Enzymes of two clostridial amino-acid fermentation pathways

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and Emil Herrmann Belloso

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Abbreviations

DTT	Dithiothreitol
DTNB	5,5'-dithiobis(2-nitrobenzoate)
ABTS	2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid
INT	Iodonitrotetrazolium chloride
$FcPF_6$	Ferricenium hexafluorophosphate
Fd	Ferredoxin
LCD	Lactyl-CoA dehydratase
BCD	Butyryl-CoA dehydrogenase
ETF	Electron transfer flavoprotein
ACL	β-Alanyl-CoA ammonia lyase
ACAD	Acyl-CoA dehydrogenase
SCAD	Short chain acyl-CoA dehydrogenase
MCAD	Medium chain acyl-CoA dehydrogenase
LCAD	Long chain acyl-CoA dehydrogenase
VLCAD	Very long chain acyl-CoA dehydrogenase
HAD	3-hydroxyacyl-CoA dehydrogenase
TBS	Tris buffer saline
PBS	Phosphate buffer saline
PBHS	Phosphate buffer high saline
HRP	Horseradish-peroxidase
PVDF	Polyvinylidene fluoride
PMSF	Phenylmethanesulphonylfluoride

Zusammenfassung

Zwei Enzyme der Alanin-Fermentation von *Clostridium propionicum* wurden biochemisch untersucht. Das Enzym (*R*)-Lactyl-CoA Dehydratase, das eine chemisch schwierige Wasserabspaltung von (*R*)-Lactyl-CoA zum Acrylyl-CoA katalysiert, konnte unter strikt anaeroben Bedingungen partiell aufgereinigt werden. Grüne Fraktionen der Komponente D und dunkle braune Fraktionen der Komponente A (Aktivator) wurden erhalten. Über MALDI Massenspektrometrie konnte Lactyl-CoA (m/z = 840), das Hydratysierungsprodukt von Acrylyl-CoA, nachgewiesen werden. Dazu wurde Acrylyl-CoA, Komponente D und Komponente A in Anwesenheit von ATP, Mg^{2+} und Dithionit inkubiert. Ein zweites Enzym der Alanin-Fermentation, das die Addition con Ammoniak an Acrylyl-CoA katalysiert, wurde identifiziert. Die spezifische Aktivität der β-Alanyl-CoA-Ammonia-Lyase ist im Extrakt von Zellen, die auf β-Alanin gewachsen waren, 143 U mg^{-1}, während auf D,L-Alanin gewachsene Zellen nur 0,44 U mg^{-1} enthielten. Daher konnte das Enzym über eine einzige Source 15-Q Säule aus β-Alanin gewachsenen Zellen leicht gereinigt werden. Das Enzym hat eine native molekulare Masse von 95 kDa und ist aus sechs 16 kDa Untereinheiten zusammengesetzt (α_6). Es zeigt eine hohe spezifische Aktivität für Acrylyl-CoA (K_m = 23 ± 4 μM; V_{max} 1000 ± U mg^{-1}) unabhängig von der Ammoniak-Konzentration (K_m = 70 ± 5 mM) Das hohe k_{cat}/K_m = 10^7 M^{-1} s^{-1} zeigt, dass die Reaktion beinahe diffusionslimitiert ist. Die Abspaltung von Ammoniak in der Rückreaktion (β-alanyl-CoA K_m = 210 ± 30 μM) in wird in Anwesenheit von 100 mM NH_4Cl bei gleichem β-alanyl-CoA K_m Wert zu 70 % gehemmt.

Das Enzym, das die Reduktion von Crotonyl-CoA zum Butyryl-CoA in der Glutamat Fermentation von *Clostridium tetanomorphum* über 3-Methylaspartat katalysiert, wurde zusammen mit einen „Electron transfer flavoprotein“ (ETF) aufgereinigt. Der Komplex Butyryl-CoA-Dehydrogenase-ETF, ist ein $\alpha_2\beta\gamma$-Heteromer (m = 360 kDa), das aus drei unterschiedlichen Untereinheiten aufgebaut ist: die 40 kDa α-Untereinheit (Butyryl-CoA-Dehydrogenase), die 36 kDa β-Untereinheit (ETF α-Untereinheit) und die 28 kDa γ-Untereinheit (ETF β-Untereinheit). Das Enzym enthält weniger als 1 mol FAD, aber es nimmt insgesamt 2-3 FAD Moleküle auf. Der gereinigte Enzym-Komplex zeigt Butyryl-CoA-Dehydrogenase Aktivität (1 U mg^{-1}) mit Ferricenium als Elektronenakzeptor und Diaphorase Aktivität unter aeroben und anaeroben Bedingungen. Wenn 50 μM FAD im Test zugesetzt worden war, konnte mit NADH bei einer gleichzeitigen geringen Diaphorase-Aktivität (1 U mg^{-1}) Crotonyl-CoA-Reduktase Aktivität bei 340 nm anaerob nachwiesen werden (20 U mg^{-1}). Mit Immunogoldmarkierung-Elektronmikroskopie war der Komplex hauptsächlich im Zytoplasma des Bakteriums lokalisiert worden. Das Ergebnis ist mit einer direkten Beteiligung der exergonen Crotonyl-CoA-Reduktion an der Energiekonservierung über eine elektrochemischen Protonengradienten unvereinbar. Darüber hinaus ebnete es den Weg zur Entdeckung einer dritten Art der Energiekonservierung.

Summary

Two enzymes of the alanine fermentation pathway of *Clostridium propionicum* were biochemically characterized. The enzyme (*R*)-lactyl-CoA dehydratase catalyzing the difficult dehydration reaction of (*R*)-lactyl-CoA to acrylyl-CoA could be partially purified under strict anaerobic conditions. Green fractions of component D and dark brown fractions of component A (activator) were obtained in separate enzyme pools. Lactyl-CoA was identified in MALDI mass spectrometry (m/z = 840) as product of the hydration reaction after assaying lactyl-CoA dehydratase activity by mixing component D and component A in presence of acrylyl-CoA, ATP, Mg^{+2} and dithionite. A second enzyme of the alanine fermentation pathway was identified catalyzing the ammonification of acrylyl-CoA in the same micro-organism. β-Alanyl-CoA ammonia-lyase activity is 300-fold increased in cell-free extracts of β-alanine grown cells (143 U mg^{-1}) as compared to their D,L-alanine grown counterparts (0.44 U mg^{-1}). Therefore the enzyme was readily purified from β-alanine grown cells and a high final specific activity (1033 U mg^{-1}) was found after one Source 15-Q anion exchange column. The enzyme has a molecular mass of 95 kDa and is composed of six 16 kDa subunits (α_6). It shows high catalytic activity towards acrylyl-CoA (K_m = 23 ± 4 μM) independently of the ammonia concentration (K_m = 70 ± 5 mM) at almost diffusion limiting rate conditions ($k_{cat}/K_m = 10^7$ M^{-1} s^{-1}). In the reverse reaction the elimination of ammonia (β-alanyl-CoA K_m = 210 ± 30 μM) is apparently 70 % inhibited at 100 mM NH_4Cl while the K_m for β-alanyl-CoA remains unchanged.

In the glutamate fermentation pathway via 3-methylaspartate, the enzyme catalyzing the reduction of crotonyl-CoA to butyryl-CoA was purified from *Clostridium tetanomorphum* in a complex with the electron transfer flavoprotein (ETF). Butyryl-CoA dehydrogenase-ETF was characterized as a 360 kDa $\alpha_2\beta\gamma$-heteromer composed of three different subunits: the 40 kDa α-subunit (butyryl-CoA dehydrogenase) the 36 kDa β-subunit (ETF α-subunit) and the 28 kDa γ-subunit (ETF β-subunit). Flavin content was less than 1 mol FAD but the enzyme could be reconstituted with additional FAD (total 2-3 mol) after one hour incubation. The purified BCD-ETF complex presents butyryl-CoA dehydrogenase activity with ferricenium as electron acceptor (1 U mg^{-1}) and diaphorase activity in both oxic and anoxic atmospheres. Crotonyl-CoA reduction (20 U mg^{-1}) could be measured anoxically at 340 nm only in the presence of additional FAD (50 μM) and the concomitant diaphorase activity was approximately 1 U mg^{-1}. *In situ* localization using immunogold- and electron microscopy techniques revealed that the complex is evenly distributed over the cytoplasma of the bacteria. This result excludes a direct involvement of the exergonic crotonyl-CoA reduction in energy conservation via an electrochemical ion gradient. Further it paved the way to the discovery of a third mode of energy conservation.

Introduction

1 Energy metabolism of anaerobic bacteria

In bacterial metabolism there are two basic mechanisms in which redox reactions are coupled to energy conservation: substrate level phosphorylation (SLP) and electron transport coupled to phosphorylation (ETP) or more generally ion gradient phosphorylation. The latter works in combination with an ATP synthase and a multienzyme electron transport chain integrated in the bacterial membrane, where ATP synthesis is linked to the translocation of ions ($\Delta\mu H^+$ or $\Delta\mu Na^+$). The majority of organisms use oxygen as the terminal acceptor, but anaerobic bacteria are able to use other inorganic or organic compounds to respire. Some of the exogenous electron acceptors are fumarate, dimethyl sulfoxide (DMSO), trimethylamine N-oxide (TMAO), nitrite and nitrate. The fermentation process is classically defined as an anaerobic redox process where ATP is generated by substrate level phosphorylation. Though the definition excludes the participation of an electron transport chain, some strict anaerobic bacteria like *Clostridium perfringes* and species of *Propionibacterium* have been reported to contain components of dissimilatory nitrate reduction providing an electron sink for oxidative substrate-level phosphorylation. In general, fermentation cannot be completely separated from a membrane electron transport; many redox processes cause proton extrusion from the cytoplasma and therefore contribute to an electrochemical potential, which enables energy conservation in a respiratory-like process (Lengeler et al**,** 1999; Anderson & Wood, 1969). Recently, a membrane-bound NADH:ferredoxin oxidorreductase possibly responsible for generation of a proton motive force and linked to a *V*-type ATPase, was identified in *Clostridium tetanomorphum* (Boiangiu et al, 2005).

In respiratory chains, dehydrogenases and reductases are functionally linked but do not form a tight complex; the electrons are mobilized within the membrane by quinones, and many exogenous organic compounds like dimethyl sulfoxide (DMSO), nitrite and fumarate can serve as electron acceptors. Such a system is found in the reduction of fumarate to succinate catalysed by a membrane-bound fumarate reductase which is similar to nitrate reductase, having FAD as cofactor rather than molybdopterin (Kröger et al, 1992). Fumarate is a common metabolite found in diverse metabolic processes as a co-substrate for example in anoxic catabolic pathways of hydrocarbons (Heider & Fuchs, 1997) or as an intermediate in *Propionibacterium* B_{12}-dependent lactate fermentation (Random-Pathway), where pyruvate

accepts a carboxyl groups from methylmalonyl-CoA in a transcarboxylation reaction leading to the formation of oxaloacetate and propionyl-CoA. Whereas fumarate, a C_4 dicarboxylic enoate, is in general a more feasible metabolite in the citric acid cycle and related reactions, its C_3 monocarboxylic counterpart acrylate, a very unstable strong nucleophile, must be rapidly converted to the inert end product propionate. A system analogous to fumarate reductase was initially proposed with acrylyl-CoA as electron acceptor. Similar to crotonyl-CoA (E_0' = -10 mV), the redox potential of the pair acrylyl-CoA/propionyl CoA (E_0' = + 69 mV) is even more positive to be coupled to NADH oxidation in an electron transport chain (Sato et al, 1999). These assumptions could not be supported primarily because the enzymes from *C. propionium*, *C. homopropionicum* and *V. parvula*, responsible for acrylyl-CoA reduction, were localised in the soluble fraction (Hetzel et al, 2003; Seeliger et al, 2002).

Domain Bacteria

Phylum BXIII Firmicutes		Phylum BXX Bacteroidetes	Phylum BXXI Fusobacteria
Class Clostridia Order Clostridiales		Class Bacteroides Order Bacteroidales	Class Fusobacteria Order Fusobacteriales
Fam. *Clostridiaceae*	Fam. *Acidaminococcaceae*	Fam. *Porphyromonadaceae*	Fam. *Fusobacteriaceae*
Genus *Clostridium*	Genus *Accidaminococcus* Genus *Megasphaera*	Genus *Porphyromonas*	Genus *Fusobacterium*
C. tetani *C. pascui* *C. acetobutylicum*	*A. fermentans* *M. elsdenii*	Cluster XIVb *C. propionicum*	

Fig. 1 Members of Bacteria domain fermenting amino-acids classified in *Bergey's Manual of Systematic Bacteriology*, 2nd edition (Boone et al, 2001).

A great number of amino acid fermenting micro-organisms are members of the genus *Clostridium* (Fig. 1); these are Gram-positive rods like *C. tetani*, *C. acetobutylicum* or *C. sporogenes*, belonging to the phylum Firmicutes. Among the order Clostridiales there are other farther related species like those of the family *Accidaminococcaceae e.g.* the Gram negative diplococcus *A. fermentans* and the Gram negative sphere *Megasphaera elsdenii*. A representative of the next phylum Bacteroidetes, is the genus *Porphyromonas*. Members of the class Bacteroides as well as of the class Fusobacteria also use amino acids as the energy source. The genus *Fusobacterium* is classified in another distant Phylum created for its own. Earlier, all *Clostridium* species were grouped in one big family containing 19 Clusters. That classification was revised based solely on 16S rRNA analysis. However, *C. propionicum* is still classified as a member of the XIVb cluster (Collins et al, 1994).

