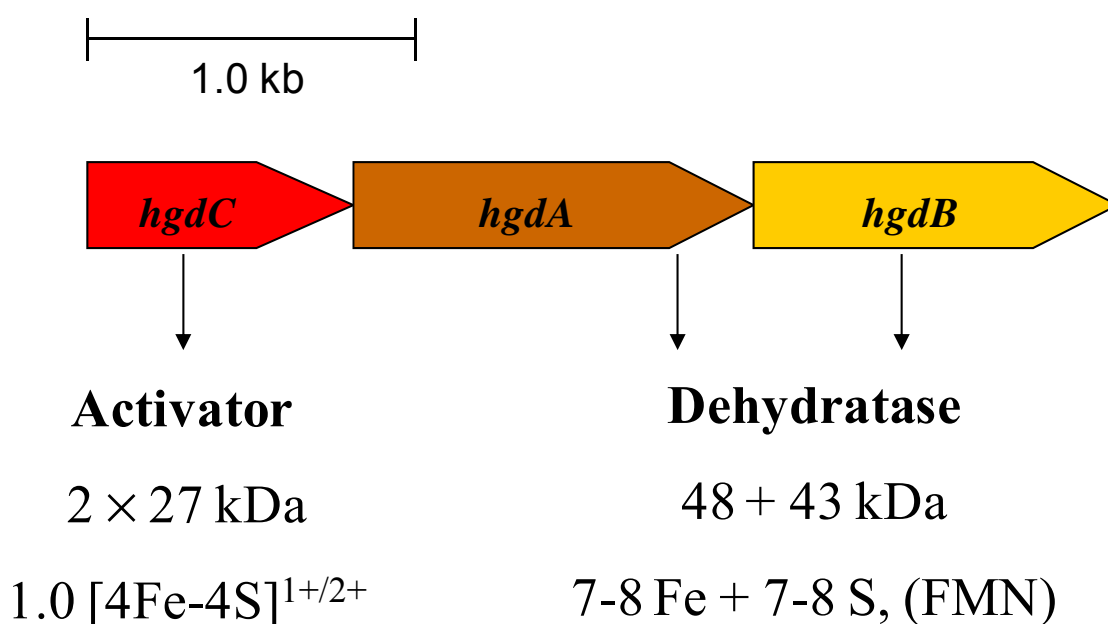


Fig 8. Overview of the (R)-2-hydroxyglutaryl-CoA dehydratase system from *C. symbiosum*.

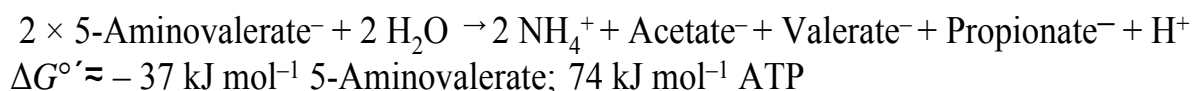
The extremely oxygen-sensitive homodimeric component A (HgdC, $2 \times 27 \text{ kDa}$) contains one $[4\text{Fe-4S}]^{2+}$ cluster between the two subunits and acts as an activator or initiator of dehydration. The moderately oxygen-sensitive heterodimeric component D (HgdAB, $\alpha\beta$, $55 + 45 \text{ kDa}$), the actual dehydratase, also carries one $[4\text{Fe-4S}]^{2+}$ cluster per heterodimer and exhibits one reduced riboflavin 5'-monophosphate as an additional prosthetic group, as well as 0.3

riboflavin and 0.1–0.2 molybdenum (Hetzel, M., PhD thesis, Philipps University, Marburg). Dehydratase activity requires both components A and D, MgCl₂, catalytic amounts of ATP and a reducing agent. *In vivo*, reduced ferredoxin or flavodoxin (Thamer, et al., 2003) and *in vitro* Ti(III) citrate or dithionite serve as reducing agents. The general requirements for dehydratase activity and the overall mechanism are assumed to be the same for all the 2-hydroxyacyl-CoA dehydratases.

2.1.4 The 5-aminovalerate pathway

Under osmotic stress, *Bacillus subtilis* reduces glutamate to the osmoprotective amino acid proline (Bremer and Krämer, 2000) which, in a Stickland-type fermentation, is further reduced to 5-aminovalerate by *C. sporogenes* (Stickland 1935; Barker 1961).

Finally, *C. viride* (Buckel, et al., 1994) ferments 5-aminovalerate via 5-hydroxyvaleryl-CoA and 2-pentenoyl-CoA to ammonia, acetate, propionate and valerate (Barker, et al., 1987). Hence, three different organisms are required for glutamate fermentation via 5-aminovalerate. The fifth pathway is the only one without decarboxylation, since the γ -carboxylate of glutamate is reduced to the amino group of δ -aminovalerate, which then is fermented to acetate, propionate and valerate. The energy gain is similar to the 4-aminobutyrate fermentation (Buckel, 2001).



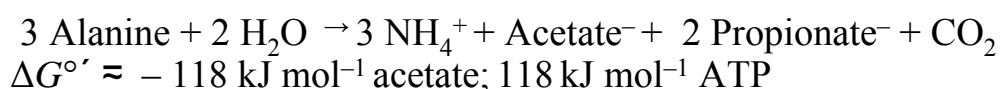
The pathway involves the oxidative dehydration of 5-hydroxyvaleryl-CoA to 2,4-pentadienyl-CoA followed by reduction to 3-pentenyl-CoA and isomerisation to 2-pentenyl-CoA.

2.2 Alanine and phenylalanine fermentation

C. propionicum ferments (*S*)-alanine to ammonia, CO₂, acetate and propionate; and the phenyl derivatives of these acids are obtained with *C. sporogenes* (Elsden, et al., 1976; Dickert, et al., 2000) which uses (*S*)-phenylalanine instead of alanine.

In both pathways, the amino acid is oxidised to the 2-oxoacid by amino-transfer to 2-oxoglutarate, followed by oxidative decarboxylation to acyl-CoA and reduction to (*R*)-lactate or (*R*)-phenyllactate. Both 2-hydroxyacids are then dehydrated in a *syn*-geometry at the CoA-ester level. Similar to (*R*)-2-hydroxyglutarate, (*R*)-lactate is converted to (*R*)-lactyl-CoA, catalysed by propionate CoA-transferase (Schweiger and Buckel, 1984). Component D of the

lactyl-CoA dehydratase from *C. propionicum* most closely resembles that of 2-hydroxyglutaryl-CoA dehydratase from *C. symbiosum*, both of which contain two $[4\text{Fe}-4\text{S}]^{2+}$ clusters, FMN and small amounts of riboflavin. Component A from *C. propionicum* has not been obtained in pure form yet (Kuchta and Abeles 1985; Kuchta, et al., 1986; Hofmeister and Buckel 1992). Acrylyl-CoA, the product of the dehydration of (*R*)-lactyl-CoA, is reduced by NADH to propionyl-CoA. The yellow FAD-containing reductase is composed of three subunits. The *N*-terminal sequence of the α -subunit (45 kDa) resembles that of an acyl-CoA dehydrogenase, whereas the other two subunits seem to be related to an electron-transferring flavoprotein (Hetzel, et al., 2003). The resulting propionyl-CoA is finally converted to propionate by another (*R*)-lactate, catalysed by propionate CoA-transferase. A similar pathway has been detected in *Megasphaera elsdenii*, formerly called *Peptostreptococcus elsdenii* (Tung and Wood, 1975). An enzyme catalysing the whole overall dehydration of (*R*)-phenyllactate to (*E*)-cinnamate (phenylacrylate) has been isolated from *C. sporogenes* (Dickert, et al., 2000).



The energetics for alanine and phenylalanine fermentations are reportedly the same (Buckel, 2001). Shown below is the fermentation scheme for alanine in *C. propionicum*.

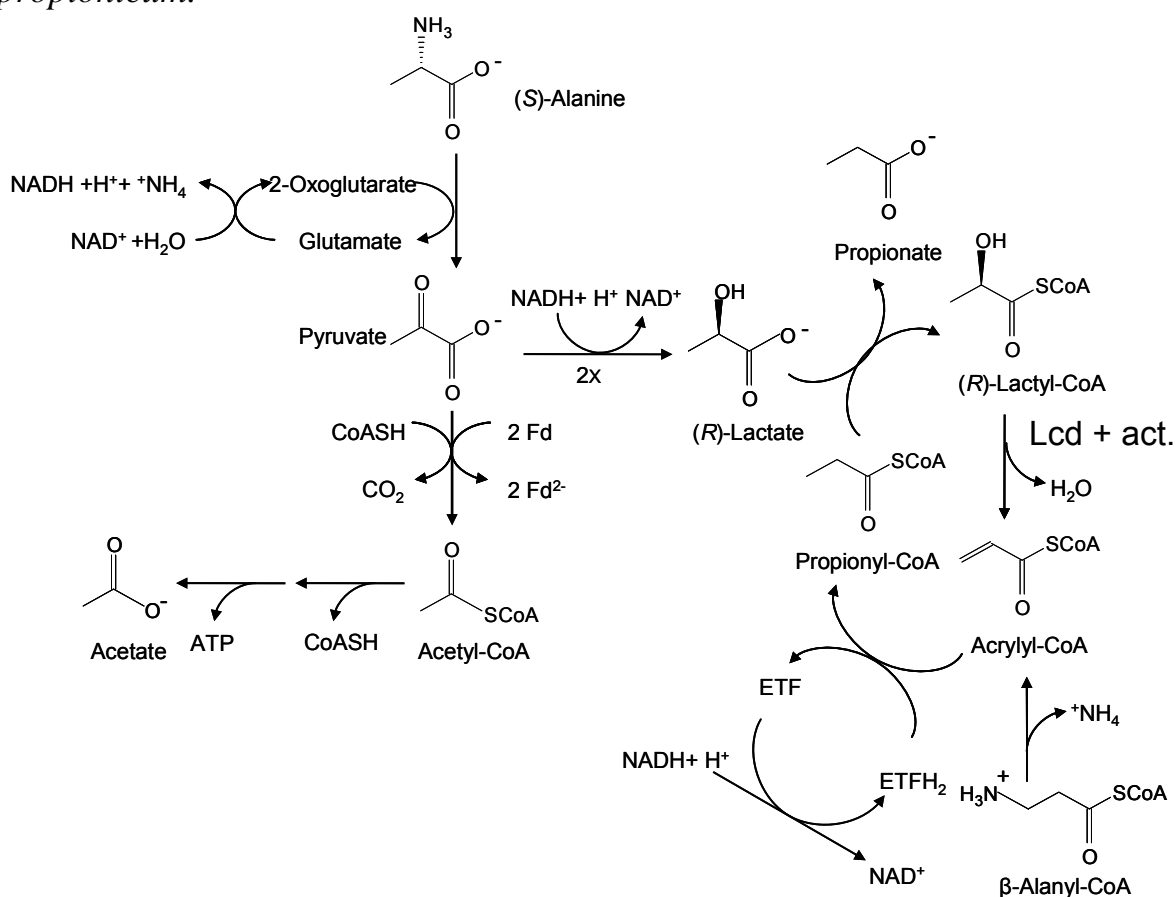


Fig 9. Alanine fermentation pathway in *C. propionicum*

3. Mechanism of the dehydration of 2-hydroxyacyl-CoA to enoyl-CoA

3.1 The chemical challenge of dehydrations at unactivated positions

2-Hydroxyacyl-CoA derivatives are the substrates of the dehydratases of clostridial amino acid fermentation pathways discussed in this work (Kim, et al., 2004; Buckel, et al., 2004). In such cases, the β -hydrogen to be removed during α,β -elimination of water has an approximate $pK_a = 40$, which is too high for an acid/base residue of an enzyme. Therefore, classical acid-base catalysis is not possible.

The requisite activation of this proton is achieved by transient addition of one high-energy electron to the thioester carbonyl forming a ketyl radical. This allows the elimination of the α -hydroxyl group (Buckel and Keese, 1995; Kim, et al., 2008) and lowers the pK_a of the β -hydrogen in the resulting enoxy radical intermediate by at least 26 units (Smith, et al., 2003). The presence of a ketyl radical was demonstrated only in case of the (*R*)-2-hydroxyisocaproyl-CoA dehydration in *C. difficile* (Kim, et al., 2008). The radical generator is the ATP-dependent activator enzyme, which is initially reduced by ferredoxin or dithionite. The proposed mechanism is depicted in fig 10.

A similar ketyl radical mechanism has been proposed for the 4-hydroxybutyryl-CoA dehydration entails the removal of an unactivated proton at the β -position. The mechanism involves one-electron oxidation of the enolate of 4-hydroxybutyryl-CoA by FAD, which makes the β -hydrogen acidic enough for deprotonation to a ketyl radical anion. The now facile elimination of the hydroxyl group leads to the dienoxyl radical which, upon reduction to the dienolate and protonation, yields the product crotonyl-CoA. The addition of crotonyl-CoA to the enzyme from *C. aminobutyricum* raises the redox potentials of the flavin and the $[4Fe-4S]^{2+}$ cluster by about 200 mV, necessary for the transient one-electron oxidation of the substrate.

Under these conditions, the enzyme is able to stabilise flavin semiquinones and the iron–sulfur clusters can be readily reduced to $[4Fe-4S]^+$ by dithionite. By EPR spectroscopy, the reduced clusters exhibit a spin 3/2 signal (Näser, et al., 2005) which is also found with the cluster bridging the two subunits of the activator from *A. fermentans* (Hans, et al., 2000). However, none of the proposed substrate radicals have been detected by EPR spectroscopy.

In the dehydration of 5-hydroxyvaleryl-CoA to 4-pentenyl-CoA in *C. viride*, the unactivated γ -hydrogen has to be removed as a proton. The activation of this hydrogen can be achieved by the introduction of a double bond, through the use of FAD as a prosthetic group (Eikmanns and Buckel, 1991). The green enzyme contains FAD, which forms an unproductive charge-transfer complex with the

proposed intermediate 5-hydroxy-2-pentenoyl-CoA. Hence, the enzyme is bifunctional, being both an acyl-CoA dehydrogenase and a hydroxyacyl-CoA dehydratase. 5-Hydroxy-2-pentenoyl-CoA can be regarded as a vinylogous 3-hydroxyacyl-CoA, whose dehydration is achieved by simple acid–base catalysis. The product, 2,4-pentadienoyl-CoA, is slowly reduced to 4-pentenoyl-CoA, whereby oxidised FAD is regenerated (Eikmanns and Buckel, 1991).

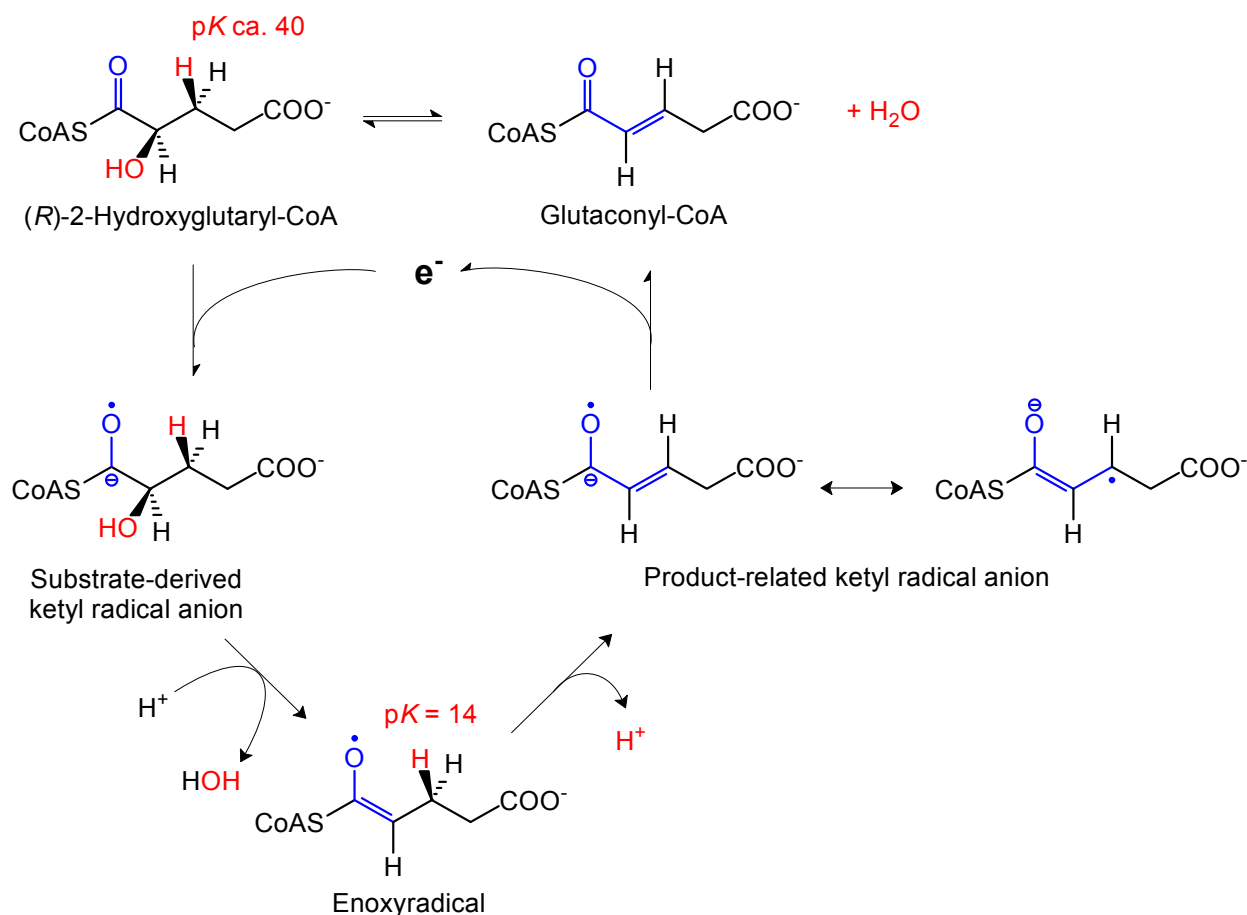


Fig 10. Proposed ketyl radical mechanism for the dehydration of 2-hydroxyacyl-CoA, with (R)-2-hydroxyglutaryl-CoA as example.

3.2 Protein interaction model for the electron transfer

Component A with a C-terminal Strep-tag has been obtained by production of HgdC in *Escherichia coli*. The enzyme was crystallised and its structure determined by X-ray crystallography at 3 Å resolution (Locher, et al., 2001). Each subunit contains a noncovalently bound ADP molecule. Thus the two form a bridge between the two subunits with the [4Fe-4S] cluster in the middle. The angle between the two helices is 105°. The [4Fe-4S]²⁺ cluster is easily reduced to a [4Fe-4S]⁺ cluster, which is required for active dehydratase, whereas the incorporation of a second electron by an excess of Ti(III) citrate leads to the inactive, all-ferrous form [4Fe-4S]⁰ (Hans, et al., 2008). In the oxidised [4Fe-4S]²⁺ state, component A exhibits high ATPase activity (up to 6 s⁻¹), which is completely abolished in the reduced [4Fe-4S]⁺ state. The iron–sulfur cluster is

exposed to the solvent, as shown by destruction of the cluster through chelation with *o*-phenanthroline. By addition of ATP to the reduced or oxidised state, the chelation rate is enhanced eight-fold, demonstrating a higher exposure to the solvent, probably induced by a conformational change through the replacement of ADP by ATP. The ATP- and redox-induced conformational changes were also inferred from chelation experiments on the highly similar activator from *C. difficile* (Kim, et al., 2007).

It was assumed that ATP enlarges the angle between the two helices, from 105° upto 180°, whereby the cluster moves about 13 Å towards component D and thus facilitates the electron transfer. Indeed, the addition of ATP to a mixture of reduced component A and component D results in partial oxidation of component A, as revealed by the resumption of ATP hydrolysis and by analysis of the iron oxidation state by Mössbauer spectroscopy. Complete oxidation of component A was achieved by the further addition of the substrate, (*R*)-2-hydroxyglutaryl-CoA. Until now, the extra electron located either on component D or on the substrate has escaped detection by EPR spectroscopy (Hans, Bill and Buckel, unpublished).

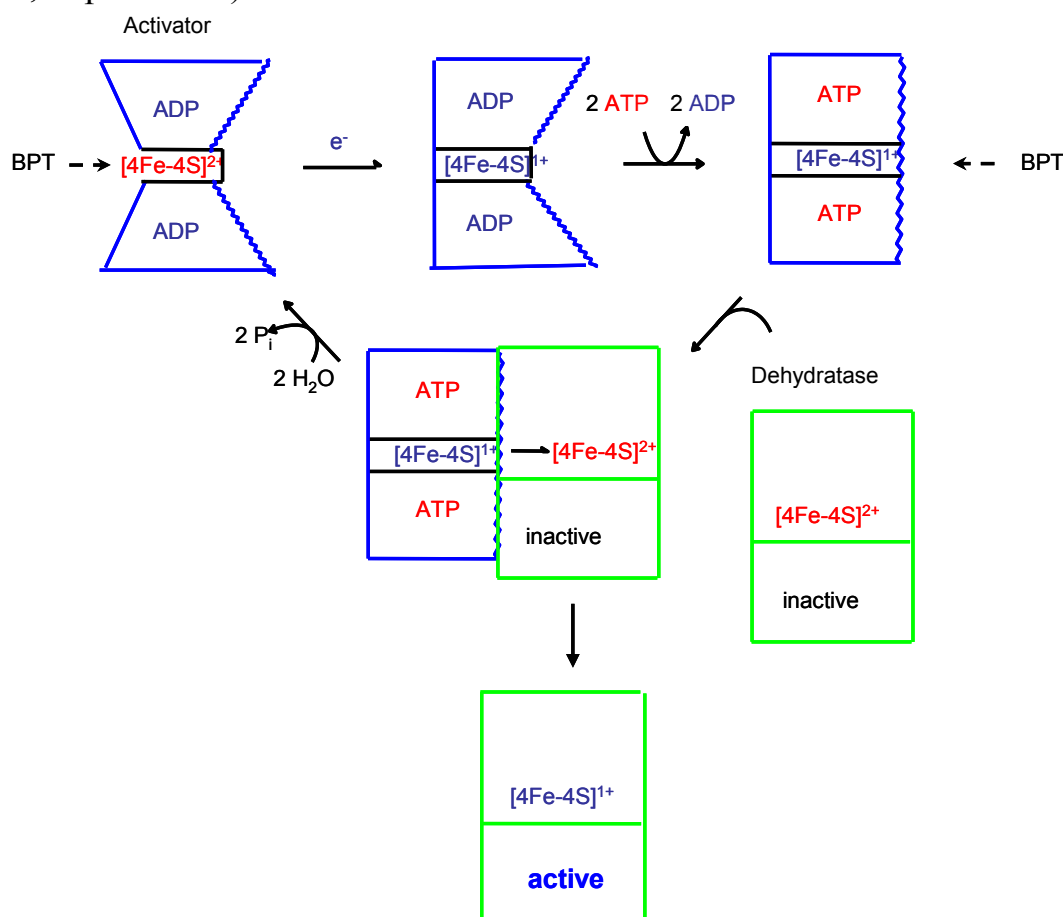


Fig 11. Interaction between the interaction between dehydratase and activator in the dehydration of (*R*)-2-hydroxyacyl-CoA to (*E*)-enoyl-CoA; BPT denotes the iron-chelator bathophenanthroline.

4. (*R*)-2-hydroxyglutarate dehydrogenase (HgdH) from *A. fermentans*

The physiological role of NAD⁺- dependent (*R*)-2-hydroxyglutarate dehydrogenase (HgdH) is the reduction of 2-oxoglutarate to (*R*)-2-hydroxyglutarate coupled with the oxidation of NADH to NAD⁺. This reaction occurs in the second step of the 2-hydroxyglutarate pathway of glutamate fermentation. The reaction equilibrium constant ($K_{eq} = 1.47 \times 10^{-12}$ M) lies on the side of (*R*)-2-hydroxyglutarate production (Buckel and Miller, 1987).

The optimum pH is 8.0 and the specific activity is reported to be 4800 U/mg with a K_m value of 134 μ M at 100 μ M NADH (Bresser, J., unpublished). During catalysis, the 4 *Re* hydrogen (Prelog/Helmchen *Re/Si* system) at the C4N atom of the nicotinamide moiety of the reduced coenzyme (NADH) is transferred as a hydride (Berk, et al., 1996), making HgdH an 'A-side' specific dehydrogenase (Oppenheimer, et al., 1978; Benner, et al., 1982). HgdH belongs to the NAD⁺-dependent D-2-hydroxyacid dehydrogenase protein family (Grant, 1989).

5. Goals of this work

Previous work in the group on the 2-hydroxyacyl-CoA dehydratases was focussed on purification, characterisation and mechanistic aspects of these enzymes. This work was the first to explore the substrate specificity of the 2-hydroxyglutaryl-CoA dehydratase from *C. symbiosum*. Another goal was to purify the lactyl-CoA dehydratase from *C. propionicum*. The third aim was the need to understand the thermodynamic equilibria between (*R*)-2-hydroxyacyl-CoA and 2-enoyl-CoA in different 2-hydroxyacyl-CoA dehydratase systems.

Other objectives included studying the activator of (*R*)-2-hydroxyglutaryl-CoA dehydratase; and the substrate specificity and inhibition of the (*R*)-2-hydroxyglutarate dehydrogenase, which also occur in the 2-hydroxyglutarate pathway of glutamate fermentation.

Materials and methods

1. Chemicals, biochemicals and reagents

All chemical compounds, reagents and biochemicals 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), MP Biomedicals (Heidelberg), Boehringer (Mannheim) or Serva (Heidelberg).

2. Bacterial growth

2.1 Anaerobic cultures of *Clostridium symbiosum*

Clostridium symbiosum DSMZ strain 934 was grown anaerobically on glutamate medium composed of the following components

100 mM Sodium glutamate \times H₂O

Yeast extract 0.5% (w/v)

Sodium thioglycolate 0.1% (w/v)

10 mM Potassium phosphate buffer pH 7.0

0.4 mM D-Biotin

Trace element solution (SL10) 0.1% (v/v)

SL 10 solution (m/v): 0.15% FeCl₂, 0.007% ZnCl₂, 0.01% MnCl₂, 0.0006% H₃BO₃, 0.02% CaCl₂, 0.0002% CuCl₂, 0.0024% NiCl₂, 0.0036% Na₂MoO₄

In each serum bottle of 120 ml volume, 50 ml medium was filled and sealed with rubber stoppers and aluminium seals. The media were boiled and then 500 µl of the oxygen indicator resazurin (0.2% stock solution) was added per litre medium. The bottles were degassed with a vacuum pump till the solutions were cooled and the atmosphere was exchanged to N₂. Then, the media were autoclaved. The sterile media were inoculated with 1% culture and incubated overnight at 37°C with shaking. The thickest grown cultures were stored at 4°C. These cultures were used to inoculate 1 l cultures. These in turn were precultures for 10 litre cultures. The final step was fermentation in 100 l medium. The yield was 400 g wet weight of cells, which were stored at -80°C till further use.

2.2 Anaerobic cultures of *Clostridium propionicum*

Clostridium propionicum DSMZ strain 1682 was grown in alanine /threonine medium composed of

Yeast extract 0.4% (w/v)

50 mM D, L-alanine /threonine
3 mM L-cysteine-HCl
5 mM Potassium phosphate buffer pH 7.4
VRB solution 0.4% (v/v)

VRB solution (m/v) (Rogosa, 1969):

2.4% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% CaCl_2 , 0.05% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025% NiCl_2 , 0.04% ZnSO_4 , 0.025% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.025% CoCl_2 , 0.025% NH_4VO_3 , 0.0125% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.025% Na_2MoO_4 .

The cultures were anaerobised with the same procedure *C. symbiosum*, 3 mM of sterile cysteine hydrochloride was added later. The same growth sequence was adopted, the final yield from 30 litre culture being 40 g wet cells.

2.3 Culture of recombinant *E. coli* producing the activator of 2-hydroxyglutaryl-CoA dehydratase from *Acidaminococcus fermentans*

Escherichia coli strain XL1-blue MRF' (Stratagene, Heidelberg, Germany) harbouring *hgdC* from *A. fermentans*, which codes for the activator of 2-hydroxyglutaryl-CoA dehydratase, on the plasmid pMH6 derived from pASK-IBA3, (IBA-GmbH, Goettingen, Germany) was used (Hans, et al., 2002).

These cells were cultivated aerobically in Standard 1 medium buffered with MOPS buffer pH 7.4 containing 50 $\mu\text{g/l}$ of carbenicillin at 37°C with shaking. The second precultures of 50 ml each were grown in 100 ml serum bottles. For production of recombinant protein, the cells were grown in 10 l of the same medium anaerobically. When the optical density of the culture reached 0.25, the cells were induced with 100 μM of AHT for 3 hours and harvested under anaerobic conditions. Anaerobic handling was done in an atmosphere of 95% N_2 and 5% H_2 in a Coy anaerobic chamber (Ann Arbor, Michigan). The cells were stored at -80°C till further use.

2.4 Culture of recombinant *E. coli* producing glutaconate CoA-transferase from *A. fermentans*

The recombinant *E. coli* cells carrying the pJF118HE plasmid coding the genes for the glutaconate CoA-transferase (Gct) subunits from *A. fermentans* (Mack et al., 1994), were cultivated aerobically in Standard 1 medium containing 50 $\mu\text{g/l}$ of carbenicillin at 37°C with shaking. For production of the transferase, the cells were grown aerobically in 2 l of the same medium till the A_{578} of the culture reached 2. The cells were then induced with 300 μM of β -IPTG and grown until A_{578} reached 5.5. The cells were then harvested and stored at -80°C till further use.

2.5 Growth experiment with *C. symbiosum*

Cultures of *C. symbiosum* were grown in the glutamate medium with as mentioned with 50 mM glutamate, in the presence of 1mM DFGA. Two controls were made one with 50 mM glutamate only, and the other with 50 mM glutamate containing 1mM oxoglutarate. The optical densities of the three sets of cultures were measured every hour.

3. Chemical and enzymatic synthesis

3.1 Chemical synthesis of carboxylic acids

3.1.1 2-Hydroxymuconic acid/ Oxalocrotonic acid (2-oxo-hex-4-ene-1,6-dioic acid)

Synthesis of oxalocrotonic acid (2-hydroxymuconic acid) was done modifying published procedures (Lapworth 1901, Whitman et al., 1991). Potassium tertiary butoxide (35.5 mmol) was added under inert atmosphere to a reaction flask maintained at 0°C. Ethyl crotonate (32.4 mmol) and sufficient dry ether were added and the mixture stirred for 15 minutes. Then, 32.4 mmol of ethyl oxalate were introduced under anhydrous conditions and the mixture was stirred at the same temperature for 30 minutes. The reaction mixture was left overnight at 0-4°C. The resulting bright yellow mixture was filtered. The filtrate was collected and dissolved in ice-water. Acetic acid (50% v/v), was added, the precipitate collected and washed with cold water to obtain diethyl oxalylcrotonate. The purity was confirmed by ^1H and ^{13}C NMR spectra.

Pure diethyl oxalylcrotonate dissolved in 200 ml of cold 30% sodium hydroxide was left standing at room temperature, until no more precipitate formed with acetic acid. The solution was then diluted with water to 700 ml, filtered, cooled and made acidic with dilute acid (pH 2). After standing overnight at room temperature, the crude 2-hydroxymuconic acid separated. This was removed by filtration, washed with a small amount of water and dried on a Buechner funnel. The purity was confirmed by ^1H and ^{13}C NMR spectra as before.

The precursor compound, diethyl oxalocrotonate was pure as determined by its NMR spectra. The yield was 85%.

^1H nmr (CDCl_3) δ ppm: 7.7 (1H, t), 6.3 (1H, d), 6.0 (1H, d), 4.4 (2H, q), 4.2 (2H, q), 1.5 (3H, t), 1.3 (3H, t)

^{13}C nmr (CDCl_3) δ ppm: 169 (C-1), 167 (C-6), 143 (C-2), 136 (C-3), 123 (C-5), 108 (C-4), 63 (CH_2), 59 (CH_2), 13 (CH_3), 10 (CH_3)

The pure precursor was hydrolysed as described in methods and the structure of the resulting product confirmed by the following spectra. The yield was 70 %.

^1H nmr (CD_3OD) δ ppm: 7.16 (1 H, dd), 5.95 (1H, d), 5.84 (1H, d)

^{13}C nmr (CD_3OD) δ ppm: 109.0 (C-3), 122.2 (C-5), 139.6 (C-4), 148.2 (C-2), 166.6 (C-1), 170.5 (C-6).

3.1.2 (*R, S*)-2-Hydroxyadipic acid (2-hydroxyhexane-1,6-dioic acid)

Synthesis was performed starting from (*RS*)-2-aminoadipate modifying the literature method (Blaser et al., 1991). To a solution of 6.2 mmol of commercial DL-aminoadipate in 6M HCl at -5°C , 9.2 mmol NaNO_2 was added dropwise with stirring. The mixture was stirred overnight at room temperature. The residue was then extracted with ethylacetate. The solvent was evaporated and the residue dissolved in absolute ethanol under highly acidic conditions and reacted overnight under dry conditions. The resulting mixture was chromatographed on a silica-gel (200-mesh Sigma-Aldrich) column with a 1:1 solvent mixure of petrol 40/60 and diethyl ether. Two fractions separated and only the less polar first fraction was concentrated by rotary evaporation and de-esterified with 1 M NaOH. The product was then recrystallised from ether-petrol, dried and its purity was analysed by its ^1H and ^{13}C spectra.

The resulting product was confirmed as 2-hydroxyadipate by analyzing its NMR spectrum.

^1H nmr (CDCl_3) δ ppm: 4.3 (m, 1H), 2.3 (t, 2H), 1.8 (m, 2H), 1.6 (dt, 2H)

^{13}C nmr (CDCl_3) δ ppm: 180 (C-6), 178 (C-1), 73 (C-2), 34 (C-5), 32 (C-3), 22 (C-4).

3.1.3 DFPA or 2,2-difluoroglutaric acid (2,2-difluoro-pentan-1,5-dioic acid)

Synthesis of DFPA was performed by a modification of the published method (Laurent, et al., 2005). Ethyl bromodifluoroacetate (6.32 mmol) and acrylonitrile (12.5 mmol) were added to a suspension of activated copper powder (15 mmol) in 30 ml dry DMSO. After being stirred overnight at 65°C , the reaction mixture was quenched with ice-cold saturated NH_4Cl and extracted with ether. The combined organic phase was concentrated under reduced pressure, and the residue obtained was purified by silica-gel (200-mesh Sigma-Aldrich) column chromatography with petroleum ether-ethyl ether as solvents in a 4:1 ratio, giving a yellow oil. To this product, 20% NaOH was added slowly at 0°C , and the reaction mixture was then warmed to room temperature and left overnight. The reaction mixture was then extracted with ether to remove any impurities. The aqueous phase was acidified to a pH 1 and extracted with ethyl acetate. The combined organic phase was concentrated under reduced pressure and dried *in vacuo*, giving DFPA as a yellow-white solid. The product was crystallised from

ether-petrol at 0-4°C. The mother-liquor was seeded repeatedly until no more crystals appeared. The product purity was ascertained by the peak at -107.5 ppm in the ^{19}F NMR and the molecular ion peak at 191 mass units in the ESI mass spectrum.

Activated copper was prepared as follows.

5 g of commercially available 99% copper granules were stirred with 6 N HCl for a few minutes. The acid waste was decanted and the copper washed 8-10 times with double distilled water, each time discarding the washings. Then, the water was removed by similarly rinsing with acetone 3-5 times, and finally the acetone was removed by washing with dichloromethane 3-5 times. The brightly coloured copper powder was carefully dried by rotary evaporation till a thin film of dichloromethane remained, and was used immediately for synthesis.

3.1.4 3-Pentynoic acid

3-Pentynoic acid was synthesised by the literature procedure (Fendrich and Abeles, 1982), by isomerisation of the commercially available 2-pentynoic acid. Rearrangement of 2-pentynoic acid to 3-pentynoic acid was accomplished by heating the former in 6 N NaOH at 60°C for 90 minutes. The acid was isolated by acidic ether extraction and recrystallised from toluene. Sodium pent-3-ynoate was prepared and the measured ^1H nmr spectrum matched with the reported spectrum (Bushby and Whitham, 1969). The yield was about 75%.

^1H nmr (D_2O) δ ppm: 6.84 (q, 3H, J 2.5 Hz) and 8.23 (t, 2H, J 2.5 Hz).

3.1.5 2-Oxadipate (2-oxohexane-1,6-dioic acid)

2-Oxadipate was synthesised by the condensation of diethyl glutarate with diethyl oxalate (Matos and Wong, 1986). To 4 mL of diethyl glutarate in 50 ml of anhydrous ether and 15 ml of ethanol was added 3.25 g of sodium ethoxide. The mixture was refluxed for 60 min to allow for dissolution of sodium ethoxide followed by rapid addition of diethyl oxalate (8 ml) while vigorously stirring the solution for 3.5 hours and then the mixture was concentrated to an oil.

The oil was added to 60 mL of 3.5 N HCl and the solution quickly extracted with ether until the aqueous layer was almost colourless. The ether was dried over anhydrous Na_2SO_4 , then concentrated under vacuum. The resulting oil was dissolved in 100 ml of concentrated HCl and the mixture kept at room temperature for 18 hours. The solution was evaporated to dryness, the solid was dissolved in acetone and decolourised with activated carbon. The acetone was evaporated and the solid dissolved in water and adjusted to pH 4 with NaOH. 2-propanol was added until complete precipitation. The mixture was then stored for 1 h at 0-4°C, the precipitate was filtered off and dried. The product was

recrystallised from petrol / ether. The yield was 85-90% and the measured spectrum matched those in the literature (Friedman and Kosower, 1955).

¹H nmr (D₂O) δ ppm: 2.48 (t, 2 H), 2.45 (t, 2 H), 1.87 (m, 2 H).

3.2 Chemical synthesis of CoA esters

3.2.1 from chlorides

Acrylyl-CoA was synthesised by reacting 3-fold molar excess of acrylyl chloride in dry acetonitrile with free CoA dissolved in minimum volume 0.5 M NaHCO₃ under a stream of N₂. The solution was mixed at room temperature until no yellow colour of free thiol was obtained with DTNB (Ellman, 1959). The pH was adjusted to 2 and frozen at -20°C until purification. In view of the instability of the compound, it was prepared and purified just prior to use.

2-Hydroxyglutaryl-CoA was synthesised starting from commercial (*R*)-2-oxo-tetrahydrofuran-carboxylic acid. This acid was converted to the corresponding acid chloride by reacting it with excess oxalyl chloride at 60°C for 3 hours. The excess oxalyl chloride was removed by evaporation at reduced pressure. A 3-fold molar excess of the acid chloride was dissolved in dry acetonitrile and reacted with CoASH in 0.5 M NaHCO₃ at room temperature and the pH was lowered to 6 to get the lactone-CoA. Finally, the lactone-CoA was incubated at pH 0-1 for 2-3 hours to obtain (*R*)-2-hydroxyglutaryl-CoA.

3.2.2 from mixed anhydrides

Pentanoyl-CoA, 2-pentenoyl-CoA and 2,4-pentadienoyl-CoA were synthesised according to the mixed anhydride method (Goldman and Vagelos, 1961). To about 250 μmol of free acid in 2 ml of dry toluene, were added 300 μmol of fresh dry triethylamine and 300 μmol of ethyl chloroformate were mixed under an N₂ atmosphere for 1 hour. Then, excess toluene, triethylamine and ethyl chloroformate were removed by a stream of N₂.

The residue was dissolved in 5 ml of dry THF. A 2-3 fold excess of the resulting anhydride was added to an aqueous solution of CoA at pH 8 under an N₂ atmosphere and stirred for 10 minutes, after which the reaction was stopped by acidifying to pH 2.

3.2.3 using dicyclohexylcarbodiimide

3-Pentynoyl-CoA was synthesised by the following procedure used for making pentynoyl pantetheine (Gomes, et al., 1981). 40 μmol of CoA was suspended in 5 mL of acetone dried over CaSO₄.