Many anaerobic organisms ferment amino acids pair-wise, which is called Stickland reaction. In this process one amino acid (electron donor) is oxidised and the reduction equivalents are taken up by two different amino acids (electron acceptor), e.g. *C. sporogenes* oxidises alanine and reduces glycine, both to acetate (Stickland, 1934; Barker, 1981; Bader et al, 1982). In single amino acid fermentations, the same amino acid is used in the oxidative and in the reductive branch. L-Alanine and other C_3 amino acids like L-serine and L-cysteine are fermented by *C. propionicum* to ammonia, CO_2, H_2, propionate and acetate; L-threonine serves also as a fermentation substrate but the fatty acids derived are propionate and butyrate (Cardon & Barker, 1947). In contrast to the B_{12} dependent lactate fermentation carried out by *Propionibacteria*, *C. propionicum* and also *M. elsdenii*, ferment alanine by the 2-hydroxy acid pathway with acrylyl-CoA as intermediate (Fig. 3). In the first step of this pathway the amino group is oxidised to an oxo group. L-Threonine is deaminated by elimination to 2-oxobutyrate, while alanine is oxidatively deaminated to yield ammonia and pyruvate which disproportionates to acetate and propionate, to the overall redox equilibrium (eq. 1):

3 L-alanine + 2 H_2O → 3 NH_4^+ + CO_2 + acetate$^-$ + 2 propionate$^-$ (eq 1)

Pyruvate is oxidised via acetyl-CoA to acetate, where ATP is formed via acetyl-phosphate. The reductive pathway begins with an initial stereo-specific reduction of pyruvate to (*R*)-lactate, which is followed by CoA-activation catalysed by propionate-CoA transferase. The resulting (*R*)-2-lactyl-CoA undergoes dehydration to acrylyl-CoA, which is then reduced to propionyl-CoA; finally propionate is liberated by the CoA transfer reaction (Schweiger & Buckel, 1984; Hofmeister & Buckel, 1992). The dehydration of lactyl-CoA by *syn*-elimination is of particular interest due to the chemically difficult elimination of the hydrogen from the β position (pK_a ca. 40) (Buckel, 1996; Kim et al, 2004). Lactyl-CoA dehydratase is one representative of an enzyme family that catalyses the reversible dehydration of 2-hydroxyacyl-CoA derivatives to the respective enoyl-CoA's. The reaction proceeds by a radical mechanism involving an enoxy radical intermediate that reduces the pK_a of the leaving β-proton (pK_a= 14), which is lowered by 26 units compared to the pK_a of the 2-hydroxyacyl-CoA substrate (Smith et al, 2003). The two component system of (*R*)-lactyl CoA dehydratase, component A or activator (also refered to EI) and the component D (EII) (Kuchta & Abeles, 1985) are related to other 2-hydroxyacyl-CoA dehydratases found in related micro-organisms, including (*R*)-2-hydroxyglutaryl-CoA dehydratase from *F. nucleatum* or *A. fermentans,* or the 2-hydroxyisocaproyl-CoA dehydratase from *C. difficile* (Kim et al, 2004). The component A is a homodimer of 27 kDa subunits and contains one iron sulfur cluster [4Fe-4S] $^{1+/2+}$ cluster

(Locher et al, 2001; Hans et al, 2000). The homologous components D containing the enzyme's active site are heteromeric iron-sulfur proteins of different constitution e.g. the α,β dimer of 54 + 42 kDa from *A. fermentans*. The enzyme has to be activated by the oxygen sensitive component A requiring a strong reducing agent like dithionite *in vitro*, or ferredoxin or flavodoxin *in vivo*, and catalytic amounts of ATP and Mg^{2+}. The supplied electron energized by ATP hydrolysis is transferred from the activator (component A) to the catalytic site of component D where the dehydration reaction takes place. Once the reaction is started, the electron recycles catalysing multiple turnovers (Buckel, 1996; Hans et al, 2002; Thamer et al, 2003; Kim et al, 2004). Recently, a proposed ketyl radical intermediate was detected by EPR-spectroscopy (Kim et al, 2008). (*R*)-Lactyl-CoA dehydratase from *C. propionicum* was partially purified and characterised but in contrast to other systems only low activity could be detected in a coupled NADH oxidation assay reaction with (*R,S*)-2-hydroxybutyrate, acetyl-CoA, NAD^+ and phosphate as substrates and a protein fraction from *A. fermentans* containing the enzymes able to catalyse the overall NAD^+ dependent oxidation of crotonyl-CoA as depicted in figure 2 (Hofmeister & Buckel, 1992; Mack, 1995). The conversion of (*E*)-glutaconyl-CoA to (*R*)-2-hydroxyglutaryl-CoA was shown to occur in a *syn*-fashion (Buckel 1980) as observed with lactyl-CoA dehydratase (Hofmeister & Buckel, 1992; Brunelle & Abeles, 1993). The conversion of lactyl-CoA to acrylyl-CoA is immediately followed by the irreversible reduction to propionyl-CoA with NADH catalysed by the heterotrimeric acrylyl-CoA reductase.

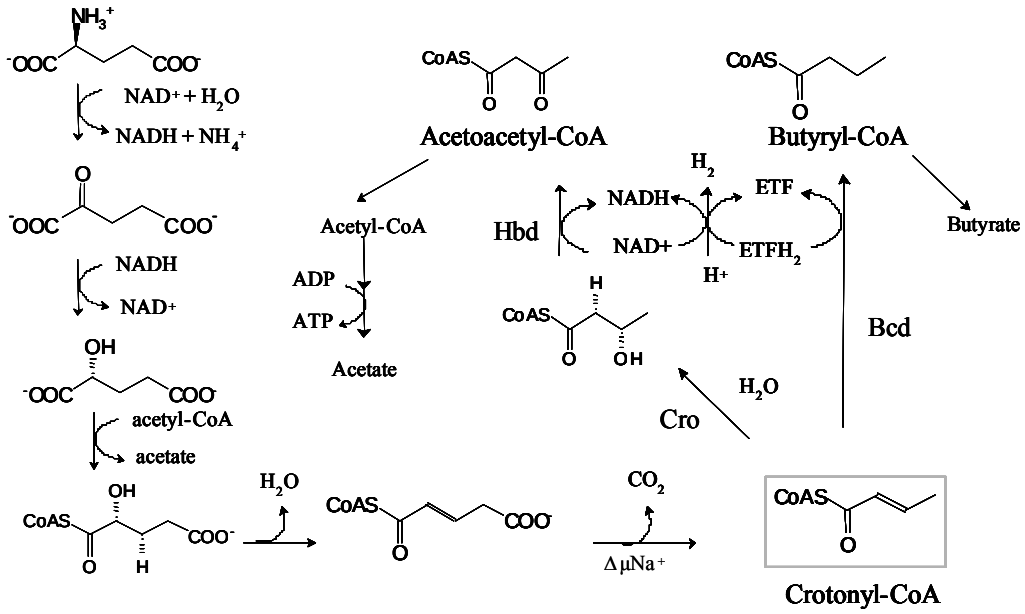


Fig. 2 Hydroxyglutarate pathway in *A. fermentans*. **Bcd**, butyryl-CoA dehydrogenase; **Cro**, crotonase; **Hbd**, 3-hydroxybutyryl-CoA dehydrogenase; **ETF**, electron transfer flavoprotein.

Another way to produce acrylyl-CoA is by deamination of β-alanine catalysed by the enzyme β-alanyl-CoA ammonia lyase (Reaction 10, Fig. 3). β-Alanine is a natural constituent of coenzyme A and an end product in the pyrimidine degradation pathway of many micro-organisms. The fermentation of β-alanine produces the same end products as those for L-alanine (eq. 1) (Vagelos et al, 1958).

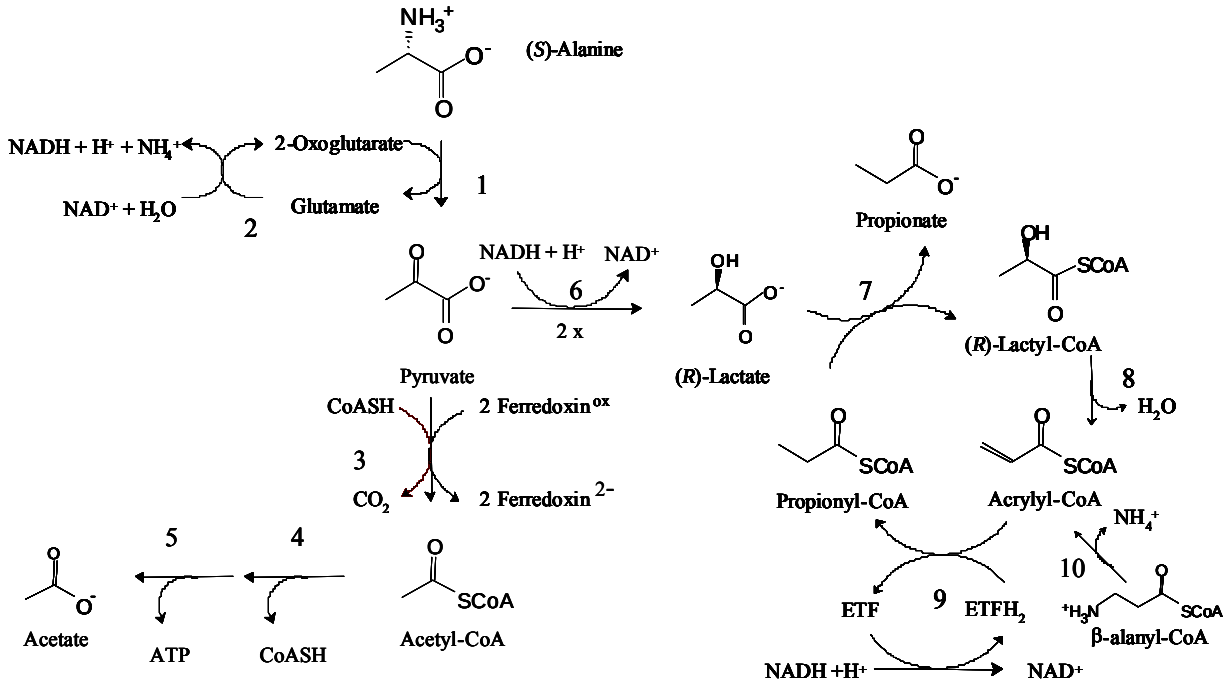


Fig. 3 L-Alanine fermentation in *C. propionicum*. The enzymes are: **1** L-alanine aminotransferase, **2** glutamate dehydrogenase, **3** pyruvate:ferredoxin oxidoreductase, **4** phosphate acetyltransferase, **5** acetate kinase, **6** (*R*)-lactate dehydrogenase, **7** lactate CoA-transferase, **8** (*R*)-lactyl-CoA dehydratase, **9** acrylyl-CoA reductase and ETF as electron transfer flavoprotein, **10** β-alanyl-CoA ammonia lyase.

Stadtman et al reported 120 times more enzyme in extracts of β-alanine grown *C. propionicum* cells than in their α-alanine grown counterpart. Either from α-alanine or β-alanine grown cultures, the purified enzyme exhibits high activity toward acrylyl-CoA in presence of relative high ammonia concentrations. The reaction was established to be freely reversible but with an equilibrium favouring the formation of β-alanyl-CoA (K = 8.19 x 10^5 M) (Vagelos et al, 1958). The study of β-alanyl-CoA ammonia lyase is of particular interest for biotechnological reasons. Among the bioengineering ways to produce 3-hydroxy propionic acid, β-alanyl-CoA may serve as an intermediate for this purpose. In this sense, the major interest was focused on the kinetic behaviour of this enzyme.

Glutamate can be fermented either by the 2-hydroxyglutarate pathway or by the coenzyme B_{12}-dependent methylaspartate pathway. The first pathway, called after the key enzyme 2-hydroxyglutaryl-CoA dehydratase (Fig. 2), leads to the product glutaconyl-CoA, the substrate for the energy-linked decarboxylation step catalysed by the membrane bound and biotin dependent glutaconyl-CoA decarboxylase (Buckel et al 1981; Buckel, 1986; Buckel, 2001).

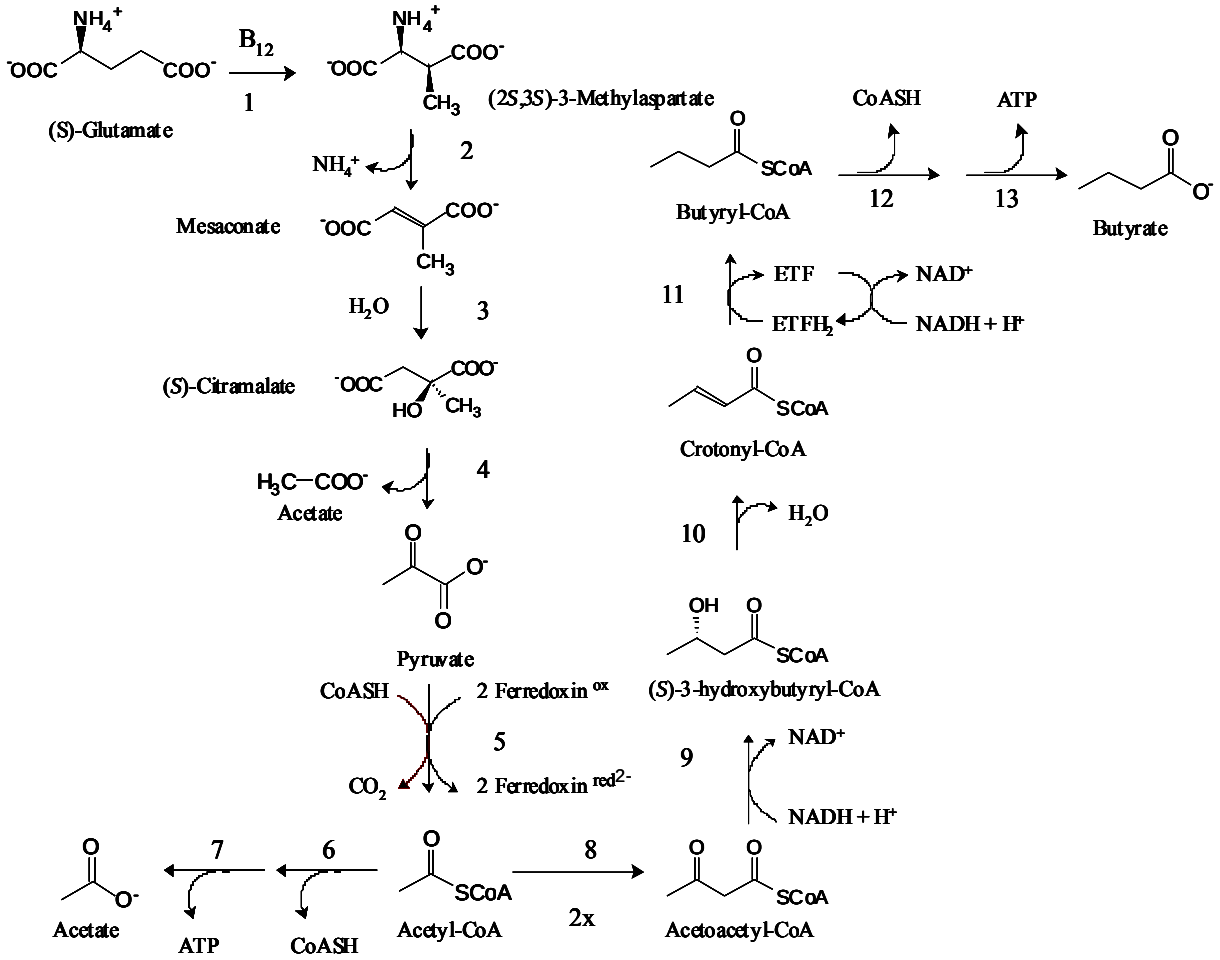


Fig. 4 Glutamate fermentation in *C. tetanomorphum*. **1** Glutamate mutase, **2** (2*S*,3*S*)-methylaspartase, **3** mesaconase, **4** citramalate lyase, **5** pyruvate:ferredoxin oxidoreductase, **6** phosphate acetyltransferase, **7** acetate kinase, **8** acetyl-CoA acetyltransferase, **9** 3-hydroxy butyryl-CoA dehydrogenase, **10** crotonase, **11** butyryl-CoA dehydrogenase-electron transfer flavoprotein, **12** phosphatebutyryl-transferase, **13** butyrate kinase.

The second pathway is named after its first intermediate: 3-methylaspartate, a compound produced from the rearrangement reaction catalysed by the B_{12}-dependent glutamate mutase (1, Fig. 4) following deamination and water addition yields (*S*)-citramalate, which is cleaved into acetate and pyruvate by catalysis of citramalate lyase. Then, pyruvate is oxidised to acetyl-CoA reducing 2mol ferredoxin. Only 20 % of the acetyl-CoA can be used for SLP

leading to acetate; the major part (80 %) is reduced to butyrate in order to regenerate the oxidised ferredoxin; thus two acetyl-CoA molecules are condensed into acetoacetyl-CoA leading to butyryl-CoA by the reverse β-oxidation. Thereby, the formed NAD^+ re-oxidises the reduced ferredoxin. Energy can be conserved mainly from SLP by the combined action of butyrate kinase/phosphotransbutyrylase and their related acetate kinase/phosphate acetyltransferase whereby overall 0.6 ATP is conserved (Buckel, 2001). The two main pathways for glutamate fermentation either via 2-hydroxyglutaryl-CoA or 3-methylaspartate end up into identical products as shown in equation 2:

$5\ Glutamate^- + 6\ H_2O + 2\ H^+ \rightarrow 6\ Acetate^- + 2\ Butyrate^- + 5\ NH_4^+ + 5\ CO_2 + H_2$ (eq. 2)

$\Delta G^{\circ\prime} = -63.5$ kJ mol $^{-1}$ glutamate; 106 kJ mol $^{-1}$ ATP. (Buckel, 2001)

2 Fatty acid oxidation and oxidative phosphorylation

Acyl-CoA dehydrogenases catalyse the first reaction of β-oxidation followed by the enzymes enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase and β-ketothiolase completing the oxidation cycle. This process takes place in mammalian mitochondria and degrades saturated and non-unsaturated short, medium and long chain fatty acids. In contrast, peroxisomal β-oxidation degrade mainly very long fatty acids (Osmundsen et al, 1980). While in mitochondria, the first step catalysed by the acyl-CoA dehydrogenase, electrons are transfered to FAD and further to the electron transport chain for oxidative phosphorylation, in peroxisomes, the first step is catalysed by an acyl CoA oxidase, also FAD dependent, which transfers the electrons directly to oxygen forming H_2O_2 (Casteels et al, 1990). The structure of peroxisomal acyl-CoA oxidases resembles that of the mitochondrial medium chain acyl-CoA dehydrogenase (Nakajima et al, 2002). The bacterial butyryl-CoA dehydrogenases (Engel & Massey, 1971a,b; Williamson & Engel, 1984) have structural similarities with the mammalian medium chain acyl-CoA dehydrogenase (MCAD) and comparable substrate specificities as the mammalian short chain acyl-CoA dehydrogenase (SCAD); these two last enzymes catalyse the first step (reaction 1, Fig. 5) of mitochondrial fatty acid β-oxidation and are part of a vast family group of enzymes performing αβ-dehydrogenation. MCADH, SCAD, and other acyl-CoA dehydrogenases (ACAD) differ with regard to substrate specificity, tissue or microbial distribution and amount. In the first oxidation step of β-oxidation catalyzed by an acyl-CoA dehydrogenase electrons are transferred to ETF; while in the second oxidation step catalyzed by the 3-hydroxyacid dehydrogenase electrons are transferred to NAD^+ (Ghisla & Mayhew, 1976; Ghisla, 2004).

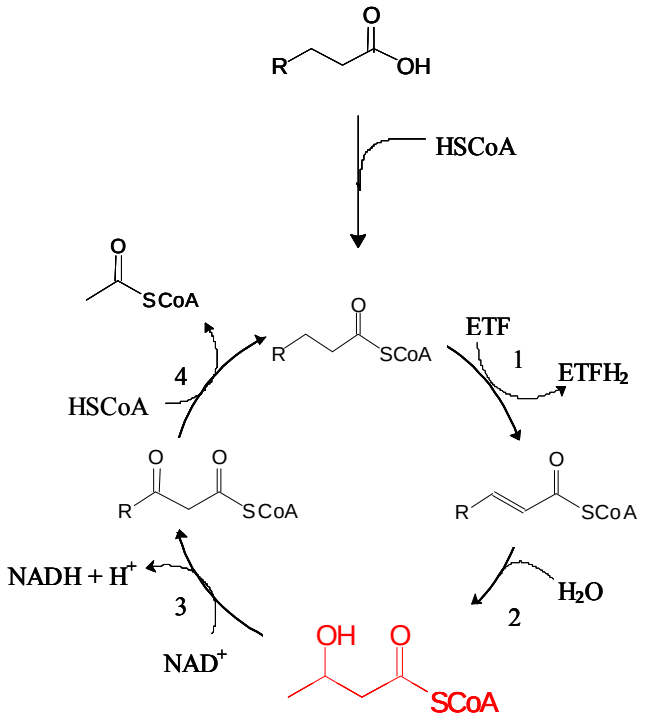
Mitochondria[1] 1. Acyl-CoA dehydrogenase SCAD, MCAD, LCAD, VLCAD 2. Enoyl-CoA hydratase [EC 4.2.1.17] 3. L-3-hydroxyacyl-CoA dehydrogenase HAD [EC 1.1.1.35] [EC 1.1.1.211] 4. β-ketothiolase [EC 2.3.1.16]	
Peroxisome[2] 1. Acyl-CoA oxidase [EC 1.3.3.6] 2-3. Bifunctional enzyme enoyl-CoA isomerase/hydroxyacyl-CoA dehydrogenase [EC 4.2.1.17] and [EC 1.1.1.35] 4. Thiolase [EC 2.3.1.16]	
Bacteria[3] 1. Butyryl-CoA dehydrogenase [EC 1.3.99.2] 2. β-hydroxybutyryl-CoA dehydrogenase [EC 1.1.1.157] 3. Crotonase [EC 4.2.1.55] 4. Thiolase [EC 2.3.1.16]	

Fig. 5 β-oxidation in mitochondria, peroxisomes and reverse β-oxidation in bacterial butyrate fermentation. [1,2] The process in respiring organisms is clockwise while in fermenting anaerobes [3] is counter-clockwise. SCAD short-chain acyl-CoA dehydrogenase, MCAD medium-chain acyl-CoA dehydrogenase, LCAD long-chain acyl-CoA dehydrogenase, VLCAD very long –chain acyl-CoA dehydrogenase, ETF electron transfer flavoprotein, HAD 3-hydroxyacyl-CoA dehydrogenase (Eaton et al, 1996).

Anaerobic respiration has been described separately from fermentation processes, because fermentative micro-organisms are supposed to lack electron transport phosphorylation (ETP).

Electron transfer flavoprotein from bacteria was initially isolated from *M. elsdenii* (earlier *Peptostreptococcus elsdenii*) as a separate dimer (Whitfield and Mayhew, 1974a) it has been associated to several dehydrogenases like butyryl-CoA-, sarcosine-, and trimethylamine-

dehydrogenases, the latter two involved in choline degradation (Ghisla, 2004). Bacterial ETF is considered as an analogous protein to the mammalian enzyme but the electrons flow in the opposite direction; in mitochondrial β-oxidation ETF participates in the oxidation of the substrate introducing a double bond to form the enoyl-CoA intermediate whereas in bacteria the electrons flow saturating the enoyl-CoA intermediate in the direction of fatty acid synthesis (Hetzel et al, 2003). There are common features in the proteins isolated from both eukaryote and prokaryote sources (Roberts et al, 1996), like a conserved heterodimer composition and similar flavin composition. The best characterized pig liver enzyme has 1 FAD and 1 AMP content similar to the enzymes isolated from *Paracoccus denitrificans* and the methylotroph W_3A_1 (Hussain & Steenkamp, 1985; Du Plessis et al, 1994; Chen & Swenson, 1994; Jang et al, 2000); while *M. elsdenii* enzyme can be saturated until 2 mol FAD and has no AMP (O'Neill et al, 1998). ETF functions as an electron acceptor intermediating between flavoproteins, but only the *M. elsdenii* enzyme is able to oxidise NADH (Engel & Massey, 1971a,b; Sato & Shiga, 1993; O'Neill et al, 1998).

3 Butyryl-CoA dehydrogenase

Butyryl-CoA dehydrogenases from bacteria have been extensively studied as a model for understanding the electron transfer mechanism in fatty acid metabolism (Fink et al, 1986; Stankovich & Soltysik, 1986). The best characterized butyryl-CoA dehydrogenase is that of the anaerobic bacteria *M. elsdenii*. The enzyme accounts for 2 % of dry weight of this organism and it was purified in variable greenish preparations similarly to mammalian butyryl-CoA dehydrogenases (Engel & Massey, 1971; Williamson & Engel, 1984). This green enzyme preparation showed a broad band at 710 nm in UV/vis spectroscopy, a property that was first observed in the pig liver enzyme and has been attributed to the presence of a tightly bound CoA persulfide (CoA-S-S$^-$) possibly involved in a charge transfer between this ligand and the protein bound flavin. The greening disappears after dithionite reduction followed by air oxidation, faster in the enzyme of *M. elsdenii* than in the mammalian one (Engel & Massey, 1971a; Williamson & Engel, 1982). The *M. elsdenii* butyryl-CoA dehydrogenase shows 33 % amino-acid sequence identity with the rat short chain acyl-CoA dehydrogenase and 44 % with human medium chain acyl-CoA dehydrogenase; it is has a molecular mass of 150 kDa, contains 3 to 4 FAD per tetrameric structure and possesses a subunit molecular mass of 43 kDa (Engel & Massey, 1971). The corresponding electron transfer flavoprotein has been separately purified from the same micro-organism as a dimer of

two different subunits the 33 kDa β-subunit and the 44 kDa α-subunit. In the first purification report, Whitfield and Mayhew already suggested a complex association of ETF with a dehydrogenase, most probably butyryl-CoA dehydrogenase (Whitfield & Mayhew, 1974a). Amino acid sequence analysis revealed that there are two possibly NAD/FAD nucleotide binding sites on the large alpha subunit and only one in the beta subunit and, in contrast to mammalian ETF, no AMP is present (O'neill et al, 1998). The enzyme possesses 1.4 mol FAD and after incubation with an excess of FAD, up to 2 mol FAD per heterodimer (Sato & Shiga, 2003). *M. elsdenii* ETF catalyzes the oxidation of NADH allowing electrons to pass from NADH to BCD and further to other electron acceptors like 2,6-dichlorophenolindophenol (DCPIP). Another interesting feature is the presence of hydroxylated derivatives of flavins like 6-OH-FAD (6-hydroxy-7,7-dimethyl-10(5'-ADP-ribityl)isolloxazine) and 8-OH-FAD (7-methyl-8-hydroxy-10(5'-ADP-ribityl)isolloxazine) (Whitfield, 1974b; O'Neill, 1998). On the other hand, there are structure similarities between butyryl-CoA dehydrogenase from *M. elsdenii* and 4-hydroxybutyryl-CoA dehydratase from *C. aminobutyricum*. Comparable three-dimensional structure and equal requirement of oxidised FAD for catalysis lmay indicate similarities in the mechanism of action of both enzymes. The dehydration of 4-hydroxybuytyryl-CoA, like in the case of 2-hydroxyacyl-CoA, is also a chemically difficult reaction due to the un-activated β-hydrogen that has to be removed. The oxygen sensitive 4-hydroxybutyryl-CoA dehydratase has been described as an homotetramer containing 4 FAD and 4 $[4Fe\text{-}4S]^{2+}$ cluster (Martins et al, 2004; Buckel et al, 2005). The proposed radical mechanism involves the participation of a flavin semiquinone and a dienolate intermediate in the water elimination reaction (Buckel & Golding, 1999). The enzyme apparently has an additional Δ-isomerase activity, catalysing the shift of the 2,3 double bond of vinylacetyl-CoA to the 3,4 position in crotonyl-CoA. The catalytic activity toward the isomer was of the same rate as for its natural substrate (Martins et al, 2004). Recently, the stereochemistry of the dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA by the 4-hydroxybutyryl-CoA dehydratase from *C. aminobutyricum* has been completely elucidated (Friedrich et al, 2008). In contrast to butyryl-CoA dehydrogenase from *M. elsdenii* the enzyme acrylyl-CoA reductase from *C. propionicum* was found in a tight complex with electron transfer flavoprotein (ETF) shows a total molecular mass of 600 ± 50 kDa, for the $\alpha_2\beta\gamma$-tetramer. The found flavin content of the enzyme was 2.4 mol per $\alpha_2\beta\gamma$-tetramer and the whole system can accept upto four FAD molecules. Propionyl-CoA dehydrogenase activity is comparably low (~ 0.8 U mg^{-1}) and the reduction of acrylyl-CoA exhibits a specific activity of 1.8 U mg^{-1}. There is no pathway known to describe the oxidation of propionyl-CoA to

acrylyl-CoA possibly due to the high redox potential of the acrylyl-CoA/propionyl-CoA pair (+ 69 mV), which is in the same order of magnitude like that of ubiquinone (+ 90 mV). On the other hand, menaquinone, a much weaker oxidant (- 74 mV) than ubiquinone, is preferred among anaerobic bacteria. The only comparable example known to carry out a similar "uphill" reaction is the proton motive force driven menaquinone dependent oxidation of succinate to fumarate (ΔE = 99 mV) (Schirawski & Unden, 1998; Hetzel et al, 2003). The only acrylyl-CoA reductase reported forms a complex with ETF in bacteria.

4 The aim of this work

Initially the mechanism of lactyl-CoA dehydratase was studied, which resulted in the re-discovery of β-alanyl-CoA ammonia lyase in *C. propionicum*.

The main aim of the present work was the study of the potential energy conservation by crotonyl-CoA reduction in *C. tetanomorphum*

Materials and methods

1 Bacterial growth

1.1 Anaerobic cultures of Clostridia

The bacteria *Clostridium tetanomorphum* strain 528 (DSMZ, Braunschweig, Germany) was grown on glutamate media supplemented with 1 % (w/v) yeast extract, while the bacterium *C. propionicum* (strain 1682 DMSZ) was grown in alanine and accordingly in β-alanine media with 0.1 % (w/v) yeast extract. The liquid media (table 1 and 2) were boiled; 50 ml each were filled into 100 ml serum bottles and finally sealed with rubber stoppers. The gas phase of the serum bottles was exchanged with nitrogen (99.99 %). All cultures were grown in shakers at 37°C.