In another flask was placed dicyclohexylcarbodiimide (60 μmol ,) and 5 ml of dry THF. To the THF solution was added 0.1 g of 3-pentynoic acid; the THF and acetone solutions were mixed quickly. The mixed solution was then stirred overnight in a sealed flask at 4°C. The solution was filtered on a sintered glass filter to remove dicyclohexylurea. The solvent was removed with a rotary evaporator to yield an oily substance, which was treated with ether and dried *in vacuo*.

3.2.4 using carbonyl-di-imidazole

Lactyl-CoA and 2-hydroxybutyryl-CoA were synthesised via an imidazole intermediate (Kawaguchi et al., 1981). The carboxylic acid (50 μmol) and carbonyl-di-imidazole (70 μmol) were mixed in 0.5 ml of dry acetonitrile. The mixture reacted for about 2 minutes after which it was dropped into 40 μmol CoA dissolved in 0.5 ml of 0.5 M NaHCO_3 . After about 30 minutes the mixture was acidified to pH 2 for purification.

3.2.5 from anhydrides

Butyryl-CoA, propionyl-CoA, crotonyl-CoA, glutaryl-CoA and acetyl-CoA were synthesised from their anhydrides by the well-known Shemin method (Simon and Shemin, 1953). CoA (25 μmol) dissolved in 1ml of 0.5 M KHCO_3 was added to 35 μmol of the respective anhydride which was dissolved in 0.5 ml acetonitrile and the mixture diluted to 5 ml with water. The mixture was reacted at room temperature till negative with DTNB and then acidified to pH 2 for purification.

3.3 Enzymatic synthesis of CoA esters

3.3.1 with glutaconate CoA-transferase (Gct)

Gct was used to synthesise glutaconyl-CoA (Buckel et al., 1981), 3-methylglutaconyl-CoA (Mack et al., 2006), butynediol-CoA, difluoroglutaryl-CoA and hydroxyadipoyl-CoA with acetyl-CoA or glutaconyl-CoA as CoA donor. 20 μmol of acetyl-CoA and a 10-20 fold excess of the respective carboxylic acid and 5 U of Gct were mixed in a volume of 3-5 ml of potassium phosphate buffer pH 7.0 and the mixture reacted at 37°C for 1 hour, after which the mixture was acidified to pH 2 and filtered through a 1 kDa cut-off membrane (Amicon, Amersham Biosciences) to remove precipitated protein.

3.3.2 modifications to the Gct method

Trans, *trans*-muconic acid and oxalocrotonic acid were converted to their potassium salts by treatment with 1M KOH and the dried salts were used instead

of the acids as in section 3.3.1. In order to obtain 3-methylglutaconyl-CoA from 100 mM 3-methylglutaconate and 1 mM acetyl-CoA, the incubation time had to be extended to 2 hours (Mack et al., 2006).

3.4 Purification of CoA esters by reverse phase chromatography

All CoA esters were purified by reverse phase chromatography through Sep-Pak C₁₈ columns (Waters, Massachusetts USA). The reaction mixtures were freed from solvents or precipitated proteins and acidified to pH 2. They were loaded on C₁₈ columns washed with methanol and equilibrated with 0.1% TFA (v/v). After loading the column was washed with 3 volumes of the same solution. Elution was performed with 0.1% TFA containing 50% acetonitrile (v/v). The eluted CoA esters were freed from acetonitrile by freezing and centrifuging on a Speed-Vac concentrator (Bachofen, Germany). They were then refrozen and vacuum-dried on a lyophiliser (Alpha1-4, Christ Instruments, USA). The lyophilised powders were stored at -80°C till further use.

4. Assays

All spectrophotometric assays were performed on Ultrospec 1100 *pro* spectrophotometers from Amersham Biosciences installed under aerobic or anaerobic conditions as needed, or a Uvikon 943 double beam spectrophotometer from Kontron Instruments, Switzerland. Quartz cuvettes were used for measurements below 320 nm and disposable plastic cuvettes for measurements above 320 nm, all of which had a path length of 1 cm and a volume of 0.5 or 1 ml.

4.1 Direct assay for 2-hydroxyglutaryl-CoA dehydratase activity

Components of the assay mixture for 2-hydroxyglutaryl-CoA dehydratase activity

Tris/HCl buffer, pH 8.0	50 mM
MgCl ₂	5 mM
Dithiothreitol	5 mM
ATP	0.4 mM
Dithionite	0.1 mM
(<i>R</i>)-2-Hydroxyglutaryl-CoA	
Activator	
Dehydratase	

The activator and the dehydratase were incubated for 5 minutes with all the assay components except the substrate, under strictly anaerobic conditions in an anaerobic chamber. The reaction was started by the addition of (*R*)-2-

hydroxyglutaryl-CoA. The volume of the reaction mixture was 0.5 ml. The formation of (*E*)-glutaconyl-CoA was measured at 290 nm ($\epsilon_{290} = 2.2 \text{ mM}^{-1}\text{cm}^{-1}$). The wavelength was chosen to avoid the competing absorption of the adenine moieties starting from 260 nm and was therefore more selective for detecting the formation or disappearance of double bonds. This method was used to detect the activity also with the other substrates.

4.2 Indirect assay for 2-hydroxyglutaryl-CoA dehydratase activity and optimisations of the parameters

The same activity described in 4.1 can be measured indirectly without the need for synthesising (*R*)-2-hydroxyglutaryl-CoA. This assay was done as follows. The dehydratase and catalytic amounts of activator were preincubated for 5 minutes in the presence of 0.4mM ATP, 5 mM DTT, 5 mM MgCl_2 , 50 mM Tris-HCl pH 8.0, and 0.4 mM sodium dithionite. Then, CoA transferase and acetyl-CoA were added and the reaction was started by addition of (*R*)-2-hydroxyglutarate.

The dehydratase activity changes with the concentration of acetyl-CoA and (*R*)-2-hydroxyglutarate. The activity also changes with the amount in Units of the helping enzyme, glutaconate CoA-transferase added. The activities were measured changing one parameter at a time.

4.3 DTNB assays

4.3.1 DTNB assay for the activity of glutaconate CoA-transferase

Glutaconate CoA-transferase activity was measured in a cuvette containing in a total volume of 1.0 ml at 25°C (Buckel, et al., 1981).

The reaction was initiated by addition of the enzyme. The increase of absorbance was followed at 410 nm ($\epsilon_{410} = 14.2 \text{ mM}^{-1}\text{cm}^{-1}$) (Riddles, et al., 1983). and the measured slope was used to calculate activity. The assay is based on reaction of free thiol of CoA with DTNB (Ellman, 1959).

Components of the assay for the activity of glutaconate CoA-transferase

Potassium phosphate buffer pH 7.0	100 mM
Sodium acetate	200 mM
Oxaloacetate	1.0 mM
DTNB	0.5 mM
Citrate synthase	0.1 mM
Glutaryl-CoA	20 μg
Glutaconate CoA-transferase	

4.3.2 DTNB assay for the estimation of CoA esters

The concentrations of CoASH, acetyl-CoA and glutaconyl-CoA (or any other CoA-ester substrate of the transferase) were determined in a single assay (Buckel, et al., 1981). A sample was added to the reaction mixture of the standard assay as in 3.3.1 without the enzymes causing a first increase in absorbance due to free CoASH. Addition of citrate synthase and finally of transferase (5 U) gave two further increases due to acetyl-CoA and glutaconyl-CoA, respectively.

4.4 Phosphate acetyltransferase assay

The formation of acetyl phosphate and free CoA from acetyl-CoA and inorganic phosphate by the enzyme phosphate acetyltransferase was assayed as follows.

100 μ M acetyl-CoA, and 1 mM DTNB were mixed in a cuvette containing 100 mM of potassium phosphate buffer pH 7.0. The assay starts with the addition of an enzyme aliquot to be tested. If there is phosphodiesterase activity, an increase in the absorption at 410 nm would be seen.

4.5 Crotonase assay

Crotonase was assayed with 0.1 mM crotonyl-CoA in 100 mM potassium phosphate pH 7.0 following the decrease in absorbance at 280 nm, ($\epsilon_{280} = 4.2 \text{ mM}^{-1}\text{cm}^{-1}$; Barker, et al., 1978).

4.6 (R)-2-Hydroxyglutarate dehydrogenase assay

α -Ketoglutarate reductase or 2-hydroxyglutarate dehydrogenase activity (Lerud and Whiteley, 1971) was determined spectrophotometrically by following changes in absorbance at room temperature at 340 nm. The reaction mixture for assaying enzymatic reduction of α -ketoglutarate contained the following components in a total volume of 1ml

Potassium phosphate buffer pH 8.0	100 mM
α -Ketoglutarate	1 mM
NADH	0.2 mM
(R)-2-Hydroxyglutarate dehydrogenase	

The purified enzyme from *A. fermentans* was a gift from Ivana Djurdjevic of our group. α -Ketoglutarate was replaced by other substrates for other measurements.

For the case of 2-hydroxymuconate / oxalocrotonate (2-oxohexene-dioate), the substrate was buffered in an appropriate volume for 1 hour at 30°C to allow for stabilisation of the absorbance (Whitman, et al., 1991; Hajipour, et al., 1993).

4.7 Malate dehydrogenase (MDH) assay

Malate dehydrogenase from Boehringer, Germany was assayed as follows: appropriate dilution of the enzyme was incubated in 20 mM potassium phosphate buffer pH 7.4 and the assay was started by adding oxaloacetate and NADH at different concentrations. The fall in the absorbance due to the formation of NAD^+ was followed at 340 nm using $\epsilon_{340} = 6.3 \text{ mM}^{-1} \text{ cm}^{-1}$ for the reduction of NADH to NAD^+ (Ziegenhorn, et al., 1976).

4.8 Methylviologen assay for acrylyl-CoA reductase activity

The enzymatic reduction of enoyl-CoA like acrylyl-CoA or crotonyl-CoA was measured anaerobically, with the help of methylviologen as a redox indicator. The reduced form of methylviologen donates electrons that are transferred during enzymatic activity, thus decreasing the absorbance at 600 nm. The enzymatic solution containing 1 mM methylviologen in 50 mM potassium phosphate buffer pH 7.5 was fixed to an initial absorbance of 0.8 at 600 nm, after titration with 10 mM Ti(III)citrate. Activity was calculated based on a extinction coefficient of $\epsilon_{604} = 27.2 \text{ mM}^{-1} \text{ cm}^{-1}$ (Mayhew, 1978). 200 μM acrylyl-CoA was added to determine the reductase activity.

4.9 Ferricenium hexafluorophosphate assay

Acrylyl-CoA reductase activity was measured with ferricenium hexafluorophosphate (FcPF_6), as an artificial electron acceptor (Hetzl, et al., 2003). The assay measured the decrease of absorbance at 300 nm in a reaction containing 0.2 mM FcPF_6 and propionyl-CoA in 50 mM potassium phosphate buffer pH 7.5. The redox indicator ferricenium hexafluorophosphate was previously prepared in a 10 mM HCl solution to a final concentration of 2 mM set at 617 nm ($\epsilon_{617} = 0.41 \text{ mM}^{-1} \text{ cm}^{-1}$; Lehman and Thorpe, 1990). During the enzymatic dehydrogenation of propionyl-CoA, the blue colour of the solution was lost concomitant with the reduction of ferricenium ions to ferrocene. This led to a decrease in the absorbance at 300 nm ($\epsilon_{300} = 8.6 \text{ mM}^{-1} \text{ cm}^{-1}$). The initial slopes were used for activity calculations.

4.10 Assay for lactyl-CoA dehydratase activity

The enzyme fractions to be tested or the purified enzyme was incubated for 30 minutes under anaerobic conditions with 5 mM of 3-pentynoyl-CoA, which is a reported inactivator of acrylyl-CoA reductase activity (Kuchta and Abeles,

1985). The protein fraction was freed from the inhibitor by passing it over a 1ml PD-10 Spintrap G-25 column (GE Healthcare, USA) equilibrated with anaerobic buffer and reconcentrating via a Centricon 30 kDa filter (Millipore Corporation, USA).

Then, the assay was performed exactly similar to the direct assay for 2-hydroxyglutaryl-CoA dehydratase activity in 4.1, except that acrylyl-CoA was used in place of 2-hydroxyglutaryl-CoA and the lactyl-CoA dehydratase was used instead of 2-hydroxyglutaryl-CoA dehydratase. The recombinant activator from *A. fermentans* could be used instead of the activator from *C. propionicum* which is very unstable and has never been purified completely.

5. Purification of proteins

5.1 Methods of cell disruption

Cells were suspended in appropriate buffers and filled into a serum bottle under anaerobic conditions and the bottle sealed with a rubber stopper and an aluminium lid. Anaerobicity was maintained by connecting the pressure cell via a needle to the serum bottle. The cell suspension was sucked into a pre-cooled French pressure cell (American Instruments, Maryland, USA) and the cells were disrupted by applying a pressure of 1.4 MPa. The broken cells were collected into the serum bottle and refilled into the pressure cell via the needle for another cycle of pressurising. The cycle was repeated 3-4 times and the cells were observed under a microscope to verify optimal cell opening.

Resuspended cells were filled into a glass Rosetta cell (under aerobic or anaerobic conditions as necessary) kept on ice-water and broken by ultrasonication on a Branson 250 Sonifier (Heinemann, Germany). The duty time was 5-10 minutes at 50% duty cycle. The process was repeated several times and the cells were observed under a microscope to verify cell opening.

All buffers, columns, Centricon filters, centrifuge tubes and other materials were anaerobised prior to use for the purification of 2-hydroxyglutaryl-CoA dehydratase from *C. symbiosum*, lactyl-CoA dehydratase from *C. propionicum* and recombinantly produced activator of 2-hydroxyglutaryl-CoA dehydratase of *A. fermentans*. All operations during these purifications were carried out anaerobically in glove-box (Coy Labs, Ann Arbor, USA). All anaerobic buffers and solutions were pre-reduced with 2 mM DTT. The homogeneity of all these proteins were established by SDS-PAGE. The columns and the FPLC system installed used for the purifications were from Pharmacia (Sweden).

5.2 Purification of 2-hydroxyglutaryl-CoA dehydratase from *C. symbiosum*

Purification of the dehydratase (48 + 43 kDa) from *C. symbiosum* was done anaerobically following the procedure of Hetzel and Kim (Hetzel, doctoral thesis, Philipps University, Marburg). 18 g of frozen cells were resuspended in 50 ml of buffer A (50 mM MOPS buffer pH 7.2) under anaerobic conditions and disrupted via ultrasonication as described before. The cell-free extract was clarified by centrifugation at $100,000 \times g$ for 1 hour at 4°C on an Optima L-90K Ultracentrifuge (Beckman Coulter, Germany) and applied to a DEAE-Sepharose column equilibrated with buffer A. The column was washed with buffer A and elution was performed by running a linear gradient of 0-0.7 M NaCl. The active fractions were eluted around 0.35 M NaCl. The pooled fractions were desalted and to the combined fractions was added solid ammonium sulfate to 1M final concentration.

This was loaded onto a Phenyl-Sepharose column pre-equilibrated with buffer A containing 1 M ammonium sulfate. After washing, the proteins were eluted with a decreasing gradient of 1-0.3 M ammonium sulfate. The active fractions were eluted starting from 0.5 M ammonium sulfate and desalted. The pooled fractions were loaded onto a Q-Sepharose column pre-equilibrated with buffer A. After washing the column, elution was done with an increasing gradient of 0-0.5 M NaCl. The active fractions eluted around 0.3 M NaCl. The most active fractions were pooled, desalted and concentrated. The purified samples were then stored at -80°C till further use. The indirect assay involving the glutaconate CoA-transferase detailed earlier, was used to identify active fractions.

5.3 Purification of lactyl-CoA dehydratase from *C. propionicum*

Buffers:

- (A) 25 mM Tris-HCl, 1 mM magnesium chloride, 1 mM EDTA, 2 mM DTT
- (B) 1.5 M NaCl in (A)
- (C) 1.5 M Ammoniumsulfate in (A)
- (D) 150 mM NaCl in (A)

Pre-equilibration of the columns allowed the purification in about one working day. About 12 g of frozen cells were resuspended in 20 mL buffer A and sonicated at 50% duty time, for 10 min on ice-water. The cell-free extract was clarified by ultracentrifugation for 45 min at $100,000 \times g$ and applied onto a Source 15Q column (1.6 x 15 cm) equilibrated with buffer A and the column was washed with 25 mL buffer A. Gradually the bound proteins were eluted in a linear gradient from 0-0.33 M NaCl in 100 mL with buffer B, 5 mL fractions were collected. The flowrate was 5 mL/min. Two brownish peaks were obtained.

The first eluting peak contained the activator (5 mM ATP/MgCl₂ was added and the fractions stored on ice), the second peak was found to be the dehydratase based on the assay described in 4.10. The relevant fractions were desalted and stored on ice.

Solid ammonium sulfate was added to a final concentration of 1.5 M to the pooled fractions of dehydratase, sterile filtered and applied on a Source 15Phe column (1.0 x 10 cm) equilibrated with buffer C. The column was washed with 20 ml buffer C and the bound proteins were eluted in a decreasing gradient from 1.5-0 M of ammonium sulfate. Fraction size was 2 ml and the flowrate was 2 mL/min. The brownish fractions were pooled and the sample concentrated to about 400 µL with a 100 kDa cut-off Centricon membrane.

A maximum of 200 µL of the concentrated sample was applied on Superdex 200 column (HR 1.0/30) equilibrated with buffer D and 0.5 mL fractions were collected. The flowrate was 0.4 mL/min. The activities of the purified enzyme were measured by assays 4.9 and 4.10. The dehydratase and the activator were not frozen. Both preparations were stable in the glove box on ice-water for 2-3 days although the activity loss was in the range of 10-15% per day in the activity assay 4.10. Due to brownish color of the dehydratase, a 'blind' purification was performed to save time and the specific activity.

5.4 Purification of recombinantly produced activator of 2-hydroxyglutaryl-CoA dehydratase of *A. fermentans*

10 g of frozen *E. coli* XL1-blue MRF' cells producing the activator of *A. fermentans* were suspended in anaerobic buffer A i.e., 50 mM MOPS pH 7.4 containing 300 mM NaCl and 10 mM MgCl₂. 2 mM solid ADP was dissolved. The cells were opened by a French pressure cell as described previously and centrifuged for 1 hour at 100,000 × g at 4°C on an ultracentrifuge.

The cell-free extract was applied onto a 5 ml Strep-tactin affinity column (IBA, Germany) that was pre-equilibrated and washed with buffer A. Elution was done with 3 mM desthiobiotin and 2 mM ADP added to buffer A. 0.5 ml fractions were collected and the brown fractions concentrated by Centricon 30 kDa cut-off membranes. The purified protein was stored at -80°C for further experiments.

5.5 Partial purification of recombinantly produced glutaconate CoA-transferase of *A. fermentans*

5 g of transformant cells of *E. coli* expressing the genes for glutaconate CoA-transferase of *A. fermentans* (Mack et al., 1994), were suspended in 17 ml 20 mM potassium phosphate, pH 7.4 and sonicated in two 15-min intervals at 0°C with a Branson sonifier. Cell debris was removed by centrifugation at 100,000 ×

g for 1 hour. Solid ammonium sulfate was added to the cell-free extract to achieve 50% saturation. After centrifugation at $25,000 \times g$ for 30 minutes, the supernatant was brought to 80% ammonium sulfate saturation and centrifuged as described above. The protein pellet was dissolved in 40 ml of 20 mM potassium phosphate, pH 7.4, and partially purified by passing through a Centricon cut-off membrane of 100 kDa. The sample was stored at 4°C for use in further experiments.

6. General biochemical methods, spectroscopy and spectrometry

6.1 SDS-PAGE

Stock solutions	Separating gel	Stacking gel
1 M Tris/HCl, pH 8.8	3000 μ l	-
1 M Tris/HCl, pH 6.8	-	470 μ l
H ₂ O	1370 μ l	2900 μ l
10 % SDS	85 μ l	40 μ l
Acrylamide/Bisacrylamide(40% / 1.6 %)	2750 μ l	585 μ l
5% TEMED	85 μ l	40 μ l
10% Ammonium peroxodisulfate	115 μ l	80 μ l

The samples to be analysed by electrophoresis were mixed with 1-2 parts of SDS-sample buffer (125 mM Tris/HCl, pH 6.8, 10% glycerin, 10% mercaptoethanol, 4% SDS, 0.2% bromophenol blue) and boiled for about 10 minutes to denature the proteins. 10-15 μ l of each sample was loaded into each well. The molecular mass standards were purchased from Fermentas, Germany. The running buffer was 25 mM Tris pH 8.8, 190 mM glycine, 0.1% SDS. The electrophoresis was run at constant voltage of 150 mV till the bromophenolblue marker ran to the end of the gel. The proteins were then stained by heating the gel with 0.1% Coomassie Brilliant blue R-250 in methanol/water/glacial acetic acid (4:5:1) shortly and leaving it at room temperature for 5 minutes. The gel was destained by heating it with ethanol/water/glacial acetic acid (4:5:1) and incubating overnight on a shaker.

6.2 Concentration, desalting and estimation of proteins

Proteins were concentrated by centrifuging them on a Hettich EBA-21 (Global Medical Instruments, USA) through Centricon filters (Millipore Corporation, USA) of 10, 30, 50 or 100 kDa cut-off depending on their molecular sizes. The same columns were also useful to desalt proteins. For very sensitive experiments, the desalting or removal of certain chemicals was done over PD-10 Spintrap G-25 columns (GE Healthcare, USA).

The protein concentrations were estimated by the Bradford method (Bradford, 1976). The method is based on the principle that the absorption maximum of Coomassie Brilliant blue shifts from 465 to 595 nm by reacting with proteins. The Biorad-Microassay reagent (Bio-Rad-Laboratories, Munich, Germany) was diluted 1:5 with water. Bovine serum albumin (Sigma, Germany) was used as a standard which was made up to a concentration of 10 µg. The protein sample to be measured was made up to 50 µl and finally made up to 1 ml with the Biorad-Microassay reagent and incubated in the dark for 30 minutes at room temperature. The absorbance at 595 nm was measured.

6.3 Enzyme studies with UV-visible spectroscopy

For enzyme studies with UV-visible spectroscopy, the concentrations of the enzymes were adjusted so that the interesting spectral regions absorbed between 0.05 and 1.3 units. For inhibition or redox studies of the activator of 2-hydroxyglutaryl-CoA dehydratase, the concentrations of oxidant or reductant were varied between 0-20 or in some cases 50 µM.

For inhibition studies of 2-hydroxyglutaryl-CoA dehydratase, the enzymes were incubated as described earlier, the possible inhibitor was added, then the natural substrate was added and any resulting activity measured. For Michaelis-Menten studies, assays were performed in duplicates and the measured data fitted to the Michaelis-Menten equation using the Microsoft Excel software. The errors were calculated by the method of least squares.

6.4 ^1H , ^{13}C and ^{19}F NMR spectra

^1H and ^{13}C NMR spectra were routinely measured with 3-30 mg sample in standard NMR solvents (Sigma-Aldrich, Germany) on a Bruker AVANCE 300 B (300 MHz) spectrometer in automated mode at the Department of Chemistry, Philipps University, Marburg. ^1H spectra were measured at 300 MHz and ^{13}C spectra at 75.45 MHz.

Chemical shifts were measured with respect to tetramethyl silane as external standard. ^{19}F MAS NMR spectra were performed manually at the Department of Chemistry, Philipps University, Marburg, on a Bruker DRX 400 spectrometer at 376.8 MHz and 9.4 teslas (Brey, 1996). To improve the resolution, samples were spun at magic angle, and the spinning rate was set at 3,500 Hz. The pulse sequence used was a single pulse experiment (SPE) with a pulse length of 1.2 s and a recycle delay of 2 s. To obtain a good signal-to-noise ratio, 30,000 scans were accumulated for each sample. Chemical shifts were referenced to an external standard of 1 M NaF aqueous solution.

6.5 Estimation and characterisation of CoA esters

UV-visible absorption spectra were recorded for those CoA esters which are not described in the literature. The concentrations were measured from the DTNB assay mentioned earlier, whenever possible. Otherwise, the yield of synthesised CoA ester was assumed as 100%. In both cases, the extinction coefficient at the maximum around 260 nm was estimated.

6.6 CD spectroscopy

CD spectra of proteins were measured in regions the 200-350 nm range for polypeptide absorption and 350-650 nm range for iron-sulfur absorbance, both at 20°C. 20 µM samples of the 2-hydroxyglutaryl-CoA dehydratase were filled in 200 µL quartz cells inside the anaerobic chamber with all assay components including 1 mM glutaconyl-CoA as substrate and the CD-spectra measured in the UV-visible region at the Department of Chemistry, Philipps University, Marburg on a JASCO J-810 Spectropolarimeter (Jasco Labs, Germany).

Concentrations of the activator of the 2-hydroxyglutaryl-CoA dehydratase used were in the 0.5-5 µM range in a quartz cell of 3 ml and the measurements were performed under the same instrument conditions. These were handled anaerobically in 100 mM potassium phosphate buffer pH 7.5 or 100 mM Tris/HCl buffer pH 8.0.

6.7 Chemical labelling studies and Nano-LC/MS

Labelling of the 2-hydroxyglutaryl-CoA dehydratase (Hgd) with the cysteine-modifying agent iodoacetamide (Schmidt, et al., 1995), was performed with the following mixtures in potassium phosphate buffer pH 7.5 containing 5 mM DTT incubated under anaerobic conditions for 15 minutes.

- a) 10 µM Hgd + 5 mM iodoacetamide
- b) 10 µM Hgd + 5 mM iodoacetamide + 2 mM glutaconyl-CoA

After the incubation period, the protein samples were separated from the small molecules, by the use of Spin-Trap PD-10 Sephadex columns from Amersham Biosciences and following the manufacturers' protocol. Then, they were digested with trypsin in Tris-HCl buffer pH 7.8 for 3 hours at 37°C and separated and analysed at the MPI for Terrestrial Microbiology, Marburg with the Nano-LC/MS system Ultimate 300 (Dionex, Idstein, Germany). The column was PepMap100, C₁₈, 3 µm, 100 Å, ID 75 µm, length 15 cm. The buffers were buffer A: 0.05% trifluoroacetic acid in water buffer B: 0.04% trifluoroacetic acid in 80% acetonitrile in water. The flow rate was 0.3 µl/min.

A protein sample without iodoacetamide subjected to the same digestion procedure was used as reference. Using the Mascot Search system (Matrix Science Ltd., UK), the sequences were matched to the known sequences of the protein available in the NCBI (National Center for Biotechnology Information) database and modified residues were identified.

6.8 MALDI-TOF mass spectrometry

The CoA samples were purified from their synthesis or enzyme reaction mixtures as described in and the lyophilised samples were dissolved in 10-40 μ l of water. Acetyl-CoA or free CoA was used as the internal standard for calibration when present in the mixture.

The matrix was CHCA (alpha-cyano-4-hydroxy cinnamic acid from Sigma) dissolved in 70% acetonitrile / 0.1% TFA (trifluoro acetic acid). 1 μ l of each sample was mixed with 1 μ l of α -cyano-4-hydroxy-cinnamic acid or α -cyano-3-hydroxy-cinnamic acid as matrix and spotted onto a gold plate in a dilution series. Measurements were performed with a 355 nm laser in positive reflector mode with a delayed extraction with a positive polarity on the Proteomics Analyzer 4800 mass spectrometer (Applied Biosystems, Framingham, USA) at the MPI for Terrestrial Microbiology, Marburg. The acceleration voltage was 20,000 V, the grid voltage 58% and the delay time 50 ns. The ratio of reflector voltage was 1 to 1.12. An average of 0.5% of acceleration was laid on the guide wire. The mass range measured was 700-1,000 Da. For each spectrum more than 1000 shots were accumulated.

6.9 ESI mass spectrometry

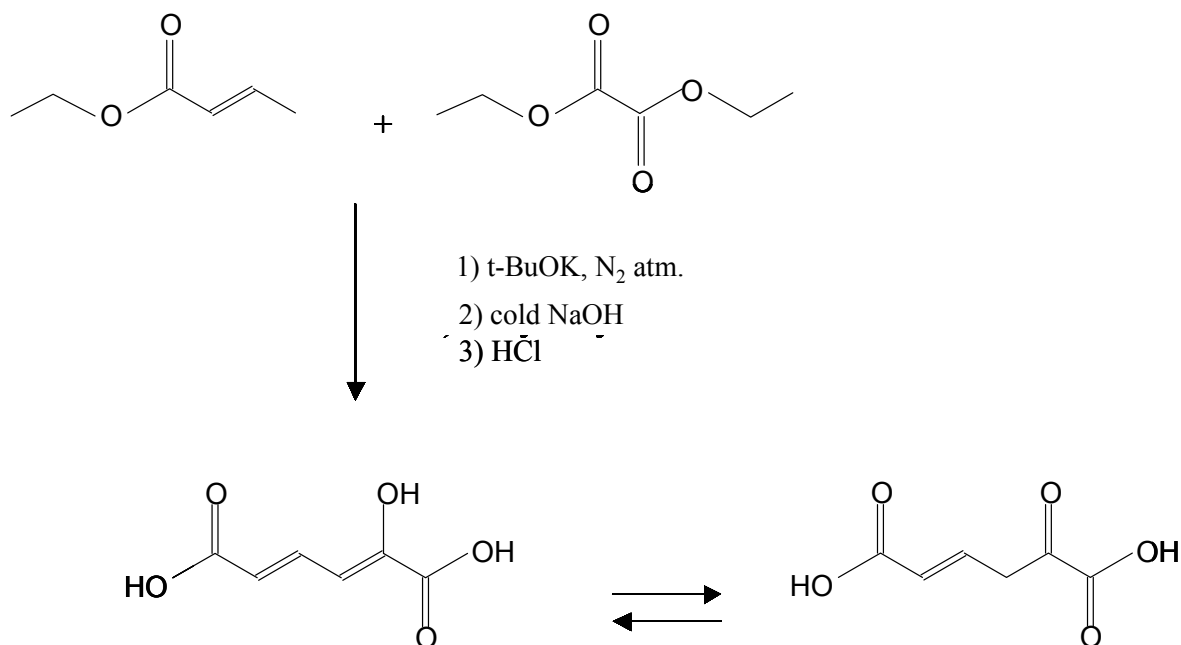
ESI mass spectrometry was performed on a Finnigan LTQ-FT (Thermo Fisher Scientific, Germany) mass spectrometer at Department of Chemistry, Philipps University, Marburg, in certain cases where the ^1H or ^{13}C NMR spectra were not enough to assess the purity of compounds. Upto 15 mg of the sample was dissolved in 0.5 ml methanol and subjected to ESI mass spectrometry in the negative ion mode.

Results

1 Chemical synthesis of carboxylic acids

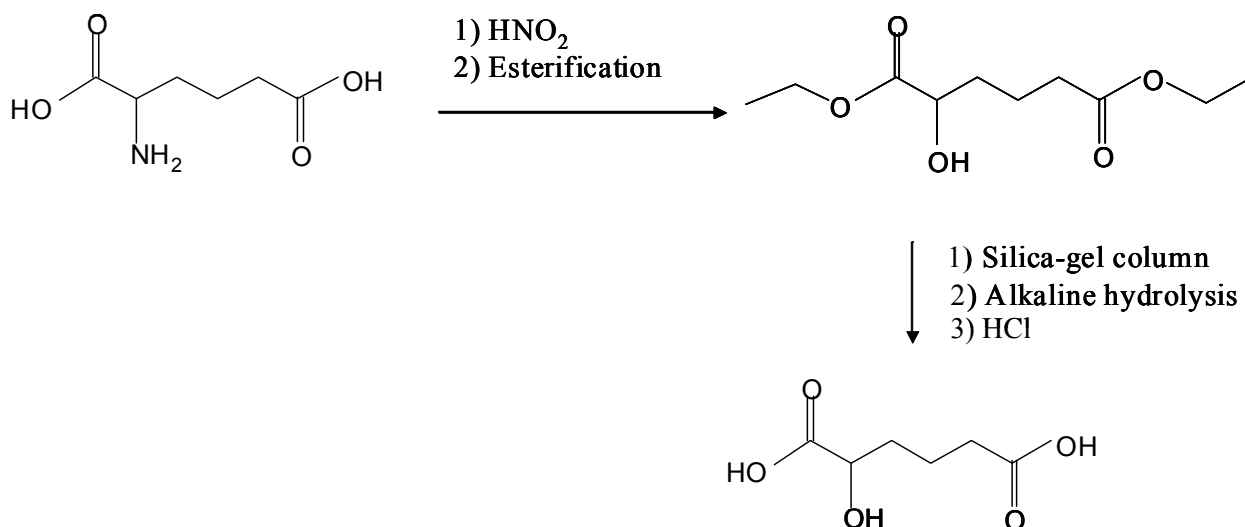
1.1 2-Hydroxymuconic acid/ oxalocrotonic acid (2-oxo-hex-4-ene-1, 6-dioic acid)

Scheme 1. Synthesis of 2-oxo-hex-4-ene-1,6-dioic acid starting from diethyl oxalate and ethyl crotonate



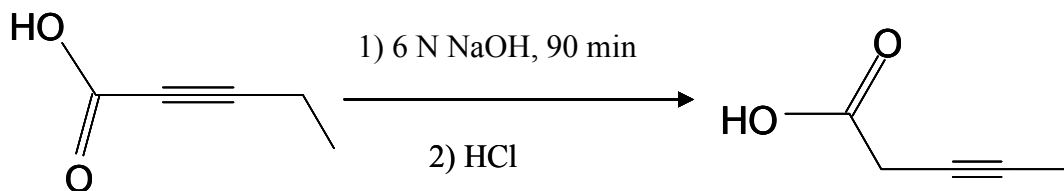
1.2 (*R, S*)-2-Hydroxyadipic acid (2-Hydroxyhexane-1, 6-dioic acid)

Scheme 2. Synthesis of (*R, S*)-2-hydroxyadipic acid (2-hydroxy-hexan-1,6-dioic acid) starting from (*R, S*)-2-aminoadipic acid



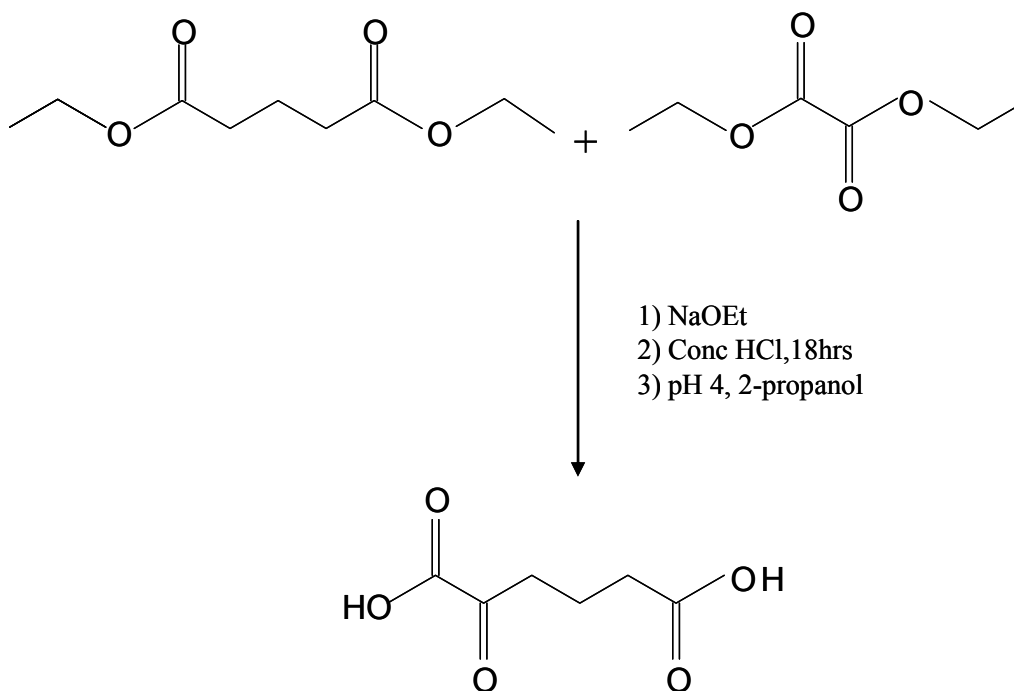
1.3 3-Pentynoic acid

Scheme 3. Isomerisation of 2-pentynoic acid to 3-pentynoic acid



1.4 2-Oxoadipic acid

Scheme 4. Synthesis of 2-oxoadipate by condensation of diethyl oxalate and diethyl glutarate.



1.5 DFPA or 2,2-difluoroglutaric acid (2,2-difluoro-pentane-1,5-dioic acid)

The ^1H and ^{13}C spectra were not characteristic enough to clearly identify this compound and therefore the peak at -107.5 ppm (in CDCl_3) in the ^{19}F nmr spectrum was used for characterisation. The absence of any other peaks showed that there were no other fluorinated compounds (Fig 12). The chemical shift differed slightly from the literature because CDCl_3 was used in place of CFCl_3 .

Further characterisation by ESI mass spectrometry revealed the presence of the monosodium salt of DFPA (Fig 13), with the structural formula $C_5F_2O_4H_6Na$ based on the molecular ion peak at 191.01 Da.

Direct fluorination of diethyl 2-oxoglutarate with 2.5 equivalents of DAST did not lead to formation of the expected product but the formation of several fluorinated products as seen from the ^{19}F nmr spectrum (data not shown).

Scheme 5. Synthesis of 2,2-difluoroglutarate via copper-catalysed Michael addition of acrylonitrile and dibromofluoroglutarate

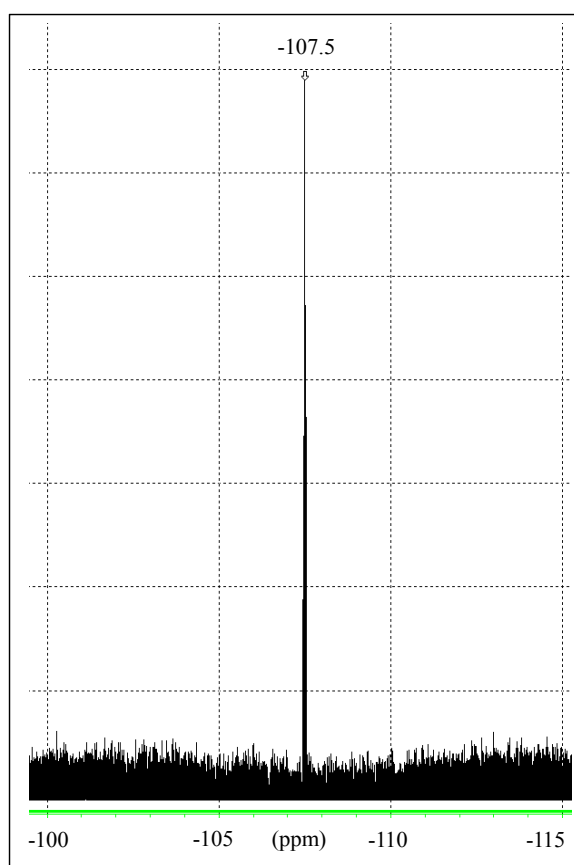
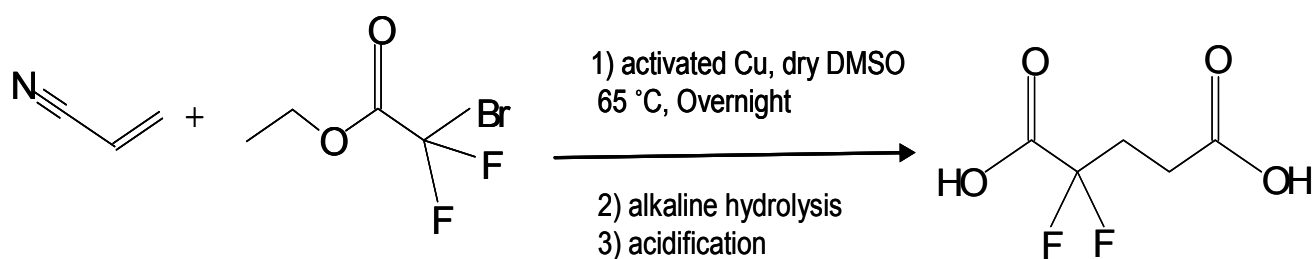


Fig 12. Identification of DFPA by its fluorine-19 nmr spectrum

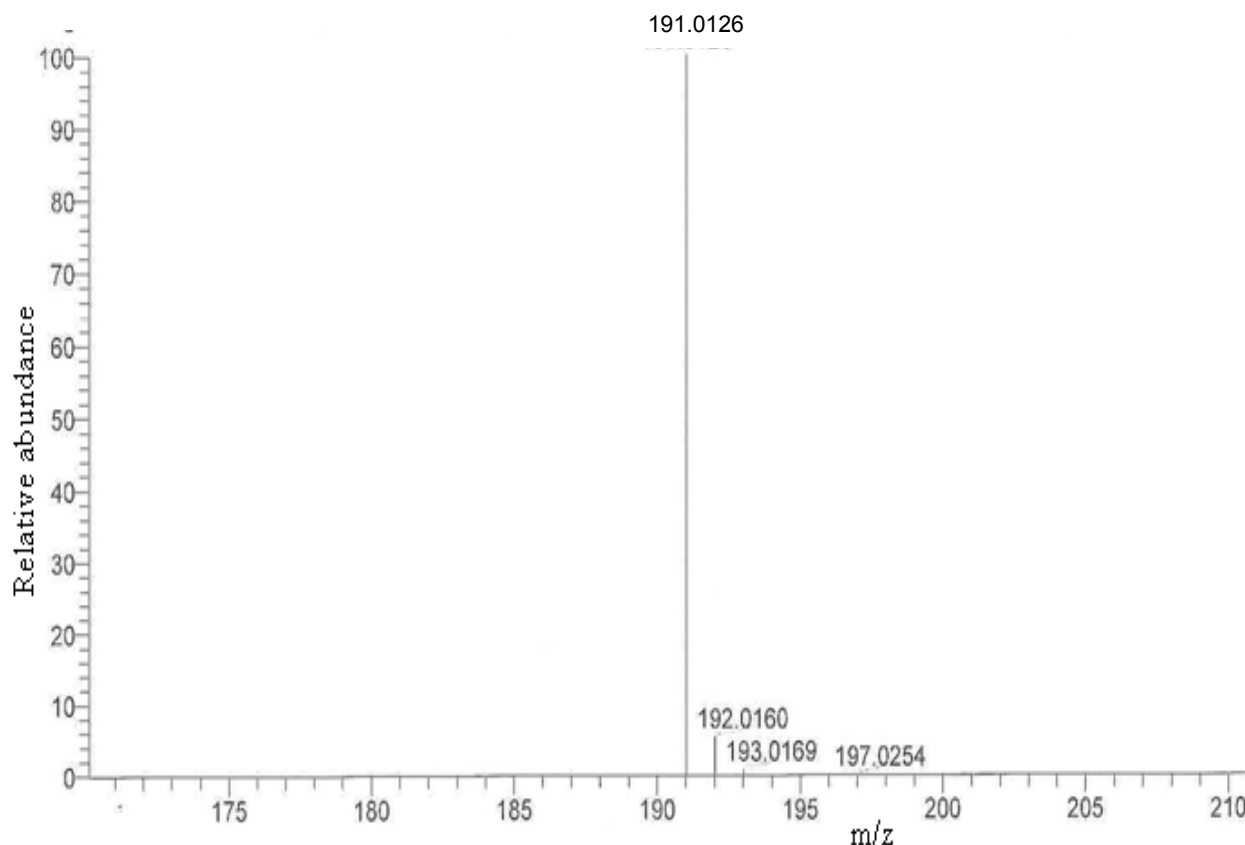


Fig 13. The ESI mass spectrum showing the molecular ion peak corresponding to monosodium salt of DFPA

2 Partial purification of glutaconate-CoA transferase, synthesis and assays

The glutaconate-CoA transferase (Gct), was partially purified as outlined in the methods section, with a specific activity of 100 U/mg using glutaryl-CoA as a substrate. A pure enzyme was not necessary for synthetic purposes i.e., the generation of CoA esters from the respective carboxylic acids. However, it was necessary to check the formation of free CoA from acetyl-CoA and phosphate which was a competing reaction.

A slight phosphate acetyltransferase activity of 1.5 U/mg was found. When the buffer was changed from 100 mM potassium phosphate pH 7 to 100 mM Tris-HCl buffer pH 7.2, the phosphate acetyltransferase activity was suppressed. The replacement of acetyl-CoA with glutaryl-CoA also circumvented the problem. For the synthesis of CoA esters a 20-30 fold excess of the carboxylic acid with respect to acetyl-CoA was used to shift the equilibrium in favour of CoA ester formation. Upto 5 U of crude enzyme was used in each synthesis.

In order to measure the concentrations of the CoA esters synthesised, the assays were performed with excess acetate and with small amounts of the Gct preparation up to 0.5 U.

3 Studies on the (*R*)-2-hydroxyglutaryl-CoA dehydratase from *C. symbiosum*

3.1 Purification of the 2-hydroxyglutaryl-CoA dehydratase

For the purification of the 2-hydroxyglutaryl-CoA dehydratase, the indirect assay described earlier was used. The substrate was generated *in situ* from 2 mM each of (*R*)-2-hydroxyglutarate and acetyl-CoA catalysed by of Gct.

The amount of Gct was varied and the 2-hydroxyglutaryl-CoA dehydratase activities measured. Fig 14 shows that the best activity was observed around 1.8-2 U of the Gct. The amounts of the (*R*)-2-hydroxyglutarate and acetyl-CoA as well as that of Gct needed to be fixed in order to find the true dehydratase kinetics, within few seconds of starting the coupled reaction. At lower amounts of (*R*)-2-hydroxyglutarate or acetyl-CoA, limiting conditions for Gct catalysis occurred and complicated the kinetics measured.

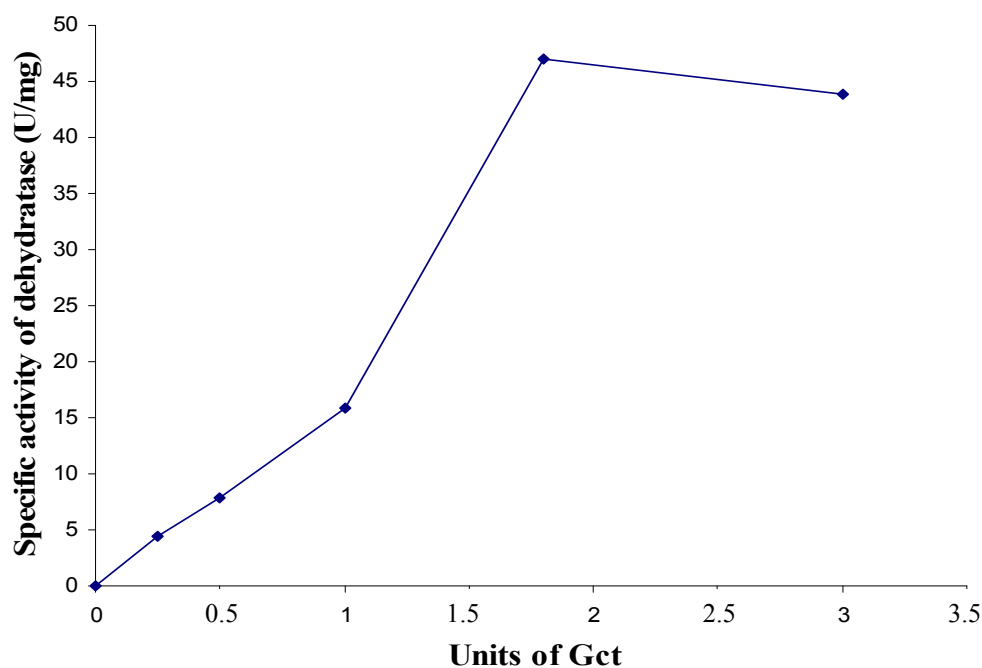


Fig 14. Variation of specific activity of 2-hydroxyglutaryl-CoA dehydratase with the enzyme units of Gct, at 2 mM each of (*R*)-2-hydroxyglutarate and acetyl-CoA, under indirect assay conditions.

As described in the methods section, the 2-hydroxyglutaryl-CoA dehydratase was purified in three chromatographic steps on an FPLC system, based on the indirect assay. The specific activity of 54 U/mg was measured for the purified enzyme. The enzyme was stable on ice under anaerobic conditions for several days and was stored at -80°C for several months.

Table 1 summarises the purification process. SDS-PAGE analysis demonstrated the apparent homogeneity of the purified protein (fig 15), showing the two subunits of the enzyme at 43 and 48 kDa approximately.

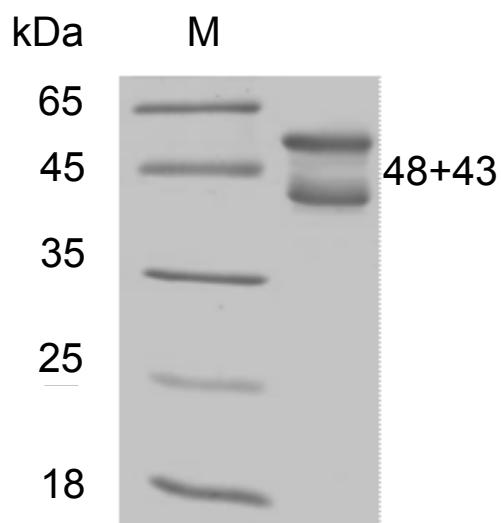


Fig 15. SDS-PAGE showing on the left lane molecular mass marker and on the right lane the purified 2-hydroxyglutaryl-CoA dehydratase.

Table 1. Purification of (*R*)-2-hydroxyglutaryl-CoA-dehydratase from *C. symbiosum* using the indirect assay for 2-hydroxyglutaryl-CoA

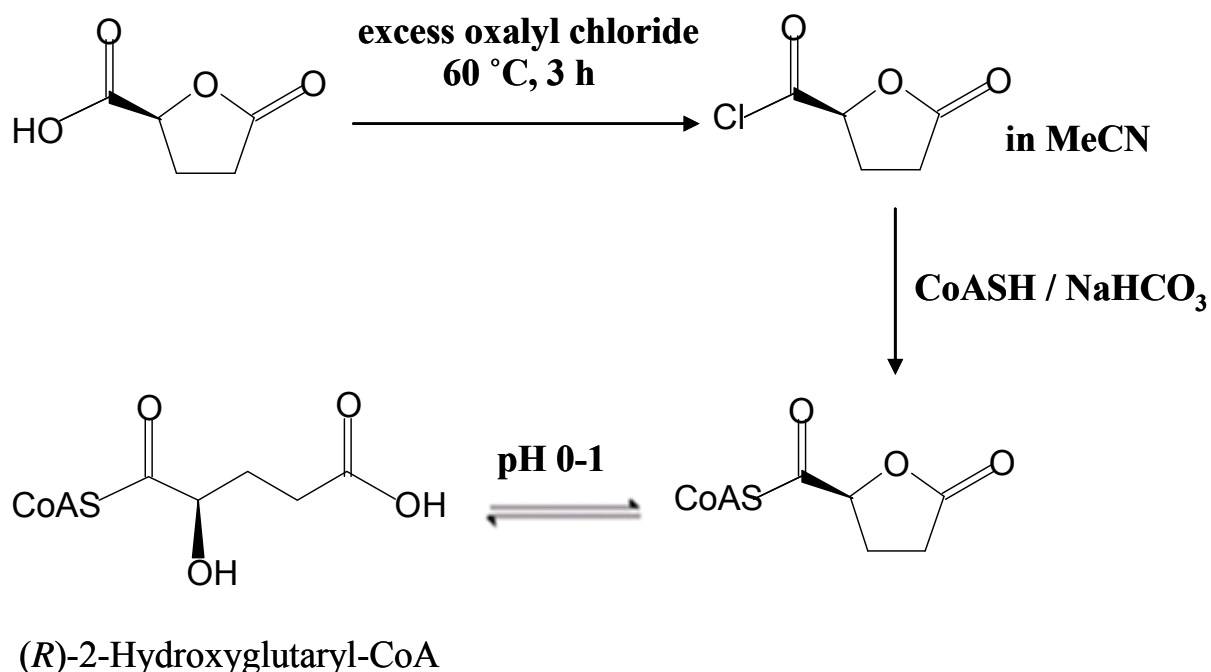
Step	U	mg protein	U/mg	Purif. Factor	Yield %
Cell-free extract	2400	2400	1.1	1	100
DEAE- Seph.	1598	1206	1.3	1.2	66
Phenyl- Seph.	1036	35	29	26	43
Q-Seph.	836	15	54	49	34

3.2 Properties of 2-hydroxyglutaryl-CoA and lactone-CoA

Enzymatic synthesis of hydroxyglutaryl-CoA with the help of Gct leads to the formation of the kinetically favored 2-hydroxyglutaryl-CoA and the thermodynamically favored 4-hydroxyglutaryl-CoA (Buckel et al., 1981).

The *in situ* generation of the substrate via the indirect assay could be used to follow the purification of the dehydratase. However, accurate measurement of the Michaelis-Menten kinetics with 2-hydroxyglutaryl-CoA using the purified enzyme, required the production of this substrate via the chemical route described in the methods section (Scheme 6). Acid hydrolysis was used to convert the lactone-CoA (**3**) into 2-hydroxyglutaryl-CoA (**4**), because alkaline hydrolysis also cleaves the CoA thiol ester. The presence of lactone-CoA was inferred from the 880 Da peak in the MALDI-TOF mass spectrum (fig 16).

Scheme 6. Synthesis of 2-hydroxyglutaryl-CoA from (*R*)-2-oxo-tetrafuran-carboxylic acid



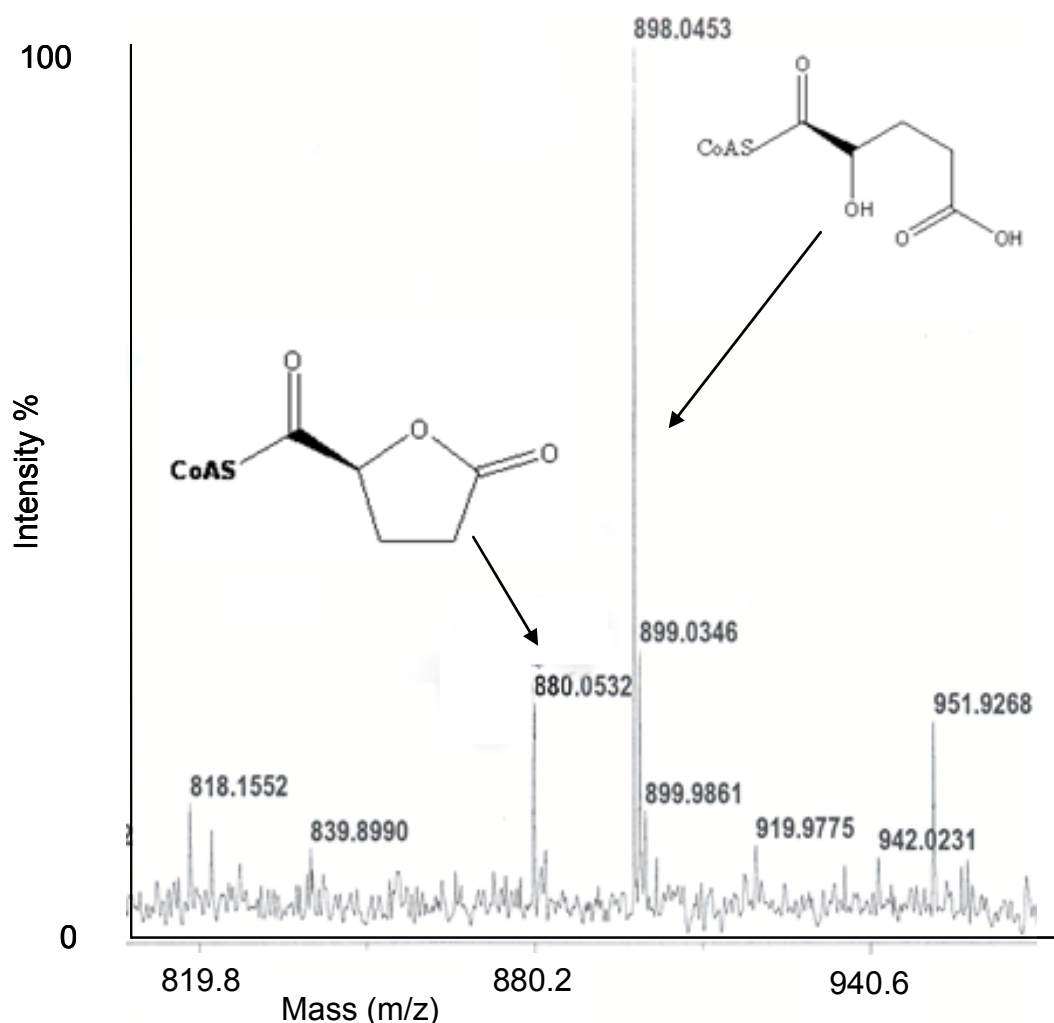


Fig 16. The MALDI-TOF mass spectrum showed that the products of method 2.2.1 were lactone-CoA (880 Da) and 2-hydroxyglutaryl-CoA (898 Da), inlays show the structures of these two compounds.

The occurrence of the lactone-CoA in the sample at pH 2 was possibly an artefact of the preparation method. At pH 2, the lactone and the hydroxyl forms likely existed in equilibrium. When the acid hydrolysis was omitted and the sample was buffered at pH 7, 7.5 or 8, there still remained between about 25 % of the lactone form between pH 7 and 8 (fig 17).

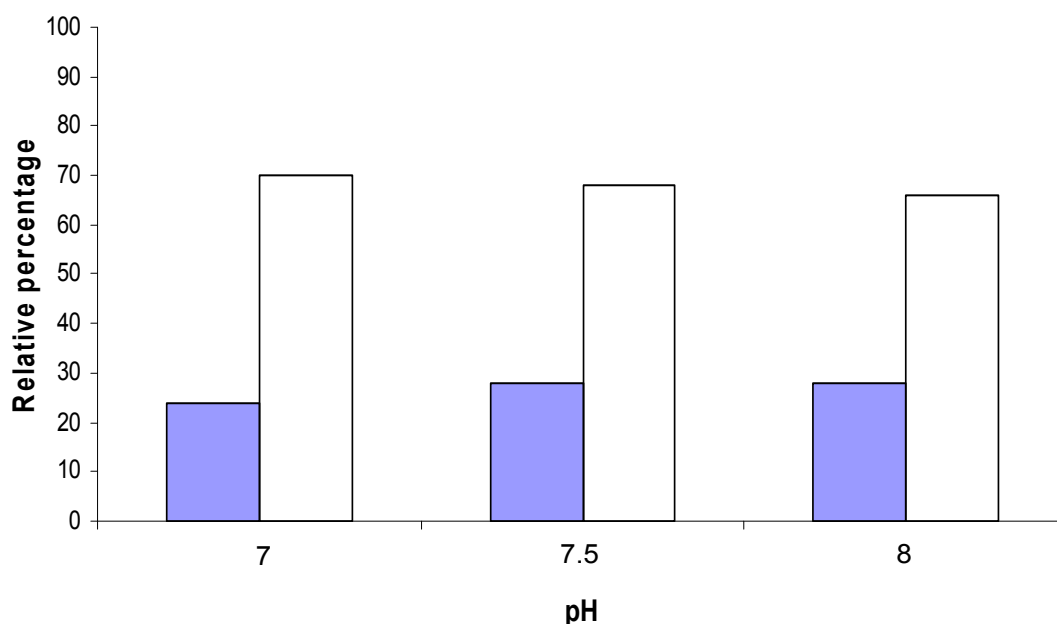


Fig 17. Relative proportions of lactone-CoA (filled blocks) and 2-hydroxyglutaryl-CoA (empty blocks) without acid hydrolysis, in samples buffered at various pH values.

Following acid hydrolysis for 2 hours at pH 0.5, aliquots were buffered in 100 mM phosphate buffer at pH 7, 7.5 and 8, and then assayed with DTNB and CoA-transferase at 410 nm as detailed in the methods section. The total CoA content was estimated by the same method, after alkaline hydrolysis of CoA esters followed by neutralisation. The proportion of free CoA stayed nearly constant at different pH values, except for a slight increase at pH 8. It was observed that the lactone did not form lactone-CoA with the CoA-transferase and acetyl-CoA at measurable rates. The increase of absorbance at 410 nm following the addition of CoA-transferase to the assay mixture could be assumed to be due to the formation of 2-hydroxyglutaryl-CoA. By subtracting the concentrations of 2-hydroxyglutaryl-CoA and free CoA from the total CoA content, the amount of lactone-CoA was inferred.

With this method, it was observed that once acid hydrolysis occurred, incubation at pH 7, 7.5 or 8.0, produced no significant amount of lactone-CoA. It was clear that there would be no lactone-CoA in the preparation to interfere with the dehydratase reaction, which was performed at pH 8. The pH dependence results are summarised below (fig 18).

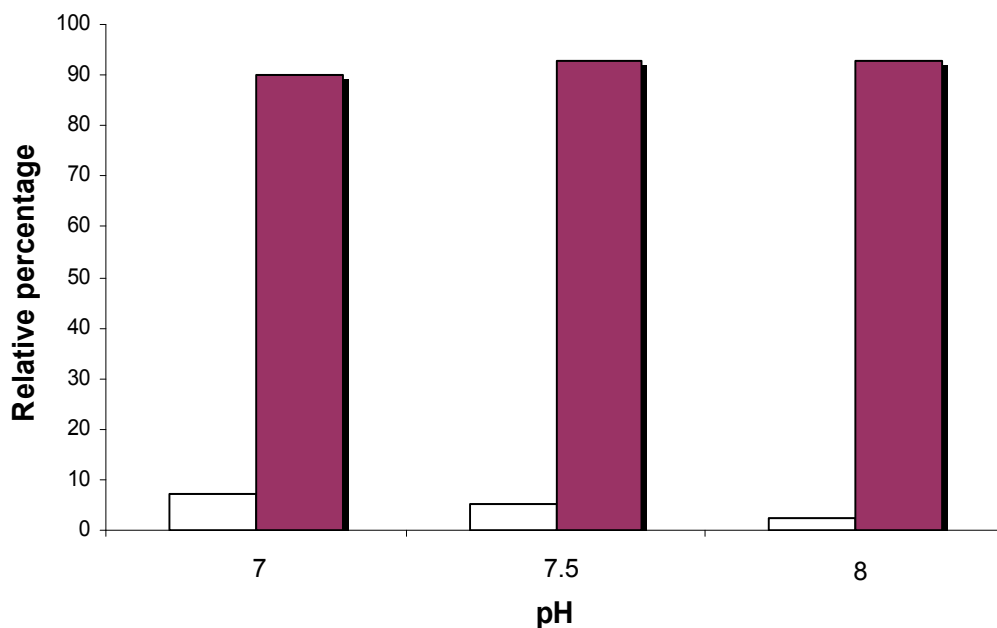


Fig 18. Relative proportions of lactone-CoA (empty blocks) and 2-hydroxyglutaryl-CoA (filled blocks) after acid hydrolysis, in samples buffered at various pH values.

3.3 Michaelis-Menten kinetics with natural substrates

The 2-hydroxyglutaryl-CoA dehydratase activity was assayed directly with the chemically synthesised (*R*)-2-hydroxyglutaryl-CoA as described before. The Michaelis-Menten parameters were $V_{\max} = 55 \pm 5$ U/mg and $K_m = 52 \pm 3$ μ M respectively (fig 19). The kinetic parameters for the hydration of (*E*)-glutaconyl-CoA were measured to be $V_{\max} = 4.5 \pm 0.5$ U/mg and $K_m = 250 \pm 20$ μ M (fig.20).

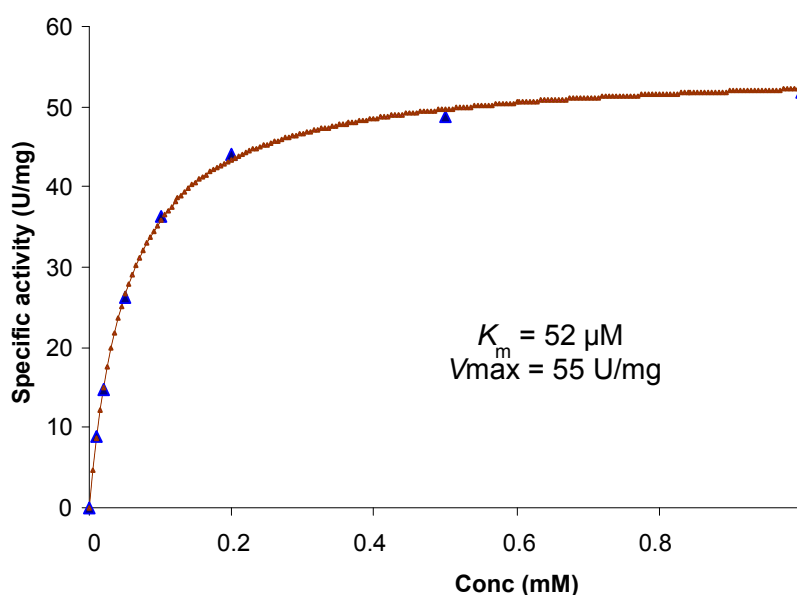


Fig 19. Michaelis-Menten kinetics of the dehydration of 2-hydroxyglutaryl-CoA

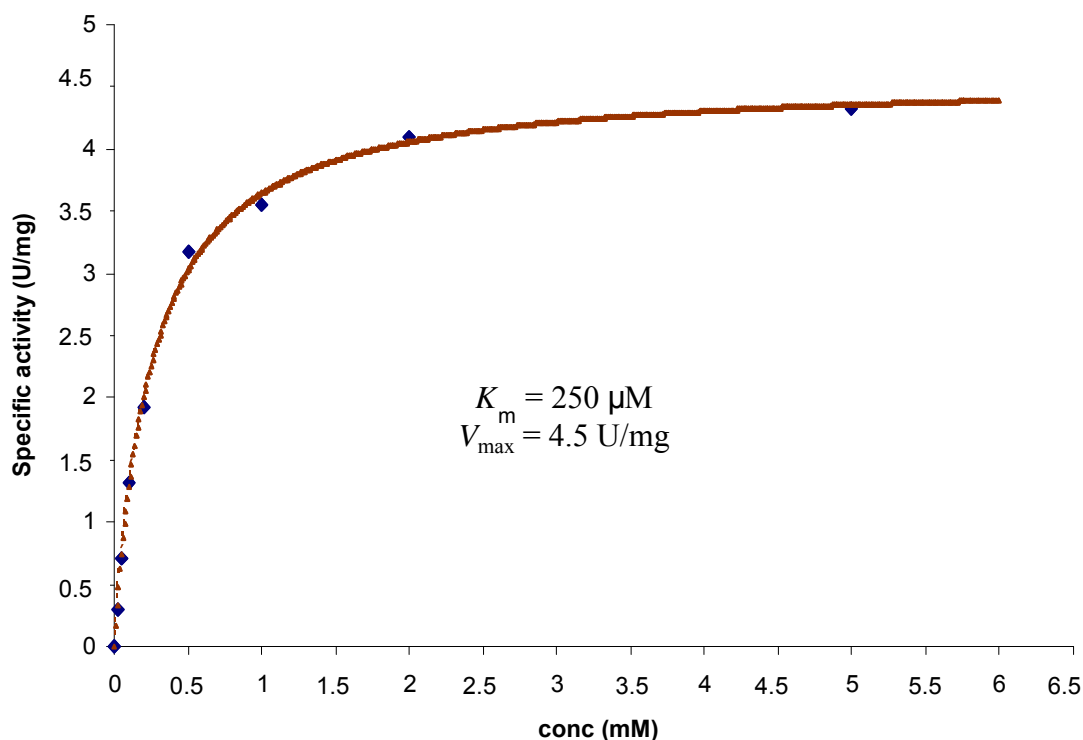


Fig 20. Michaelis-Menten kinetics of the hydration of glutaconyl-CoA

3.4 Substrates of the 2-hydroxyglutaryl-CoA dehydratase, their reactions and kinetics

3.4.1 (*R*, *S*)-2-Hydroxyadipoyl-CoA

The UV-visible spectra of (*R*, *S*)-2-hydroxyadipoyl-CoA and acetyl-CoA were quite similar except that the former had a maximum absorbance at 263 nm (fig 21). The extinction coefficient at 263 nm was estimated to be $17 \text{ mM}^{-1} \text{ cm}^{-1}$. The concentrations were compared by means of the DTNB assay. MALDI-TOF mass analysis of the reaction mixture of a direct assay with the dehydratase showed the presence of hydroxyadipoyl-CoA at 913 Da and a reaction product at 895 Da (fig 22).

This suggested that the substrate was dehydrated which correlated to the increase of absorption at 290 nm in the direct assay. The use of Gct for the synthesis of this substrate would possibly also yield the 5-hydroxy isomer. However the dehydration occurred, showing the presence of the 2-isomer. Only the *R*-isomer was possibly accepted by the enzyme and not the *S* isomer. These factors and the longer carbon chain made hydroxyadipoyl-CoA a poorer substrate than (*R*)-2-hydroxyglutaryl-CoA. The Michaelis–Menten parameters were measured to be $K_m = 100 \pm 10 \text{ } \mu\text{M}$ and $V_{\text{max}} = 29.0 \pm 0.3 \text{ U/mg}$ as in fig 23.

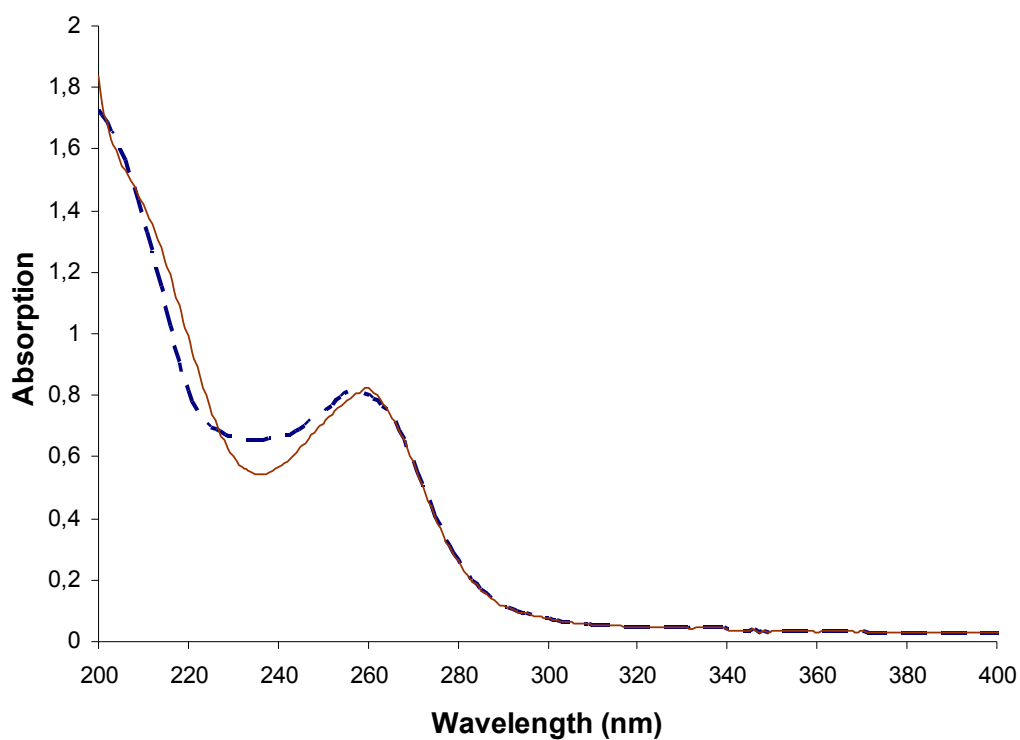


Fig 21. The UV-visible spectra of (*R, S*)-2-hydroxyadipoyl-CoA (solid line) and acetyl-CoA (dotted line), both concentrations at around 0.05 mM

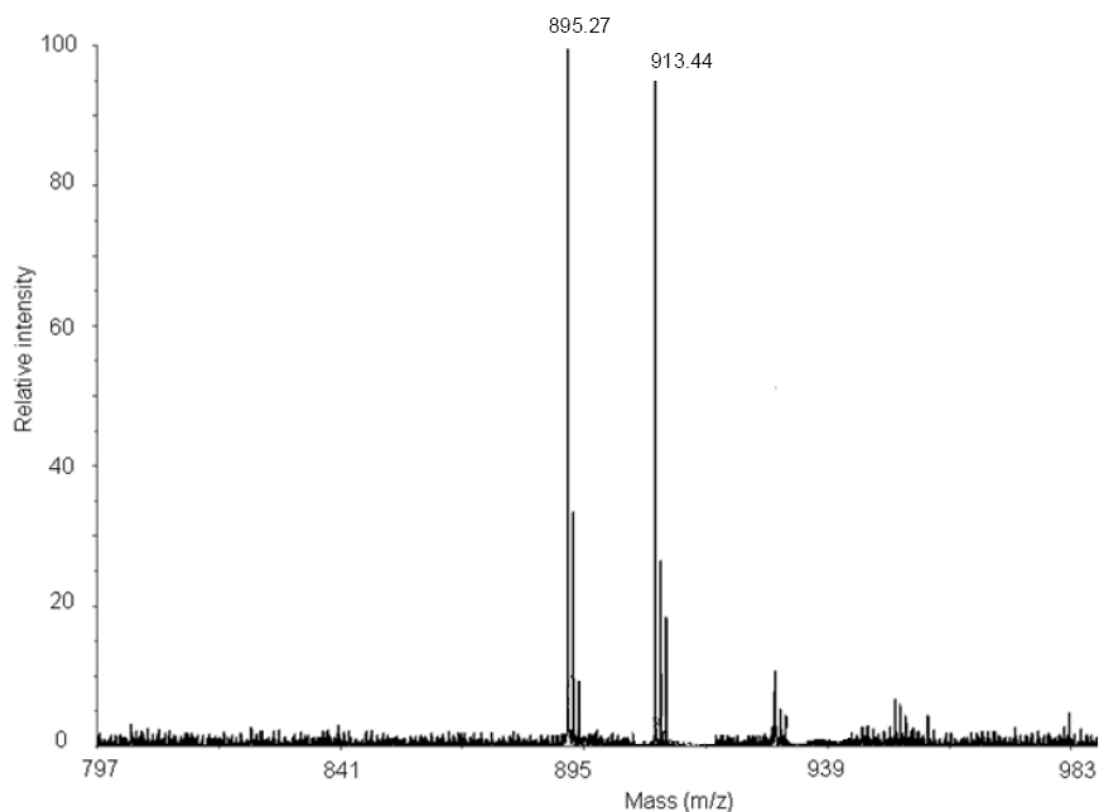


Fig 22. MALDI-TOF mass spectrum showing 2-hydroxyadipoyl-CoA (913 Da) and its dehydration product at 895 Da.

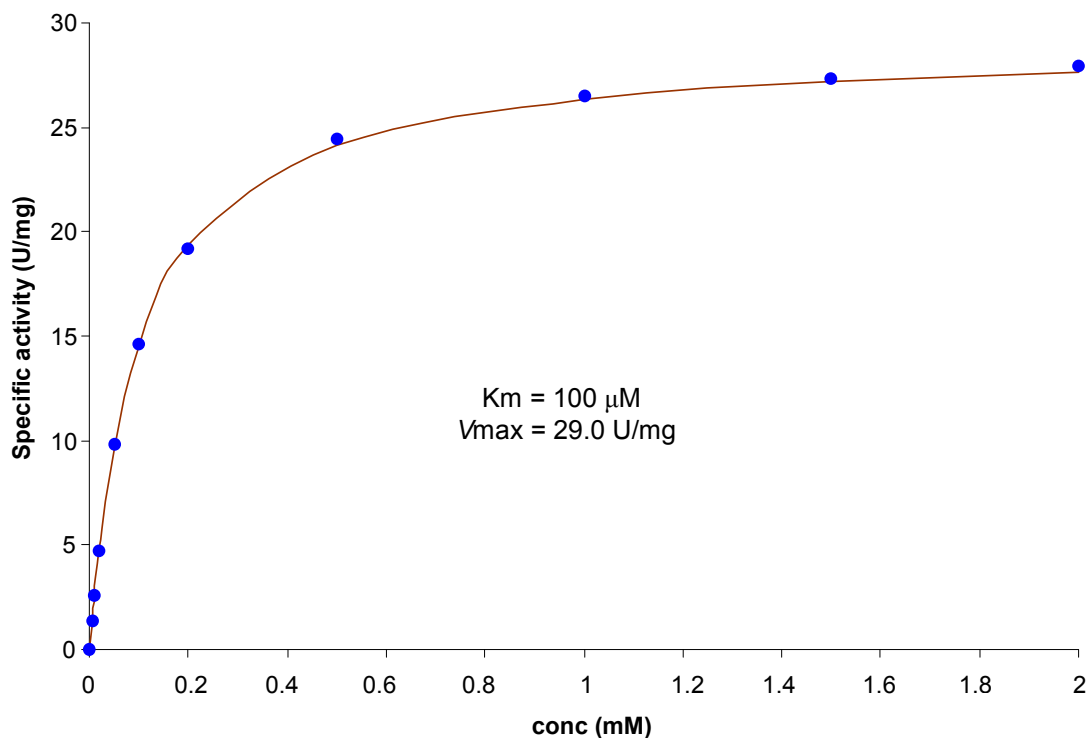


Fig 23. Michaelis-Menten kinetics of dehydratase activity with the substrate 2-hydroxyadipoyl-CoA

3.4.2 Muconyl-CoA

This substrate was characterised by its UV-vis spectrum exhibiting a peak at 262 nm (fig. 24). The extinction coefficient at 262 nm was estimated to be $26 \text{ mM}^{-1} \text{ cm}^{-1}$. The direct assay of muconyl-CoA with 2-hydroxyglutaryl-CoA dehydratase at 290 nm showed apparent hydration.

Analysis of the MALDI-TOF mass spectrum of the assay mixture showed two peaks, one corresponding to muconyl-CoA at m/z 893 Da and the other to the reaction product at 911 Da (fig 25). This demonstrated that the product was hydrated as indicated by the optical assay. The kinetic parameters calculated for this substrate were $K_m = 570 \pm 50 \mu\text{M}$ and $V_{\text{max}} = 3.1 \pm 0.4 \text{ U/mg}$ as seen in fig 26. Muconyl-CoA was expected to give a stable conjugated ketyl radical which may be detected by EPR spectroscopy at low temperature. However, no radical could be detected.