Table 1: Components of 1 liter liquid media

C. propionicum Alanine / Threonine medium	*C. propionicum* β-Alanine medium	*C. tetanomorphum* Glutamate medium
Yeast extract 0.1 % (w/v) 50 mM D,L-alanine / threonine 3 mM L-cysteine-HCl 5 mM Kpp pH 7.0 VRB solution 0.4 % (v/v)	Yeast extract 0.1 % (w/v) Peptone 0.1 % (w/v) 50 mM β-alanine 3 mM L-cystein-HCl 5 mM Kpp pH 7.0 VRB solution 0.4 % (v/v)	Yeast extract 1 % (w/v) 200 mM Na-glutamate Na-thioglycolate 0.1 % (w/v) 50 mM Kpp pH 7.0 SL 10 solution 2 % (v/v)

Table 2: Components of minimal mineral VRB and SL-10 salts solutions

VRB solution (m/v):	2.4% $MgSO_4.7H_2O$, 0.05% $CaCl_2$, 0.05% $FeSO_4.7H_2O$, 0.025% $NiCl_2$, 0.04% $ZnSO_4$, 0.025% $MnSO_4.H_2O$, 0.025% $CoCl_2$, 0.025% NH_4VO_3, 0.0125% $CuSO_4.5H_2O$, 0.025% Na_2MoO_4.
SL 10 solution (m/v):	0.15% $FeCl_2$, 0.007% $ZnCl_2$, 0.01% $MnCl_2$, 0.0006% H_3BO_3, 0.02 % $CaCl_2$, 0.0002% $CuCl_2$, 0.0024% $NiCl_2$, 0.0036% Na_2MoO_4

Growth curves were followed after two sequential passages for pre-cultures in the same media. Inoculation was done by transferring 1 % (v/v) of a stock culture stored at 4 °C. Inoculated cultures were incubated at 37 °C under constant shaking. For growth curves, 1 ml probes were taken with a sterile syringe every hour, from witch 200 µl were centrifuged at 9000 x *g* in a Biofuge. Cell pellet was smoothly mixed in saline solution. The optical density (OD) of a proper dilution with the same saline solution was read at 578 nm against water as a reference blank. For the growth experiment in presence of cylclopropane carboxylic acid, media containing 14 mM L-alanine and 0.2 % yeast extract was prepared and complement with cyclopropane carboxylic acid to 15, 20, 30 and 50 mM final concentrations under sterile conditions. Two separately experiments were developed in the cyclopropane growth study recording only the final optical density lectures at 4 and, in a second experiment, at 24 hours of growth.

1.2 Glutamate fermentation by *Clostridium tetanomorphum*

C. tetanomorphum was grown in glutamate media with 1 % w/v yeast extract (YE) and 0.2 M sodium glutamate (standard medium). Additionally poor medium cultures with limited fermentable substrates containing 0.1 % w/v yeast extract and 0.02 M sodium glutamate were also prepared with and without supplementation of 0.05 M sodium crotonate. Cultures containing crotonate were denominated with the letter B while the corresponding reference cultures with A. Serial passages from cultures A and B were inoculated in parallel as shown in the following inoculation schedule:

I. Poor-to-poor media passage: from 0.1 % YE and 0.02 M glutamate to 0.1 % YE and 0.02 M glutamate

II. Poor-to-rich media passage from 0.1 % YE and 0.02 M glutamate to 1 % YE and 0.2 M glutamate

Growth and OD measurement procedures for each of these 4 cultures was applied similarly as described in anaerobic cultures of clostridia, taking probes before and after inoculation, in periods of 60 minutes until completing 20 hours of growth with one exception, the re-inoculation from 0.1 % w/v yeast extract to 0.1 % w/v yeast extract poor medium second passage, which was grown overnight for 10 hours before start of the experiment in order to ensure measurements at prolonged incubation time.

2 General biochemical and immunological methods

2.1 Determination of protein concentration

Protein concentration was photometrically measured after a wavelength shift from 465 to 595 nm upon protein binding to the Bradford reagent: Coomasie G-250 dye in phosphoric acid solution (Bradford, 1976). The assay method was developed in a micro scale range, applying 0.0625 to 2 µg protein in 10 µl probe volume in a titer plate mixed with 200 µl of 1/5 dilution of the Bradford reagent. The absorbance lecture was done on a Dynex MRX II reader. Bovine Serum Albumin (Sigma) was used as a standard.

2.2 Synthesis, isolation and identification of CoA thioesters.

MALDI probes

Acetyl-, butyryl-, crotonyl-, isobutyryl- and valeryl-CoA thioesters were synthesized from their correspondent anhydrides after the Shemin method (Simon & Shemin, 1953). A 1.2-fold excess of anhydride over CoA-SH was applied. The anhydride was disolved in 1 ml acetonitrile and dropped into a solution of 80 mg coenzyme A tri-lithium salt (ICN Biochemicals, Eschwege Germany) in 10 ml of a 100 mM $KHCO_3$ solution. After 15 minutes incubation time at room temperature the solution was acidified to pH 2 with 1 M HCl. The enzymatic synthesis of propionyl- and β-alanyl-CoA was performed by incubating 1 mM acetyl-CoA with 100 mM of the salt of the fatty acid in 50 mM Kpp pH 7.5 in presence of 1 Unit of propionyl-CoA:acetate CoA-transferase or β-alanyl-CoA:acetate CoA-transferase. Mixtures were incubated 15 to 45 minutes and acidified to stop the reaction. Freshly synthesized acyl-CoA esters, as well as all the enzymatic reaction mixtures involving CoA derivates as a substrate, were purified by reversed phase method using solid phase extraction C18 Sep-Pak cartridges (Waters, Massachusetts USA). The columns varying from 1 ml/50 mg material to 20 ml/2g material were weted with methanol prior use. The 20 ml columns used for the isolation of 100 µmol synthesized CoA ester were washed with 10 ml TFA 0.1 % (v/v) before and after probe loading. At the end of the process 5 ml 50 % (v/v) acetonitrile in TFA 0.1 % (v/v) was added for CoA-esters elution. Solvent free probes were obtained after evaporation. The final solutions were dissolved in water (pH 3) and stored at –20°C. The probes analysed by MALDI were dropped directly on a gold grid platform and mixed 1:1 with the matrix alpha-Cyano-4-hydroxycinnamic acid to a final volume of 1 µl.

2.3 DTNB test for quantification of CoA and its derivates

The free thiol group of CoA esters reacts with 5,5'-dithiobis(2-nitrobenzoate) Ellman's reagent. The splitting product of DTNB forms a new disulfide bond between a CoA molecule and one 2-nitro-5-thiobenzoate (TNB^-). For every CoA molecule reacting, 1 mol of the yellow TNB^- anion is formed absorbing at a maximum wavelength of 412 nm (ε_{412} = 14.0 $mM^{-1}cm^{-1}$) (Riddles et al, 1983). This assay coupled to citrate synthase, can detect the release of free CoA-SH from acyl-CoA esters in an enzymatic reaction containing oxalacetate, free acetate and propionyl-CoA:acetate CoA-transferase (Table 3). The absorbance difference at the end point of each reaction were recorded in order to calculate the concentration of free-CoA, acetyl-CoA and CoA-derivates in this order of succession.

Table 3: Contents of the enzymatic coupled DTNB assay (Volume of 1.0 ml, d = 1cm)

	50 mM Kpp pH 7.0		
DTNB Assay	100 mM Acetate		
	1 mM DTNB	+	propionyl-CoA:acetate CoA-transferase
	1 mM Oxalacetate	+	citrate synthase

2.4 SDS-PAGE

Identification of the mass and subunit number of isolated proteins was achieved by electrophoresis under denaturing conditions. For the preparation of two 14 x 16 mm gels with 15 % polymerisation grade, two consecutives mixtures of stacking and running gels were prepared after Laemmli method (Laemmli, 1970) putting together the following volume specifications:

Running gel	**Stacking gel**	
Buffer A [(1)]	2.5 ml	-
Buffer B [(2)]	-	2.5 ml
Acrylamide 30 % [(3)]	5.0 ml	1.1 ml
Water	2.5 ml	6.5 ml
TEMED	42 µl	20 µl
APS [(4)]	60 µl	50 µl

[(1)] Buffer A	1.5 M Tris-HCL (pH 8.8) / 0.4 % SDS
[(2)] Buffer B	0.5 M Tris-HCl (pH 6.8) / 0.4 % SDS
[(3)] Acrylamide 30 %	(37.5:1) Acrylamide : bisacrylamide, Rotiphorese Gel 30
[(4)] APS	10 % (W/V) Ammonium persulfate (fresh)

The protein solution to be analyse was mixed 1 to 4 with sampling buffer (0.24 M Tris-HCl pH 6.8) 8 % SDS 40 % glycerol, 1 % bromophenol blue, 12 % DTT) heated at 95 °C for 5 minutes. The temperate and denaturised protein solutions, in volumes of 10 µl, were loaded into readily polymerised gel, assembled into a Biorad apparatus, witch was placed into a buffer chamber filled with running buffer (30 mM Tris/HCl, 200 mM glycine, 0.02 % SDS) and connected to a power supply at 120 V constant current. Protein was stained with Coomassie Brillant Blue R-250 (Serva, Heidelberg, Germany) and detained with a mixture of 30 % Methanol/ 10 % acetic acid.

2.5 Blue native gel electrophoresis

The polyacrylamide gel for blue native electrophoresis was prepared in a similar form like reported for PAGE but excluding denaturing components like SDS. The two parts of the PAGE, a 3 % sample gel and a 8 % running gel were made by mixing the solutions written in table 4. The volumes for one analytical gel were adapted to 14 x 14 x 0.16 cm glass plates

Table 4: Gel preparation for blue native PAGE

	3 % T Sample gel	8 % T Running gel
AB mix [(1)]	0.5 ml	2.5 ml
Gel buffer [(2)] (3x)	2 ml	5.0 ml
Glycerol	50 µl	3.0 g
Water	3.5 ml	4.5 ml
APS 10 %	50 µl	75 µl
TEMED	5 µl	7.5 µl

[(1)] acrylamide-bisacrylamide (49.5 % T, 3 % C): 48 g acrylamide ,1.5 g bisacrylmide/100ml

[(2)] Gel buffer: 75 mM imidazol/HCL (pH 7.0) 1.5 M 6 aminohexanoic acid

A butyryl-CoA dehydrogenase-ETF protein aliquot was loaded at different concentrations, not exceeding 50 µg of total protein. Loaded samples contained 5-15 % glycerol, 200-500 mM 6-aminocaproic acid and Coomasie dye from a 5 % suspension in 500 mM 6-aminohexanoic acid. The electrophoresis was completed after approximately 4 hours run between cathode and anode buffers (Table 5) at a constant current of 250 V, 0.2 A.

Table 5: Buffers needed for blue native PAGE

Deep blue cathode buffer B	50 mM Tricine, 7.5 mM imidazole, 0.02% Coomasie-blue G-250 (pH 7.0)
Slightly blue cathode buffer B/10	Cathode buffer B, 0.002 % Coomasie-blue G-250
Anode Buffer	25 mM imidazol/HCl (pH 7.0)
5 % Coomasie-Blue	5 % Coomasie blue in 500mM aminohexanoic acid

2.6 Western blot

Purified protein aliquots and/or cell-free extract samples were transfered from a SDS-PAGE to a **PVDF** Westran S^{TM} membrane (Schleicher & Schuell GmbH Dassel, Germany) by the semi-dry method. The polyacrylamide gel containing proteins was placed onto a PVDF membrane previously soaked in methanol, and laid between 11 layers of filter papers wetted with transfer buffer (25 mM Tris, 192 mM glycin, 10 % methanol). The sandwiched material was located in a Trans-Blot SD semi-Dry electrophoretic transfer cell from Biorad, and subjected to 200 mA constant current for 1 hour.

2.7 Antibody production and immune serum screening

Previous to immunization, four different rabbit candidates were tested to be free of positive signals at the size range where the protein under study is expected to appear (28-40 kDa) in the antibody detection method explained below. The antigen consisted of butyryl-CoA dehydrogenase-ETF protein aliquots of 200 µg/150 µl in 0.7 % sterile saline solution. The selected candidate was subjected to subcutaneous injections of the antigen, conjugate to 0.05-0.1 µg/ml adjuvant, for three sequential times in periods of 4 weeks between every event. Blood samples were collected at every immunization time and the antiserum was checked for the antibody titer level, screened against pure protein probes of butyryl-CoA dehydrogenase.

To establish the antibody titre in blood samples, antiserum dilutions were assayed against a butyryl-CoA dehydrogenase-ETF protein fixed in a PVDF membrane. Blood samples were coagulated overnight (4°C), and centrifuged at 10,000 *g* for one hour to separate the blood clot. SDS-PAGE of 200 ng pure butyryl-CoA dehydrogenase-ETF was prepared and subjected to Western Blot analysis by the described semi-dry technique. The membranes were treated with the chemicals described in table 6, as follows:

1. - 10 minutes TBS (Tris buffer saline) wash (2 times) at room temperature
2. - One hour incubation in blocking solution at room temperature
3. - 10 minutes TBS-Tween/Triton wash (2 times) at room temperature

4. - 10 minutes TBS wash
5. - Overnight incubation in blocking buffer containing the dilution of the antiserum at 4° C
6. - 10 minutes TBS-Tween/Triton wash (2 times) at room temperature
7. - 10 minutes TBS buffer wash
8. - One hour incubation with secondary antibody goat anti-rabbit IgG-HRP, 1:20000 diluted.
9. - 10 minutes TBS-Tween/Triton buffer wash (4 times) at room temperature
10. - Chemoluminescence reaction

Table 6: Contents of the solutions employed for antibody/antigen incubation reaction

	TBS buffer	**TBS Tween/Triton buffer**	**Blocking solution**
Tris-HCl pH 7.5	10 mM	20 mM	10 mM
NaCl	150 mM	500 mM	150 mM
Tween 20	-	0.05%	0.1 %
Triton X-100	-	0.2 %	-
Milk powder	-	-	10 %

The chemoluminescence reaction was developed in the dark room mixing solution 1 (luminol /p-coumaric acid) and solution 2 (H_2O_2) during three minutes, followed by a exposition on a Fuji X-Ray film, which was finally developed with Kodak processing chemicals (Table 7).

Table 7: Reactives for bioluminescense reaction

Solution 1	**Solution 2**
100 µl 250 mM Luminol in DMSO	6 µl 30 % H_2O_2
44 µl 90 mM p-coumaric acid in DMSO	1 ml Tris-HCl pH 8.5
1 ml Tris-HCl pH 8.5	9 ml H_2O
8.85 ml H_2O	.