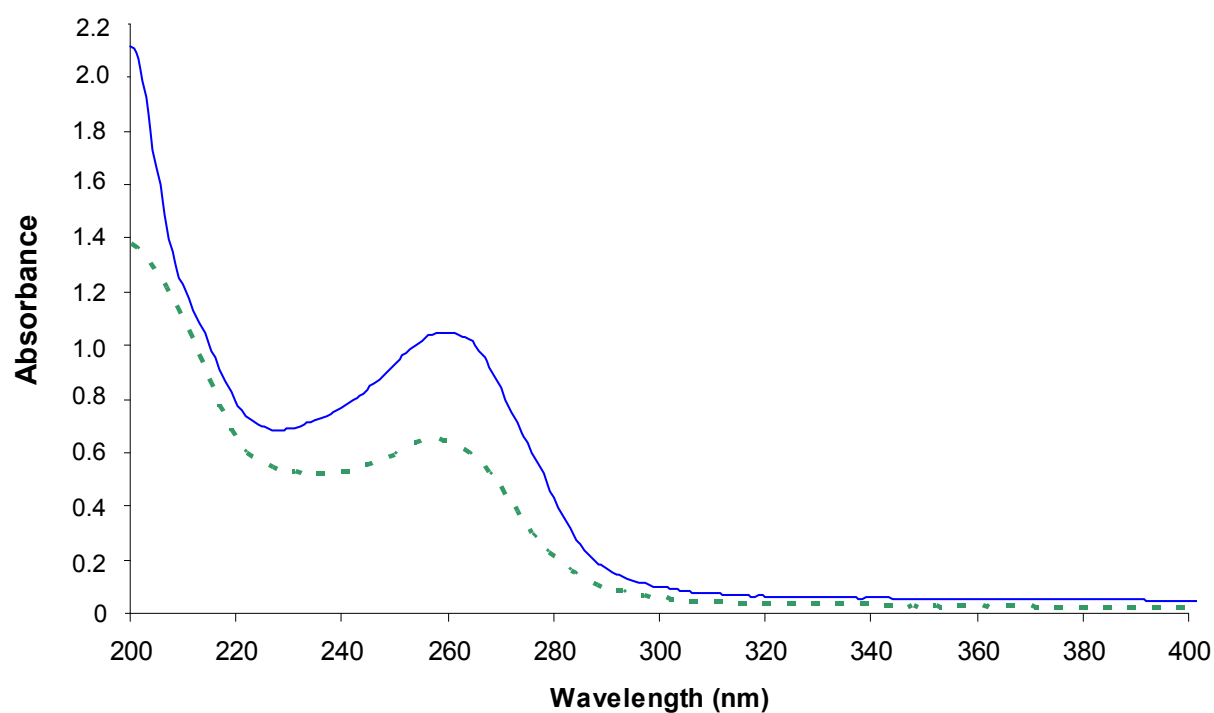


Fig 24. UV-visible spectra of muconyl-CoA (solid) vs. acetyl-CoA (dotted) at ~ 0.04 mM, concentrations determined by the DTNB assay.

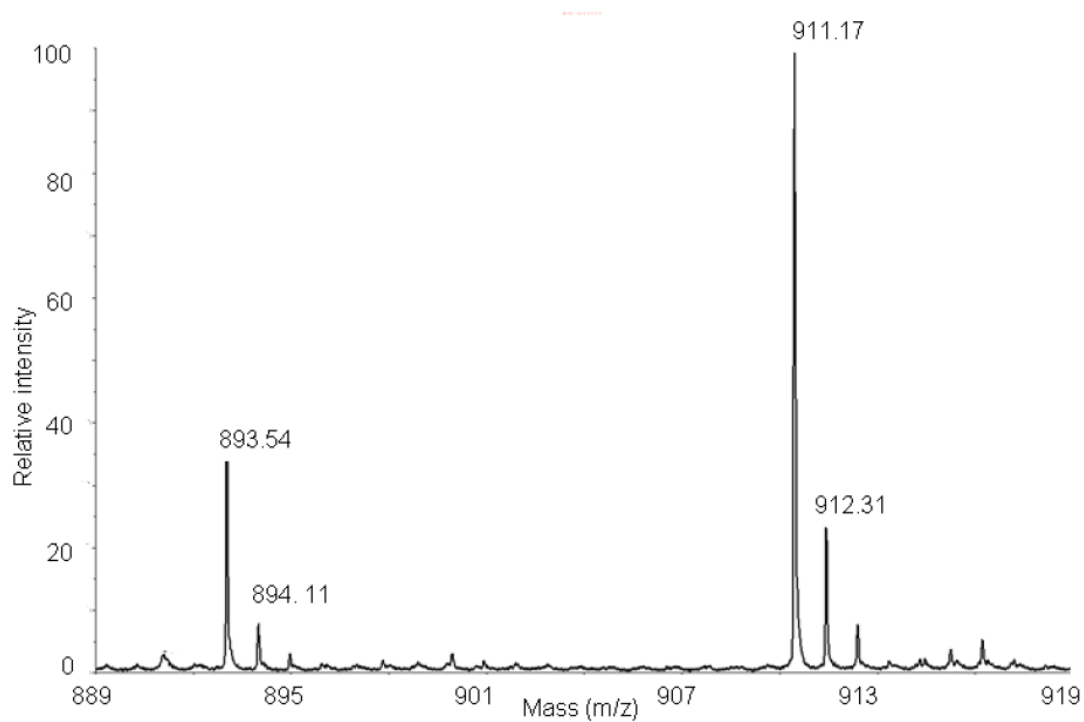


Fig 25. MALDI-TOF mass spectra showing muconyl-CoA (893 Da) and its hydration product (911 Da)

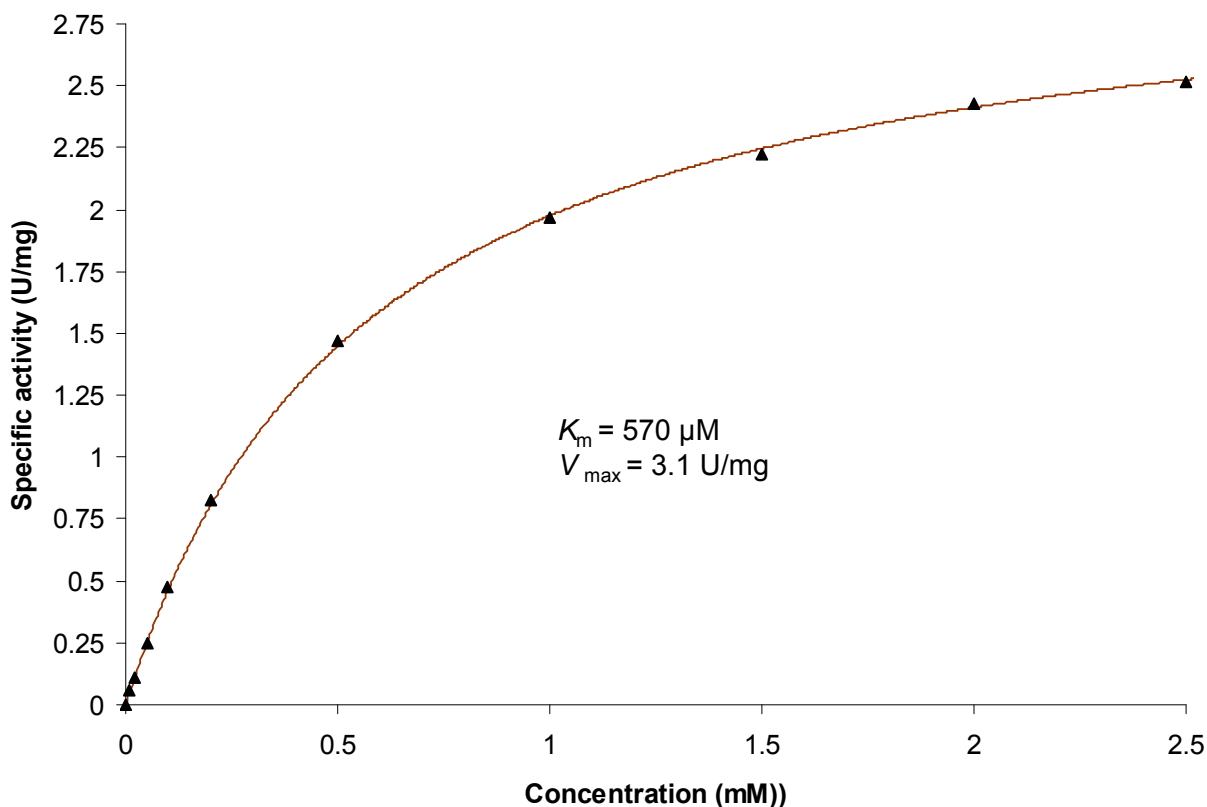


Fig 26. Michaelis-Menten kinetics of hydratase activity with muconyl-CoA

3.4.3 Oxalocrotonyl-CoA

This substrate analogue was characterised by the UV-visible spectrum with a maximum at 265 nm and a shoulder at 300 nm seen in fig 27. The extinction coefficient at 265 nm was estimated to be $20 \text{ mM}^{-1}\text{cm}^{-1}$.

A detailed study of the tautomerism and other properties of hydroxymuconate as a free acid based on UV-visible and NMR has appeared elsewhere (Whitman et al., 1991); it was stated that the *keto* form predominates over the *enol* below pH 10.5. The CoA ester may therefore reasonably be supposed also to be the oxo-ester, possibly the 4-oxo isomer, since the 2-oxo isomer would hydrolyse rapidly.

The MALDI-TOF spectrum of the dehydratase assay mixture exhibited two peaks at masses of 908 and 926 Da (fig 28). The first one corresponded to oxo-hexenoyl-CoA and the second one, to its hydration product. This correlated well with the observations in the assay at 290 nm. The Michaelis-Menten parameters were $K_m = 1.1 \pm 0.1 \text{ mM}$ and $V_{\text{max}} = 1.5 \pm 0.6 \text{ U/mg}$ (fig. 29).

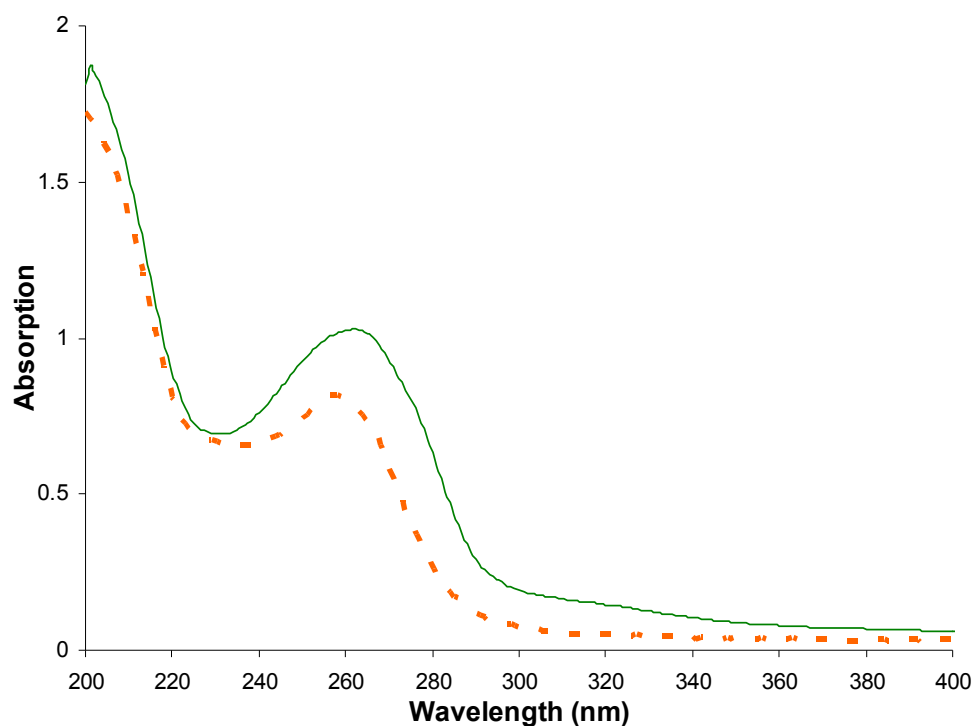


Fig 27. UV-vis spectra of oxalocrotonyl-CoA (solid) vs. acetyl CoA (dotted) at ~ 0.05 mM, concentrations determined by the DTNB assay

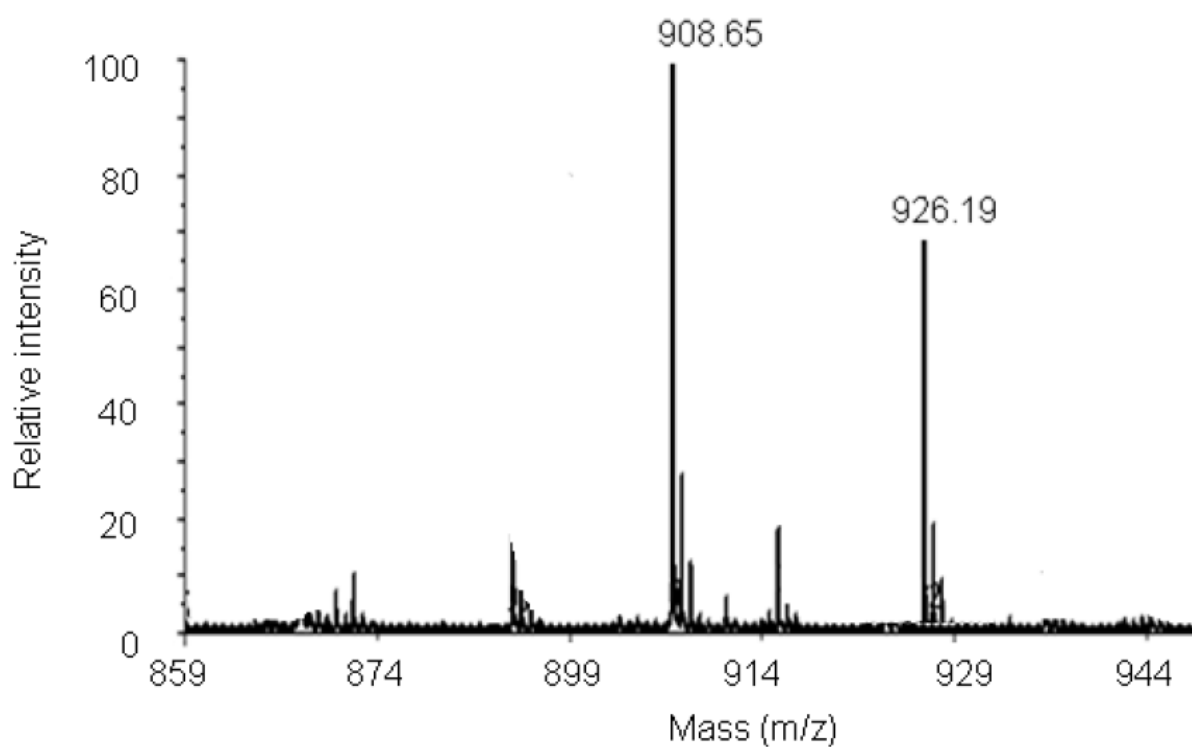


Fig 28. MALDI-TOF mass spectra showing oxalocrotonyl-CoA (908 Da) and its hydration product (926 Da)

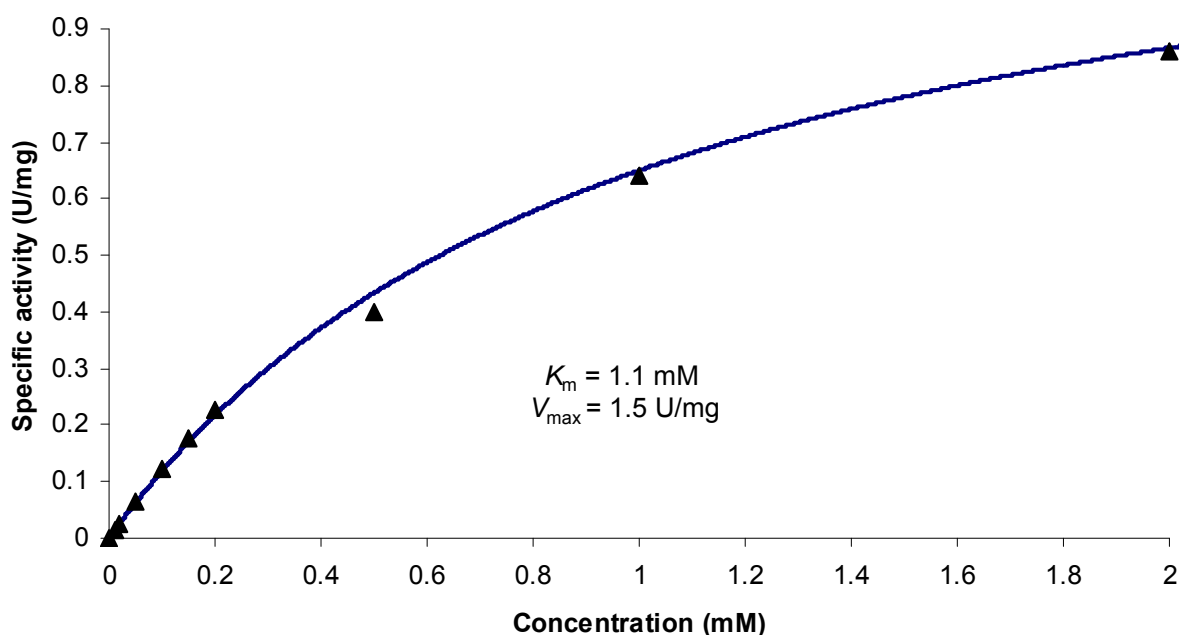


Fig 29. Michaelis-Menten kinetics of the 2-hydroxyglutaryl-CoA dehydratase activity with oxalocrotonyl-CoA.

3.4.4 Butynedioyl-CoA

This substrate was characterized by its UV-visible spectrum which shows a peak at 266 nm shown in fig 19. The extinction coefficient at 266 nm was estimated to be $26 \text{ mM}^{-1}\text{cm}^{-1}$. The hydration of this substrate by dehydratase was assayed as described in methods. It was also detected by MALDI-TOF mass (fig 31) from its m/z peak at 863 Da. The enzymatic hydration lead to an unusual product, free CoA (fig 19). The Michaelis-Menten kinetic parameters were measured to be $K_m = 2.0 \pm 0.1 \text{ mM}$ and $V_{\max} = 1.88 \pm 0.11 \text{ U/mg}$, (fig 32).

If this unusual C_4 substrate with a linear geometry was really hydrated, the presumable product would be hydroxyfumaryl-CoA or hydroxymaleyl-CoA. This would then tautomerise to the keto-form, oxaloacetyl-CoA. Oxaloacetyl CoA (2-oxosuccinyl-CoA) would be unstable and hydrolyse to give oxaloacetate (2-oxosuccinate) and free CoA (fig 33).

Free CoA was detected by the continuous DTNB method – an aliquot from the assay of dehydratase with all the usual components and 1mM butynedioyl-CoA as substrate was introduced into an anaerobic mixture containing 0.1 mM DTNB and 200 mM acetate in 50 mM potassium phosphate buffer pH 7.0. The assay (fig 34) showed that the increase in absorbance was enzyme-catalysed. The fairly high initial absorbance was due to the presence of DTT in the reaction mixture. Taking into account the dilution factor of the aliquot, the concentration of free CoA in the original mixture was calculated to be about 0.19 mM. The

amount of the substrate consumed in the dehydratase assay in about 10 minutes (starting with 2 mM) was 0.2 mM.

The oxaloacetate was detected by the malate dehydrogenase assay. The unknown sample was assayed against different concentrations of NADH until an optimal response could be obtained. The best response was at 0.04 mM NADH (fig 35). When the dilution was factored in, the original oxaloacetate concentration was back-calculated to be 0.196 mM. Thus, the amount of butyryl-CoA consumed was quantitatively converted to free CoA and oxaloacetate within limits of experimental error.

A control without the dehydratase did not show significant hydrolysis during a similar time period (data not shown). A control without activation (no ATP in the reaction mixture) was also performed and showed no activity. That this reaction occurred due to contamination of crotonase was ruled out because butyryl-CoA gave no response with the crotonase assay.

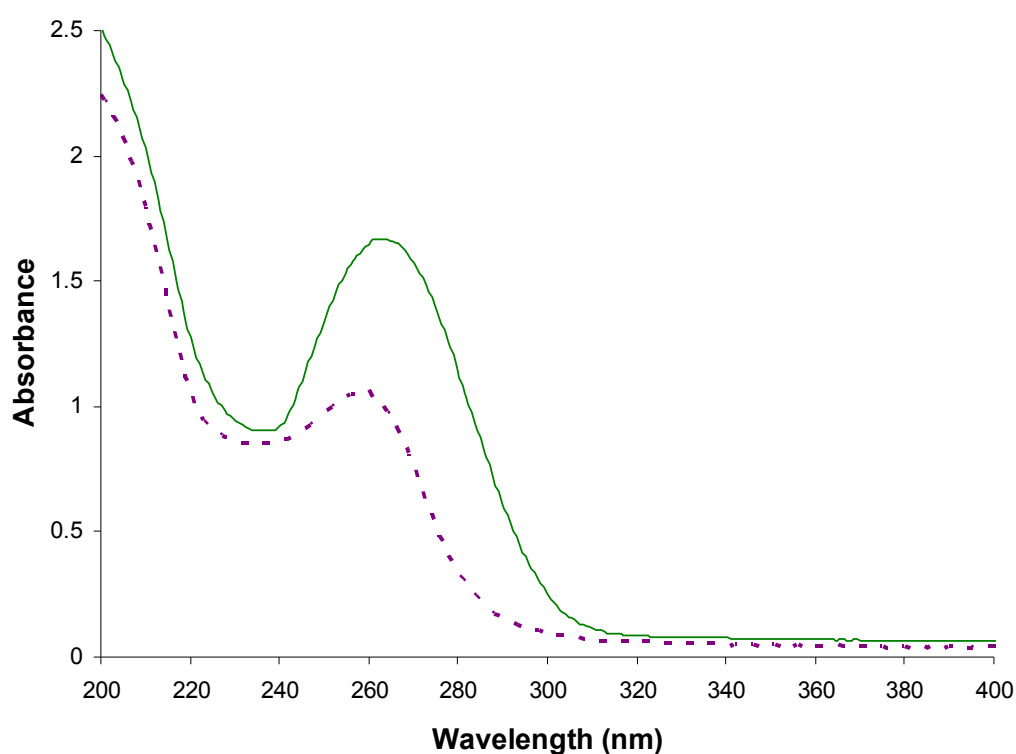


Fig 30. UV-visible spectra of butyryl-CoA (solid) vs. acetyl-CoA (dotted) at ~0.06 mM

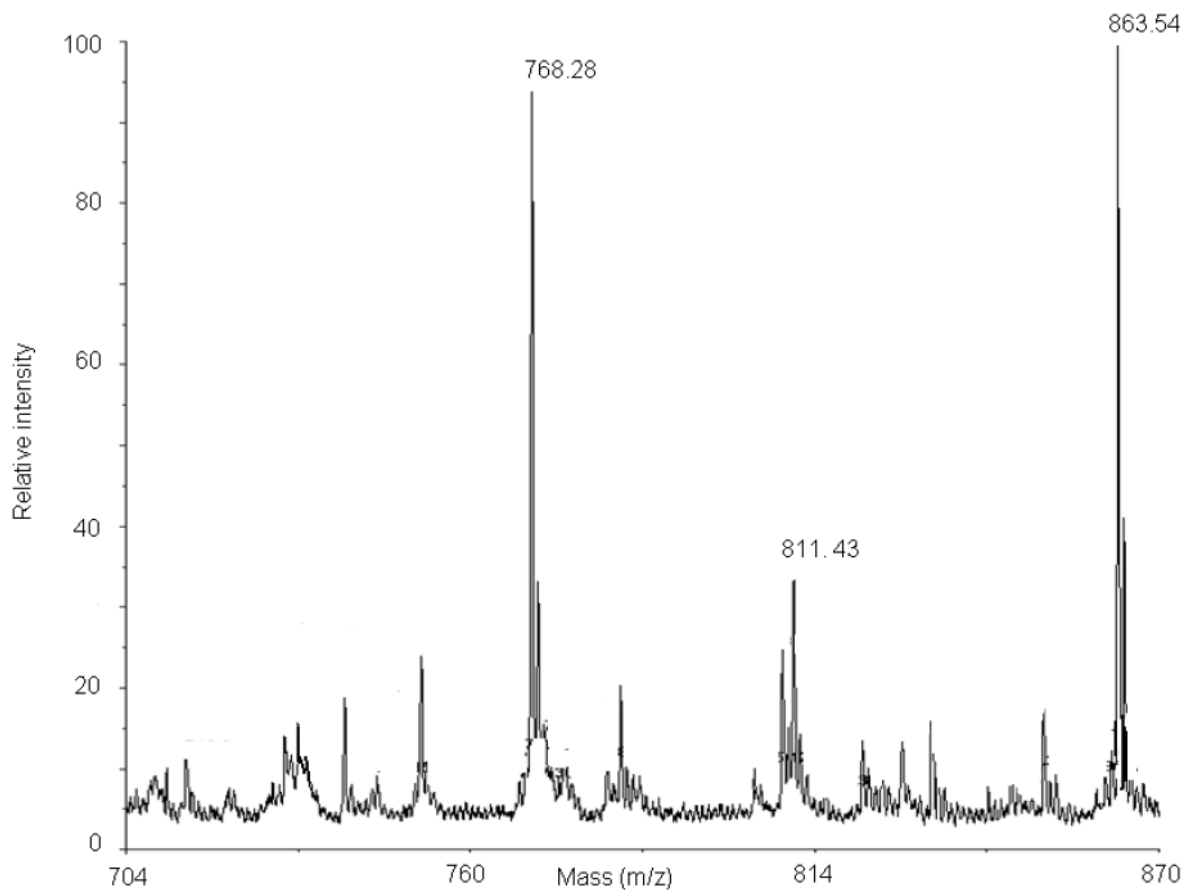


Fig 31. MALDI-TOF mass spectra showing the product of butynedioyl-CoA (863 Da) hydration was free CoA (768 Da).

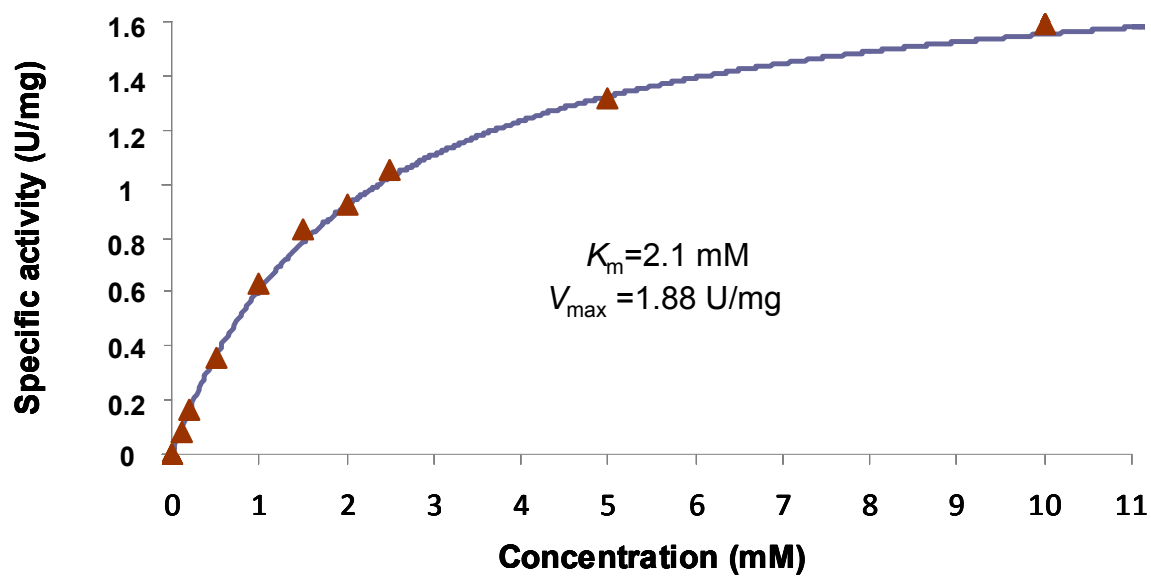


Fig 32. Michaelis-Menten kinetics of hydratase activity with butynedioyl-CoA.

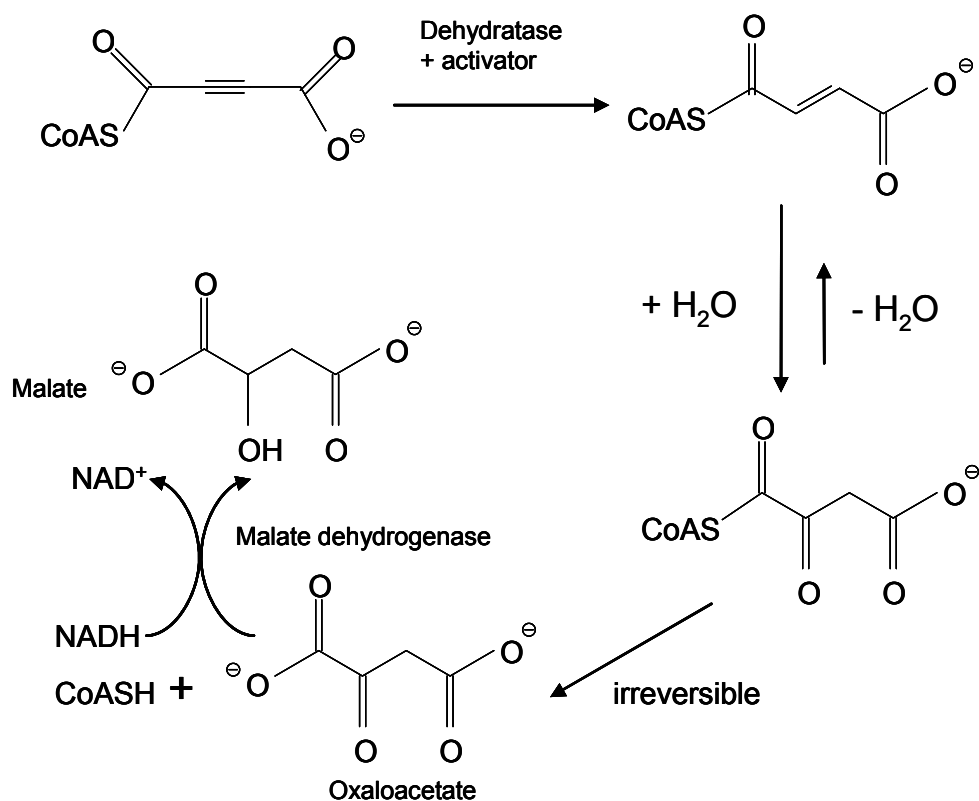


Fig 33. Proposed reaction scheme involving butynedioyl-CoA

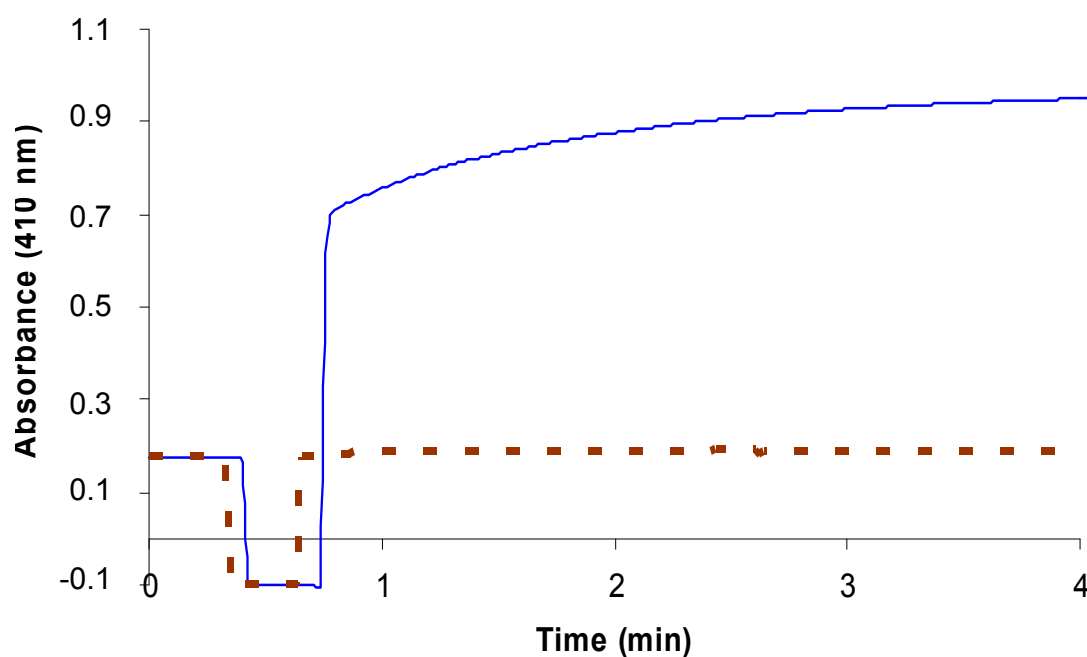


Fig 34. Generation of free CoA from butyne-dioyl-CoA (from dehydratase reaction) assayed by DTNB (solid). Sample without the substrate as control (dotted).

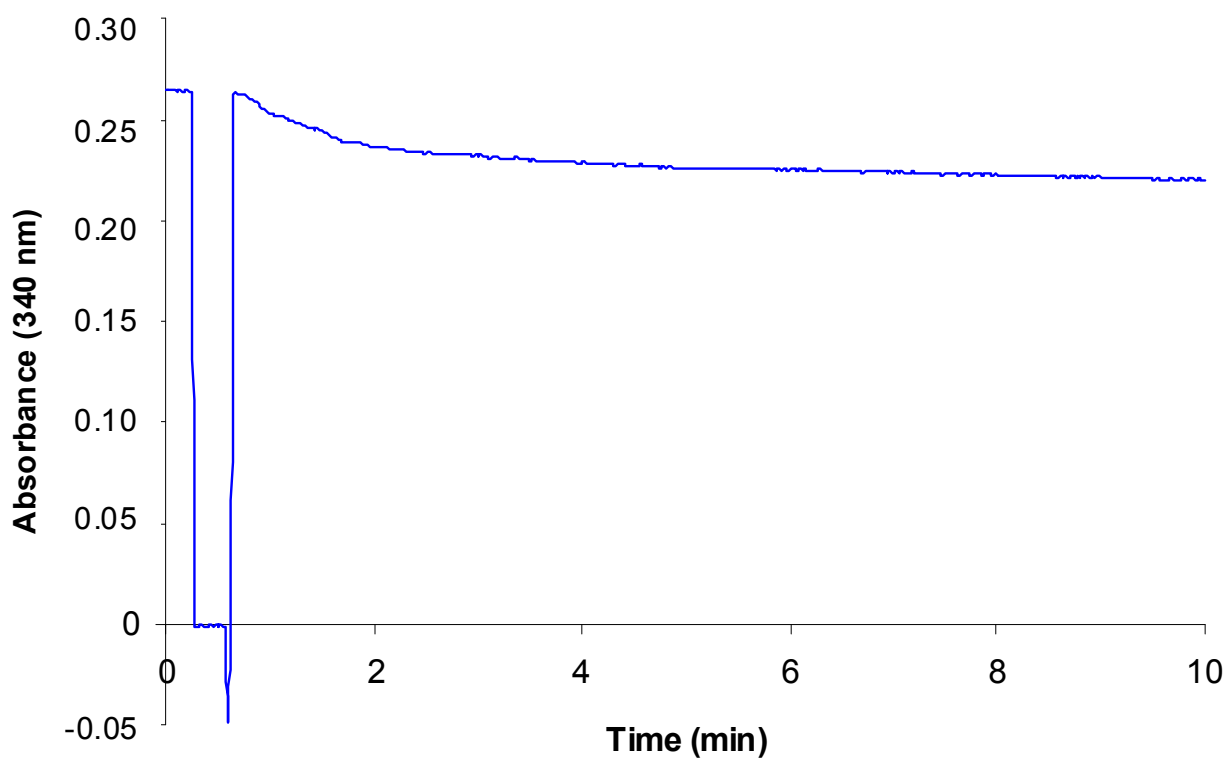
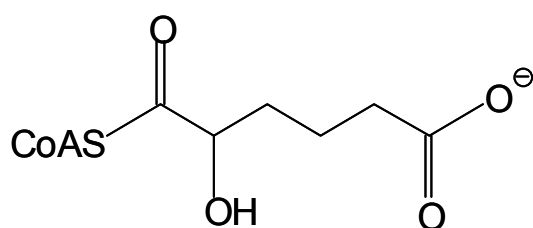
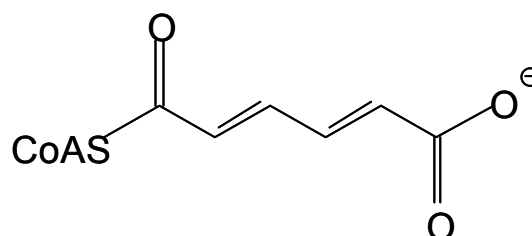


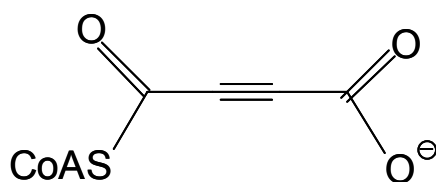
Fig 35. Malate dehydrogenase assay with the products of dehydratase-catalysed hydration of butynediol-CoA



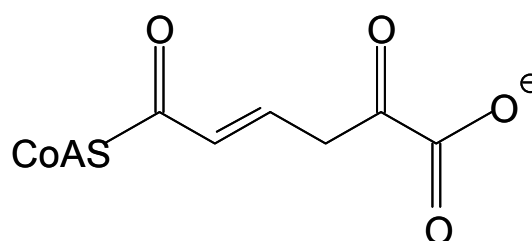
2-Hydroxyadipoyl-CoA



Muconyl-CoA



Butynediol-CoA



Oxalocrotonyl-CoA

Fig 36. Summary of the structures of non-natural substrates of 2-hydroxyglutaryl-CoA dehydratase

Table 2. Summary of Michaelis-Menten parameters with various substrates of 2-hydroxyglutaryl-CoA dehydratase

Substrate	K_m (μM)	V_{max} (U/mg)
2-Hydroxyglutaryl-CoA	52 ± 3	55 ± 5
Glutaconyl-CoA	250 ± 20	4.5 ± 0.5
2-Hydroxyadipoyl-CoA	100 ± 10	29.0 ± 0.3
Butynedioyl-CoA	2100 ± 100	1.88 ± 0.11
Muconyl-CoA	570 ± 50	3.1 ± 0.4
Oxalocrotonyl-CoA	1100 ± 100	1.5 ± 0.6

3.4.5 Other possible substrates

(*R*)-2-Hydroxybutyryl-CoA, 3-methylglutaconyl-CoA, 2-pentenoyl-CoA, and crotonyl-CoA gave no appreciable response with the direct dehydratase assay. Therefore, these compounds were not utilized as substrates (fig. 37).

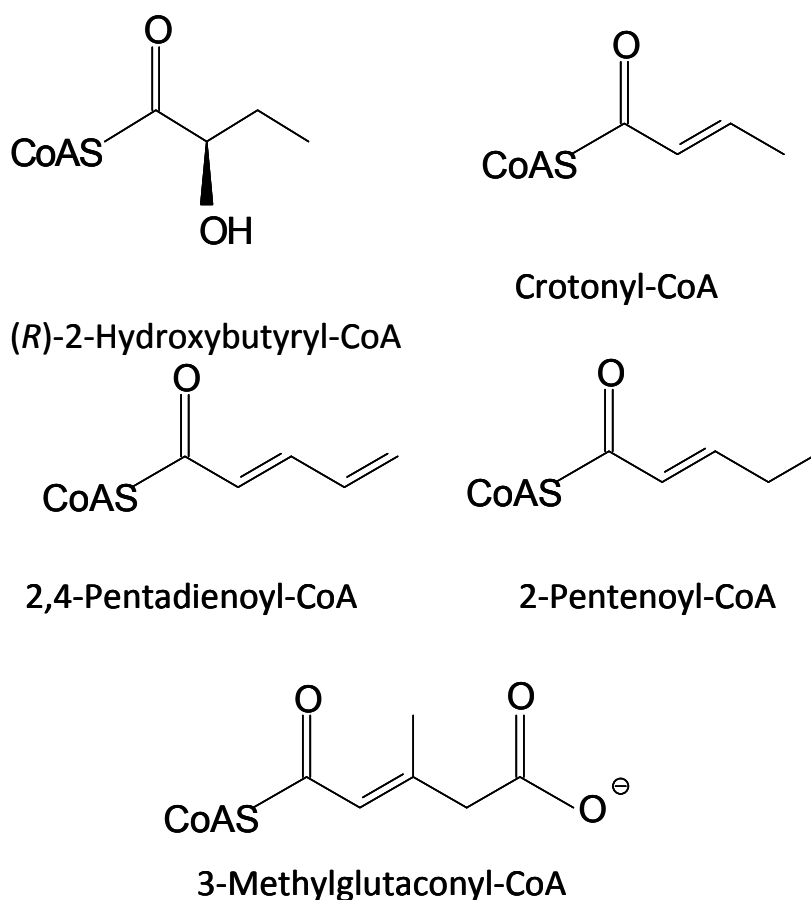


Fig 37. Summary of the structures of non-substrates of 2-hydroxyglutaryl-CoA dehydratase

3.5 Inhibition of dehydratase activity

3.5.1 with metronidazole

Metronidazole is a known inhibitor of 2-hydroxyglutaryl-CoA dehydratase from *Clostridium symbiosum*. The activity was measured with the normal components of the direct assay system including dithionite and 2-hydroxyglutaryl-CoA as substrate. All operations were performed under anaerobic conditions.