2.8 Affinity purification of specific Ig G antibodies

Polyclonal immune serum containing antibodies against butyryl-CoA dehydrogenase-ETF complex was purified from contaminants and other unspecific immunoglobulins present in the

original rabbit serum, using the buffers described in table 8 and following a two step procedure:

I. General purification of Ig G antibodies by preparative protein A Sepharose:

The total content of Ig G was separated from albumin and other serum components by binding to protein A from *Staphyloccocus aureus*. The self-made column, packed with 2.5 ml of protein A Sepharose™ CL-4B (Amersham-Pharmacia, Freiburg Germany) material was washed with PBHS (Phosphate buffer high saline) and then loaded with 1 ml immunsera collected after 14 weeks of immunization time. The elution of immunoglobulin is achieved after lowering the pH with 50 mM glycine/HCl pH 2.8. Those fractions containing highest protein amounts were pooled together and concentrated using a 3 kDa cut off Centricon device (Millipore Corporation, MA, USA).

II. Specific purification. of butyryl-CoA dehydrogenase-ETF Ig G antibodies by affinity interaction:

One epoxi-potein-coupling mini spin column from the company Vivascience (Sartorius group, Hannover Germany) was used to prepare an affinity column with bounded butyryl-CoA dehydrogenase-ETF as the antigen. Following several wash and centrifugation steps at 2,000 x *g*, the epoxi membrane was loaded with a coupling solution containing the antigenic protein, butyryl-CoA dehydrogenase-ETF in 1.5 mg/ml concentrated PBS (Phosphate buffer saline) solution. After 3 hours binding incubation time, the column was dry centrifuged and washed to remove unbounded protein. The new created affinity column was then used to selectively purify only those Ig G antibodies against butyryl-CoA dehydrogenase-ETF from the Protein A purified total Ig G solution. PBHS buffer was used for equilibration and wash steps. Then elution proceeded with 0.1 M glycine/HCl pH 2.8 in receptor caps containing high molarity Tris-HCL pH 8.0 in order to get it immediately neutralize to a final pH 7.0.

Table 8: Buffers for affinity purification of specific IgG

0.5 M NaCl in 10 mM Kpp pH 7.4 (PBHS)
50 mM glycine/HCl pH 2.8
0.1 M glycine/HCl pH 2.8
1 M Tris pH 9.0
0.5 M Tris/HCl pH 8.0

3 Protein isolation and characterization

The following anaerobic procedures were done in a glow box (Coy laboratories, Ann Arbor MI, USA) filled with 90 % Nitrogen/5 % formic gas. For that purpose, all solutions were degassed and flushed with nitrogen.

3.1 Purification of lactyl-CoA dehydratase from *Clostridium propionicum*

Wet packed cells of *C. propionicum* (28 g) suspended in 50 ml buffer A (25 mM Tris/HCl pH 7.5, 5 mM $MgSO_4$ 1 mM DTT, 1 mM dithionite) were opened by sonication with a Branson sonifier, during 15 minutes at 50 % duty cycle, under anaerobic conditions. The cell-free extract was obtained after 100,000 *g* centrifugation, then loaded on a Q-sepharose 26/10 column (Pharmacia), pre-equilibrated with buffer A. The chromatography was developed in the FPLC programmed at a flow rate of 5 ml/min, set to an initial wash with 50 ml buffer A, followed by a 33 % gradient of buffer B (1.5 M NaCl in buffer A). Dark green fractions corresponding to lactyl-CoA dehydratase component A were eluted at 0.2 M NaCl salt, while pale green fractions of lactyl-CoA dehydratase component D eluted at 0.4 M NaCl salt. The fractions containing component A of lactyl-CoA dehydratasse were immediately frozen at –20°C. The dehydratase fractions involving component D were pooled and concentrated for further purification on a Phenyl Sepharose HR 10/10, set to 1.0 M ammonium sulfate final concentration with buffer C (1.0 M NH_4SO_4 in buffer A). Finally, a pool of pure component D of lactyl-CoA dehydratase in buffer A was kept at –20 °C.

3.2 MALDI-TOF assay for lactyl-CoA dehydratase

A semi-analytical assay was developed in order to identify the final products from lactyl-CoA dehydratase anaerobic catalysis by MALDI TOF detection procedure. The assay carried out under strict anaerobic conditions, was done mixing equal parts of assay- and substrate-buffer (Table 9) with 1:100 cell free extract to a final volume of 1 ml lactyl-CoA dehydratase activity assay reaction. Assay buffer was supplied with 10 µl of each of both fractions containing component D and component A from lactyl-CoA dehydratase. After 1 min the reaction was stopped with 10 % trifluoracetic acid (TFA). The probes were desalted by reverse phase before analysed in the mass spectrometer following the same procedure already described for acyl-CoA identification.

Table 9: Assay components for lactyl-CoA dehydratase activity

Assay buffer (500µl)	**Substrate buffer** (500µl)
50 mM Tris-HCl pH 7.5	2 mM acrylyl-CoA/ lactyl-CoA
5 mM $MgSO_4$	50 mM Tris-HCl pH 7.5
1 mM Na-dithionite	5 mM MgSO4
2 mM ATP	1 mM Na-dithionite,
	2 mM ATP

3.3 β-Alanyl-CoA ammonia lyase purification from *C. propionicum*

C. propionicum wet cells (2 g) obtained from 2 liter β-alanine grown cultures were re-suspended in 40 ml 25 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM EDTA. Cell disruption was done by sonication at 70-80 % duty cycle, step 6, for 15 minutes. The supernatant obtained after centrifugation at 100.000 x *g* was applied to a Source 15-Q (1.6/20) column (Amersham Pharmacia, Freiburg Germany), equilibrated with 25 mM Tris-HCl pH 7.0. After performing a linear gradient of 0-500 mM NaCl, pure β-alanyl-CoA ammonia lyase active fractions were collected. The final protein pool was concentrated using Millipore Amicon equipment with a corresponding 30,000 kDa cut off membrane. Desalted protein aliquots in 50 mM Tris-HCl pH 7.0 was concentrated to final 20 mg/ml and stored at –20°C.

3.4 Superdex 200 size exclusion chromatography

The size exclusion column Superdex 200 HR 10/30 (Amersham Pharmacia, Freiburg Germany) was washed and run with 100 mM NaCl in 100 mM Kpp pH 7.0. Protein was loaded using a 100 µl loop at 0.4 ml/min flow rate. The standards were purchased from Sigma, having the following molecular masses in Daltons: aldolase (158,000) catalase (232,000); ferritin (440,000); thyrogloblin (669,000). Final results were obtained from calculating the partition equation after the formula $K_{av} = (V_E - V_0) / (V_T - V_0)$, where V_E is the elution volume, V_0 and V_T respective the void and total volume of the column.

3.5 Membrane preparation from *C. tetanomorphum* cell pellet

Next experiments involving membrane fractions were all done under anaerobic condition. In order to obtain a dense extract, cell suspensions of 0.5 to 1 g *C. tetanomorphum* wet packed cells per ml were prepared after suspension in anaerobic 50 mM Tris-HCL buffer, pH 7.5, with added 1 mM DTT, 5 % glycerol, 25 % sucrose, 0.5 mM EDTA, 1 mM PMSF. Lyzozyme was also added to a final concentration of 0.3 mg/ml (if not otherwise stated) and the whole mixture was incubated for 30 minutes at 25 °C in the anaerobic tent. After addition of 5 ml 50 mM Tris-HCl, pH 7.5 + 10 mM $MgCl_2$, the membrane preparation was incubated with DNAse (1 µg/ml) for 15 min. Followed one passage through a French press and two consequent centrifugation steps, first at low revolutions to separate cell debris and second at 100,000 *g* to get a membrane pellet. After a 50 mM Kpp pH 7.0 wash, the pellet was treated in sequential steps while probe aliquots were taken to be later assayed. The first membrane preparation, in 20 mM Kpp buffer pH 7.0, consisted of pottered pellet obtained by use of a potter-Elvehjem mortar and pestle instrument to smoothly homogenise the proteolipid components layered at the bottom of the centrifugation tube. Then 0.1 % (w/v) deodecyl maltoside (DM) (Glyco Biochem Lückenwalde, Germany) was added to remove hydrophobic proteins from the membranes, resulting in the second 1 % DM whole membrane preparation. The third preparation was made up from the supernatant of the dodecyl maltoside treated second preparation after final centrifigation in a Hettich table centrifugue at maximal power.

3.6 Partial isolation of membrane proteins

The ferricenium assay was used to find butyryl-CoA dehydrogenase activitiy in membrane fractions of *C. tetanomorphum* cell pellet preparations. The loading material, membrane extracts obtained from processing 28 g *C. tetanomorphum* wet cells in a similar manner like previously described, except for the solving buffer that changed to 20 mM Kpp pH 7.0 plus 2 mM DTT (without addition of PMSF), was readily prepared after the dodecyl maltoside solubilization step and consequently centrifuged at 100,000 *g* to separate the supernatant containing the solubilized proteins from the hydrophobic resting compounds. The probe of brown-greenish colour was weakly bound to a Reactive Blue-Agarose (Sigma) column pre-equilibrated with buffer A (Mops 20 mM pH 7.2 + 0.15 mM NaCl /0.01% dodecylmaltoside /0.5 mM EDTA) but eluted in the wash step with buffer B (2.0 M NaCl in buffer A). The first fractions were collected, concentrated through a 5 kDa cutt-off membrane and reloaded on a 10 ml self-made column filled with 2 g Red Reactive Sepharose © (Sigma). After a wash with 50 ml buffer A and application of a linear gradient to 50 % of buffer B, butyryl-CoA

dehydrogenase active fractions following the $FcPF_6$ assay (described below) were detected at low salt concentration (~20 mM NaCl) elution.

3.7 Purification of butyryl-CoA dehydrogenase-ETF from *C. tetanomorphum*

The *C. tetanomorphum* cells source for all purification procedures was obtained from the same harvest, from a 100 liter fermentor (glutamate medium) at the end of the exponential phase. The cell suspension was carefully prepared from 40 g readily thawed wet cells in 700 ml 50 mM Kpp buffer pH 7.5. In a cooled ice box, cell disruption took place by sonication using a Branson Sonifier 250 adjusted to 60 % duty cycle, output step 7. The ultrasonic treatment was applied four times during 3 minutes, having 5 minute cooling time in between. The cell-free extract was obtained after recovering the supernatant from centrifugation at 6,000 *g* in a Sorvall RC 5B plus centrifuge, SLA-300 rotor. The cell-free extract was subjected to 33 % ammonium sulfate precipitation, stirring at 4°C for 30 minutes, then 2,000 *g* centrifuged in SLA-300 rotor. The green pellet was dissolved smoothly in 10 mM Kpp pH 7.5 and dialysed through a dialysis hose of cellulose 8,000-10,000 kDa cut-off (Roth, Karlsruhe, Germany) in 5 liter of the same buffer containing 10 µM riboflavin (Sigma). The dialysis process was performed in a dark room slowly stirring at constant 4 °C temperature. The chromatographic fractionation of the protein was performed in a Pharmacia FPLC (GP-250 pump-500) system using first an ionic exchange DEAE XK26/10 column (Pharmacia), applying a 100 % salt gradient of 1M NaCl in 10 mM Kpp pH 7.5, 0.5 mM EDTA plus 10 µM riboflavin. Protein eluted between 0.25 and 0.3 M NaCl. Fractions with highest butyryl-CoA dehydrogenase activity were pooled, concentrated and desalted to a low salt solution (10-50 mM) using a 10 kDa cut-off ultrafiltration membrane of regenerated cellulose (Amicon Milllipore). As a second column, a self-packed XK16 column with 20 µm hydroxyapatite material from Biorad was used. Pure protein fractions were collected after applying 33 % salt gradient of 1.0 M Kpp pH 7.5 in 10 mM Kpp pH 7.5, devoid of EDTA. Butyryl-CoA dehydrogenase-ETF complex came down from the column in a salt concentration range between 135 and 155 mM. The complex underwent a final run through a preparative Superdex 200 35/600 column using 10 mM Kpp pH 7.0 + 120 mM NaCl. Final protein solutions were instantly frozen when submerging the 1 ml aliquots in dry ice/acetone bath before final storage at –80 °C.

3.8 Superose 6 size exclusion chromatography

Size and molecular conformation from butyryl-CoA dehydrogenase-ETF protein complex was partially determined complementing the native gel result to size exclusion chromatography.

For size exclusion chromatography column, a Superose 6 Gel filtration of 24 ml bed volume from Pharmacia was used. Setting conditions in the FPLC at maximal 1 bar pressure with a flow rate of 0.5 ml/min, the separation was performed in 0.12 M NaCl, 10 mM Kpp pH 7.0. The protein sample, thawed shortly before use to prevent complex dissociation, was loaded on the column through a 200 µl loop. The following protein standards (in Daltons) purchased from Sigma Aldrich were used for the calibration curve: thyroglobulin (669,000), apoferritin (440,000), β-amylase (200,000), alcohol dehydrogenase (150,000) and albumin (66,000). The elution volumes were evaluated with the given formula for partition coefficient (K_{AV}).

3.9 N-terminal sequencing of butyryl-CoA dehydrogenase-ETF subunits

Aiming at isolated peptide fragments of each protein subunits for N-terminal sequencing via Edman degradation, a variation of the Western Blot method using a Biorad transfer Blot cell (Biorad –Laboratories, München, Germany) was employed. Having completed a 10 % SDS-PAGE electrophoresis the gel containing 20 µg of pure butyryl-CoA dehydrogenase-ETF protein was placed on a methanol-soaked PVDF Westran S^{TM} membrane (Schleicher & Schuell GmbH Dassel, Germany). The latter was packed between 6 layers of gel blotting filter paper, previously soaked in transfer buffer and finally assembled in the Biorad transfer-blot cell (16 x 20) cm, filled with transfer buffer (25 mM Tris-glycine, 20 % methanol). The system was connected to a power supply set to a constant current of 300 mA, during 1 hour at 4 °C. After a short Coomasie blue R-250 stain, and immediate de-stain in 80 % methanol/ 10% acetic acid, 3 bands were assigned for N-terminal sequencing and thus send to Dr. Linder at the Biochemisches Institut des Fachbereichs Humanmedizin, Justus-Liebig-Universität in Gießen, Germany .

3.10 Flavin cofactor investigation in butyryl-CoA dehydrogenase-ETF

3.10.1 Spectrophotometric flavin identification

The flavin content was spectroscopically investigated in the native form and in the supernatant of butyryl-CoA dehydrogenase-ETF after denaturation. For UV-visible light analysis, heat denaturation of 0.3 to 3 µg protein in 20 mM Kpp buffer pH 7.5 was

accomplished incubating 500 µl protein solution at 70 °C for 15 minutes. By centrifugation at 16,000 *g*, the yellow supernatant was separated from the white protein pellet. The probe filling a 0.5 ml quartz cuvette was scanned in a spectrometer (Kontron Instruments, Switzerland) between 200 and 600 nm wavelength.

3.10.2 Flavin quantification by HPLC

Butyryl-CoA dehydrogenase-ETF flavin content was detected by HPLC. To afford denaturation, the protein solutions in 20 mM Kpp pH 7.5 was heated in presence of 0,8 % SDS at 75 °C for 20 minutes. Then, 20 µl of the supernatant of a 14 mg/ml precipitated protein solution was injected into a Sykam HPLC equipped with a LiCroCART 250-4 column. Standards of FAD, FMN and Riboflavin (Sigma) were use at concentrations ranging from 50 to 200 µM. The system was run with ammonium acetate/methanol buffer in a 75:25 ratio with a flow rate of 2 ml/min.

4. Enzyme assays

4.1 β-Alanyl-CoA ammonia lyase assay

β-Alanyl-CoA ammonia lyase catalyses the addition of ammonia to the double bond of acrylyl-CoA.The β-alanyl-CoA ammonia lyase activity was measured recording the decrease of the absorbance at 259 nm (or at 280nm) due to the double bond of acrylyl-CoA ($\Delta\varepsilon_{259\,nm}$ = 6.4 $mM^{-1}cm^{-1}$; $\Delta\varepsilon_{280\,nm}$= 3.5 $mM^{-1}cm^{1}$). The assay was developed in 50 mM triethanolamine-HCl, pH 8.0 containing 100 µM acrylyl-CoA in presence of 100 mM ammonium chloride in a total volume of 500 µl. The reaction was followed in a Kontron spectrophotometer (Kontron Instruments, Switzerland).

4.2 Ammonia donors for β-alanyl-CoA ammonia lyase

There were different ammonia donors for β-alanyl-CoA ammonia lyase activity tested. Incubating for 60 and 30 minutes, every reaction consisted of a mixture of 100 µM acrylyl-CoA, and 100 mM of either hydroxylamine, methylamine or glycine as substitutes for ammonium chloride in 50 mM Kpp pH 7.5. The reaction mixtures were analysed by MALDI spectrometer, after purification through Water™ columns as described before.

4.3 Butyryl-CoA dehydrogenase-ETF aerobic assays

4.3.1 Ferricenium hexafluorophosphate assay

Butyryl-CoA dehydrogenase (BCD) activity was measured with ferricenium hexafluorophosphate ($FcPF_6$) as artificial electron acceptor. The assay measured the decrease of absorbance at 300 nm in a reaction containing 0.2 mM $FcPF_6$ and butyryl-CoA in 50 mM Kpp pH 7.5. The redox indicator ferricenium hexafluorphosphate was previously prepared in a 10 mM HCl solution to a final concentration of 2 mM set at 617 nm (ε_{617nm} = 0.41 $mM^{-1}cm^{-1}$) (Lehman & Thorpe, 1990). During the enzymatic dehydrogenation of butyryl-CoA, ferricenium ions of blue colour get reduced to ferrocene displaying a decrease in the absorbance (ε_{300nm} = 8.6 $mM^{-1}cm^{-1}$). The slopes of maximal initial activity, measured as absorbance difference per min (ΔE/min), were recorded for enzymatic activity calculation. One unit of butyryl-CoA dehydrogenase-ETF ativity is equivalent to 1 µmol butyryl-CoA consumed per minute.

4.3.2 pH and buffer influence on butyryl-CoA dehydrogenase activity

The activity of butyryl-CoA dehydrogenase-ETF was aerobically measured at different pH and buffer conditions. Citric acid (pH 4.0-6.6), Mops (pH 6.6-7.8), potassium phosphate (pH 6.5-7.5), di-phosphate (pH 8.5-12.5) buffers were all tested at 50 mM, in the ferricenium assay. Enzyme was incubated for 5 minutes in an assay mixture of buffer and enzyme. The reactions were started with addition of butyryl-CoA as the substrate.

4.3.3 Butyryl-CoA oxidation at 290 nm

Butyryl-CoA oxidation to crotonyl-CoA was followed in a spectrophotometer measuring the increase of the absorbance at 290 nm in the presence of butyryl-CoA dehydrogenase-ETF in 50 mM Kpp pH 7.5.

4.3.4 NADH activity by INT

Iodonitrotetrazolium chloride (INT) was used as a coloured indicator to evidence the NADH mediated transfer reaction of reducing equivalents. In the assay butyryl-CoA dehydrogenase-ETF enzyme was added to 50 µM INT in 50 mM Kpp pH 7.5. The INT-formazan product formation was followed at 496 nm using the extinction coefficient ε_{496nm}=19.2 $mM^{-1}cm^{-1}$ (Möllering et al, 1974) for activity calculation. Exactly the same assay was also performed

under anaerobic conditions.

4.3.5 NADH consumption and peroxide formation in the ABTS test

Reaction of 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid (ABTS) with hydrogen peroxide was measured at 422 nm (412 nm if otherwise stated) under non-limiting conditions. The substrate consumption and the simultaneous product formation experiments were performed starting from the NADH reaction mixture shown on table 10.

Table 10: Composition of NADH / ABTS reaction mixtures. Peroxidase from horseradish

	λ = 340 nm		λ = 412 nm
NADH Mix	200 µM NADH	**ABTS Mix**	50 µM ABTS
(1ml final vol)	50 mM Kpp pH 7.0	(1ml final vol)	50 mM Kpp pH 7.0
	10 µl BCD-ETF [1mg/ml]		5 µl peroxidase [5 mg/ml]

The reaction was started after addition of butyryl-CoA dehydrogenase-ETF enzyme to the NADH mix. Instantly a time dependent extinction of NADH was recorded at 340 m in a Kontron UV-VIS spectrometer, while aliquots of probes were taken in time periods of one minute, stopped with 0.5 M KOH and inmediatly tested for indirect H_2O_2 product formation in a second Kontron spectrometer. Peroxide final concentration was calculated after the extinction coefficient value of ε_{412nm} = 5.3 $mM^{-1}cm^{1}$ obtained from a calibration curve made under the working conditions at 412 nm (alternative measurements at 422 nm were done with an ε_{422nm}= 4.6 $mM^{-1}cm^{-1}$).

4.4 Butyryl-CoA dehydrogenase-ETF anaerobic assays

4.4.1 Ferricenium hexafluorophosphate assay in membrane fractions

During the processing of cell pellets from *C. tetanomorphum*, several aliquots of each sequential step were taken and kept at 4 °C inside the anaerobic chamber. Butyryl-CoA dehydrogenase activity was measured in the fractions before and after dodecyl maltoside solubilization by the ferricenium hexafluorophosphate assay described before. Unspecific absorbance decrement (ΔE/min) due to background cross reactions in cell extracts in the absence of the substrate were also noted and subtracted from the values recorded after adding butyryl-CoA.

4.4.2 Methylviologen assay

In order to follow spectrometrically the enzymatic consumption of acrylyl- or crotonyl-CoA, an anaerobic test has been developed to measure the enoyl-CoA reduction on behalf of methylviologen as redox indicator. The reduced form of methylviologen donates electrons that are transferred during enzymatic activity, thus exhibiting a decrease in the absorbance at 600 nm that was detected in the spectrophotometer Ultrospec 400. The enzymatic solution containing 1 mM methylviologen in 50 mM Kpp pH 7.5 was fixed to an initial absorbance of E_{600nm} = 0.8 after titration with 10 mM Ti(III)citrate. Activity was calculated based on a extinction coefficient of ε_{604nm} = 27.2 $mM^{-1}cm^{-1}$ (Mayhew, 1978). The enzyme to be tested was pre-incubated in the reaction buffer containing 1 mM methylviologen in 50 mM Kpp pH 7.5, previous titration with Ti(III)citrate. Various specific enzymatic activities were measured adding 100-500 µM of the correspondent CoA-substrate as shown in table 11. The K_m values were obtained at fixed substrate and protein concentration and at constant 20 °C temperature.

Table 11: Methylviologen assay for the measurement of various enzyme activities.

Reaction	Enzyme	Substrate	Helping enzyme	Co-substrate
Acrylyl-CoA → propionyl-CoA	Acrylyl-CoA reductase	Acrylyl-CoA or Na-acrylate and propionyl-CoA	Propionyl-CoA:acetate CoA-transferase	-
β-Alanyl-CoA → acrylyl-CoA → propionyl-CoA	β-Alanyl-CoA ammonia lyase	β-Alanyl-CoA	Acrylyl-CoA reductase	-
Lactyl-CoA → acrylyl-CoA → propionyl-CoA	Lactyl-CoA dehydratase	D-Lactate or lactyl-CoA	Acrylyl-CoA reductase, propionyl-CoA:acetate CoA-transferase	2 mM ATP 5 mM $MgSO_4$
Crotonyl-CoA→ butyryl-CoA	Butyryl-CoA-ETF dehydrogenase	Crotonyl-CoA	-	-

For complementation of the β-alanyl-CoA ammonia lyase assay with acrylyl-CoA reductase, a *C. propionicum* helping enzyme pool was elaborated. The partially purified acrylyl-CoA reductase pool was obtained after running a Phenyl Sepharose column loaded with a 35 % ammonium sulfate precipitate from 10 g *C. propionicum* wet cells. Then followed a second Mono Q fractionation using 1 M NaCl in 20 mM Kpp pH 7.5 as high salt buffer, green fractions were collected and aliquoted before stored at –20 °C.

4.4.3 NADH/FAD crotonyl-CoA reduction assay

A crotonyl-CoA dependent oxidation of NADH carried out by butyryl-CoA dehydrogenase-ETF can be measured under when adding FAD to the enzymatic reaction. Freshly reconstituted anaerobe enzyme preparations with FAD were assayed in a spectrometer at 340 nm using as extinction coefficient $\varepsilon_{340nm} = 6.3\ mM^{-1}\ cm^{-1}$ (Ziegenhorn et al, 1976). Three consecutive procedures were performed before measuring specific enzyme activity:

I. Anaerobe flavin reconstitution (solution I)

Freshly thawed butyryl-CoA dehydrogenase-ETF enzyme aliquots were incubated in six fold excess FAD (300 µM) in presence of 100 µM NADH at pH 7.5 in anaerobic 50 mM Kpp solution (Buffer A) inside the anaerobic chamber.

II. Enzyme elution by gel filtration (solution II)

After 10 to 60 minutes flavin reconstitution incubation time, the preparation was poured into a 5 ml G25 filtration column (Biorad), equilibrated and eluted with a 100 mM Kpp pH 7.5 anaerobic solution. The new pale yellow enzyme eluate was immediately used for activity and spectra analysis.

III. Pre-mix enzyme preparation (solution III)

Small-scale enzyme preparations (200 µl) were prepared to follow 10 consecutive enzyme assays under the same conditions (100 µM NADH and 50 µM FAD).

III. Long incubation enzyme assay (solution IV)

Every assay was developed having completing a 10 minute pre-incubation time of the enzyme in reaction buffer containing 100 µM NADH and 50 µM FAD (R-buffer, Table 12) in the absence of any acyl-CoA substrate. Once an assay was started with 20 µl of the pre-mix enzyme preparation (solution III) in new prepared R-buffer, 10 minutes incubation time was followed while monitoring the extinction at 340nm in an Ultrospec 4000 photometer. The substrate, 100 µM crotonyl-CoA, was added only when the background absorption was less than 10 % of the CoA dependent activity corresponding to 1 U mg^{-1} NADH oxidation activity.

Table 12: Components of the reaction buffer in the NADH/FAD assay

Reaction-buffer	100 µM NADH
	50 µM FAD
	20 mM Kpp pH 7.5
	0.5 ml final volume

5 Immuno-gold labelling and electron microscopy techniques

To further evaluate the cellular compartmentalization of butyryl-CoA dehydrogenase activity in *C. tetanomorphum*, polyclonal antibodies raised against the complex butyryl-CoA dehydrogense-ETF was incubated on fine cuts of bacterial cells embedded in a matrix of Lowacryl polymer. The procedure was done in collaboration with Dr. Erhard Mörschel and the technical assistance of Frau Marianne Johannsen and the student Ana Möbues, at the laboratory for cell biology, Philipps-University, Marburg. The cell source came from a 50 ml glutamate culture of *C. tetanomophum* anaerobically grown during 10 hours. The dense cell culture was slightly centrifuged to recollect most of the cells (70 %) at the lowest centrifugal force. The cell pellet was carefully washed with Sorensen´s phosphate buffer pH 7.5 at semi-anaerobic conditions. Cells were fixed with 5 % glutaraldehyde, incubated for 3 hours at room temperature, washed 3 times with Sorensen buffer and embedded in Lowacryl medium. Sections of 80–100 nm meters were cut with an Ultracut (Reichert-Jung) Mikrotom and fixed to cupper grids. The immunolabelling process was done after incubation with butyryl-CoA dehydrogenase-ETF IgG antibody solution purified by affinity interaction and a colloidal gold conjugates as second antibody solution as follows: platforms grids were submerged in water for 7 to 8 minutes and incubated with bovine 1% (w/v) bovine serum albumin in TBS for 15 minutes at room temperature to block non-specific protein binding. The butyryl-CoA dehydrogenase-ETF specific IgG antibody solution was one hour incubated at room temperature at 1:400 and 1:1600 dilutions. Further on the grids were 3 times washed with 0.1% TBS-Triton x 100 and H_2O. Incubation of the second colloidal-gold-conjugated antibody was performed with a 1:140 dilution at room temperature for one hour. A final washing step in TBS-Triton x 100 and H_2O preceded the examination in a Phillips EM301 electron microscope.

Results

I. Butyryl-CoA dehydrogenase-ETF from *Clostridium tetanomorphum.*

In the beginning of the experiments, investigations on NADH-dependent dehydrogenase activity in membrane fractions of *C. tetanomorphum* lead to three proteins that were partially purified and identified by N-terminal sequencing: acyl-CoA/butyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydrogenase and acetyl-CoA acetyltransferase. In addition, four copies of the gene encoding for butyryl-CoA dehydrogenase in *C. tetani* genome were found (Brüggemann et al, 2003). These preliminary findings motivated us to purify and characterize the enzyme butyryl-CoA dehydrogenase from the soluble fraction of *C. tetanomorphum.*

1 Purification of the butyryl-CoA dehydrogenase-ETF complex

The purification protocol described as follows is derived from the first attempt to purify acyl-CoA dehydrogenases from liver mitochondria (Ikeda et al, 1985); it is simplified in a three step procedure that was followed up measuring activity with the ferricenium assay. The whole process was performed under aerobic conditions. In the first stage of purification, a highly diluted cell-free extract of *C. tetanomorphum* was carefully precipitated with ammonium sulphate (33 % saturation), stirred at 4 °C for 45 min and immediately centrifuged to recover the green pellet. The colour of this pellet varied slightly from bright green to pale yellow at the end of the purification (Table 13).