When the activator was treated with different concentrations of metronidazole, then purified by passage through a SpinTrap (Sephadex G-25 column) equilibrated with 100 mM Tris-HCl pH 8.0 and then added to the dehydratase assay mixture, a concentration effect on the activity was observed. A minimum of 20 μ M metronidazole was needed to abolish the activity (fig 38).

The time of exposure of the activator to 20 μ M metronidazole was also shown to have an effect on the activity. An exposure time about 10 minutes abolished the activity nearly completely (fig 39).

Other experiments were performed by changing the order of addition of the various components as described below. In these cases, the concentration of metronidazole was fixed at 20 μ M. When the dehydratase was incubated together with metronidazole and activator, and the small molecules removed by passage through a SpinTrap column, addition of substrate resulted in poor activity. However, direct treatment of the dehydratase with metronidazole, followed by desalting, and incubation with the activator gave the full activity. The result was the same when the dehydratase was pre-reduced with the activator for 5 minutes and metronidazole added directly afterwards.

In summary, if the activator alone or both the dehydratase and the activator were exposed to metronidazole, there was no activity. But if the dehydratase was pre-reduced with the activator prior to metronidazole exposure, there was no inhibitory effect. The exposure of dehydratase alone also had no effect on the activity as well (fig 40).

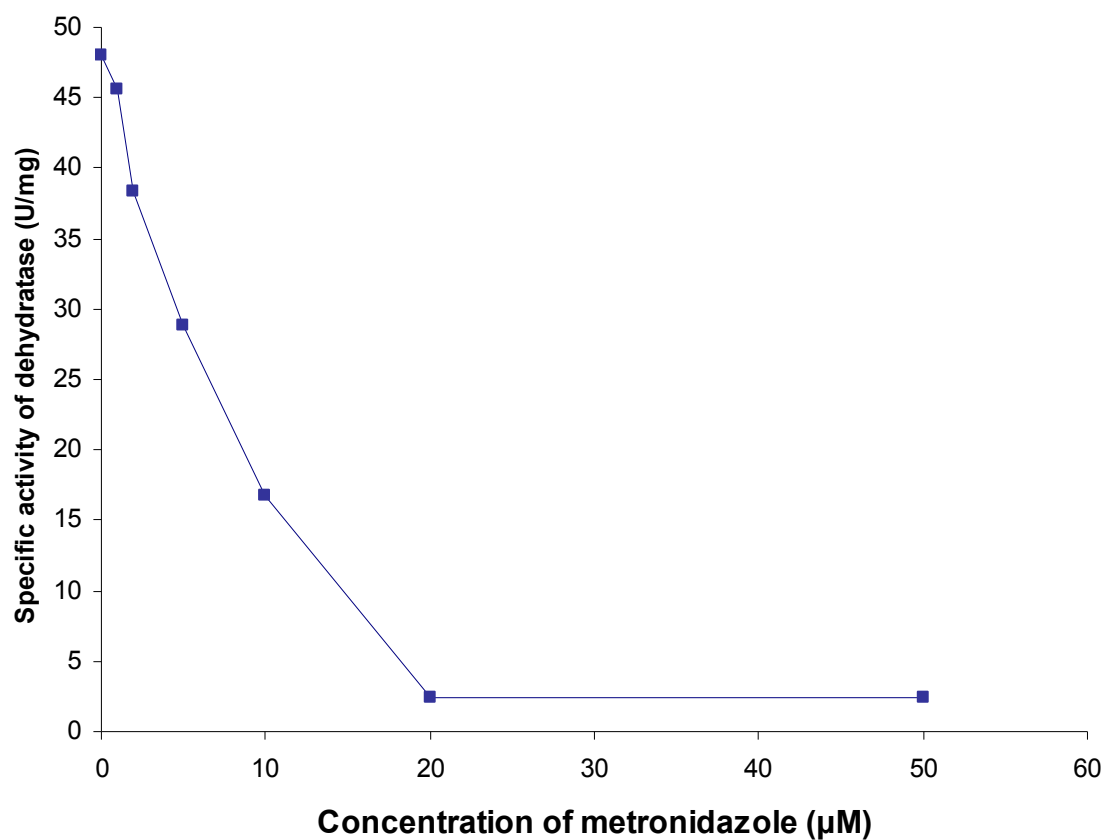


Fig 38. The inhibition of the dehydratase activity depends on the concentration of metronidazole

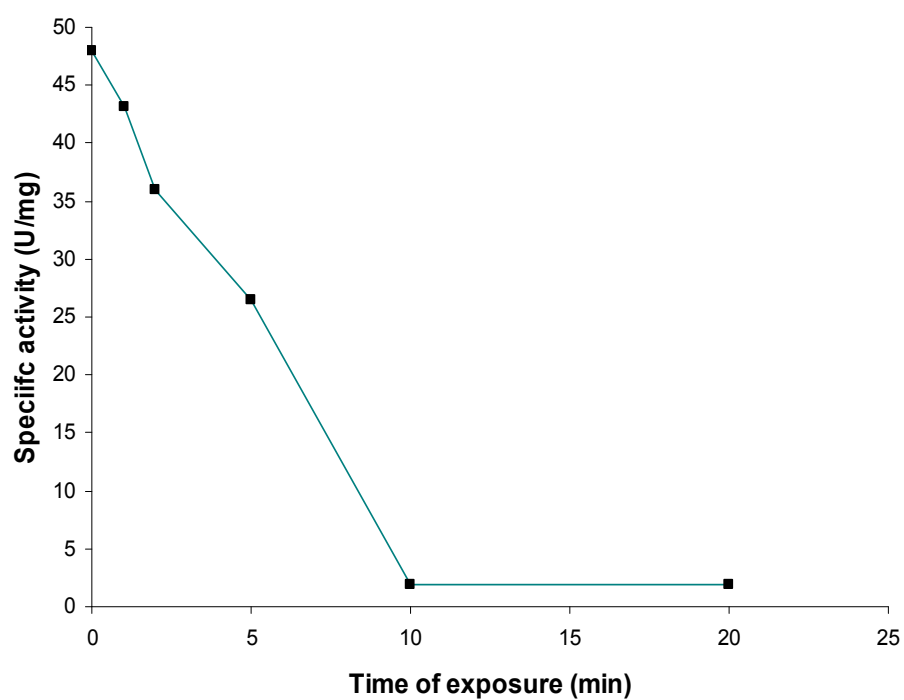


Fig 39. Effect of exposure-time of the activator to metronidazole on the dehydratase activity

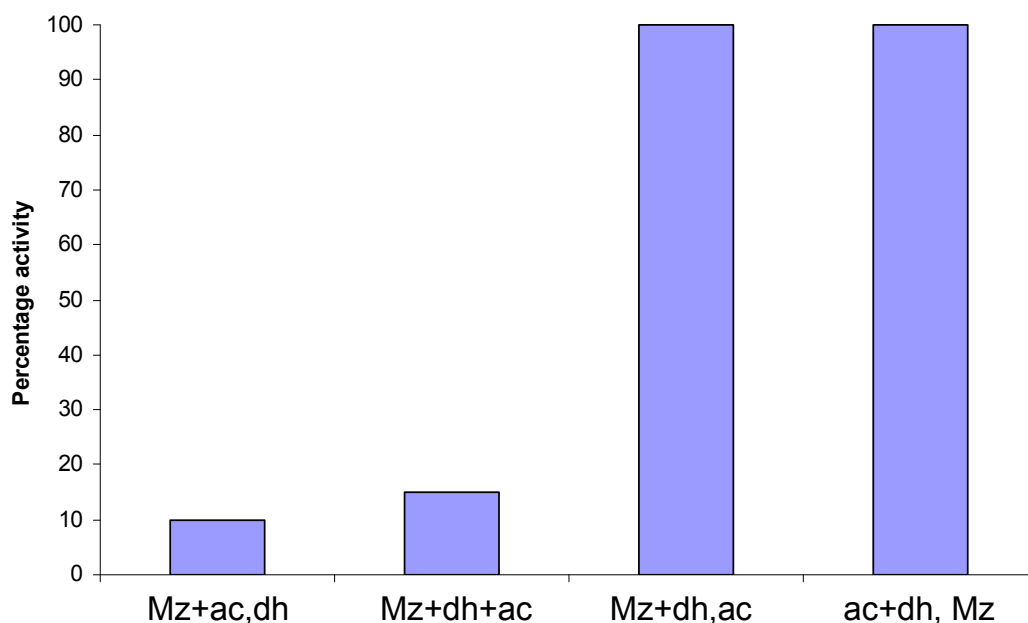


Fig 40. Effect of Mz (metronidazole) addition on the percentage activity: Mz+ac, dh indicates incubation of Mz and activator first followed by dehydratase and substrate; Mz+dh+ac indicates incubation of Mz, activator and dehydratase together followed by addition of substrate; Mz+dh,ac indicates incubation of Mz with dehydratase, followed by desalting and addition of activator and substrate; ac+dh, Mz indicates pre-incubation of activator and dehydratase, followed by the addition of Mz and then substrate.

3.5.2 with CoA esters

Glutaryl-CoA, pentanoyl-CoA and difluoroglutaryl-CoA were tested as inhibitors of the 2-hydroxyglutaryl-CoA dehydratase from *C. symbiosum*. The limiting of activity by the action of 0.1 mM of each of these was tested. In all the cases, the inhibitory effect was reduced by addition of sufficient amounts of the substrate 2-hydroxyglutaryl-CoA. However, the recovery of activity with a fixed amount of natural substrate was lowest in the presence of difluoroglutaryl-CoA (Table 3).

Table 3. Inhibitory effect of various CoA esters at 0.1 mM concentration

	Substrate concentration (mM)	Specific activity (U/mg)	Substrate concentration (mM)	Specific activity (U/mg)
Without inhibitor	0.1	36.4	0.2	44.1
Difluoroglutaryl -CoA	0.1	15.8	0.2	23.1
Pentanoyl-CoA	0.1	28.4	0.2	35.3
Glutaryl-CoA	0.1	18.6	0.2	25.4

Difluoroglutaryl-CoA was identified from the 918 Da peak in the MALDI-TOF mass spectrum (fig 41). The simulation of the Michaelis-Menten plots yielded kinetic constants $K_m = 0.052$ mM and $V_{max} = 55$ U/mg for the uninhibited and $K_m = 0.132$ mM and $V_{max} = 39$ U/mg for the inhibited reaction, respectively (fig 42).

The inhibitory constant K_i value was calculated from the apparent K_m values at the inhibitor concentration of $[I]$, by the formula

$$K_m^{app} = K_m (1 + [I] / K_i)$$

The calculated K_i was 0.069 mM, which showed it was a good inhibitor.

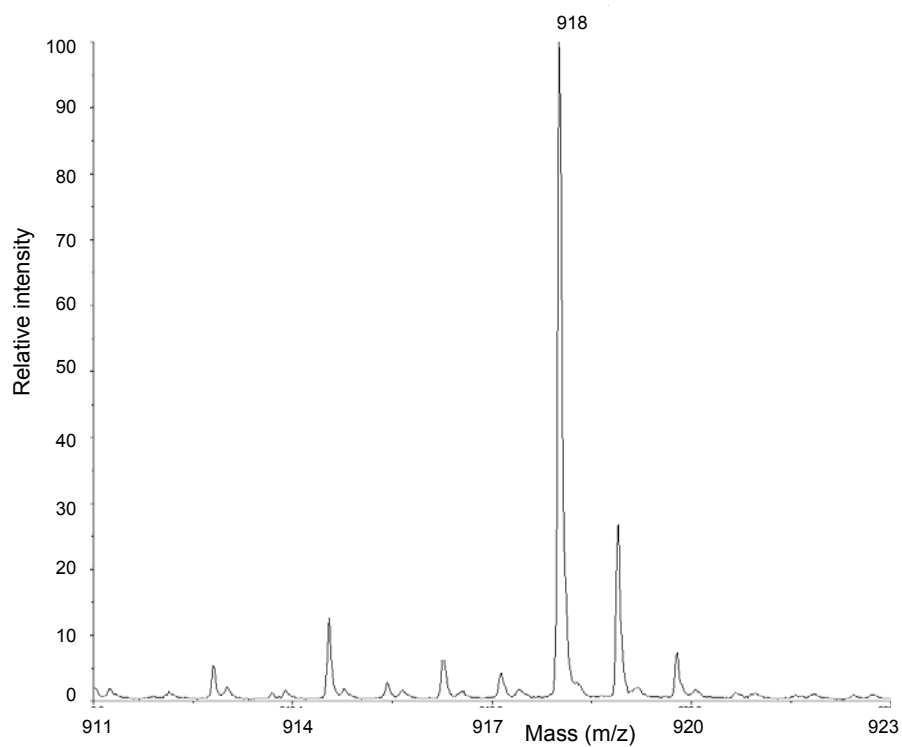


Fig 41. MALDI-TOF mass spectrum showing the peak at 918 Da corresponding to difluoroglutaryl-CoA.

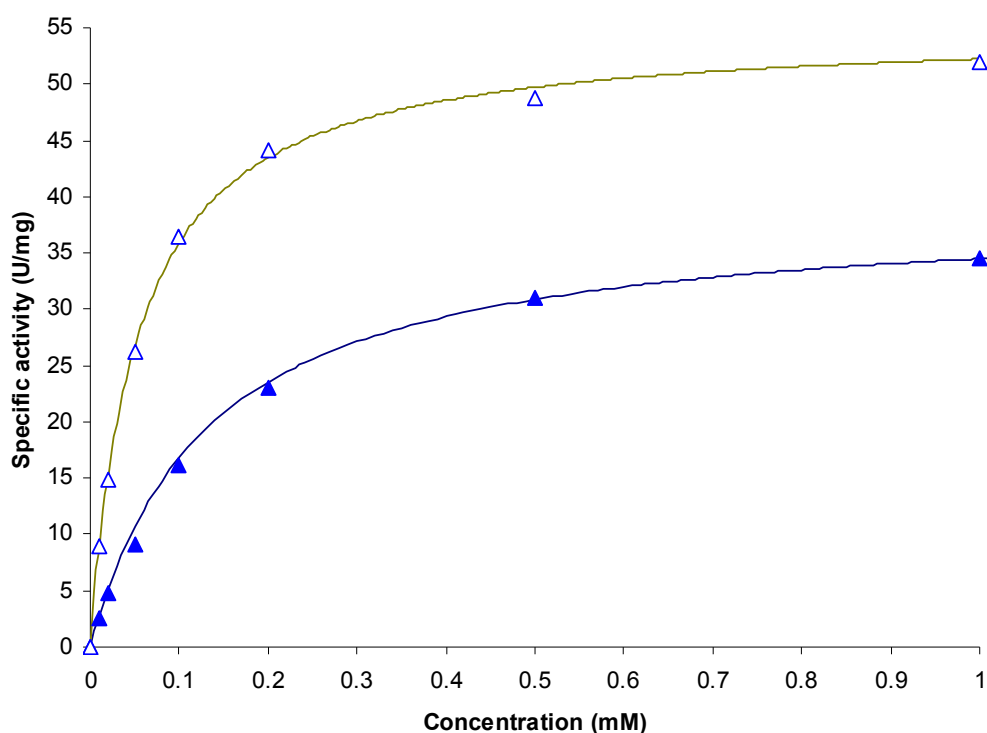


Fig 42. Michaelis–Menten plots of the difluoroglutaryl-CoA inhibited (filled triangles) and uninhibited (empty triangles) enzymatic dehydrations of 2-hydroxyglutaryl-CoA. The solid lines represent the simulated curves and the concentration of the inhibitor was 0.1 mM.

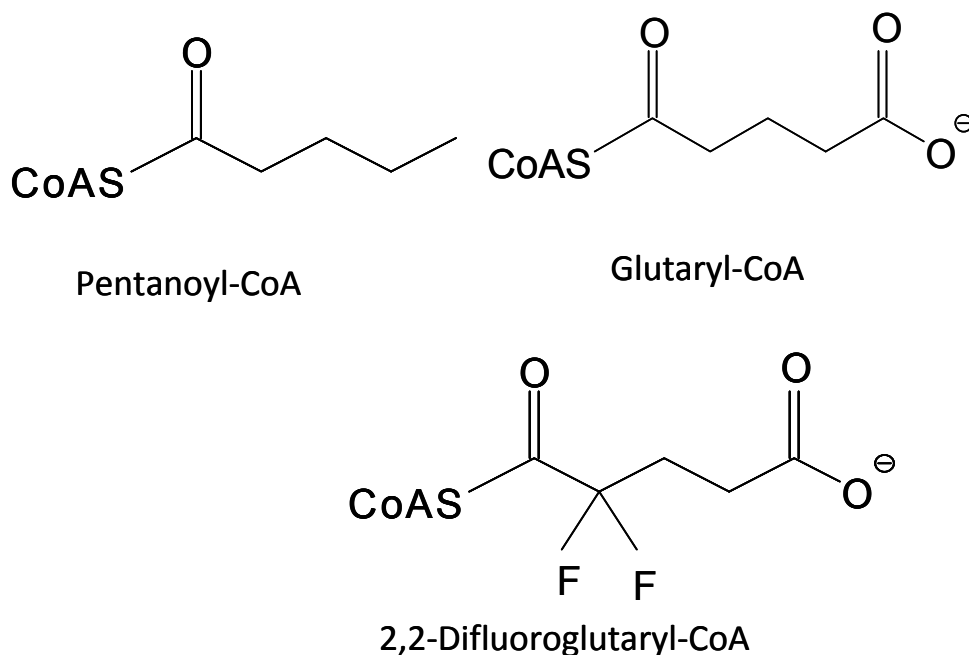


Fig 43 Structures of the inhibitors of 2-hydroxyglutaryl-CoA dehydratase

3.6 Structural aspects

3.6.1 Chemical labelling

Chemical modification with iodoacetamide of the related HgdAB (2-hydroxyglutaryl-CoA dehydratase) from *C. symbiosum* was performed as mentioned earlier, with and without the substrate. Whereas the cysteines of HgdB or second subunit were unaffected by iodoacetamide, one of the conserved cysteines in the first subunit or HgdA, Cys 85 was modified, as analysed by Nano-LC-MS (Joerg Kahnt, MPI Marburg). The pattern was unchanged when the modification was performed in the presence as well as the absence of substrate. However, the non-conserved residues Met 426, Met 379 and Cys 441 were modified only in the presence of the substrate (fig 44). The coverage of the residues, i.e. the fraction of residues identified from the tryptic digest was 72 %. The cysteines were carboxymethylated and the methionines oxidized.

1 **MAKQVSPGVL ALRKVDDVH KEAREAKRG ELVGWSSSKF PCELAADF**
 51 **NVMYPENQAA GIAANRYGEH MCQAAEDLGY DNDICGYARI SLAYAAGVRV**
 101 **SRKYDAETGE YIDPATGKP LKDAEGNVM DEATGPKKD PKTQTPYLV**
 151 **DNLLBEALP DGPEKERRLE AISPIRQMRI PQPDFVLCN NICNCMTKWY**
 201 **ENIARNMCNMP LIMIDIPYNN TVEVHDDNVK YVRAQFDKAI KQLEELTGKK**
 251 **FDEKKEKAC SNANRTAQAW LKVC DY LQYK PAPYSGFDLF NHADAVTAR**
 301 **ARVEAAEAFE LLAD DLEETV KQGETTTPFP EKYRVMFEGI PCWPKLPNLF**
 351 **KPLKEHGVNV TAVVYAPAFG FVYNIDEHA RAYYKAPNSV CIEQGVOWRE**
 401 **GICRONKVDG VLVHYNRSCK PWSGYHAEMQ RRFTE DLGVP CAGFDGDQAD**
 451 **PRNFNAAQYE TRVQGLVEAH EANKQAKEAK**

Subunit A/HgdA

1 **MSINALLDEF KVKAATPKQQ LAEYKAQGKK VIGVLPYYAP EELVYAAGMV**
 51 **PMGMWGSNNK TISRAKEYCA TFYCTIAQLA LEMLDGTMD QLDGIITPTI**
 101 **CDTLRPMSQN FRVAMGDKMA VFLAQPNR FEDFLQFSV DQYTNMKEL**
 151 **EKVAGKEITN EAIQDAIKYY NKSRAARRKF VELASAHCDV ITPTKRSAYL**
 201 **KSFFFFMEKPE YIEKLEELNA ELEKLPVCDW QGTVVTSGI ICDNPKLLEI**
 251 **FEENNIAJAA DOVGHESSSF RVDAPEDPAD ALMALAKQFA NMIDYDVLLYDP**
 301 **KSTENRRGEF IANMVKESGA QGLVLFMQQF CDPEEMEYPY LKALNNAGI**
 351 **PHIKLGIDQQ MRDFGQASTA IQAFA DYLEM QK**

Subunit B/HgdB

Fig 44. The amino-acid sequence of HgdAB showing in green the residues modified in all cases; six conserved Cys in large italics underlined – the one in green was modified while the five in blue were not. The yellow-brown residues were modified only in the presence of the substrate; tryptic peptides with red/green/yellow-brown residues were found by Nano-LC-MS while the black ones were not.

3.6.1 Circular dichroism spectroscopy

The CD spectra of the dehydratase were measured in the UV region between 220-320 nm. The changes in the CD in this region correlate to structural changes of the polypeptide part of the protein.

The spectra of the enzyme in the unreduced state (without activator) and in the reduced state (with catalytic amounts of the activator) in the presence of 1mM of the substrate were not significantly different (fig 45). Therefore it could be concluded that there was no major structural change of the protein backbone due to reduction of the dehydratase by the activator or due to the binding of the substrate. The CD spectrum in the visible region (350-700 nm) was featureless (data not shown); therefore it was not possible to detect any structural changes around the iron-sulfur clusters of the enzyme.

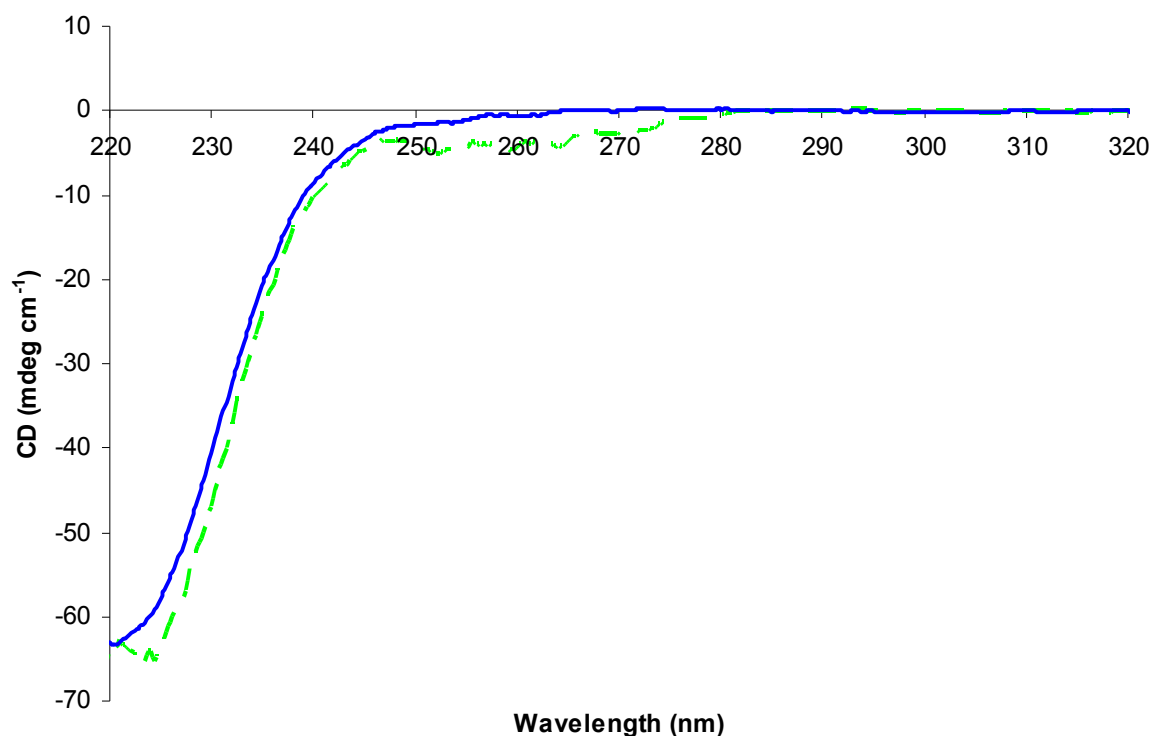


Fig 45. Comparison of the CD spectra of the dehydratase in the unreduced state (solid) and in the reduced state with 1mM of the substrate (broken).

4 Studies on the activator of (*R*)-2-hydroxyglutaryl-CoA dehydratase from *A. fermentans*

4.1 Purification of the activator

The activator of (*R*)-2-hydroxyglutaryl-CoA dehydratase from *A. fermentans* was purified by Strep-tactin affinity chromatography as described earlier (Hans and Buckel, 2000). The SDS-PAGE of the purified protein showed a single band around 27 kDa, demonstrating its apparent homogeneity (fig 46).

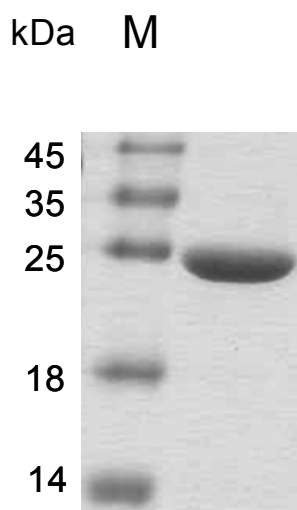


Fig 46. SDS-PAGE showing the molecular mass standard on the left lane and a single band at 27 kDa of the purified activator on the right lane.

4.2 Studies on the activator

4.2.1 with metronidazole and other oxidants

The results summarised in figure 40 suggested that metronidazole interfered with the reduction of the dehydratase by the activator. To investigate the direct effects of metronidazole on the activator, it was necessary to measure its spectra in the 350-600 nm region under oxidised (10 fold excess of thionine acetate or 3,7-diamino-5-phenothiazinium acetate, structure see fig 63b), reduced (10 fold excess of dithionite) and native conditions. These and other spectra were measured after removing excess oxidant or reductant or other chemicals by desalting over a Sephadex G-25 column and amplified to correct for the protein concentrations. The spectrum of native activator shows a shoulder at around 400 nm due to the iron-sulfur cluster. This feature is flattened or shifted out when the protein is oxidised or reduced (fig. 47).

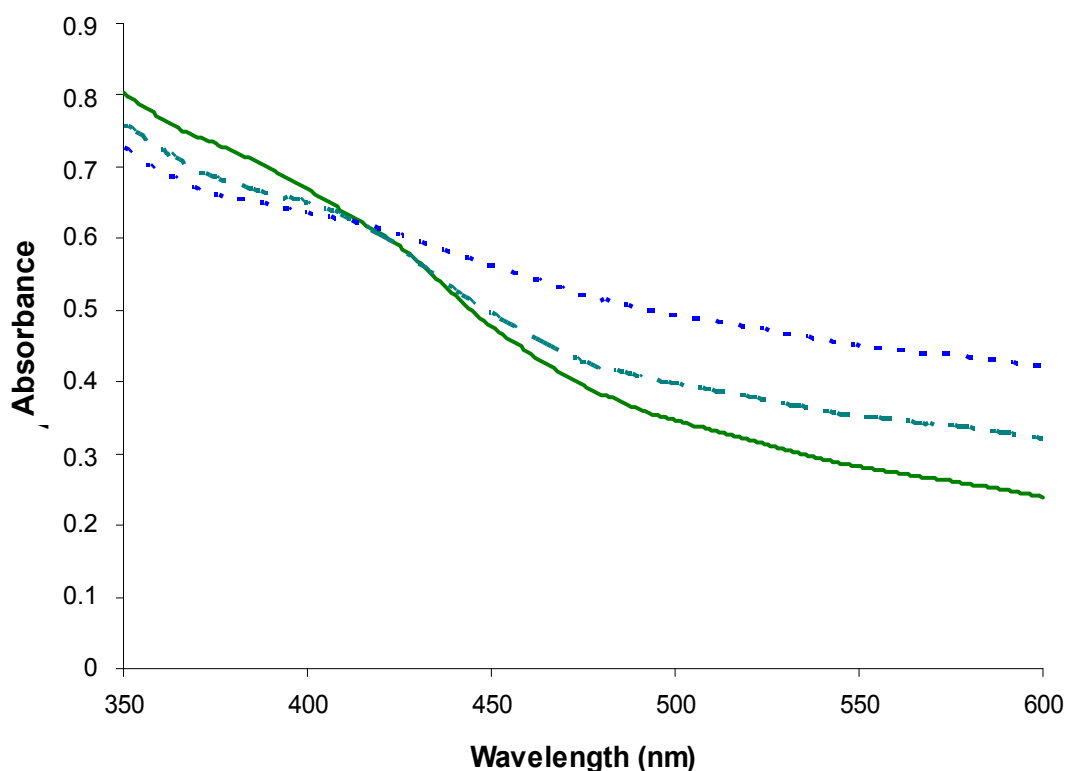


Fig 47. Comparison of visible spectra of native (solid), reduced (4- fold amplified, dotted) and oxidised (4-fold amplified, dashed) activator.

When the enzyme was treated with 20 μM of metronidazole, the spectrum was similar to the oxidised spectrum in fig. 47 (data not shown). To examine the effect on the spectrum of other known inhibitors, 12 μM of 2-nitrophenol was added to the activator and the spectrum after exposure and desalting was quite similar to that of the oxidised spectrum in fig 47. The same effect was also

reproduced by other nitrophenols like 4-nitrophenol and 2,4-dinitrophenol (data not shown). To answer the question if the nitro group was responsible for the oxidation, the same experiments were repeated with 12 μM of 2-aminophenol and the spectra were compared. It could be clearly seen that the oxidising effect was lost in the absence of the nitro group (fig. 48). All spectra were corrected for the protein concentrations. The inhibitory effect of nitrophenols on the dehydration reaction was already known (Müller and Buckel, 1995) but the mechanism was unclear.

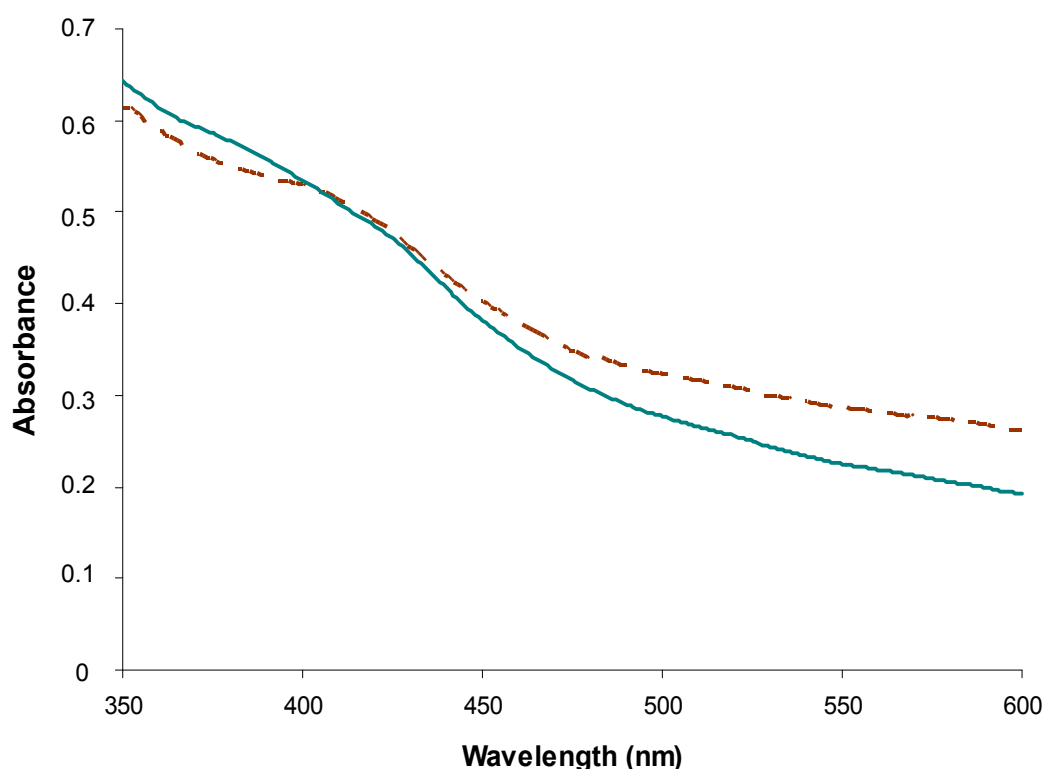


Fig 48. Spectra after treatment with 12 μM of 2-nitrophenol (dotted) and 12 μM of 2-aminophenol (solid) resembled oxidised and native spectra, respectively.

4.2.2 Circular dichroism spectroscopy

CD spectra of the activator under oxidised conditions bound to ADP and under reduced condition bound to ATP or ADP were similar as shown below (fig 49). There appeared to be only a slight change of the protein conformation upon reduction of the [4Fe-4S] cluster by dithionite. Further, addition of 5mM ATP to the enzyme did not cause a significant change in the pattern. There appeared to be no large change of conformation involving ATP hydrolysis. Further, the activator exhibits a featureless CD spectrum in the iron-sulfur region between 350 and 600 nm, under reducing conditions (data not shown).

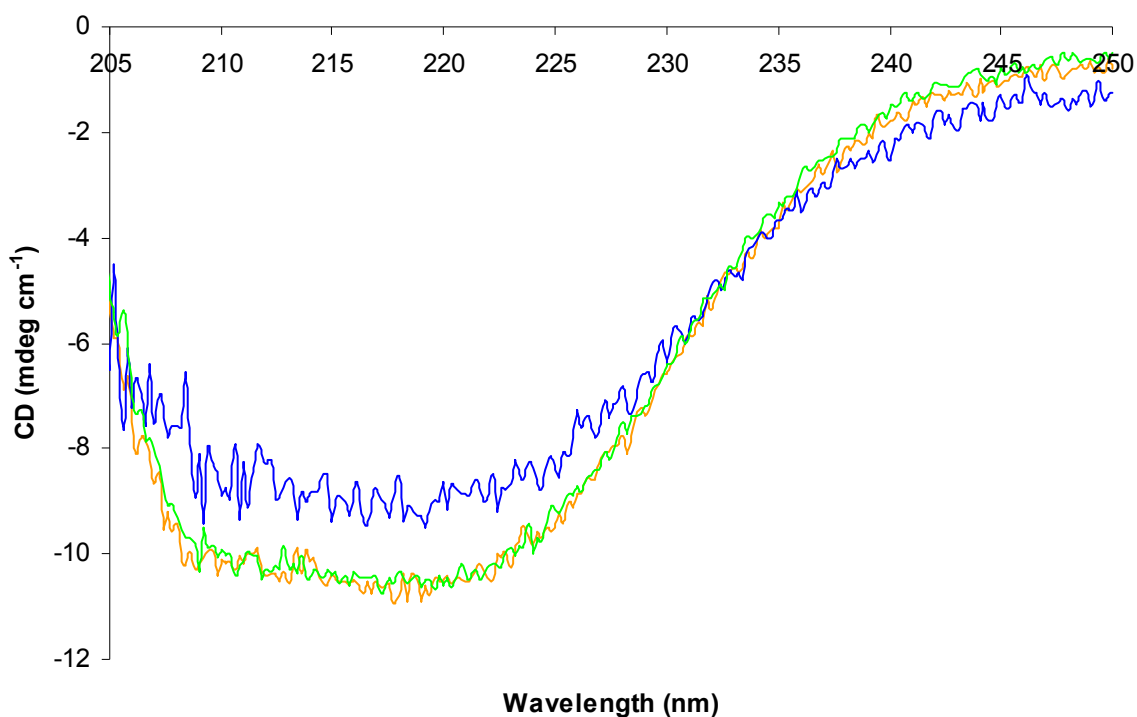


Fig 49. The circular dichroism spectra in the UV region of the unreduced/oxidised activator bound to ADP (blue), reduced activator bound to ADP (green) and reduced activator bound to ATP (yellow).

5 Lactyl-CoA dehydratase from *C. propionicum* and the comparison of certain equilibria

5.1 Purification of lactyl-CoA dehydratase and kinetic measurements

The chromatographic steps were done following the greenish-brown colour of the enzyme. The relevant fractions were identified on the basis of the direct assay for the lactyl-CoA dehydratase, based on the hydration of acrylyl-CoA. The suppression of the acrylyl-CoA reductase activity was achieved by the inhibitor 3-pentynoyl-CoA. Also the reductase activities of the same fractions were also measured without the inhibitor. These enabled the separation of the two activities. An apparently homogenous enzyme was purified as seen by SDS-PAGE analysis (fig 50). Two brownish peaks were obtained after the first column. The first eluting peak contains the activator (5 mM ATP/Mg were added and the fractions stored on ice), the second peak was the dehydratase around 0.45 M NaCl. The further two columns were used to obtain higher purity and specific activity, using the greenish-brown colour of the dehydratase to follow the purification. The crude activator fraction from the first column

(around 0.3 M NaCl) was used. Later, we found the recombinant activator from *A. fermentans* could be used. The purification scheme is summarised in table 4.

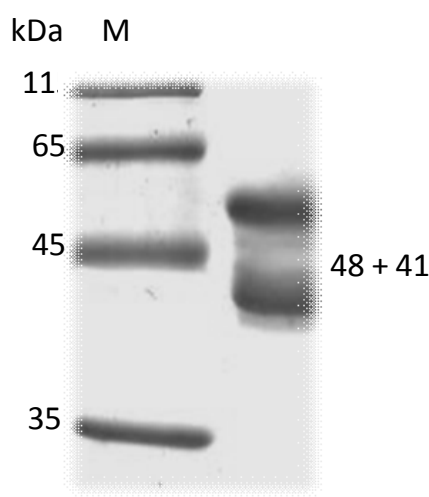


Fig 50. SDS-PAGE gel showing molecular mass marker on the left lane and, on the right lane, the purified lactyl-CoA dehydratase (48+41 kDa).

Table 4. Purification of lactyl-CoA dehydratase from *C. propionicum* using the assay for acrylyl-CoA hydration

Step	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Purification factor	Yield %
Cell-free extract	270	900	0.3	1	100
Source-15Q	249	31	8	27	92
Source-15Phe	192	9.6	20	67	71
Superdex-200	85	1	85	283	32

The kinetic parameters for acrylyl-CoA hydration by the lactyl-CoA dehydratase were measured to be $K_m = 150 \pm 4 \mu\text{M}$; $V_{\max} = 85 \pm 6 \text{ U/mg}$ (fig 51) under assay conditions mentioned earlier.

The optical measurements for lactyl-CoA dehydration were less accurate because of the low specific activity below 0.1 mM of substrate. At higher concentrations of substrate, changes in the overall activity were small. The estimated values are $K_m = 320 \pm 20 \mu\text{M}$ and $V_{\max} = 3.0 \pm 0.4 \text{ U/mg}$ (fig 52).

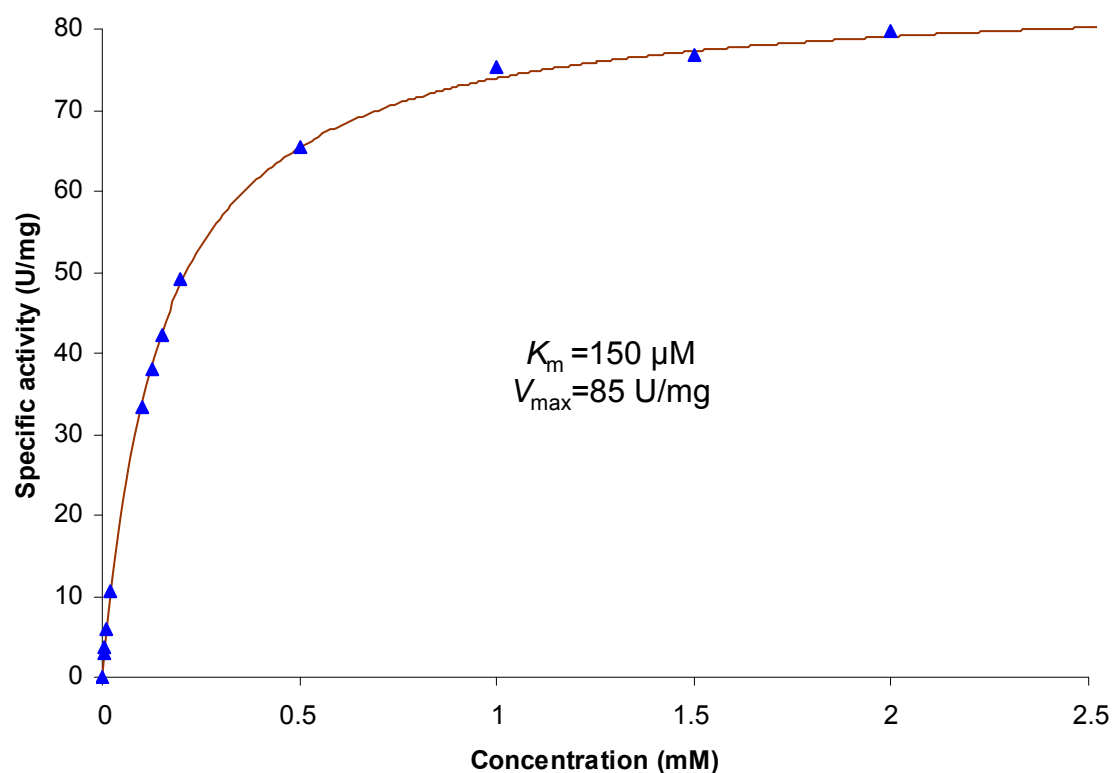


Fig 51. Michaelis-Menten kinetics of lactyl-CoA dehydratase with acrylyl-CoA as substrate

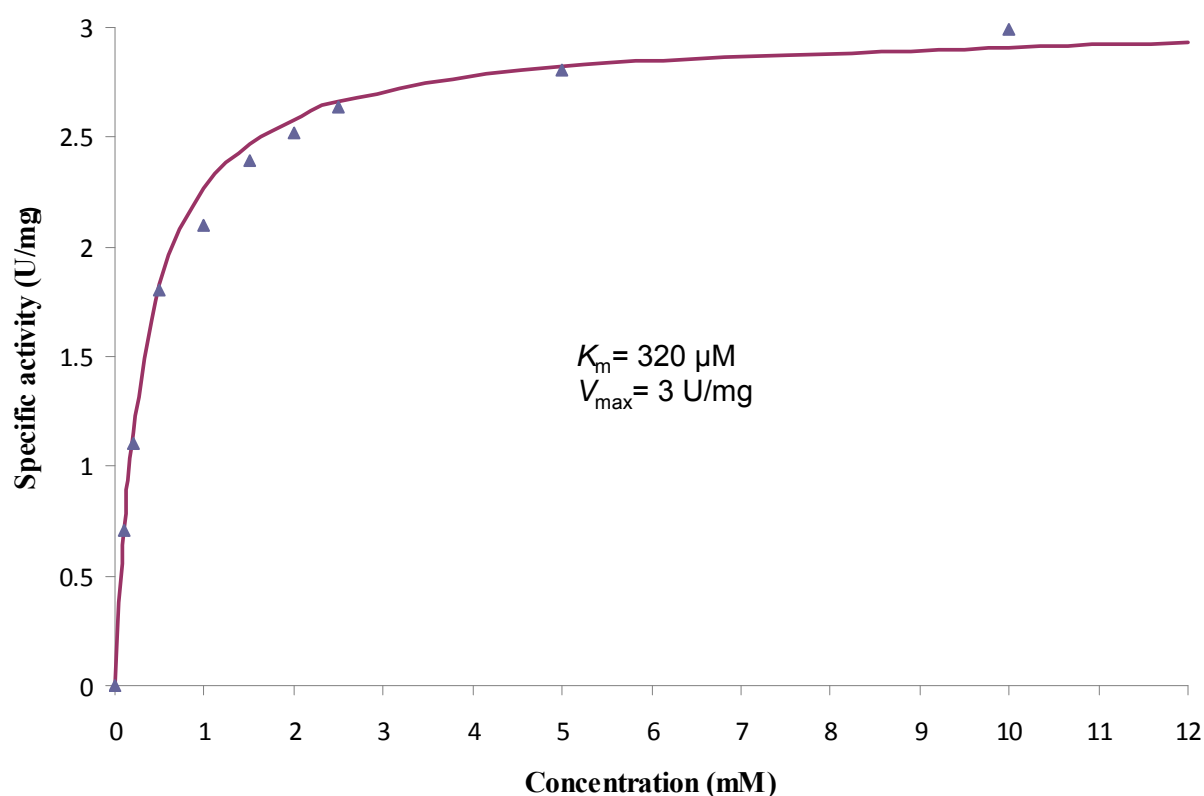


Fig 52. Michaelis-Menten kinetics of lactyl-CoA dehydratase with lactyl-CoA as substrate

5.2 Calculation of thermodynamic equilibria

5.2.1. with 2-hydroxyglutaryl-CoA dehydratase from *C. symbiosum*

The activity measurements between 0-1 mM (*R*)-2-hydroxyglutaryl-CoA (**1b**) and 0-5 mM glutaconyl-CoA (**2b**) gave smooth Michaelis-Menten curves, from which K_m and k_{cat} values could be calculated by simulation. In experiments starting with (*R*)-2-hydroxyglutaryl-CoA (**1b**), we obtained values of $K_m = 0.052 \pm 0.003$ mM and $k_{cat} = 83 \pm 8$ s⁻¹, resulting in a specificity constant [k_{cat}/K_m (**1b**)] of 1600 ± 300 s⁻¹mM⁻¹. Beginning the reaction instead with (*E*)-glutaconyl-CoA (**2b**) resulted in $K_m = 0.25 \pm 0.02$ mM and $k_{cat} = 7.0 \pm 0.7$ s⁻¹, associated with a specificity constant [k_{cat}/K_m (**2b**)] of 28 ± 6 s⁻¹ mM⁻¹. K^b was subsequently calculated from the Briggs-Haldane equation:

$$K^b = [k_{cat}/K_m (\mathbf{1b})] / [k_{cat}/K_m (\mathbf{2b})] = 1600/28 = 57 \pm 1.5$$

5.2.2. with lactyl-CoA dehydratase from *C. propioncum*

The kinetic parameters for the hydration of acryloyl-CoA (**2c**) to (*R*)-lactyl-CoA (**1c**) were measured as $K_m = 0.150 \pm 0.004$ mM and $V_{max} = 85 \pm 6$ U/mg, yielding $k_{cat} = 126 \pm 10$ s⁻¹ and [k_{cat}/K_m (**2c**)] = 0.84×10^6 s⁻¹M⁻¹. Because the equilibrium concentration of acryloyl-CoA (**2c**) is very low, it was more difficult to determine the kinetics of (*R*)-lactyl-CoA dehydration (**1c**→**2c**). Reasonable estimates are given by: $K_m = 0.32 \pm 0.02$ mM and $V_{max} = 3.0 \pm 0.4$ U/mg, with $k_{cat} = 4.5 \pm 0.6$ s⁻¹ and [k_{cat}/K_m (**1c**)] = 1.41×10^4 s⁻¹M⁻¹). Substitution of these data into the Briggs-Haldane equation yields:

$$K^c = [k_{cat}/K_m (\mathbf{1c})] / [k_{cat}/K_m (\mathbf{2c})] = 0.017 \pm 0.007$$

The same equilibria were modelled theoretically by our collaborator D.M. Smith (Parthasarathy, A. Buckel, W. and Smith, D.M., to be submitted), substituting the CoA thiol ester moiety with an S-methyl group in all cases. This was done to enable easier calculations. The experimentally measured values are in fairly good agreement with the calculated values. Fig 42 compares the calculated and measured equilibrium constants for these two cases as well as for case A. Case A was for the equilibrium of hydroxyisocaproyl-CoA (**1a**) and isocaprenoyl-CoA (**2a**), which pertain to the *C. difficile* system (Kim, J., et al, 2004).

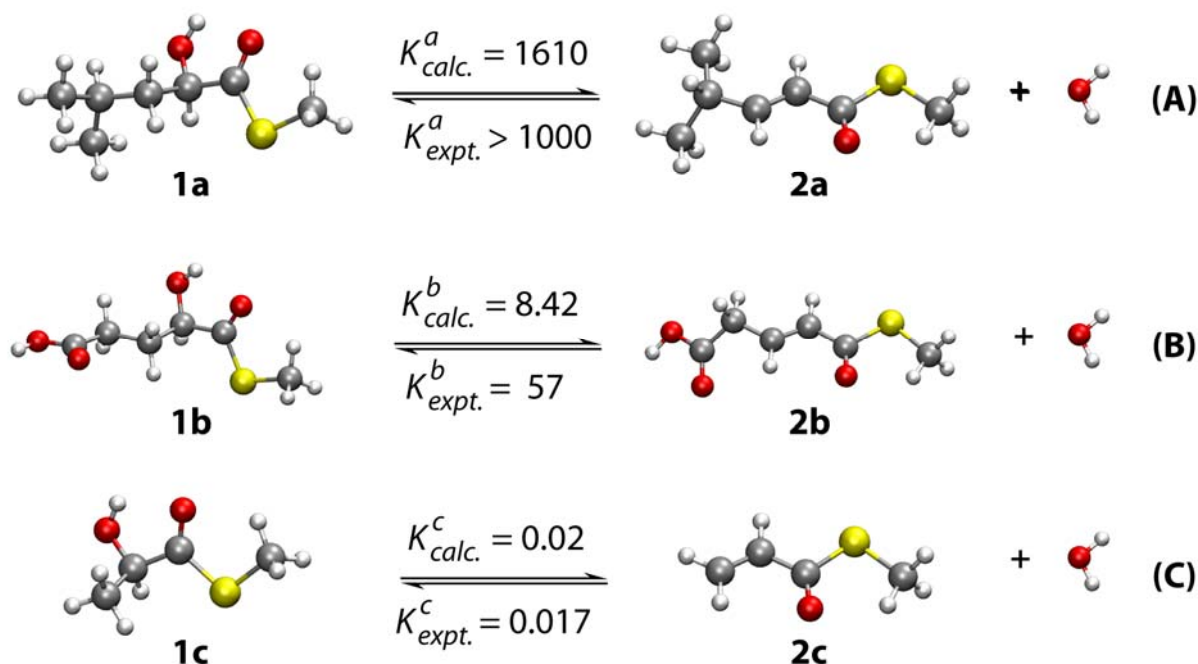


Fig 53. Thermodynamic equilibria between
 (A) (R) -2-hydroxisocaproyl-CoA (**1a**) and isocaprenoyl-CoA (**2a**)
 (B) (R) -2-hydroxyglutaryl-CoA (**1b**) and glutaconyl-CoA (**2b**)
 (C) (R) -lactyl-CoA (**1c**) and acrylyl-CoA (**2c**)

$K_{calc.}$ Refer to the calculated values in each case and $K_{expt.}$ to the values derived from experimentally measured kinetics.

6 Substrates and inhibition of the (R) -2-hydroxyglutarate dehydrogenase (HgdH) of *A. fermentans*

6.1 Substrates of the (R) -2-hydroxyglutarate dehydrogenase

The maximum activity of the dehydrogenase measured by the NADH-dependent assay with the substrate 2-oxoglutarate was 2600 ± 15 U/mg at 0.1 mM of NADH, while K_m was 0.14 ± 0.04 mM.

Two dicarboxylic acids synthesised in this work, oxalocrotonic acid or 2-oxohexene-dioic acid and 2-oxoadipic acid were tested as possible substrates. The kinetic parameters measured at 0.1 mM of NADH for 2-oxoadipic acid were $V_{max} = 800 \pm 30$ U/mg and $K_m = 0.88 \pm 0.03$ mM (fig 54), and for 2-oxohexene-dioic acid were $V_{max} = 392 \pm 5$ U/mg and $K_m = 1.1 \pm 0.3$ mM (fig 55).

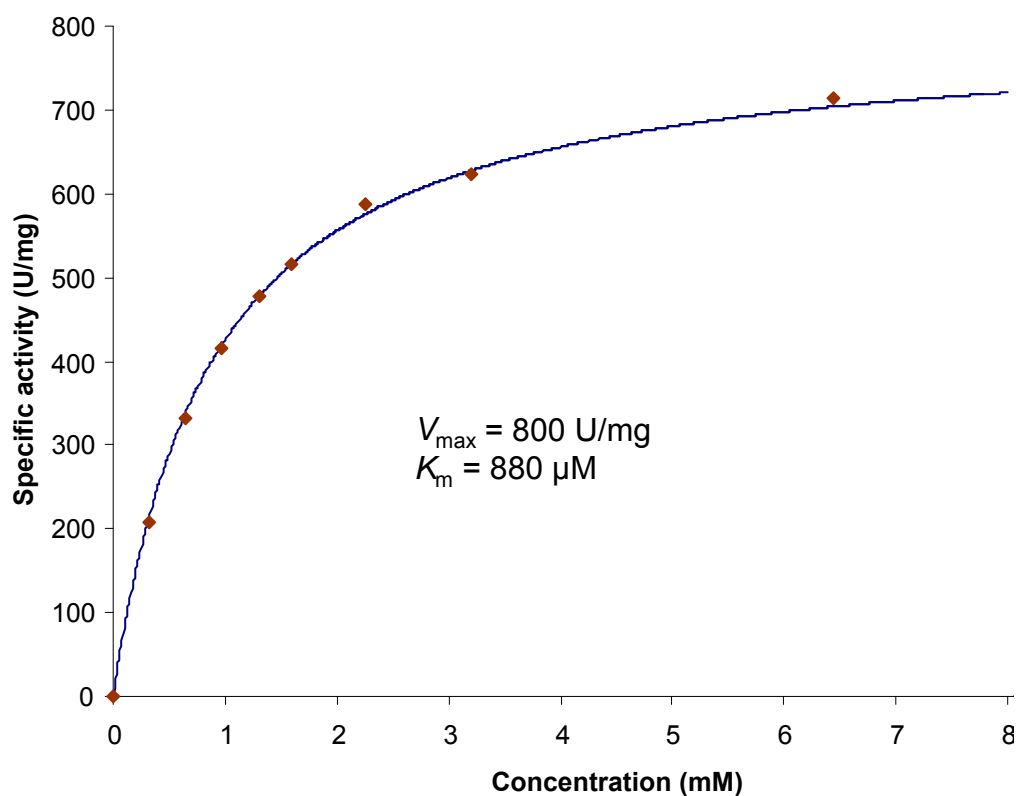


Fig 54. Michaelis-Menten kinetics of HgdH with the substrate 2-oxoadipic acid,

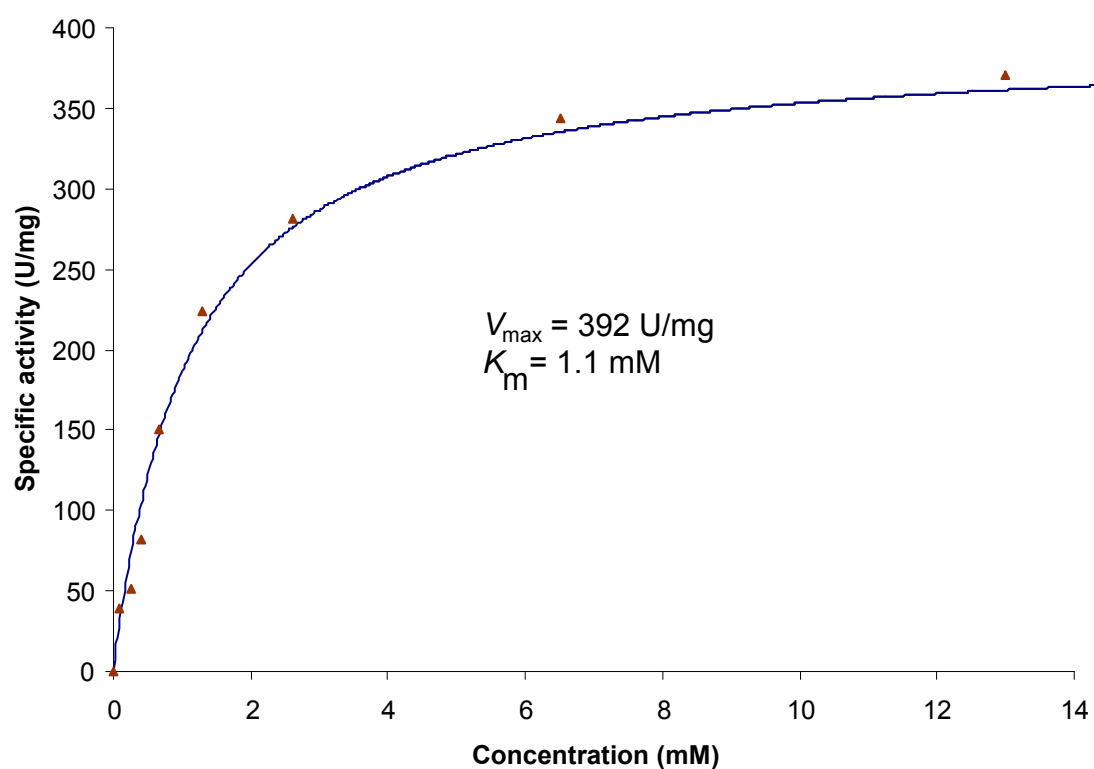


Fig 55. Michaelis-Menten kinetics of HgdH with the substrate 2-oxohexenedioate, measured at 0.1 mM of NADH

6.2 Inhibition of (*R*)-2-hydroxyglutarate dehydrogenase

When DFPA (2,2-difluoroglutarate) was added to the 2-hydroxyglutarate dehydrogenase assay mixture, NADH was not oxidised to NAD⁺. The responses to equal amounts of the natural substrate 2-oxoglutarate and DFPA were compared. The trials summarised in table 5 revealed a probable inhibitory effect of the latter compound on the enzyme. When the assay was started after pre-incubation of the enzyme with 1.5 mM the putative inhibitor, the activity was suppressed. Activity could however be recovered by the addition of sufficient amounts of the natural substrate. The NADH concentration was always maintained at 0.1 mM.

Table 5. Suppression of NADH-dependent 2-hydroxyglutarate dehydrogenase activity by 1.5 mM of DFPA

Concentration of 2- oxoglutarate (mM)	Specific activity of uninhibited reaction (U/mg)	Specific activity of inhibited reaction (U/mg)
0.05	695	266
0.1	1110	491
0.2	1581	725

The kinetics of the enzyme with the natural substrate 2-oxoglutarate were measured at different concentrations of the inhibitor and the results are shown in the Michaelis-Menten plots fig 56 and and the double reciprocal plots in fig 57. The inhibitory constant K_i were values calculated from the apparent K_M values at the inhibitor concentration of [I], by the formula

$$K_m^{\text{app}} = K_m (1 + [I] / K_i)$$

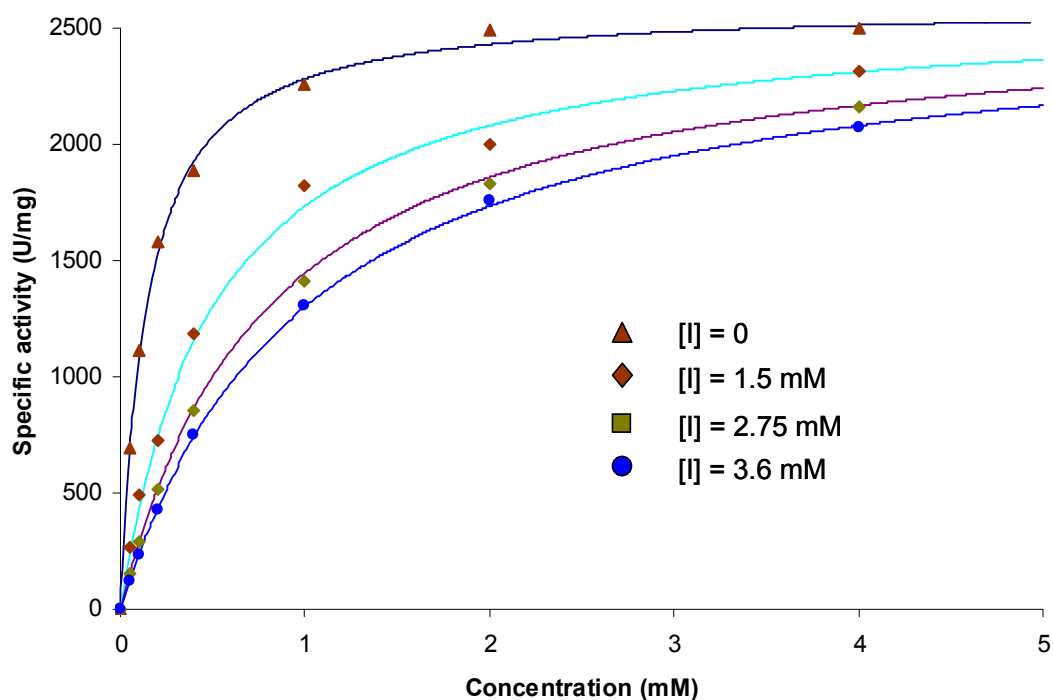


Fig 56. Comparison of Michaelis-Menten plots of the HgdH activity with 2-oxoglutarate as substrate, at different concentrations of the inhibitor DFPA. Inset shows the legend used for each concentration. The solid lines represent the respective simulated curves.

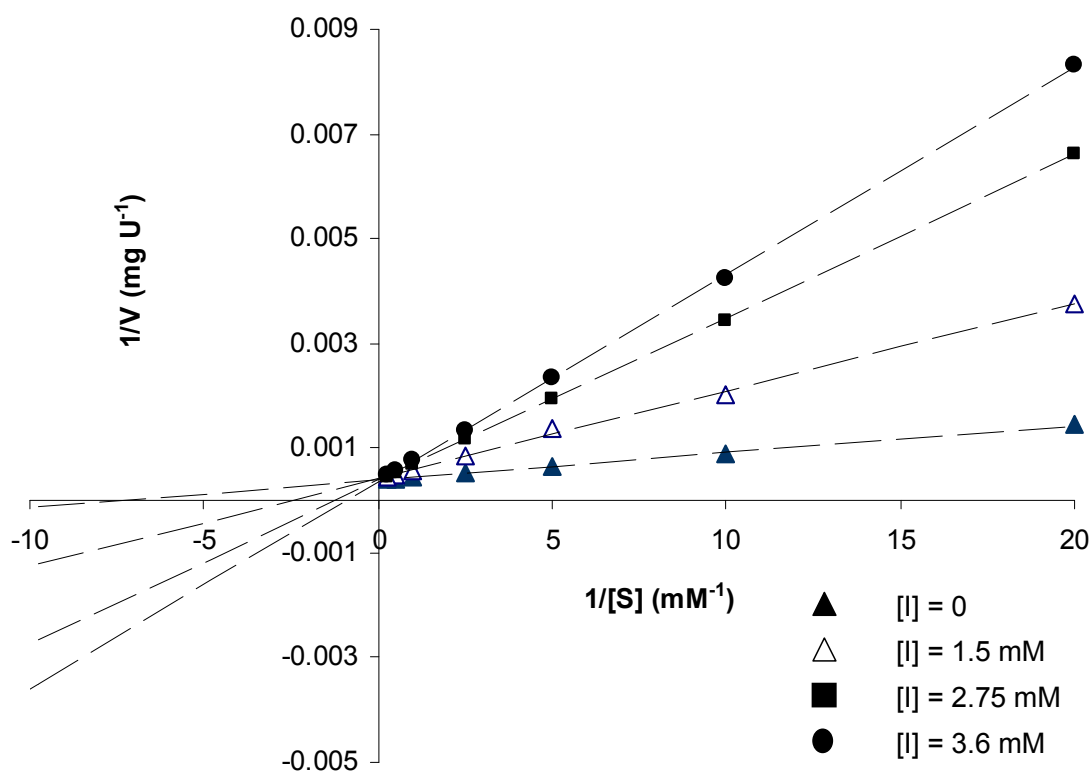


Fig 57. Comparison of double reciprocal plots of the HgdH activity with 2-oxoglutarate as substrate, at different concentrations of the inhibitor DFPA. Inset shows the legend used for each concentration. The dashed lines represent the respective theoretical plots.

Table 6. Dependence of the apparent K_m on the concentration of the inhibitor DFPA

Concentration of 2,2-difluoroglutarate (mM)	Apparent K_m (mM)
0	0.14
1.5	0.52
2.75	0.83
3.6	1

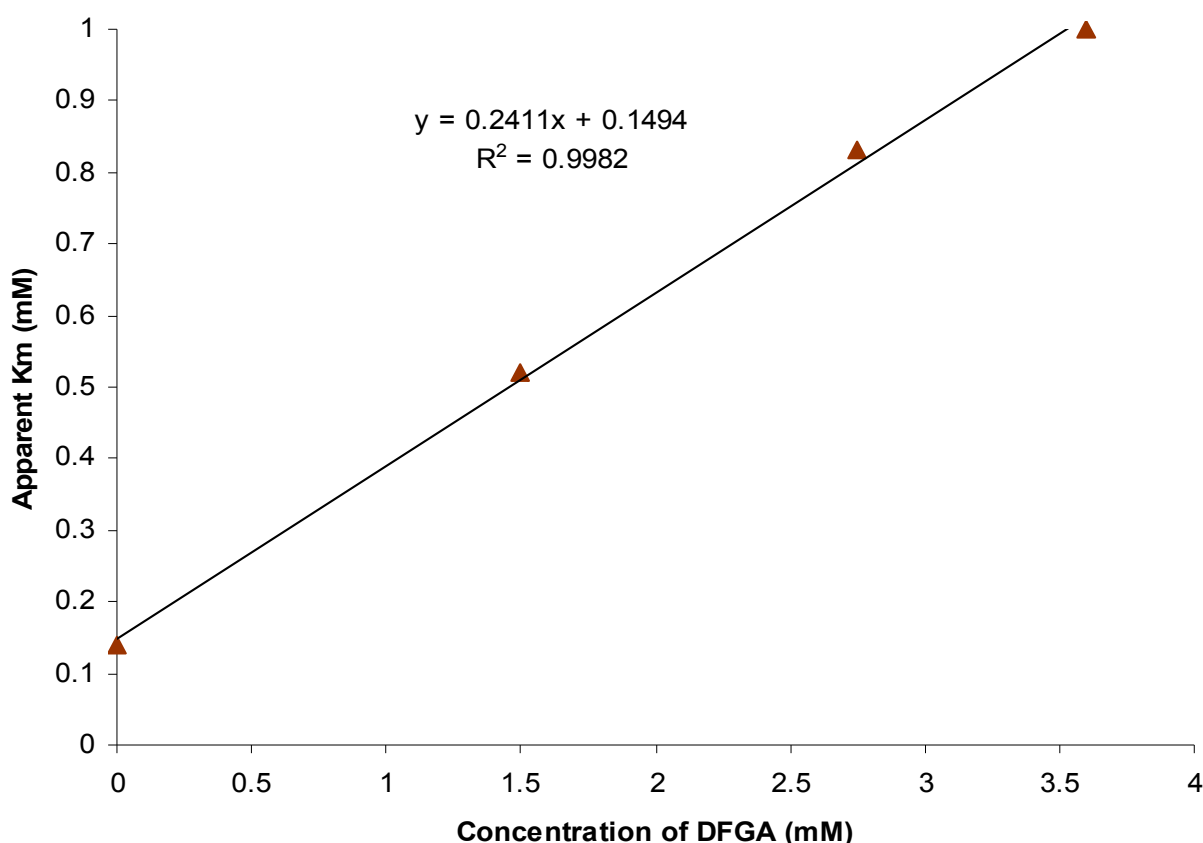


Fig 58. The apparent K_m values and the concentration of the inhibitor DFGA show a linear dependence.

The calculated K_i for difluoroglutarate was about 0.619 ± 0.006 mM and analysis of the plots in figs 41 and 42 showed that the nature of the inhibition was competitive. There was a linear dependence of the apparent K_m values on the inhibitor concentration as shown in the fig 58 and table 6.

6.3 Growth experiments

Growth experiments were performed to determine if there was an effect of difluoroglutarate (DFGA) on the whole cells of *C. symbiosum*, indirectly due to

the inhibition of the 2-hydroxyglutarate dehydrogenase. Compared to growth on 50 mM glutamate or on 50 mM glutamate supplemented with 1 mM 2-oxoglutarate, the growth on 50 mM glutamate with 1 mM of DFGA occurred with a longer lag phase (fig 59).

The stationary phase was reached about 2 hours later than the other cultures. Also, the final optical densities attained were also lower than the controls. The growth patterns on glutamate with or without 2-oxoglutarate were very similar, although the optical densities attained were higher in the latter case. An inoculum of 5% was used in all the cases to start the growth of all the cultures, which were grown for 14 hours.

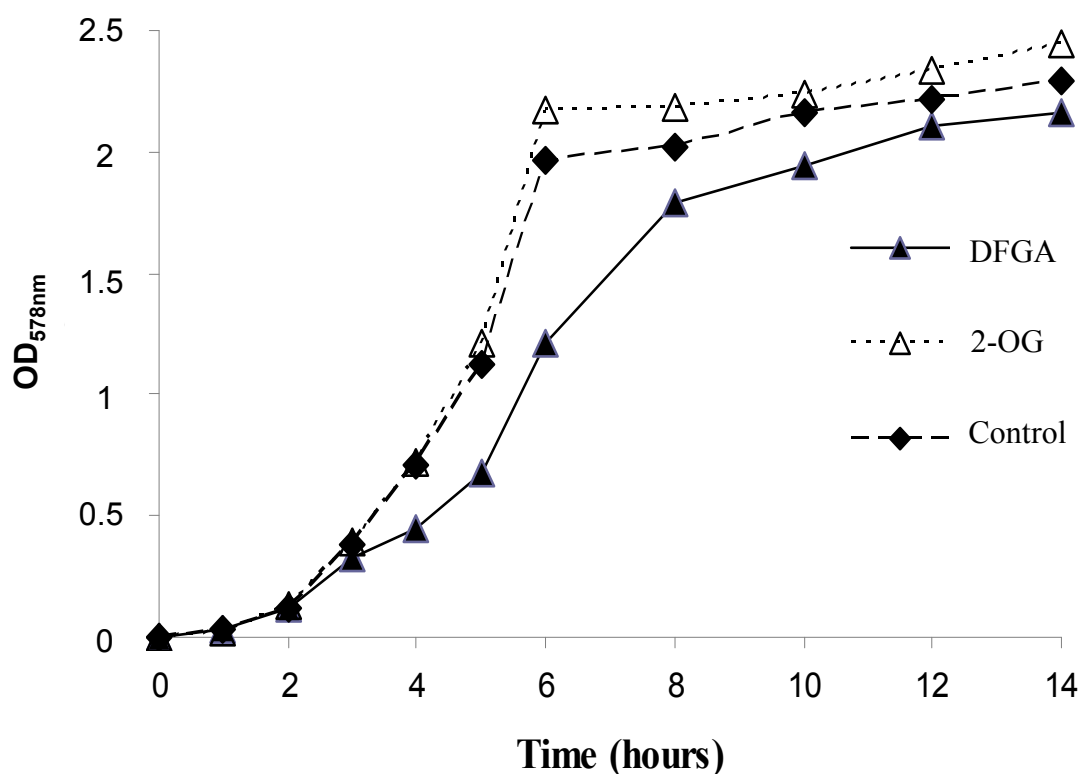


Fig 59. Comparison of the growth of *C. symbiosum* on three variants of the glutamate medium; Control = 50 mM glutamate; 2-OG = 50 mM glutamate + 1 mM 2-oxoglutarate and DFGA = 50 mM glutamate + 1 mM DFGA

Discussion

Various carboxylic acids were synthesised during the course of this work and some of their CoA esters characterised as substrates of the (*R*)-2-hydroxyglutaryl-CoA dehydratase from *C. symbiosum* as described in the results section. Results of the studies on the activator of the (*R*)-2-hydroxyglutaryl-CoA dehydratase and (*R*)-2-hydroxyglutaryl-CoA dehydratase from *A. fermentans*, and the lactyl-CoA dehydratase from *C. propionicum* are also discussed.

1 Chemical and enzymatic synthesis

1.1 Difluoroglutaric acid

Many different fluorinating reagents have been described in the literature (Shimizu and Hiyama, 2004). Among them, diethylaminosulfur trifluoride or DAST (Singh and Shreeve, 2002), a nucleophilic fluorinating agent, can be used under relatively mild conditions and is easy to handle. It is known to smoothly replace hydroxyl or keto groups with fluoro groups (Vu, et al., 2003). Many protocols for biologically useful fluoro-compounds have appeared elsewhere (Shi, and Cai, 1995, Ocampo, et al; 2002).

DAST fluorination of diethyl 2-hydroxyglutaric acid resulted in mixtures of mono- and di-fluoroglutarate esters, which were difficult to separate by silica-gel chromatography. It was decided to synthesise 2,2-difluoroglutaric acid as an alternative using the same strategy, starting from diethyl 2-oxoglutarate. The yields were poor and 60-70% of the starting material remained unreacted. Then, it was decided to use a slight modification of the published procedure (Laurent, 2005) which involves a Michael-type reaction of acrylonitrile with ethyl bromodifluoroacetate.

The carbon–fluorine bond length is typically about 1.35 Å (O' Hagan, 2008). The high electronegativity of fluorine (4.0 as compared to 2.5 for carbon) gives the carbon–fluorine bond a significant polarity. The electron density is concentrated around the fluorine, leaving the carbon relatively electron poor. This introduces ionic character to the bond through partial charges ($C^{\delta+}-F^{\delta-}$). The partial charges on the fluorine and carbon contribute to the unusual bond strength of the carbon–fluorine bond. The bond is labeled as "the strongest in organic chemistry" because fluorine forms the strongest single bond to carbon. Carbon–fluorine bonds can have bond-dissociation energies (BDE) of upto 116 kcal mol⁻¹ or 485.6 kJ mol⁻¹ (Cox, 1962; March, 1985; Lemal, 2004) and are therefore not labile under biological conditions. The electronic effects of

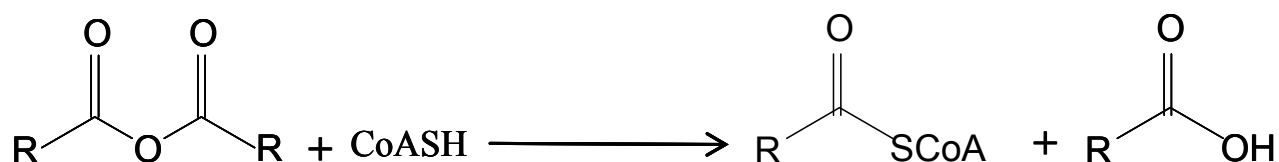
fluorine on the acidities of O-H, N-H and C-H groups have been discussed elsewhere (Schlosser, 1998), although the size effect of fluorine is negligible.

Therefore, replacing C-H, O-H or keto groups of biologically active molecules with fluoro or difluoro groups creates non-utilisable analogues. There is an extensive literature of biologically active fluorine compounds (Welch, 1987) and enzyme inhibition by fluoro-analogues (Gelb, et al., 1985; Abeles, 1990, Gumina, et al., 2001).

1.2 CoA thioesters

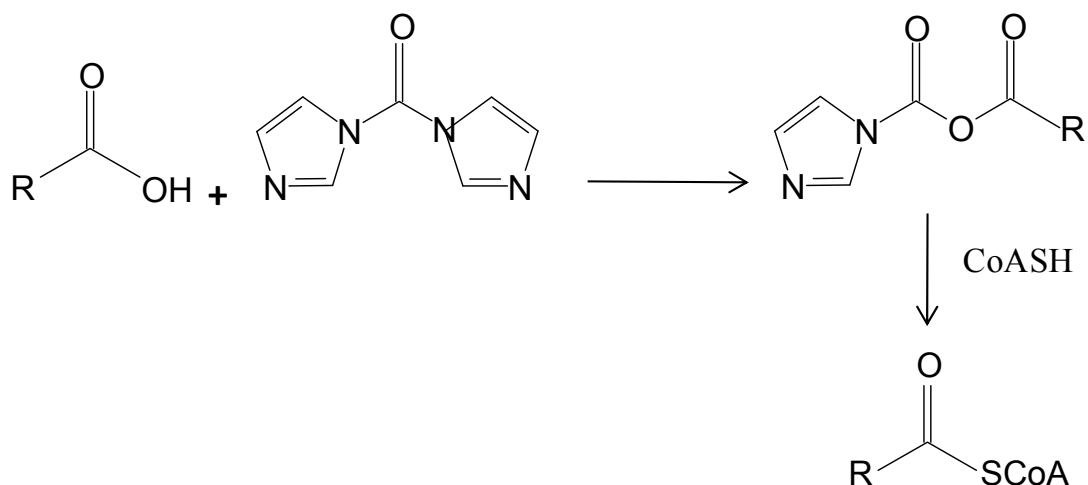
An exhaustive review of coenzyme-A chemistry and biochemistry appeared elsewhere (Mishra and Drueckhammer, 2000). For the synthesis of CoA ester of a particular carboxylic acid, the choice of the activation method depends on the structure of the latter. In all cases, the free acid (or its salt) needs to be chemically activated to a more reactive derivative before esterification can proceed. The CoA dissolved in bicarbonate (pH = 8) exists mostly as thiolate (pK = 10). Fig 1 shows a list of CoA esters synthesised in this work by known chemical methods. When anhydrides were available, the CoA ester could be obtained by the anhydride method (Simon and Shemin, 1953). CoA esters **1-5** (fig 1) were synthesised by this method, which involves the direct reaction between CoA and the anhydride (Scheme 6).

Scheme 6. The anhydride method of Simon and Shemin



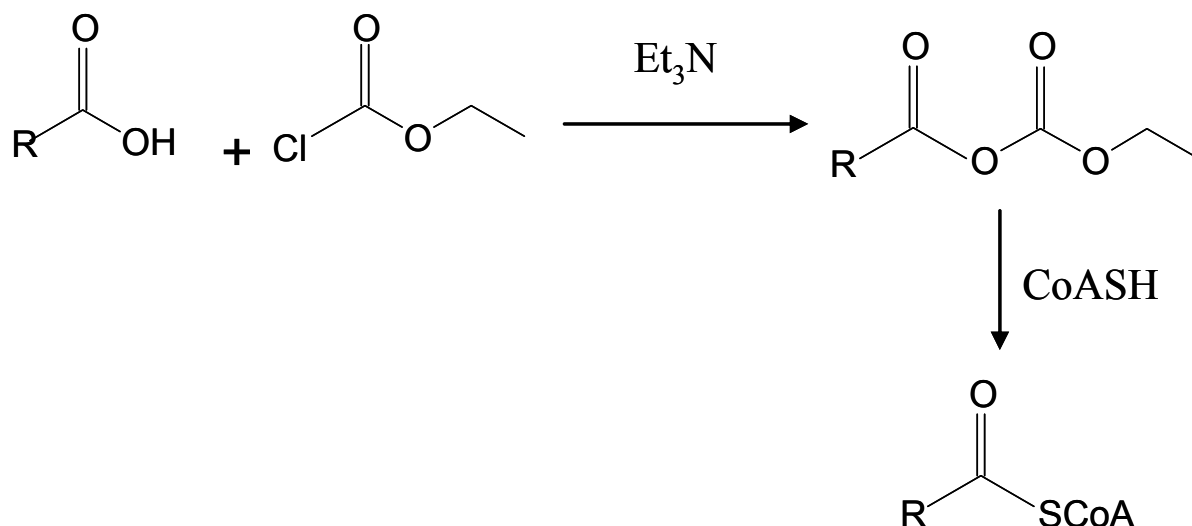
In case of monocarboxylic acids with hydroxy substituents, the carbonyl-diimidazole method (Kawaguchi, et al., 1981) was useful. CoA esters **6** and **7** (fig 1) were obtained by this method. The initial intermediate is an anhydride-like adduct of the acid with the carbonyl-imidazole, which later undergoes nucleophilic attack by the CoA thiolate to yield the CoA thioester (Scheme 7).