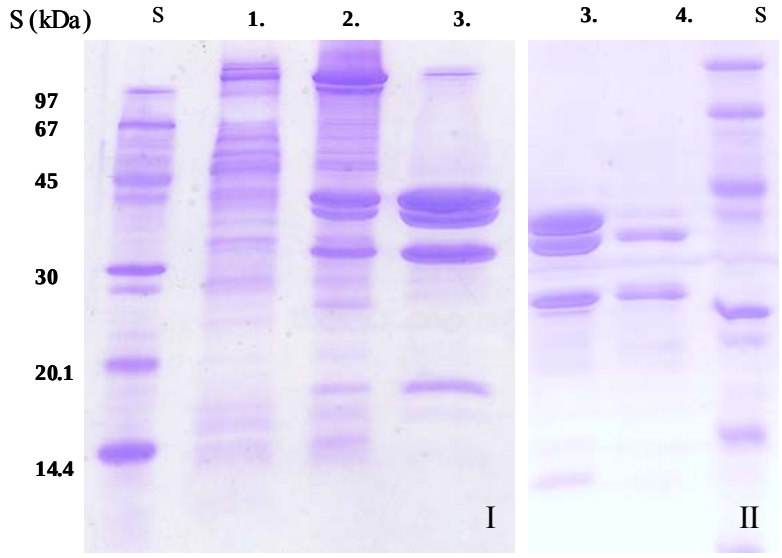


Fig. 6 SDS PAGE with the purified butyryl-CoA dehydrogenase-ETF complex. **1** Cell-free extracts from *C. tetanomorphum*, **2** $(NH_4)_2SO_4$ precipitation, **3** purified butyryl-CoA dehydrogenase-ETF complex, **4** electron transfer flavoprotein (ETF) after Superdex 200, **S** protein standard.

Starting from 100 units of butyryl-CoA dehydrogenase activity in *C. tetanomorphum* crude extracts, 0.5 units were finally 10-fold purified until apparent homogeneity. Purified butyryl-CoA dehydrogenase-ETF complex was obtained after chromatography through a hydroxyapatite column, with a specific activity ranging between 1.1 and 1.5 U mg^{-1}. Efforts to remove low levels of residual impurities resulted in loss of more than 50 % activity after polishing on a preparative Superdex 200^{TM} 36/600 column. As shown by SDS-PAGE (Fig. 6), three bands between 28 and 40 kDa mass corresponding to the purified butyryl-CoA dehydrogenase-ETF complex were observed. After gel filtration, ETF subunits were separatly found from the dehydrogenase 40 kDa protein band (Fig. 6, lane 4).

Table 13: Purification table of butyryl-CoA dehydrogenase-ETF complex

	Volume ml	Protein mg/ml	Total protein mg	Volume activity U ml^{-1}	Total activity U	Specific activity U mg^{-1}	Yield %	Purif. Factor X
Cell-free Extract	530	2.0	1060	0.2	106	0.1	100	1
35 % NH_4SO_2	65	2.0	130	0.5	32	0.25	30	2
DEAE Sepharose	35	1.2	42	0.7	24	0.58	22	5
Hydroxy-apatite	7	1.8	12.6	2.0	14	1.1	13	9
Superdex200	0.4	1.0	0.4	1.35	0.5	1.35	0.47	11

2 N-Terminal sequencing of butyryl-CoA dehydrogenase-ETF complex

The enzyme complex butyryl-CoA dehydrogenase-ETF is composed of three subunits: α-subunit 40 kDa, β-subunit 36 kDa and γ-subunit 28 kDa. Each subunit was individually sequenced by Edman's degradation chemistry. The resulting amino acid sequences were:

α-subunit: M N F A L T R E Q E F V K Q M V R E F A

β-subunit: M Q P A D Y K G V W V F A E Q R D G Q L

γ-subunit: M N I V V C L R Q V P D T N E V K I D P

The alignment of these 20 amino acid long peptide fragments with similar proteins was obtained from a BLAST search in a web data bank (Fig. 7). Two out of four copies of butyryl-CoA dehydrogenase protein sequences found in *C. tetani* genome showed 95 % similarity with the α-subunit of *C. tetanomorphum* butyryl-CoA dehydrogenase.

α-subunit: Acyl-CoA / Butyryl-CoA dehydrogenases

C. tetanomorphum(1)	MNFALTREQEFVKQMVREFA	
C. tetani CTC 2426	MNFALTREQEFVRQMVREFA	ID 95%
C. tetani CTC710	MNFALTREQEFVRQMVREFA	ID 95%
C. perfringens	MNFQLTREQELVKQMVREFA	ID 85%
C. beijerinckii	MNFQLTREQQLVQQMVREFA	ID 80%
C. acetobutylicum	MDFNLTREQELVRQMVREFA	ID 80%
G. kaustophilus	MNFSLTKEQQMIKEMVRDFA	ID 65%
T. tengcongensis	MDFSFTREQEMVRKVVREFA	ID 65%

β-subunit: Electron transfer flavoprotein alpha subunit / Fix B

C. tetanomorphum(1)	MQPADYKGVWVFAEQRDGQL	
C. acetobutylicum	MNKADYKGVWVFAEQRDGEL	ID 85%
C. tetani CTC2424	MNIADYKGVWVFAEQRDGEL	ID 85%
C. beijerinckii	MNIADYKGVWVFAEQREGEL	ID 80%
C. saccharobutylicum	MNIADYKGVWVFAEQREGEL	ID 80%
C. tetani CTC2082	MKVKDYKDVWVFIEQREGRI	ID 65%

γ-subunit: Eletron transfer flavoprotein beta subunit / Fix A

C. tetanomorphum(1)	MNIVVCLRQVPDTNEVKIDP	
C. tetani CTC2425	MNIVVCVKQVPDTTEVKIDP	ID 90%
C. tetani FixA	MNIVVCVKQVPDTTEVKIDP	ID 85%
T. tengcongensis	MNIIVCIKQVPDTSEVKIDP	ID 85%
C. tetani ctc2083	MNIVVCIKQVPDTTEVRIDP	ID 80%
P. gingivalis	MNIVVCIKQVPDTTEIKLDP	ID 80%

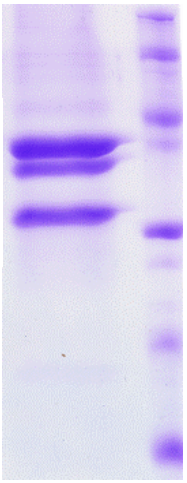


Fig. 7 N-terminal sequence of butyryl-CoA dehydrogenase-ETF complex. *C. tetanomorphum* sequences (1) in alignment with proteins after blast search in protein data bank. (P) *Porphyromonas*, (G) *Geobacillus*, (T) *Thermoanaerobacter*. ID Identity in percentage.

The 36 beta and 28 kDa gamma subunits from the purified complex of *C. tetanomorphum* were identified as electron transfer flavoproteins from the 85 to 90 % identity as compared with proteins from *C. tetani* and *C. acetobutylicum*. Electron transfer proteins (ETF) are known to be highly conserved in prokaryotes as well as in eukaryotes; even so, the N-terminal sequence of ETF alpha subunit (β subunit of the complex) shows in general less similarity than the ETF β-subunit (Roberts et al, 1996). Results also confims previously observed

similarites to the ETF-like protein products of *fix AB* genes that are involved in nitrogen fixation (Earl et al, 1987; Edgren & Nordlund, 2004). For instance, the γ-subunit of the complex seems to have greater resemblance to the *fixA* gene product of *C. tetani* than to other bacterial ETF. In fact, the overall sequence of ETF beta and alpha subunits exhibits high similarity with proteins of far related micro-organisms (O'Neill et al, 1998).

3 Size and molecular composition of the butyryl-CoA dehydrogenase-ETF complex

The size of the butyryl-CoA dehydrogenase-ETF complex was established by Superose^{TM} 6 HR10/30, with an exclusion limit of 4 x10^7. The result was investigated in a molecular range from 66 to 669 kDa after applying the following standards: thyroglobulin (669,000 Da), ferritin (440,000 Da) β-amylase (200,000 Da), alcohol dehydrogenase (150,000 Da) and bovine serum albumin (66,000 Da). The estimated size of the enzyme is 360,000 ± 10,800 Da within 3 % standard deviation.

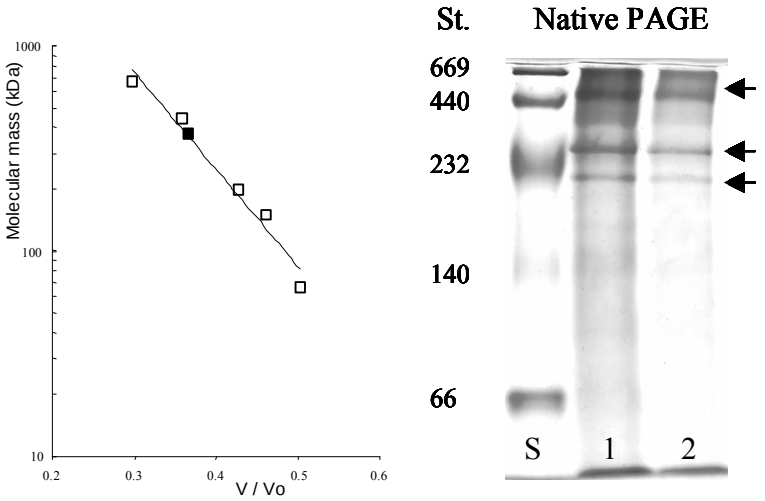


Fig. 8 Molecular size determination of butyryl-CoA dehydrogenase-ETF by size exclusion chromatography (Superose 6) and by native gel (8 % polymerisation grade). Butyryl-CoA dehydrogenase-ETF in bold square. Standards for size exclusion chromatography in hollow squares. In native PAGE, BCD-ETF in lanes 1 & 2; **S** Standard (High molecular).

The enzyme butyryl-CoA dehydrogenase-ETF is present in solution in different aggregation states judged from the results obtained from native PAGE where three bands are visible: one band around 500 kDa and two others at level of the 232 kDa (Native PAGE, Fig. 8).

Inferred from SDS-PAGE and N-terminal sequencing results, the protein complex has the minimal constitution of a heteromer composed of three different subunits: the butyryl-CoA dehydrogenase α-subunit (40 kDa), the ETF alpha subunit as β-subunit (36 kDa) and the ETF beta subunit as γ-subunit (28 kDa) of the referred complex (BCD-ETF), in a still unknown exact ratio. Considering a molecular size of 360 kDa, the most probable composition may be a $\alpha_2\beta\gamma$-heteromer (2x 40 kDa + 36 kDa + 28 kDa) of 144 kDa. Two identical heteromers would make 288 kDa; four of them 576 kDa, in accordance with the sizes shown on the native gel (Fig. 8). It may also lie in a higher molecular state containing 2 ETF molecules per α-subunit, making a complex of 336 kDa ($\alpha_2\beta_4\gamma_4$).

In the absence of more detailed information about the quaternary conformation of the enzyme, the simplest $\alpha\beta\gamma$-heterotrimer of 104 kDa mass was taken into consideration for subsequent characterization.

4 Flavin determination in butyryl-CoA dehydrogenase-ETF complex

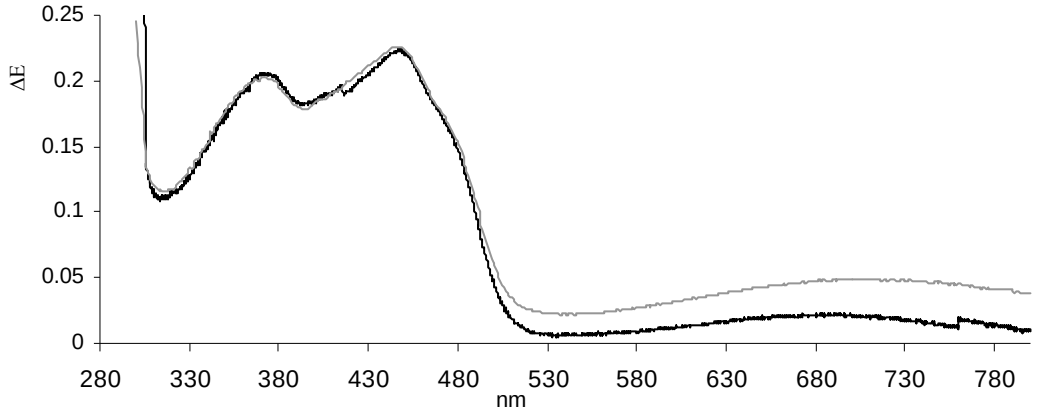


Fig. 9 Spectra (UV-visible light) of native butyryl-CoA dehydrogenase-ETF in 20 mM Kpp pH 7.5 (black line) and after one hour blue light irradiation (grey line). Peaks maximum at 374 and 447nm; wide band at 700 nm.

The native enzyme was analysed by UV-visible spectroscopy. The spectrum of figure 9 shows characteristic peaks for flavins at 374 and 447 nm. There is also one wide band extended between 600 and 800 nm with a maximum at 710 nm. After one hour of monochromatic blue light irradiation the intensity of this band increases. The same analysis was performed in the supernatant of the protein after 70 °C heat denaturation, obtaining similar spectral results with maxima at 374 and 454 mm. The characteristic greenish colour of the enzyme complex is well

documented for the butyryl-CoA dehydrogenase from *M. elsdenii* by Massey and Engel who proposed this light absorption as a result of a charged transfer reaction between the protein's bounded acyl-CoA and FAD molecules. We observed that the green colour vanishes after reduction with dithionite during anaerobic spectrophotometric analysis of the enzyme as reported elsewhere (Williamson & Engel, 1982). The shoulder near to 480 nm may be due to the presence of hydroxylated flavin derivatives like 7-methyl-8-hydroxyisolloxazine (Whitfield & Mayhew, 1974a,b; Ghisla and Mayhew 1976; Ghisla & Massey 1986). The more stable green form of the enzyme was preferred in activity assays instead of the more active yellow form. After full oxidation, the original green enzyme preparation changes to visible yellow colour and the 700 nm broad band vanishes.

The flavin content was identified and quantified by HPLC. It was found that 95 % of the total amount consisted of FAD and only residual quantities of FMN were present in the butyryl-CoA dehydrogenase-ETF complex. The total amount of FAD determined was 0.62 mol per αβγ-heteromer (Table 14).