Scheme 7. The carbonyl-diimidazole method of Kawaguchi



In case of monocarboxylic acids with unsaturation in the carbon chain, the Kawaguchi protocol gave poor yields, while the mixed anhydride method (Wieland and Rueff, 1953; Goldman and Vagelos, 1961) was successful for CoA esters **8**, **9** and **10** (fig 1). This method involves an initial adduct between the acid and ethyl chloroformate which is a type of "mixed anhydride", subsequently this reacts with the CoA thiolate to yield the CoA thioester (Scheme 8).

Scheme 8. The mixed anhydride method of Wieland and Rueff, modified by Goldman and Vagelos

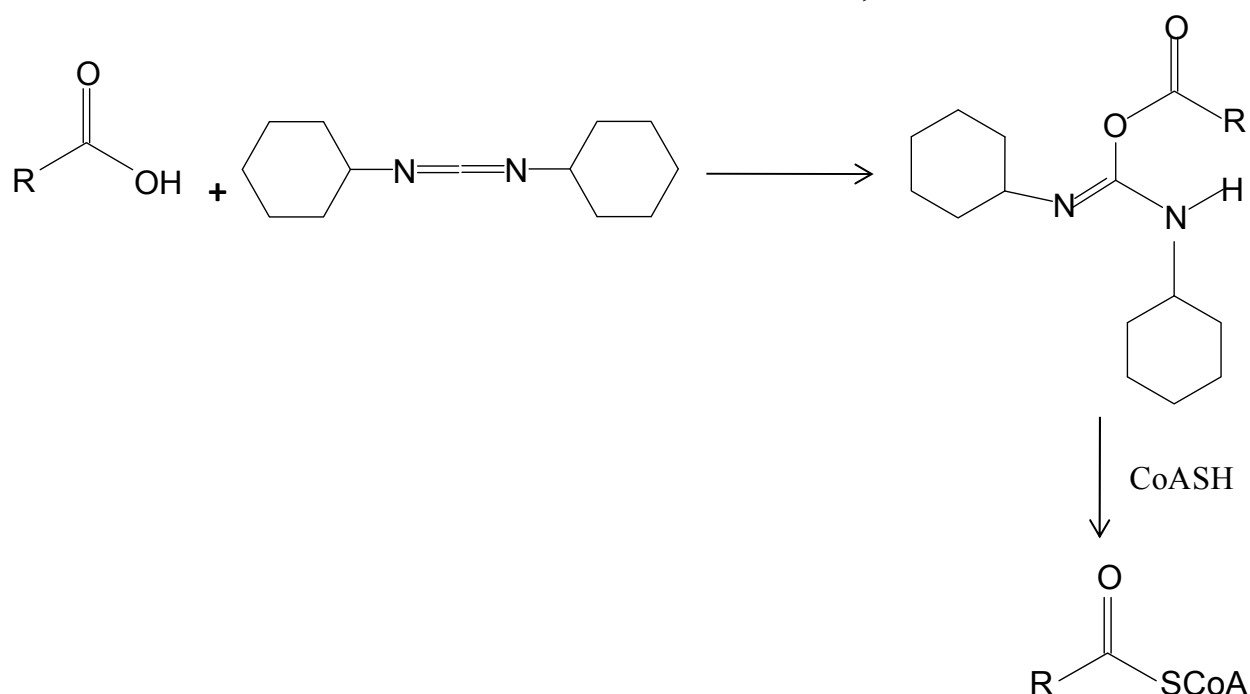


Compound **11** (fig 60), acrylyl-CoA, was made by direct reaction of acrylyl chloride and CoA as mentioned in methods. 3-Pentynoyl-CoA (compound **12**, fig 1) was synthesised by the method involving dicyclohexylcarbodiimide (Gomes, et al., 1981). Here an acylisourea intermediate is formed (Nakajima and Ikada, 1995) and further reacts with CoA thiolate to yield the CoA thioester (Scheme 9).

The novel synthesis of (*R*)-2-hydroxyglutaryl-CoA via the lactone-CoA has been described in the methods and results sections and simplifies an earlier multi-step synthesis (Klees and Buckel, 1991) which involved generating a caprolyl-cysteamine adduct that could be converted to the CoA thioester.

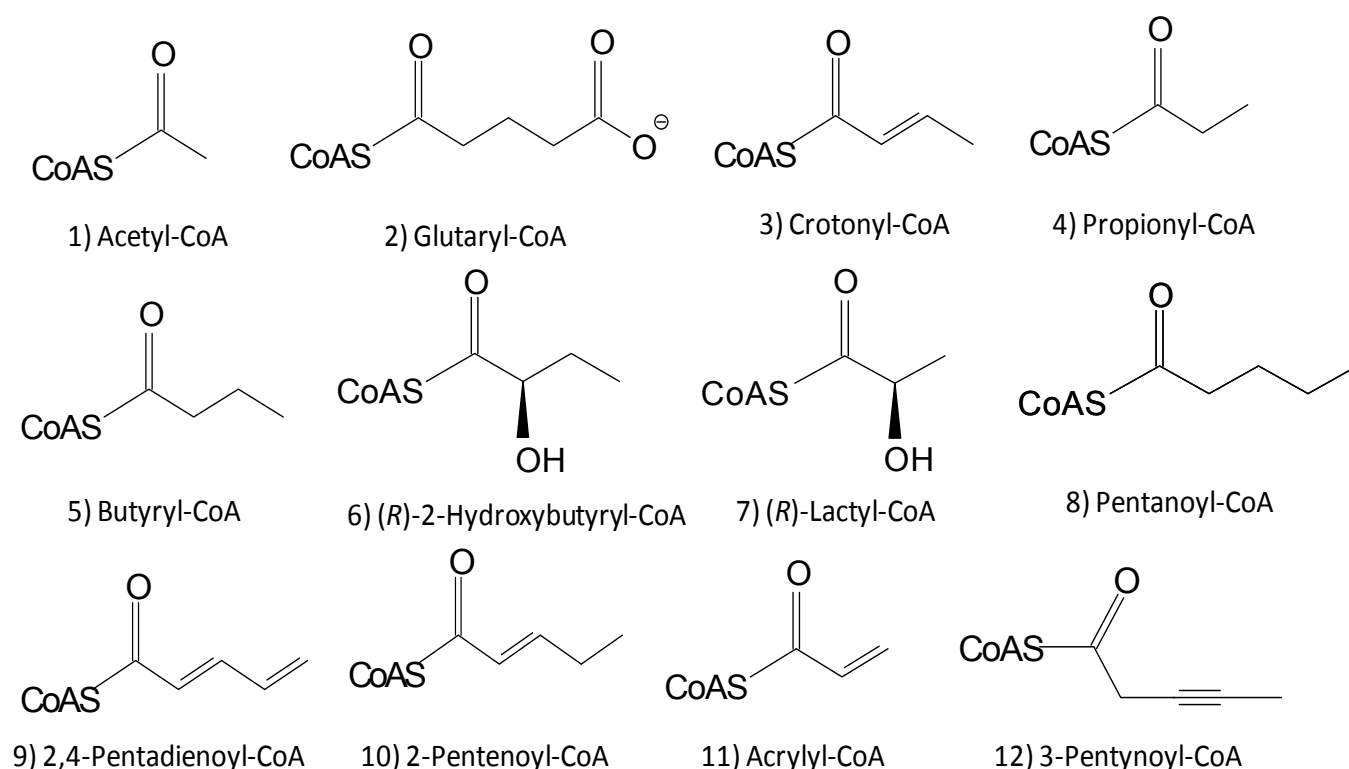
The need for this synthesis arose since the enzymatic synthesis could also generate the thermodynamically favoured 4-hydroxy isomer (Buckel, et al., 1981). Both structures are shown below in fig 61. It was observed that the 4-hydroxyisomer is not used as a substrate of the 2-hydroxyglutaryl-CoA dehydratase. The chemical method avoided this pitfall by generating the lactone-CoA first, which could be acid hydrolysed to the 2-isomer.

Scheme 9. The DCC method of Gomes, et al.



Certain dicarboxylic acids could be converted to their CoA esters using the recombinant glutaconate-CoA transferase (Buckel, 1981 and Mack, et al., 1994), which is part of the 2-hydroxyglutarate pathway in *A. fermentans*. This enzyme was earlier shown to have broad substrate specificity (Buckel, et al., 1981).

While some of these esters were known earlier (Buckel, et al., 1981), those shown in shaded backgrounds (fig 3) were synthesized for the first time in the course of this work. In all these cases, acetyl-CoA or glutaconyl-CoA was the CoA donor and the carboxylic acid was present in 10-20 fold molar excess. The enzymatic catalysis was shown to go through a "mixed anhydride" intermediate between the enzyme and acetate (Selmer and Buckel 1999). This enzyme



belongs to the Family I among the three well-known families of CoA transferases reviewed elsewhere (Heider, 2001) and most closely resembles a CoA transferase described in *E. coli* (Sramek and Frerman, 1975).

Fig 60. List of CoA esters synthesised by known chemical methods

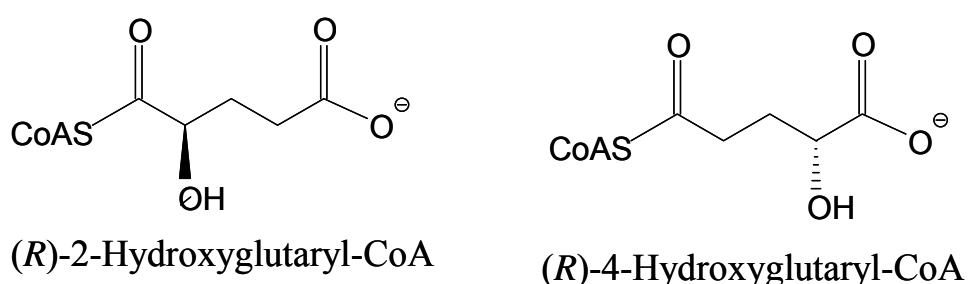


Fig 61. Structures of the isomers of hydroxyglutaryl-CoA generated from (*R*)-2-hydroxyglutarate and acetyl-CoA by the action of glutaconate-CoA transferase.

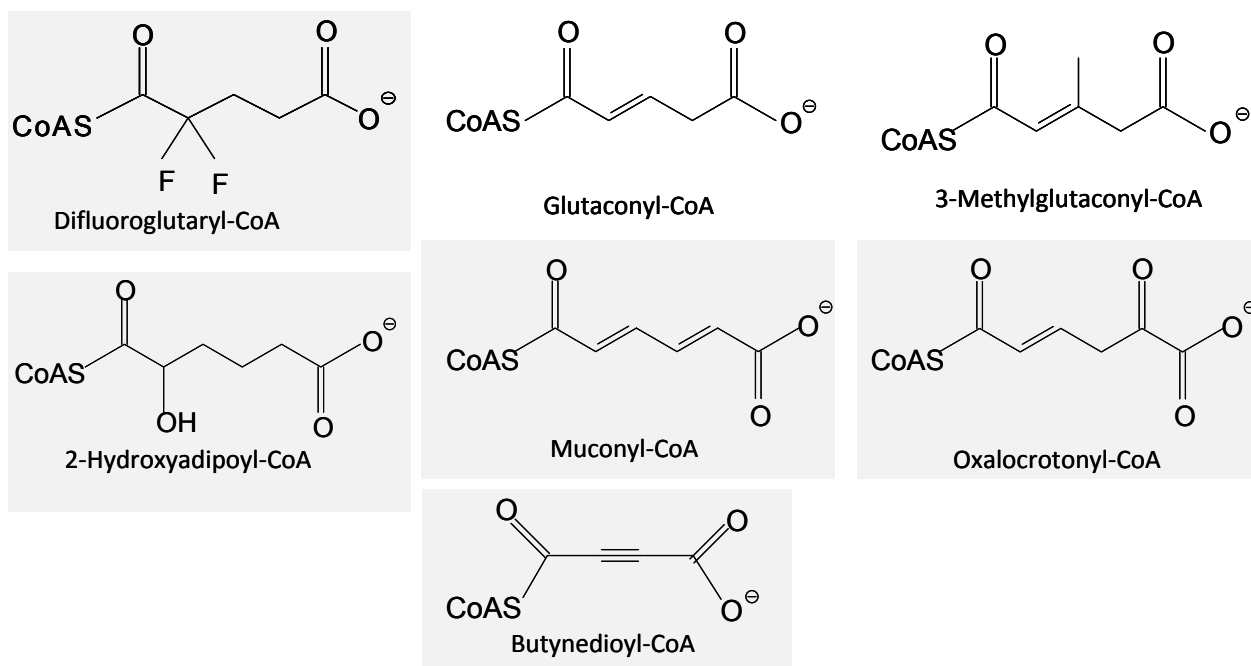


Fig 62. Structures of enzymatically synthesised CoA esters; the ones in a grey background are not described in the literature.

2 (*R*)-2-hydroxyglutaryl-CoA dehydratase system from *C. symbiosum*

2.1 Substrates

The reversible dehydration of (*R*)-2-hydroxyglutaryl-CoA to (*E*)-glutaconyl-CoA by the action of the 2-hydroxyglutaryl-CoA dehydratase from *C. symbiosum* was studied in detail by earlier investigators (Buckel, 1980), (Hans, 1999), (Hans, et al., 2002) and (Schlüter, L., Diploma thesis, Philipps University Marburg, 2000). The same enzyme was studied in two other organisms sharing the 2-hydroxyglutarate pathway, *A. fermentans* (Schweiger, 1987), (Dutsch, et al., 1989) and *F. nucleatum* (Klees, et al., 1992). However, no crystal structure data except for the β_2 dimer (Martins, Dobbek and Hetzel, unpublished) is available to date. So it was decided to "map" the active site indirectly by using substrate analogues.

Four of the substrates shown in grey backgrounds in fig 62 are not known in the literature as the CoA esters, although many of the respective carboxylic acids have been studied for a long time. For example, oxalocrotonic acid appears as the substrate of 4-oxalocrotonate tautomerase in the meta-fission pathway, a catabolic route for the conversion of catechols to Krebs cycle intermediates in

Pseudomonas putida mt-2 (Hirayama, 1984 and 1989; Johnson, 2004, Metanis, et al., 2005). Although the oxalocrotonyl-CoA could be the 4-oxo or the 2-oxo isomer, the hydration reaction with the dehydratase showed that the 4-oxo isomer was present, although the 2-oxo could also be present.

In the case of hydroxyadipoyl-CoA, the enzymatic synthesis probably yielded *R*-forms of both the 2-hydroxy and the 5-hydroxy isomers while the *S* forms were probably less favoured by the CoA transferase, similar to the case of hydroxyglutaryl-CoA (Buckel, et al., 1981). However, it is expected that only the *R*-isomer could be utilized, based on what is known about the enzyme's preference for (*R*)-2-hydroxyglutaryl-CoA.

The only mention of muconyl-CoA was in connection with a putative muconyl CoA *cis-trans* isomerase belonging to the crotonase superfamily (Ferrandez, et al., Genbank X97452, 1998), in a review of enzyme superfamilies (Gerit and Babbitt, 1998). The muconyl-CoA used in this work has *trans-trans* geometry.

2,4-pentadienoyl-CoA is generated by the action of 5-hydroxyvaleryl-CoA dehydratase/dehydrogenase (Eikmanns and Buckel, 1991; Eikmanns, 1994; Fillgrove, 2001) known from the fermentation of 5-aminovalerate by *Clostridium aminovalericum* (Barker, et al., 1987; Eikmanns and Buckel, 1991) and of the 2,4-dienoyl-CoA reductase from *E. coli*, an iron-sulfur flavoprotein that functions in fatty acid beta-oxidation (Nada, et al., 1994; Liang, et al., 2000). 2-Pentenoyl-CoA has been described in the same literature as well as in the 2-methyl branched-chain acyl-CoA dehydrogenase, an enzyme involved in NADH-dependent enoyl-CoA reduction in anaerobic mitochondria of the nematode, *Ascaris suum* (Komuniecki, et al., 1985 and 1987; Ito, et al., 1990; Li, et al., 1991).

3-Methylglutaconyl-CoA is known as a substrate of the 3-methylglutaconyl-CoA hydratase from *Acinetobacter* sp. in the oxidative degradation of (*S*)-leucine (Mack, et al., 2006).

The pattern of Michaelis-Menten kinetics with natural and alternate substrates suggests that the active site is relatively flexible. It can accommodate substrates whose acyl moieties are about 5-6 carbons long and contain 2-hydroxy or 2, 3-unsaturated groups.

There was no activity measured with (*R*)-2-hydroxybutyryl-CoA, crotonyl-CoA, *trans*-2-pentenoyl-CoA and 2,4-pentadienoyl-CoA. 2-Hydroxy-4-pentenoyl-CoA has been reported to be dehydrated by 2-hydroxyisocaproyl-CoA dehydratase and generate the mechanism-based inhibitor 2,4-pentadienoyl-CoA, whose presence was confirmed by EPR spectroscopy (Kim, et al., 2008). The question arises why 2,4-pentadienoyl-CoA does not affect the 2-

hydroxyglutaryl-CoA dehydratase. This may be because the presence of the second carboxylate may be necessary to orient the substrate in the correct position inside the active site.

The substrate butyne-dioyl-CoA probably binds to the enzyme despite its acyl part being four carbons long, probably because the strained "linear" geometry (fig 3) orients the second carboxylate in a sufficiently accessible position.

An analogue where the second carboxylate has been replaced with a nitro group has been reported to be a substrate of human glutaryl-CoA dehydrogenase (Rao, et al., 2002). So it would be interesting to see a similar effect with our system.

Attempts to synthesise 4-nitro-2-butenate by the standard nitration methods (Kornblum, et al., 1955) failed and therefore the CoA ester of this acid could also not be made.

Surprisingly, the presence of a methyl at the C-3 position hindered the activity with 3-methylglutaconyl-CoA. The steric hindrance caused by the methyl group could prevent the addition of proton from the enzyme acid/base to the double bond from the C-3 side. This correlates however, with the observation that isoleucine and valine cannot be fermented via the respective (*R*)-2-hydroxyacyl-CoA derivatives.

2.2 Inhibitors and inactivators

Among the various CoA esters which act as inhibitors, the strongest effect was with difluoroglutaryl-CoA ($K_i = 69 \mu\text{M}$) followed by glutaryl-CoA (table 3). We assume that the 2,2-difluoro isomer is present; however the presence of the 4,4-isomer cannot be ruled out. Pentanoyl-CoA was a weaker inhibitor. The inhibition by glutaryl-CoA was already observed with the 2-hydroxyglutaryl-CoA dehydratase from *F. nucleatum* (Klees, et al., 1992). The type of inhibition in this case was thought to be competitive. This supports the hypothesis that the second carboxylate is necessary to orient the substrate.

In contrast to glutaryl-CoA, there is most likely fluorine at the 2-position in difluoroglutaryl-CoA. This fluorine probably binds to [4Fe-4S] cluster of the dehydratase in the same way as the hydroxy group of (*R*)-2-hydroxyglutaryl-CoA, which is the natural substrate. The stronger binding may be attributed to the high electronegativity of fluorine compared to the proton. Also, the acidity of the C-3 proton must be much higher due to the highly electron-withdrawing *gem*-difluoro group at C-2 and may be an additional factor in the inhibitory effect.

There are other substances which inhibit the activity of the dehydratase which do not resemble the substrates, but which can be characterised as "inactivators". Some of these substances are metronidazole (fig. 63a), 2-nitrophenol, 4-

nitrophenol, 2, 4-dinitrophenol, hydroxylamine, sodium nitrite, 4-nitrobenzoate and chloramphenicol (Müller and Buckel, 1995). Nitro compounds are thought to be reduced to their nitroso derivatives, which then react with the bases of DNA, thereby leading to the death of microorganisms (Edwards, 1993).

Early studies showed that nitrophenols inhibited the conversion of lactate or acrylate to acetate and propionate (Ladd and Walker, 1959) in cell-free extracts of *Megasphaera elsdenii* (lactyl-CoA dehydratase). Hydroxylamine and 2,4-dinitrophenol are also inhibitors of the lactyl-CoA dehydratase of *C. propionicum* (Schweiger and Buckel, 1984) and of the 2-hydroxyglutaryl-CoA dehydratase from *A. fermentans* (Schweiger and Buckel, 1984).

The 2-hydroxyglutaryl-CoA dehydratase from *F. nucleatum* is inhibited by 150 μ M hydroxylamine, 10 μ M 2,4-dinitrophenol or 10 μ M chloramphenicol. The mechanism of the inhibition is obscure for all three components. Furthermore, 2,4-dinitrophenol does not act as proton carrier since all three dehydratases behave like soluble enzymes during purification. In the case of *A. fermentans* the location of the 2-hydroxyglutaryl-CoA dehydratase in the cytoplasm was demonstrated by the immuno-gold technique (Rohde, et al., 1988).

It is possible that the nitro groups of 2,4-dinitrophenol as well as hydroxylamine are reduced by the riboflavin. This could also happen to the nitro group of chloramphenicol, an inhibitor of bacterial protein synthesis. But UV-vis spectra of the dehydratase incubated with hydroxylamine or chloramphenicol showed no oxidation of the reduced riboflavin (Klees, et al., 1992).

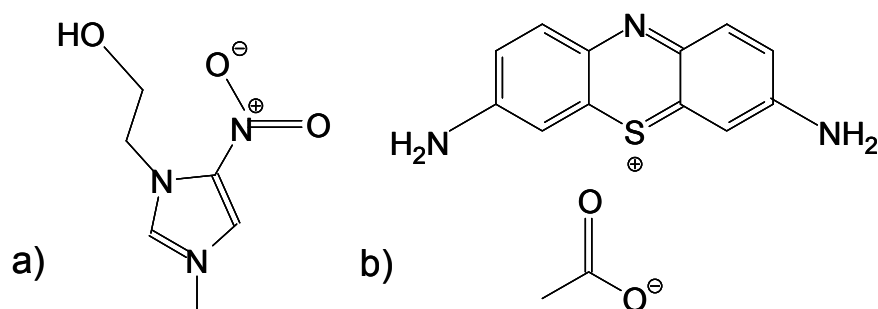


Fig 63. a) Metronidazole, 2-methyl-5-nitroimidazole-1-ethanol, a nitroimidazole derivative and **b)** thionine acetate or 3,7-diamino-5-phenothiazinium acetate, a thiazine derivative

The inactivators of the 2-hydroxyacyl dehydratases fall into two groups based on their concentration effects (Müller and Buckel, 1995). The first group consists of hydroxylamine and sodium nitrite; here high enough concentrations can inhibit the activity completely. Sigmoid curves are obtained and activity is abolished by 40 μ M of hydroxylamine or 10 μ M of sodium nitrite (Hans, et al.,

1999). This threshold was later explained by a complete non-enzymatic oxidation of the necessary *in vitro* reducing agent Ti (III) citrate to Ti (IV) by titration with hydroxylamine or nitrite.

The second group consists of nitrophenols and 4-nitrobenzoate. In the presence of these, a time-lag was observed in the conversion of substrate; higher concentrations of the inactivator lead to longer lag-phases (Mueller and Buckel, 1995; Hans, et al., 1999). Metronidazole was shown also to inhibit the growth of *C. symbiosum* although complete suppression of growth never occurred (Schlüter, L., Diploma thesis, Philipps University, Marburg). The dehydratase from this organism was inhibited by the same chemical (Hetzel, M., PhD thesis, Philipps University, Marburg). However, addition of more Ti (III) citrate (or dithionite) or of the activating enzyme or the dehydratase shortened the lag-phase in each case.

That cyclic nitro compounds can be enzymatically or chemically reduced is also known (Angermaier, et al., 1983; Lamp, 1999). Further there were reports of enzymatic reduction of hydroxylamine dependent on ATP by the *C. symbiosum* dehydratase in the presence of Ti (III) citrate (Hans, et al., 1999) and a similar observation in the benzoyl-CoA reductase with methylviologen as the reducing agent (Boll and Fuchs, 1995). It was also known that metronidazole can be converted to thiamine derivatives enzymatically (Alston and Abeles, 1987). Clostridial ferredoxin was shown to reduce metronidazole (Lockerby, et al., 1984).

It is well-known that the [4Fe-4S] cluster of the activating enzyme is reduced by dithionite or Ti (III) citrate and that the electron is then transferred to another [4Fe-4S] cluster on the dehydratase (Hans, et al., 2002). The reduced amine was characterised by HPLC, in the case of 4-nitrobenzoate inactivating the *A. fermentans* dehydratase (Müller and Buckel, 1995).

The mechanistically similar 4-hydroxybutyryl-CoA dehydratase where a catalytic flavin is involved (Müh, et al., 1996; Činkaya, et al., 1997 and Friedrich, et al., 2008) instead of an activating enzyme is not affected by metronidazole (Friedrich, P., Diploma thesis, Philipps University Marburg).

All these observations lead us to suspect that the activating enzyme might have something to do with the inactivation mechanism. It was shown in the course of this work that it is the activator which is oxidised by metronidazole and the dehydratase itself is unaffected. This correlates well with the fact that the activator has a relatively open structure where the iron-sulfur cluster is easily solvent-accessible (Locher, et al., 2001). This also explains the great oxygen sensitivity of this enzyme. By contrast, chemical labelling by iodoacetamide and crystallographic data show that the iron-sulfur clusters of the dehydratase

component are less solvent accessible and in fact, one of these clusters is nearly completely shielded from the solvent.

Spectra obtained when the activator was oxidised by thionine acetate (structure shown in fig. 63b), metronidazole or 2-nitrophenol were nearly identical. The oxidising effect was lost when 2-aminophenol was used in place of 2-nitrophenol.

The estimated standard redox potentials were reported as -415 mV for metronidazole (Scarpignato, 2004), +230 mv for thionine (Lindahl, et al., 1988), -20 mV for 4-nitrophenol, -115 mV for 2-nitrophenol and -100 mV for chloramphenicol (calculated from Chen, et al., 2006). The redox potential of the activator was estimated at -320 mV and clostridial ferredoxin at -450mV (Hans, et al., 2002, 2008). However, the enzymatic reduction of metronidazole is irreversible (Lockerby, et al., 1984), while the redox potentials mentioned are for reversible reduction. The irreversible reduction probably occurs at a much more positive potential; this would explain the observed oxidation of the activator by metronidazole.

Some oxidising or reducing agents can destroy iron-sulfur clusters as has been observed with the Mo-Fe component of nitrogenase (Lindahl, et al., 1988; Pierik, et al., 1993). Therefore, similarities in the spectrum would reflect rather destruction of the [4Fe-4S] cluster by various nitro compounds, with or without oxidation. This also accounts for the fact that the activator cannot be titrated directly with redox dyes (Hans, et al., 2002, 2008).

Therefore, we can reasonably conclude that the nitro compounds inhibit the activity of the 2-hydroxyacyl-CoA dehydratases indirectly by destroying the iron-sulfur cluster of the activator components.

2.3 Structural aspects

2.3.1 The 2-hydroxyacyl-CoA dehydratases

To date the only complete crystal structure of any 2-hydroxyacyl-CoA dehydratase available is that of the HadBC (2-hydroxyisocaproyl-CoA dehydratase) from *C. difficile* that was solved recently at a resolution of 2.2 Å (Dobbek and Knauer, unpublished). Both subunits have [4Fe-4S] clusters coordinated by 3 conserved Cys residues. In the first subunit, an OH is the fourth ligand of the [4Fe-4S] cluster (fig 64), as found in aconitase (Beinert and Thomson, 1989; Werst, et al., 1990) and in the second subunit the [4Fe-4S] cluster contains a sulfide as the fourth ligand.

The two clusters are situated about 12 Å from each other. So, electron transfer between them could occur easily. The cluster in the second or C subunit seems to be shielded from the surface of the protein, whereas a substrate channel appears to exist in the first or B subunit, leading to the cluster. The structure of HadBC with the substrate analogue (2-hydroxyisocaproate) was also solved by Dobbek, et al., where the hydroxyl group was suggested to replace the water molecule coordinating the iron atom. Chemical modification with iodoacetamide on the HgdAB (2-hydroxyglutaryl-CoA dehydratase) from *C. symbiosum* supports this idea. Whereas the cysteines of HgdB or second subunit are unaffected by iodoacetamide, one of the cysteines in the first subunit or HgdA, Cys85 is modified, as analysed by Nano-LC-MS. It has been suggested from modelling (Dobbek and Knauer, unpublished) that the CoA part lies outside the active site and studies are planned with analogues where CoA is replaced by pantetheine.

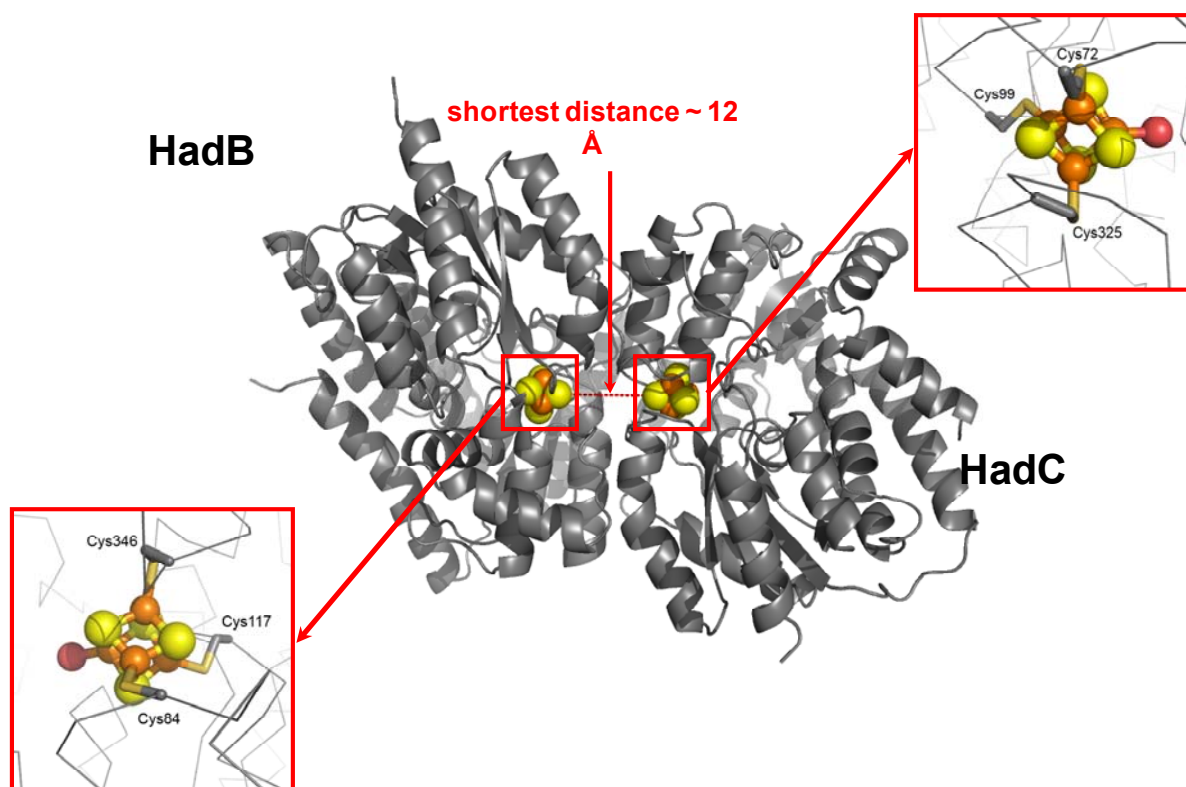


Fig 64. The crystal structure of HadBC (2-hydroxyisocaproyl-CoA dehydratase) from *C. difficile* solved recently at 2.2 Å resolution by Dobbek and Knauer.

The Mössbauer spectra of the *C. symbiosum* dehydratase, reportedly reveal that all eight iron atoms behave equally, with two cube-shaped $[4\text{Fe-4S}]^{2+}$ clusters, in each of which all four iron atoms are coordinated to cysteine residues (Hans, et al, 1999). However, an examination of the amino acid sequences of the 2-hydroxyacyl-CoA dehydratases shows only 3 conserved cysteines per subunit (shown in bold red letters and yellow background in fig. 65) and no obvious

conserved residues which might coordinate the fourth iron. None of the 3 cysteines on the second subunit is conserved in the *A. fermentans* enzyme, where there is a third subunit of unknown function (Tamannaei, A., Diploma thesis, Philipps University, Marburg) and only one $[4\text{Fe-4S}]^{2+}$ cluster. There are many conserved residues whose role is yet unknown (shown in grey background in fig. 65).

So, there is a strong reason why the fourth ligand of the iron-sulfur cluster would be a water molecule. This suggests that the substrate is unlikely to bind the iron coordinated to cysteine and likely binds that iron coordinated to the water molecule as seen in the crystal structure (fig. 66). Although the amino acid sequences of the 2-hydroxyacyl-CoA dehydratases may not be very similar, the structural motifs of the HadBC system may well be conserved in the other systems as well.

CD spectroscopy of proteins in the UV region (190-320 nm) is a classical method to detect conformational changes in the protein polypeptide chain. There were no significant changes in the spectrum upon reduction by the activating enzyme and binding of substrate. Changes in the properties of the $[4\text{Fe-4S}]$ clusters have been monitored in other systems by CD spectroscopy in the 350-650 nm region (Stephens, et al., 1979; Ryle, et al., 1996). However the spectrum was featureless and there were no changes in this region, either in the reduced, substrate-bound or in the native condition. So, there are apparently no large changes around the iron centers as well. Mössbauer spectroscopy might be helpful in detecting any change in the iron-centers.

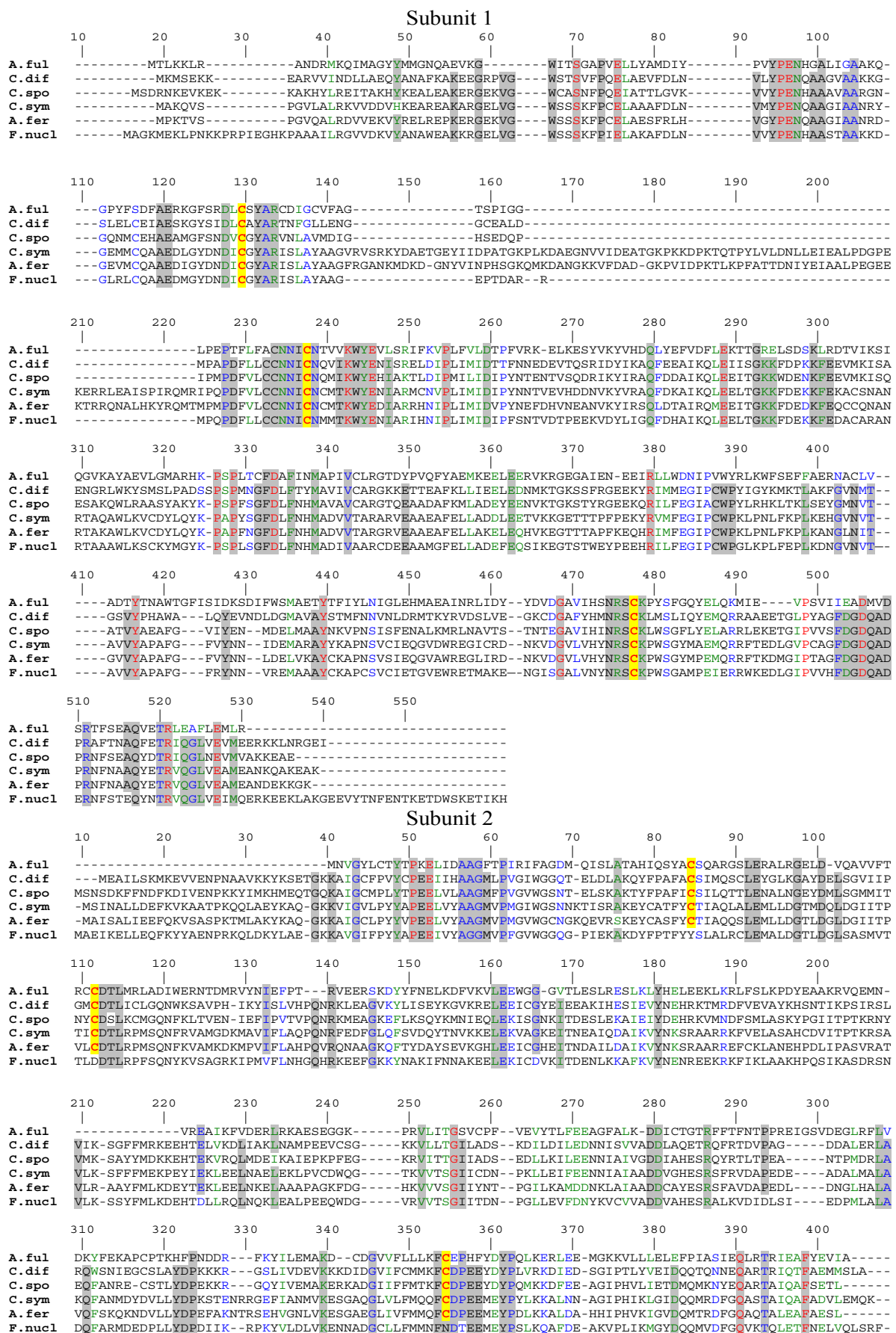


Fig 65. Amino acid sequences of the subunits of 2-hydroxyacyl-CoA dehydratases from different organisms; from top to bottom *A. fulgidus* (putative), *C. difficile*, *C. sporogenes*, *C. symbiosum*, *A. fermentans* and *F. nucleatum*.

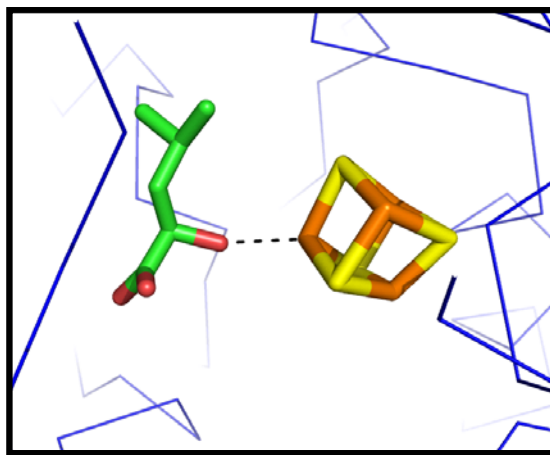


Fig 66. Model of (*R*)-2-hydroxyisocaproate coordinated to the iron-sulfur cluster of HadB based on the crystal structure of HadBC.

2.3.2 Activation of the 2-hydroxyacyl-CoA dehydratases

The Mg-ATP-dependent "activation" of the 2-hydroxyacyl-CoA dehydratases has been studied in detail (Mueller and Buckel, 1995; Hans, et al., 2002; Kim, et al., 2005) and a general review is available (Buckel, et al., 2004). All the known activators from different systems have a high sequence similarity. The activator from *A. fermentans* is a homodimeric protein with the two identical subunits bridged at one end by the [4Fe-4S] cluster (Locher, et al., 2001), structurally similar to the nitrogenase iron protein from *Azotobacter vinelandii* (Georgiadis, et al., 1992).

The general mechanism of activation is believed to resemble the classical nitrogenase system (Mortenson, et al., 1993; Howard and Rees, 1994; Lanzilotta, et al., 1996), the benzoyl-CoA reductase from *Thauera aromatica* (Boll and Fuchs, 1995) and the recently studied protochlorophyllide oxidoreductase from *Chlorobium tepidum* (Broecker, et al., 2008).

It is known that Mg-ATP hydrolysis is coupled to electron transfer between the activator and the dehydratase (Hans, et al., 2002; Kim, et al., 2005). The activator can bind two Mg-ATP molecules and that this binding is suggested to cause conformational changes essential to docking to the dehydratase. The hydrolysis of two Mg-ATP molecules leads to the transfer of an electron from the activator [4Fe-4S] cluster to another [4Fe-4S] cluster on the dehydratase for substrate reduction. Upon dissociation from the dehydratase, the oxidized activator is reduced and the two Mg-ADP molecules are exchanged for two Mg-ATP molecules. An electron is thought to be recycled for 10^4 turnovers (Kim, et al., 2005), unlike the nitrogenase system where each cycle needs 2 Mg-ATP (Hageman and Burris, 1978).

It was shown by the use of the iron-chelator bathophenanthroline that the accessibility of the [4Fe-4S] cluster to the solvent is maximum when the enzyme is unreduced and bound to Mg-ADP or when reduced and bound to Mg-ATP (Kim, J. and Lu, Y., 2007). When the enzyme is reduced with dithionite to mimic the *in vivo* reduction by ferredoxin or flavodoxin (Hans, et al., 2000; Thamer, et al., 2003), there is nearly no chelation. This and other evidence from EPR spectra suggested (Hans, et al., 2008) there may be conformational changes during the course of the catalytic cycle.

However, CD spectra of the enzyme in the UV region in the unreduced Mg-ADP, reduced Mg-ADP and Mg-ATP states were all similar. Also, the spectra in the iron-sulfur region (350-650 nm) were featureless. This is in contrast to the clear changes in the CD in this region for the nitrogenase iron protein (Lanzilotta, et al., 1996; Ryle et al., 1996). The binding of nucleotides in that protein was shown to be cooperative (Ryle and Seefeldt, 1996).

The crystal structures of the activator from *C. difficile* were solved by Dobbek and Knauer recently. The structure showed two arms with largely helical regions and a [4Fe-4S] cluster between the subunits. The overall structure is very similar to the structure of the *A. fermentans* activator (Locher, et al., 2001). There is little difference in the structures of the activators bound to ADP under reduced or oxidized conditions (fig 67). Further, the structures containing ADP or the non-hydrolysable ATP-analogue, ADPNP in the active sites are also quite similar (fig 68). Therefore the postulated nucleotide-induced conformational changes could not be proved conclusively. However, there appears to be some fairly significant differences between the structures of the *C. difficile* activator with and without nucleotides in the substrate binding region. This may indicate that ATP hydrolyses to ADP on docking to the dehydratase. ADP would then leave the active site allowing the activator to 'undock'. ATP would bind again and the cycle could continue.

Aluminium tetrafluoride $[\text{AlF}_4]^-$, induces complex formation between the activator and dehydratase is known (Hetzl, M., and Kim, J., unpublished) which represents a kind of transition-state of ATP hydrolysis reported in the nitrogenase system (Schindelin, et al., 1997). The complex formation occurred only with ADP- AlF_4^- or ADPNP, but not with ADP (Knauer, S., unpublished), showing that the dehydratase could distinguish between ADP and ATP states of its reduced activator. This suggests that there are definite changes in the dehydratase structure which may change the redox potential of its clusters. However, in view of the contradictory evidences obtained by different techniques, resolution of the crystal structure of the aluminium fluoride-induced complex of the two enzymes becomes essential.

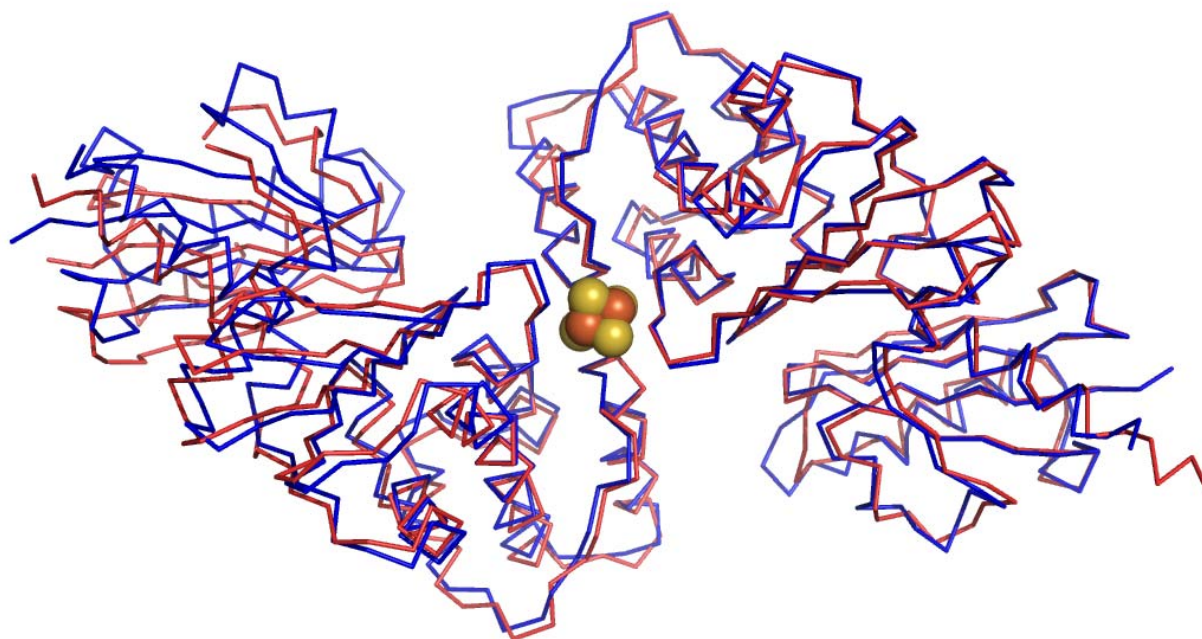


Fig 67. Structures of the reduced activator from *C. difficile* in the presence of ADP (blue) and the oxidized activator from *A. fermentans* in the presence of ADP (red).

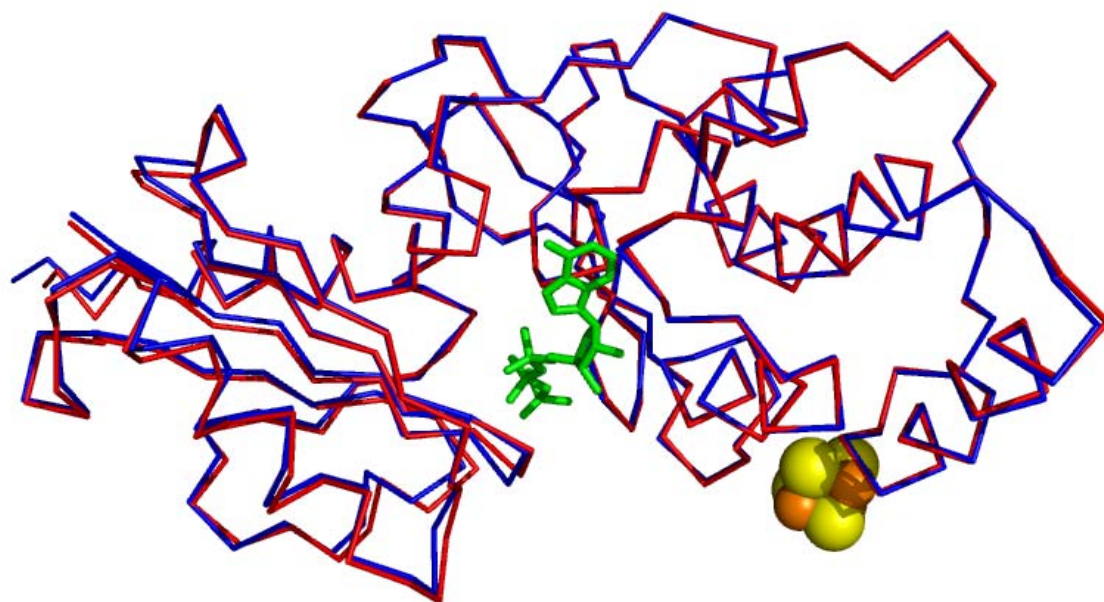


Fig 68. The monomeric structures of reduced *C. difficile* activator bound to ADP (blue) and ADPNP (red)

Based on these observations, the current model (fig. 69) is that the [4Fe-4S] cluster on the first subunit binds substrate and acts as the radical generator, while the second [4Fe-4S] cluster receives an electron from the [4Fe-4S] cluster of the activator, driven by ATP hydrolysis. The electron is 'parked' there until the binding of the substrate to the [4Fe-4S] cluster on the first subunit changes the probability of electron transfer between the two subunits of the dehydratase.

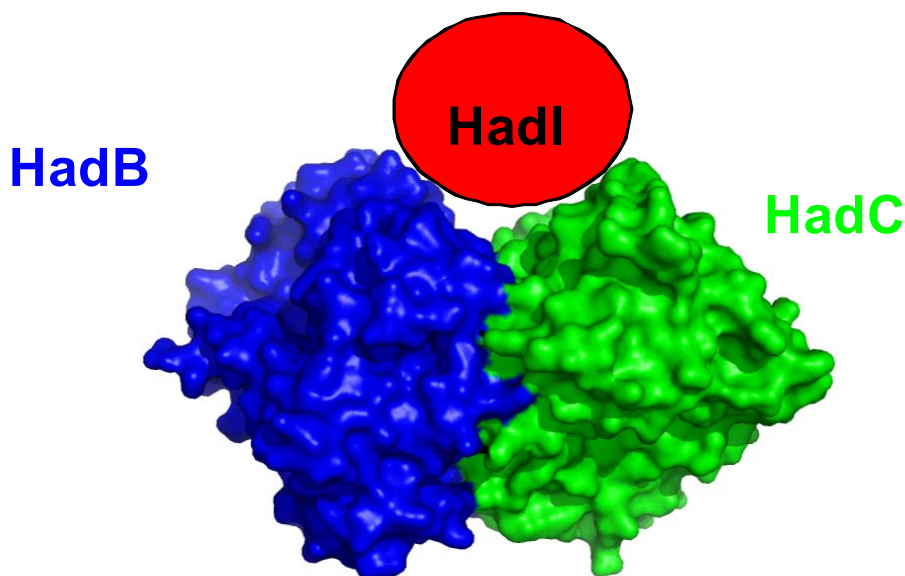


Fig 69. Model of interaction between dehydratase subunit1 (blue), subunit2 (green) and the activator (red)

3. Lactyl-CoA dehydratase from *C. propionicum*

The energetics and kinetics of the lactate fermentation has been discussed in other reports (Seeliger, et al., 2002). The lactyl-CoA dehydratase from *C. propionicum* has been purified and characterised before (Kuchta and Abeles, 1985; Hofmeister and Buckel, 1992) and shown to contain two [4Fe-4S] clusters, riboflavin and FMN (Kuchta, et al., 1986). The specific activities measured were always 3-5 Units/mg in these studies. The general mechanism and properties are reported to be similar to the 2-hydroxyglutaryl-CoA dehydratase from *C. symbiosum* (Schweiger and Buckel, 1984, 1985; Hofmeister and Buckel, 1992). That (*R*)-lactyl-CoA is converted to acrylyl-CoA and, (*R*)-2-hydroxybutyryl-CoA is converted to crotonyl-CoA have been demonstrated (Hofmeister and Buckel, 1992).

The difficulty has always been to measure acrylyl-CoA hydration reliably with an optical assay. Due to the competing reduction of acrylyl-CoA by the acrylyl-CoA reductase (Hetzl, et al., 2003; Schlien, K., Diploma thesis, Philipps University Marburg, 2008), the two activities need to be separated or the

reduction reaction suppressed. Since acrylamide is a toxic electrophile (Tareke, et al., 2000), acrylyl-CoA should be even more so due to the activation by the CoA thiol ester, the hydration and reduction of acrylyl-CoA are tightly coupled physiologically.

The use of many helping enzymes, acrylyl-CoA and NADH complicated earlier attempts to develop an optical assay (Herrmann, G., unpublished). Another problem has been that the activator from *C. propionicum* has never been purified completely and is very unstable. Since the equilibrium of the dehydration reaction has been proposed to lie far in the direction of acrylyl-CoA hydration, no direct dehydration of lactyl-CoA to acrylyl-CoA could be measured. However, the product of the subsequent reduction, propionyl-CoA could be detected by MALDI-TOF mass spectroscopy. So also, lactyl-CoA could be detected as the product of acrylyl-CoA hydration (Herrmann, G., PhD thesis, Philipps University Marburg, 2008).

2-Alkynoyl-CoA inhibitors have been used to inhibit acyl-CoA dehydrogenases and reductases (Fendrich and Abeles, 1982; Freund et al., 1985; Gomes, et al., 1981). 3-pentynoyl-CoA has been reported as an inhibitor of the acrylate reduction (Kuchta and Abeles, 1985). We used a new purification scheme that allows a 'blind purification' based on the greenish-brown colour of the dehydratase. Also, we could inhibit the reductase activity of the fractions by the use of 3-pentynoyl-CoA. Thus, we could measure solely the acrylyl-CoA hydration activity in an assay similar to the one for 2-hydroxyglutaryl-CoA dehydratase activity. Separately, the reductase activity was assayed by the ferricenium hexafluorophosphate assay with butyryl-CoA or propionyl-CoA as substrate (Hetzl, et al., 2003).

While in the earlier steps, the activator from *C. propionicum* was used, the purified enzyme could be activated by the activator from *A. fermentans*. The activity could be measured smoothly with the purified enzyme using either lactyl-CoA or acrylyl-CoA as substrate.

4. The thermodynamic equilibrium between (*R*)-2-hydroxyacyl-CoA and 2-enoyl-CoA

Whereas the equilibrium constants (*K*) for the dehydration of β , γ or δ -hydroxyacyl-CoA esters typically lie around unity (Scherf and Buckel, 1993), the situation is much less clear for the α -hydroxyacyl-CoA derivatives. For example, it has recently been determined that the enzymatic dehydration of (*R*)-2-hydroxyisocaproyl-CoA (2-hydroxy-4-methylpentanoyl-CoA, **1a** (fig. 70), derived from the amino acid (*S*)-leucine, to isocaprenoyl-CoA (4-methyl-2-pentenoyl-CoA, probably the *E*-isomer, **2a**) occurs irreversibly, within the limits of detection (Kim, et al., 2005).

In stark contrast, the equilibrium of the dehydration of (*R*)-lactyl-CoA to acrylyl-CoA (**1c**→**2c**) strongly favors the hydroxy compound. Under physiological conditions (*R*)-lactyl-CoA is only effectively dehydrated, because the very small equilibrium concentration of the unsaturated compound (**2c**) is irreversibly trapped by the consecutive reductase, resulting in propionyl-CoA (Schweiger and Buckel, 1984; Kuchta and Abeles, 1985). With (*R*)-2-hydroxyglutaryl-CoA (**1b**) and (*E*)-glutaconyl-CoA (**2b**) as substrates, the equilibrium appears to lie more in the middle, (Buckel, 1980), although the value of K^b , like those of K^a and K^c , was unknown.

The values of K^b and K^c could be derived from the experimentally measured kinetics in this work are $K^b = [k_{\text{cat}}/K_m(\mathbf{1b})] / [k_{\text{cat}}/K_m(\mathbf{2b})] = 1600/28 = 57 \pm 1.5$ and $K^c = [k_{\text{cat}}/K_m(\mathbf{1c})] / [k_{\text{cat}}/K_m(\mathbf{2c})] = 0.017 \pm 0.007$, respectively. The precise experimental determination of K^a remains experimentally challenging. The theoretically calculated values of K^a , K^b and K^c are respectively 1600, 8.4 and 0.02 respectively (Smith, D. M., unpublished).

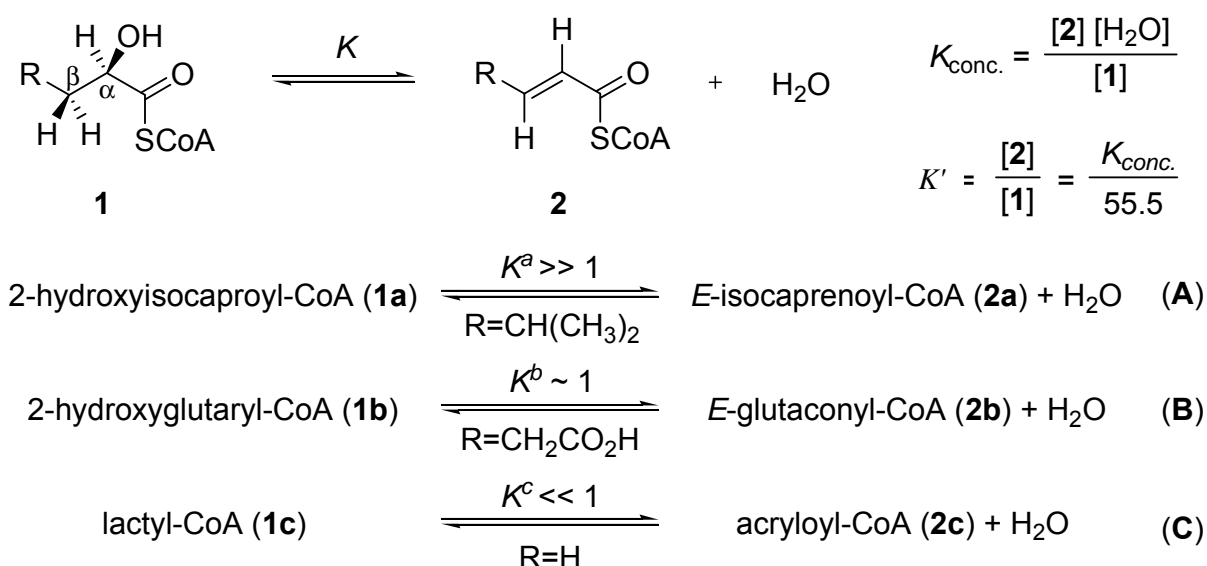


Fig 70. Dehydrations of the α -hydroxyacyl-CoA derivatives. As the biochemical experiments are performed in dilute aqueous conditions, we employ K' , as opposed to $K_{\text{conc.}}$, for convenience.

This low value of K^c corroborates with the high redox potential (Sato, et al., 1999) of the acrylyl-CoA/propionyl-CoA pair ($E_0' = +69$ mV) as compared with those of the higher homologues of 2-enoyl-CoA/acyl-CoA ($E_0' = -10$ mV). The relative magnitudes of K^b and K^c confirm an unexpectedly large (ca. 20 kJ mol⁻¹) substituent effect on the dehydration equilibrium, arising from the presence of a carboxymethyl group on the β -carbon in **1b** in place of a hydrogen atom in **1c**. The similarly large effect apparent upon further substitution by the isopropyl

group in **1a**, were rationalised by *ab initio* molecular orbital calculations (Smith, D. M., unpublished).

To enable high-accuracy calculations, the adenyolphosphopantetheine chain of CoA was replaced by the S-CH₃ group, resulting in the model systems shown in fig. 70. Only a minor effect on the individual equilibrium constants is expected and, because it is adopted uniformly, there should be virtually no effect on the relative equilibrium constants.

Table 7 shows the standard (referenced to 1 atm of pressure) free energy change for each gas phase reaction, $\mathbf{1}_{(g)} \rightarrow \mathbf{2}_{(g)} + \text{H}_2\text{O}_{(g)}$; ($\Delta G^\circ_{(g)}$) and the standard free energy changes in aqueous solution $\mathbf{1}_{(aq)} \rightarrow \mathbf{2}_{(aq)} + \text{H}_2\text{O}_{(aq)}$; ($\Delta G^*_{(aq)}$), referenced to 1 mol L⁻¹.

Table 7. Experimentally determined and calculated values for the equilibria represented by fig 70

Equilibrium	Calculated			Experimental	
	$\Delta G^\circ_{(g)}^{[a,b]}$	$\Delta G^*_{(aq)}^{[a,c]}$	$K^{[d]}$	$\Delta G^*_{(aq)}^{[a,c]}$	$K^{[d]}$
A	-9.0	-28.3	1610	<-27.1	>1000
B	-1.6	-15.2	8.42	-19.9	57
C	2.9	0.1	0.02	0.1	0.017

[a] kJ mol⁻¹ at 298 K, [b] 1 atm reference, [c] 1 mol L⁻¹ reference
[d] dimensionless, $K=K_{conc.}/55.5$ (see fig.11)

In the gas phase, the dehydration of **1c** is found to be mildly (2.9 kJ mol⁻¹) endergonic. The small associated substituent effect (4.5 kJ mol⁻¹) can be rationalised by the mild (net) electron-donating capabilities of the alkyl substituent to the electron deficient β -carbon in **2c**. Indeed, acrylamide with no substituent at the β -position has been shown to act as a toxic electrophilic agent (Tareke, et al., 2000), an effect that should be more pronounced in acrylyl-CoA (**2c**).

The introduction of a carboxymethyl substituent at the β -carbon is found to preferentially stabilize the 2-enoyl species (**2b**) such that dehydration of **2a** is exergonic by 1.6 kJ mol⁻¹. The more electron-donating isopropyl substituent

results in a larger preferential stabilization for the enoyl species (**2a**), such that the dehydration of **1a** is exergonic by 9.0 kJ mol⁻¹.

The standard free energy changes in aqueous solution $\Delta G^*_{(aq)}$, could preferentially favor the products, because of the sizeable solvation free energy of water, $\Delta G^*_s(\text{H}_2\text{O}) = -26.5 \text{ kJ mol}^{-1}$ (Ben-Naim and Marcus, 1984). This preference for the bimolecular products is reduced by the reference state correction (from 1 atm to 1 mol L⁻¹) of $RT\ln(RT)$ which is 7.9 kJ mol⁻¹ (at 298 K) for each species, to -18.6 kJ mol⁻¹.

Table 8. Calculated free energies of solvation (ΔG^*_s) for the species shown in fig.70.

	1a	2a	1b	2b	1c	2c	H₂O
ΔG^*_s [a]	-4.4	-6.8	-43.7	-40.6	-16.4	-2.5	-24.7

[a] The calculated free energy of solvation (in kJ mol⁻¹) for $\mathbf{x}_{(g)} (1 \text{ mol L}^{-1}) \rightarrow \mathbf{x}_{(aq)} (1 \text{ mol L}^{-1})$. The final $\Delta G^*_{(aq)}$ values in Table 7 are corrected by $RT\ln(RT)$ for each species.

In the case of (*R*)-lactyl-CoA, the inherent product preference in solution is partially compensated for by the relatively large (absolute) value of $\Delta G^*_s(\mathbf{1c})$ compared to $\Delta G^*_s(\mathbf{2c})$ (Table 8). The result of these competing effects is that $\Delta G^*_{(aq)}(\mathbf{C})$ is only 2.8 kJ mol⁻¹ less than the corresponding gas-phase value. Despite the potential uncertainties involved, the final calculated value for $\Delta G^*_{(aq)}(\mathbf{C})$ (0.1 kJ mol⁻¹) is in very good agreement with that derived from the measured equilibrium constant of $K^c = 0.017$ (0.1 kJ mol⁻¹).

The absolute magnitude of the free energies of solvation of **1b** and **2b** are much larger than those of **1c** and **2c** because of the presence of the hydrophilic carboxylic acid groups. Even though the hydroxyacyl species (**1b**) species is again solvated more strongly than the enoyl one (**2b**), the difference between them, and hence the associated effect on $\Delta G^*_{(aq)}(\mathbf{B})$, is much smaller than for reaction C. The favorable contribution from $\Delta G^*_s(\text{H}_2\text{O})$ is therefore counteracted to a much smaller extent and results in the value for $\Delta G^*_{(aq)}(\mathbf{B})$ being 13.6 kJ mol⁻¹ more negative than $\Delta G^\circ_{(g)}(\mathbf{B})$. The corresponding value of $K^b = 8.42$ deviates somewhat from the measured value of $K^b = 57$. In terms of energy, however, the discrepancy of 4.7 kJ mol⁻¹ is certainly within acceptable limits.

The more hydrophobic nature of species **1a** and **2a** is reflected in their less favorable solvation free energies (Table 2). In this case, however, it is the enoyl species (**2a**) that is better solvated than the hydroxyacyl one (**1a**). This serves to slightly reinforce the favorable effect of $\Delta G^*_s(\text{H}_2\text{O})$ rather to counteract it, as

occurred for equilibria B and C. The final result is that equilibrium A is predicted to lie very far to the right, with the associated values of $\Delta G^*(\text{aq})$ (A) = $-28.3 \text{ kJ mol}^{-1}$ and $K^a = 1610$.

The good agreement between theory and experiment obtained for equilibria B and C indicates that these values also provide a reasonable description of the thermodynamics of equilibrium A (2-hydroxyisocaproyl-CoA dehydration). The values correlate well with the fact that the equilibrium concentration of 2a was not detectable in experiments concerning its conversion into 2b. The precise experimental determination of K^a still remains a challenge for the future.

In summary, and in agreement with previous qualitative observations, the measurements presented here confirm an unusually large effect of the substituent at the β -carbon in the dehydration reactions shown fig 70. Molecular orbital calculations show that the inherent substituent effects, as reflected in the gas-phase data, are less drastic. The condensed-phase calculations reproduce the measured values and reveal that the large effects are primarily due to a complex interplay of competing effects connected to the solvation process. The calculations predict that the, as yet, unmeasured equilibrium involving (*R*)-2-hydroxyisocaproyl-CoA strongly favors the product. Indeed, in this context, it seems reasonable to postulate that it is this marked preference for the enoyl species that has enabled the observation of the proposed penultimate intermediate in the dehydration mechanism, the allylic ketyl radical anion of **2a**, (Kim, et al., 2008) for reaction A and not for reactions B or C.

5 Substrates and inhibition of (*R*)-2-hydroxyglutarate dehydrogenase (HgdH) from *A. fermentans*

The crystal structure of 2-hydroxyglutarate dehydrogenase (HgdH) from *A. fermentans* was solved at 1.98 Å resolution (Martins, et al., 2005). The HgdH dimer contains substrate- and nucleotide-binding domains. The two nucleotide-binding domains build the central core of the dimer, whereas the two substrate-binding domains are at opposite sides. The active site is located at the bottom of the interdomain cleft at approximately 12 Å below the surface of the protein with the catalytic triad Arg235, Glu264 and His297 facing into the cleft (Fig 12). The residues lining the active site are all well defined except for Arg235, which displays residual electron density for two possible conformations, one pointing towards His297 (used in the structural analysis) and another pointing towards Asp81. The proton-relay system built up by the histidine-glutamate dyad (His297-Glu264 in HgdH) is conserved in most D-2-hydroxyacid dehydrogenases except formate dehydrogenase (Lamzin, et al., 1985).

Both residues are involved in a hydrogen bonding interaction where the negative charge of the glutamate stabilizes the protonated state of the histidine (positively

charged). Glu264 shifts the pKa of His297 to a higher value, enabling it to act as an acid-base catalyst at the pH value required for the proton transfer (Kochhar, et al., 1992). Apart from participating in the proton transfer reaction, histidine forms a hydrogen bond with the carbonyl group of the substrate (or hydroxyl group in the reverse reaction) and stabilizes the reaction transition state (Taguchi and Ohta, 1993).

The third residue of the catalytic triad is Arg235. Its positive guanidinium moiety plays an essential role in the polarization of the substrate's carbonyl group, increasing its susceptibility for a nucleophilic attack (Vinals, et al., 1995). Additionally, this residue is involved in binding the substrate's γ -carboxylate group (Stoll et al., 1995; Dengler, et al., 1997) and in the activation of the enzyme (Lamzin, et al., 1985). A positively charged patch at the top right side of the active site is formed by Arg9, Arg52 and Arg76. In contrast to the well conserved Arg9 and Arg76, residues in the D-2-hydroxyacid dehydrogenase family corresponding to Arg52 are extremely variable.

The HgdH active site architecture is structurally optimized to recognize and bind the negatively charged substrate 2-oxoglutarate. The structural position of the side chain of Arg52, and its counterparts in other family members, strongly correlates with substrate specificity towards substitutions at the C3 atom (linear or branched substrates). Arg235 interacts with the substrate's α -carboxylate and carbonyl groups, having a dual role in both substrate binding and activation, and the γ -carboxylate group can dock at an arginine cluster. The proton-relay system built up by Glu264 and His297 permits His297 to act as acid-base catalyst and the 4*Re*-hydrogen from NADH is transferred as hydride to the carbonyl group *Si*-face leading to the formation of the correct enantiomer (*R*)-2-hydroxyglutarate.

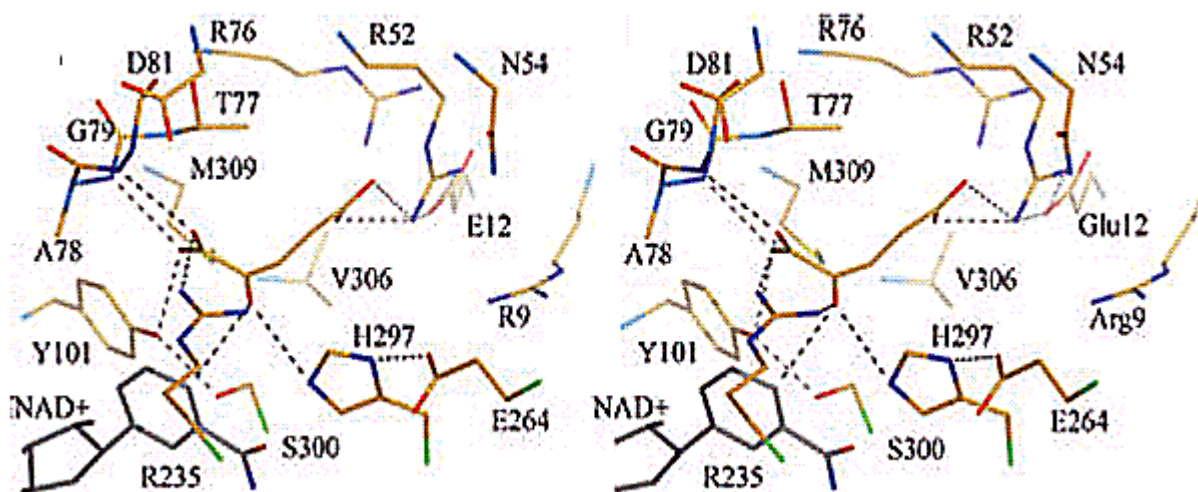


Fig 71. Stereo representation of the HgdH active site pocket with modelled NAD^+ and 2-oxoglutarate, based on the superposition of the available holo and ternary complex structures from the D-2-hydroxyacid dehydrogenases family members). Colour code: gold for carbon, blue for nitrogen, red for oxygen and yellow for sulphur, except for NAD^+ (grey with the carboxamide oxygen and nitrogen atoms in red and blue, respectively). The hydrogen bonding interactions between Glu264 and His297, Ser300 and Tyr101 and Glu12 and Arg52 are indicated by dashed lines, as well as possible hydrogen bonding interactions between 2-oxoglutarate and active site residues.

It is known that the reduction of 2-oxoglutarate by HgdH is inhibited by the substrate analogue oxalylglycine, a weak competitive inhibitor; $K_i = 5 \text{ mM}$ (Bresser, J., Diploma thesis, Philipps University, Marburg). Substitution of the 3-methylene group by an amide reduces the electrophilicity of the carbonyl group due to the partial double bond character of the amide linkage. This is expected to cause the inhibitory effect of this compound.

Further, modelling of the alternative substrate used in this work, 2-oxoadipate is shown in fig 71. This substrate fits into the active site in a manner similar to the natural substrate. Another substrate, oxalocrotonate (2-oxo-hex-4-ene-dioate) is more rigid due to the double bond at the 4 position, but the arrangement within the active site pocket should be similar to the 2-oxo-adipate (fig. 72).

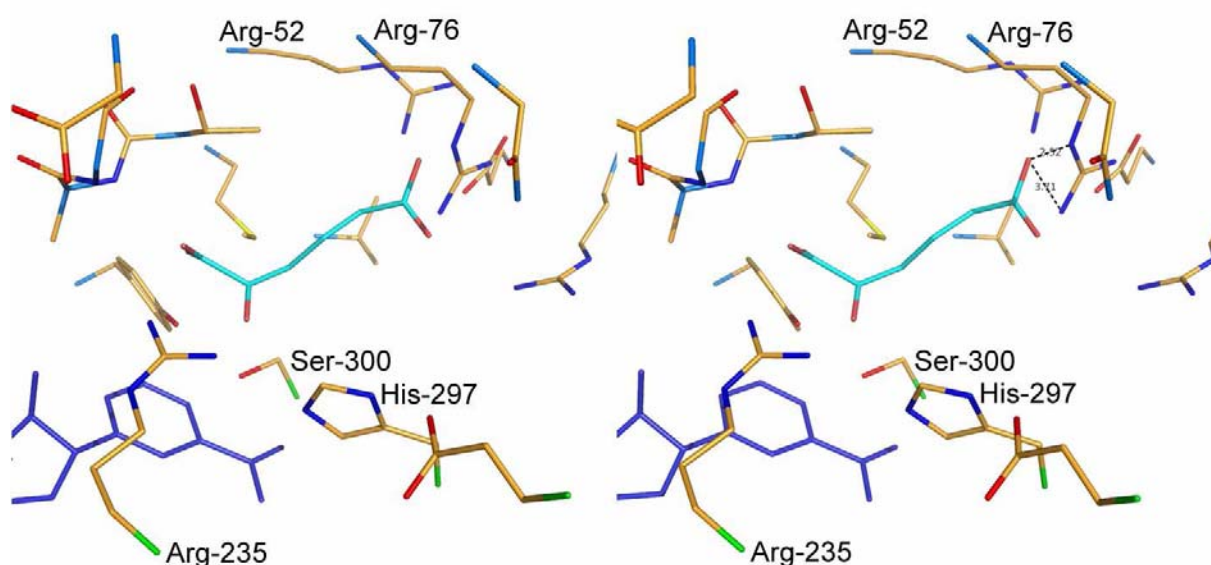


Fig 72. Stereo view of the active site pocket of HgdH, modelled showing the substrate 2-oxo-adipate using the framework of 2-oxoglutarate (as in the crystal structure manuscript). The carboxyl group (pointing towards Arg-76) was rotated not to clash with that residue and Arg-52. The distances from the carboxyl group to Arg-76 are 2.5 and 3.7 Å. Color code of substrate sky-blue for carbon and red for oxygen.

Colour code of enzyme gold for carbon, blue for nitrogen, red for oxygen and yellow for sulphur.

It was shown in this work that DFPA (2,2-difluoropentanedioic acid) competitively inhibited the NADH-dependent reduction of 2-oxoglutarate by HgdH with a K_i of 620 μM . 2,2-difluoroglutaric acid appears to be a fluorinated isostere of 2-oxoglutaric acid because the X-ray structure of DFPA resembles that of 2-oxoglutarate and the pK_a values of DFPA (2.03 and 4.37) are also similar (Laurent et al., 2005) to those of 2-oxoglutaric acid (2.35 and 4.85).

Structural modelling of the DFPA inside the active site of the HgdH (fig. 73), shows that it makes the same contacts to the enzyme as 2-oxoglutarate (Martins, B., unpublished); the γ -carboxylate is bound to the Arg 52 and Arg 235 binds the C1 carboxylate and the carbonyl at C2. Other interacting residues include Met 309, Tyr 101 and Ser 300. This makes DFPA in the anionic form a good structural analogue of 2-oxoglutarate. Further, the C-F bonds cannot be biologically reduced. So, DFPA is a better competitive inhibitor than oxalylglycine.

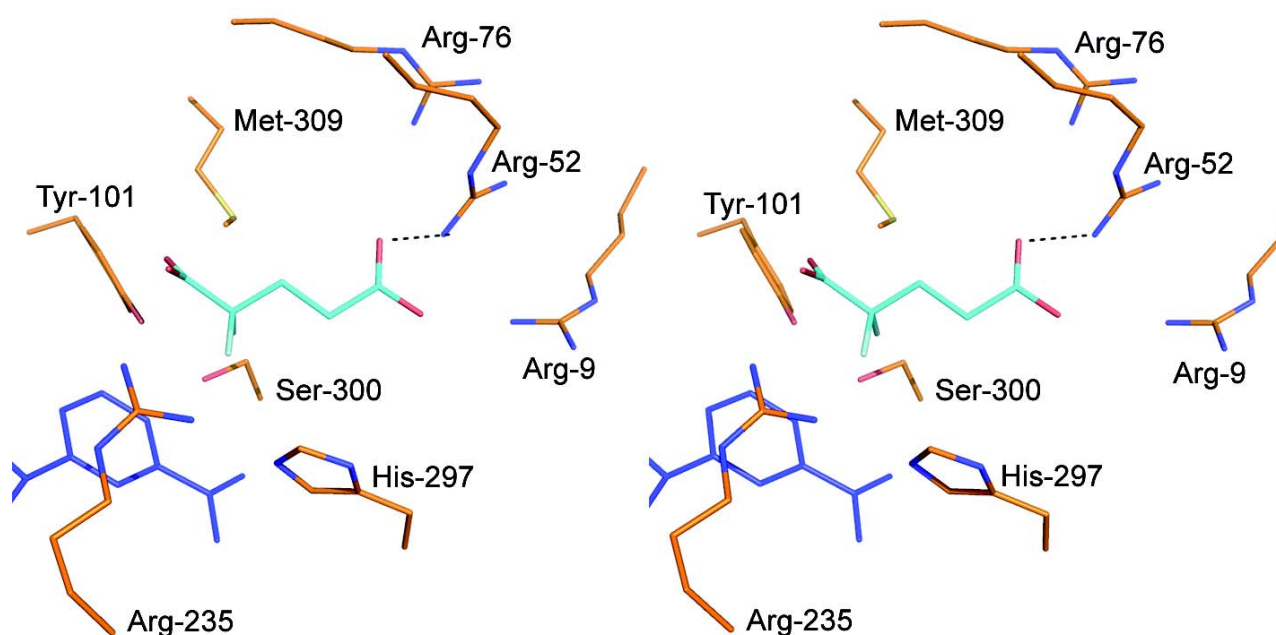


Fig 73. Stereo view of the active site pocket of HgdH with DFPA modelled. The substrate was modelled using the framework of 2-oxoglutarate. The carboxyl group is pointing towards Arg-52. The dash line indicates hydrogen bond interaction between the carboxyl group and Arg-52 (distance 3.6 Å). Color code of substrate: sky-blue for carbon and red for oxygen. Colour code of enzyme gold for carbon, blue for nitrogen, red for oxygen and yellow for sulphur.

This inhibitor did not stop the growth of cultures of *C. symbiosum* on 50 mM glutamate, which also shares the enzymes of the 2-hydroxyglutarate pathway with *A. fermentans*, although a longer lag-phase and lower final optical densities were observed. This could mean that the cultures recover when the reversible inhibition is relieved by formation of sufficient amounts of 2-oxoglutarate.

6. Outlook

Crystallographic work on the dehydratase from *C. difficile* indicated that the CoA moiety of the substrate lies outside the active site (and by extension also in other 2-hydroxyacyl-CoA dehydratases). We need to test this hypothesis by using N-acetylcysteamine derivatives as possible substrates.

A substrate- or product-like radical has never been observed in the case of the *C. symbiosum* enzyme; so efforts are underway to perform EPR experiments with this enzyme and different substrates at liquid helium temperatures. Further, it would be interesting to study the substrate specificity of the lactyl-CoA dehydratase from *C. propionicum*.

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Erklärung

Hiermit versichere ich, dass ich meine Dissertation mit dem Titel

Substrates and mechanism of 2-hydroxyglutrayl-CoA dehydratase from
Clostridium symbiosum

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die vorliegende Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen deutschen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

(Ort, Datum)

(Unterschrift)