Table 14: Flavin determination by HPLC

FAD	FMN	Total flavin
83 ± 3.8 µM	9.3 ± 4.2 µM	86 ± 3.9 µM
0.62 mol/ mol αβγ-heteromer	0.07 mol/ mol αβγ-heteromer	

5 Enzyme assays

5.1 Aerobic catalysis of the complex butyryl-CoA dehydrogenase-ETF

5.1.1 Butyryl-CoA dehydrogenase activity by the ferricenium assay

The electron acceptor ferricenium hexafluorophosphate is well established for measuring activity of acyl-CoA dehydrogenases (Lehman & Thorpe, 1990). This assay allows a sensitive detection of butyryl-CoA dehydrogenase activity for its purification but, because the dye serves as an artificial electron acceptor, it does not reflect the catalytic properties of the enzyme as a complex with ETF. The enzyme showed high affinity for its natural substrate butyryl-CoA.

During the transfer of electrons from the substrate via FAD, the blue ferricenium salt complex (Fc^{2+}) is converted into the reduced colourless ferrocene form (Fc^0) (eq. 3).

$$\text{Butyryl-CoA} + 2\ Fc^{+} \rightarrow \text{Crotonyl-CoA} + 2H^{+} + 2\ Fc^{0} \quad \text{(eq. 3)}$$

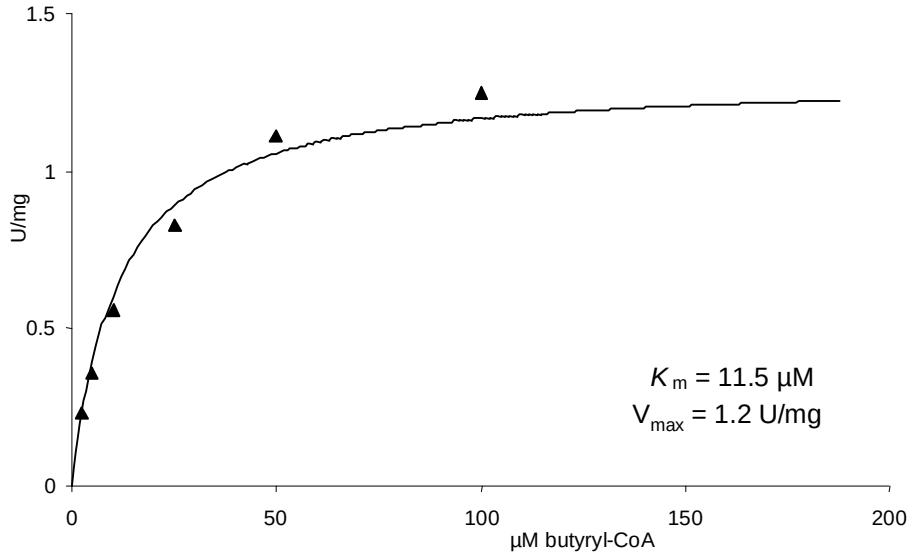


Fig. 10 Michaelis-Menten kinetics of butyryl-CoA dehydrogenase activity of the purified BCD-ETF complex for the substrate butyryl-CoA in presence of 200 µM ferricenium hexafluorophosphate.

The calculated K_m for butyryl-CoA was 11.5 ± 6.4 µM at a V_{max} of 1.3 ± 0.4 U mg^{-1} (Fig.10) and was the result of testing substrate concentrations between 2.5 and 100 µM in presence of 200 µM ferricenium hexafluorophosphate. This value is at the same range as the reported one for the native enzyme from *Megasphaera elsdenii* (K_m = 14 µM) (Du plessis et al, 1998; Williamson & Engel, 1994); and comparable to the K_m of the ETF-free enzyme from *Clostridium acetobutylicum* (K_m = 6 µM) (Diez-Gonzalez et al, 1997).

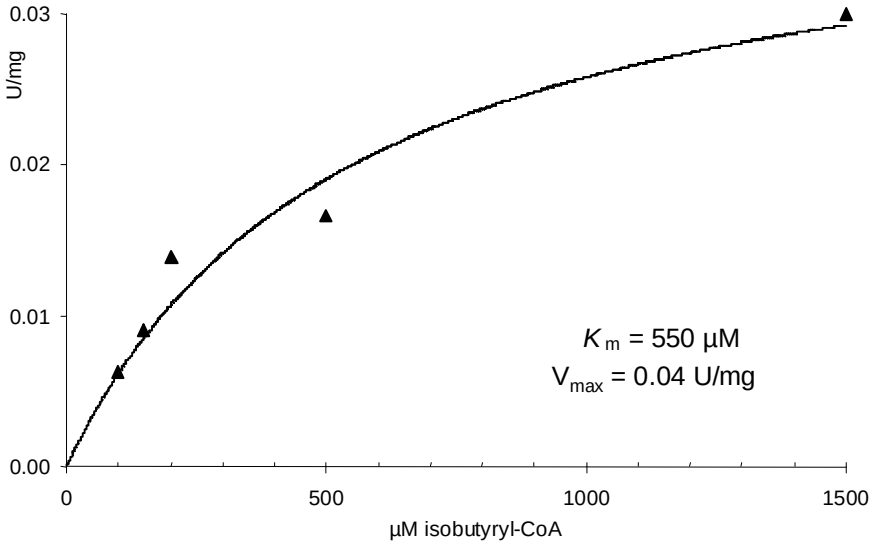


Fig. 11 Michaelis-Menten kinetics of butyryl-CoA dehydrogenase activity of the purified BCD-ETF complex for the substrate isobutyryl-CoA in presence of 200 µM ferricenium.

Under the same conditions the enzyme specificity to other subtrates was tested resulting in a much lower activity for the branched CoA-derivate, isobutyryl-CoA with a V_{max} of 0.04 U mg^{-1} and a K_m = 550 ± 250 µM (Fig. 11); in contrast, there was good activity towards the C5 substrate valeryl-CoA found with a V_{max} of 2.7 ± 0.45 U mg^{-1} and a K_m of 220 ± 50 µM (Fig. 12).

The turnover of the enzyme for its normal substrate was 2 s^{-1}; for the one carbon longer CoA-derivative this rate doubles in value. In the case of *M. elsdenii* butyryl-CoA dehydrogenase, a K_m = 161 µM for isobutyryl-CoA was determined in a phenazine ethosulfate assay, indicating that straight chains acyl-CoA thioesters act as better substrates than their branched chain counterparts (Williamson and Engel, 1984). Whereas the K_m values for isobutyryl-CoA and valeryl-CoA are similar, the main difference lies in V_{max} (Table 15).

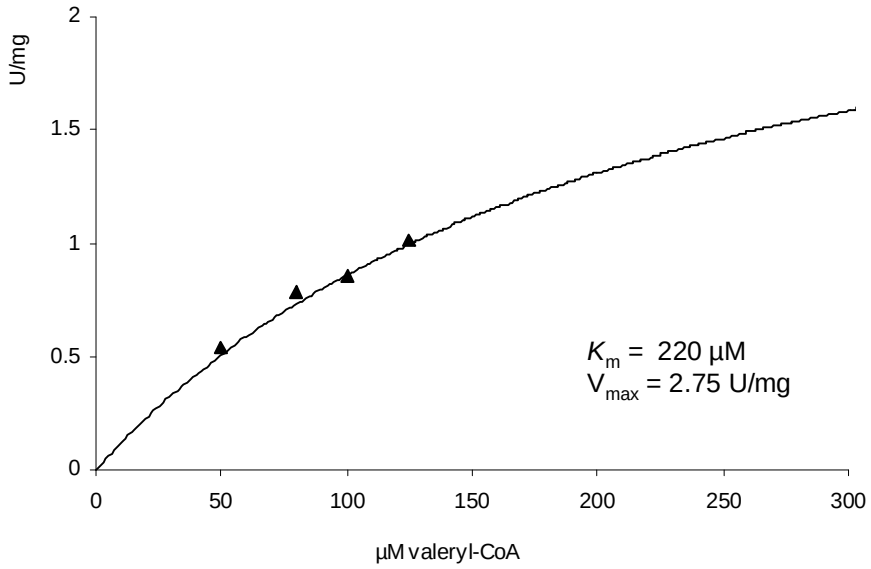


Fig. 12 Michaelis-Menten kinetics of butyryl-CoA dehydrogenase activity of the purified BCD-ETF complex for the substrate valeryl-CoA in presence of 200 µM ferricenium hexafluorophosphate.

Table 15: Summary of kinetic parameters of the dehydrogenase activity of BCD-ETF

Substrate	**Cofactor**	K_m **µM**	V_{max} **U mg-1**	k_{cat} s^{-1}
Butyryl-CoA	200 µM FcPF6	11.5 ± 6.4	1.3 ± 0.4	2.3
Valeryl-CoA	200 µM FcPF6	220 ± 50	2.7 ± 0.45	4.7
Isobutyryl-CoA	200 µM FcPF6	550 ± 250	0.04 ± 0.006	0.1

5.1.2 pH and buffer influence on the BCD-ETF activity

Under slightly alkaline conditions the enzyme butyryl-CoA dehydrogenase-ETF shows maximal activity. As presented in figure 13, the optimum pH was determined at 7.5. Mops (pK_a = 7.1) and potassium phosphate (pK_a 7.2) are good buffers for the enzyme since high and stable activity was observed in enzyme working in 50 mM of the corresponding solutions ranging between pH 7.0 and 8.0. On the contrary, the activity of the *M. elsdenii* enzyme decreases already above pH 7.0 (Fink et al, 1986).

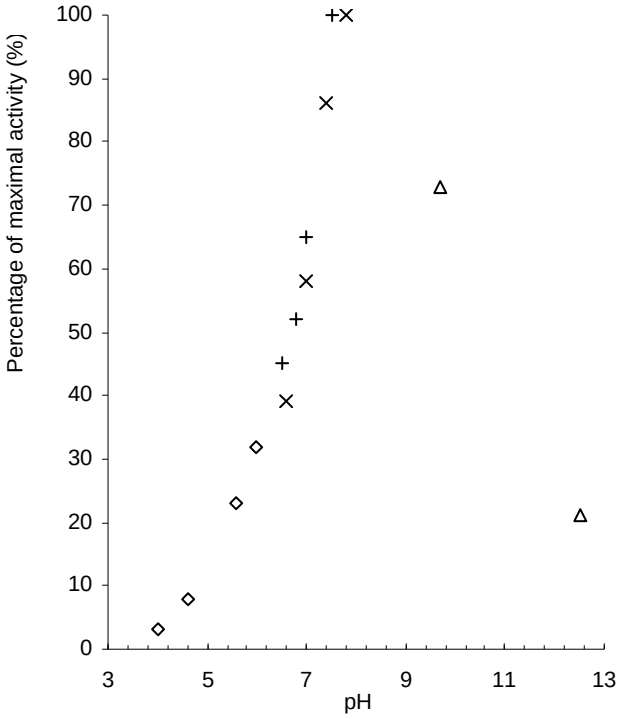


Fig. 13 pH influence on the butyryl-CoA dehydrogenase-ETF activity. The Buffers used are citric acid (◊), Mops (x), potassium phosphate (+), di-phosphate (Δ).

It should be noted that the buffers presenting hydroxyethyl- or hydroxymethyl side chains like Hepes [4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid)], Bicine [(N,N-bis(2-hydroxyethyl)glycine)] or Tris (tris(hydroxymethyl)aminomethane) could not be used in the ferricenium assay above pH 7.0. Addition of one of those buffers to ferricenium caused a slow steady decrease of absorbance at 300 nm.

5.1.3 Butyryl-CoA dependent oxidase activity at 290 nm

The enzyme butyryl-CoA dehydrogenase-ETF has a butyryl-CoA dependent activity in the presence of no other electron acceptor than oxygen from air. The residual enzymatic activity was 0.0042 ± 0.0012 U mg^{-1}, measured directly at 290 nm wavelength (ΔE_{290nm} = 2.8 $mM^{-1}cm^{-1}$) because the adenine absorption does not interfer with the enoyl, unlike at 260 nm.

5.1.4 NADH oxidation by INT

NADH-oxidation activity by iodonitrotetrazolium chloride (INT) is present in anerobic preparations of the butyryl-CoA dehydrogenase-ETF complex. The rate of NADH oxidation (diaphorase activity) was determined as INT-formazan product formation (eq. 4). The INT activity under anaerobic atmosphere was 0.15 ± 0.016 U mg^{-1} at 100 µM NADH while under

aerobic conditions it was 0.073 ± 0.1 U mg $^{-1}$. Maximal activity was found adding 50 µM FAD to the enzymatic reaction (0.36 ± 0.05 U mg^{-1}). In the same assay with an alternate tetrazolium, 2,3,5-triphenyltetrazolium (TT), no absorbance change was detected applying the same enzyme preparation under both aerobic and anaerobic conditions.

$$NADH + H^+ + INT \rightarrow NAD^+ + INT\text{-}Formazan \quad \text{(eq. 4)}$$

5.1.5 Hydrogen peroxide formation in correlation with NADH oxidation

To further investigate the oxidase catalysis of butyryl-CoA dehydrogenase as an ETF-complex, the formation of peroxide as a by-product from NADH oxidation was tested. *C. propionicum* propionyl-CoA dehydrogenase-ETF enzyme has a significant diaphorase activity (12 s $^{-1}$) at 100 µM NADH under anoxic conditions. H_2O_2 production was detected under oxic conditions by peroxidase in the ABTS assay (Hetzel et al, 2003).

In the peroxide catalysed reaction the dye ABTS (2,2'-azino-di (3-ethylbenzthiazoline-6-sulfonate) functions as electron acceptor forming a radical cation (eq. 5) that absorbs between 400 and 422 nm (Kim et al, 2004).

$$ABTS - 1e \rightarrow ABTS^{\cdot +} \quad \text{(eq. 5)}$$

Under constant pH conditions and excess of the dye all the peroxide in the sample reacts until completion. The concentration of the $ABTS^{\cdot +}$ radical cation, interpreted as a measure of the peroxide concentration, was calculated from the $\Delta E = E_f - E_i$ (E_f final maximal absorbance after the end of the reaction, E_i initial absorbance of all the reactants without sample addition) determined at 412 nm (ε_{412nm}= 53 mM^{-1}cm^{-1}). The ABTS assay was sensitive enough to detect H_2O_2 concentrations ≥ 0.5 µM. As presented in equations 6 to 8, the enzymatic reaction catalysed by the butyryl-CoA dehydrogenase-ETF accomplished the complete oxidation of NADH forming H_2O_2 as the by-product of NADH oxidation. In the first experiment (Fig. 14), starting from 200 µM NADH, one mol of NAD is formed per mol H_2O_2. It is noteworthy that the peroxide formation is apparently delayed in time and it is rapidly induced after about 2 min, and ends at the same time when NADH oxidation is complete (Fig.14).

$$NADH + H^+ + ETF \rightarrow ETFH_2 + NAD^+ \quad \text{(eq 6)}$$

$$ETFH_2 + O_2 \rightarrow ETF + H_2O_2 \quad \text{(eq 7)}$$

$$H_2O_2 + 2\ ABTS \rightarrow 2\ ABTS^{\cdot +} + 2\ OH^- \quad \text{(eq 8)}$$

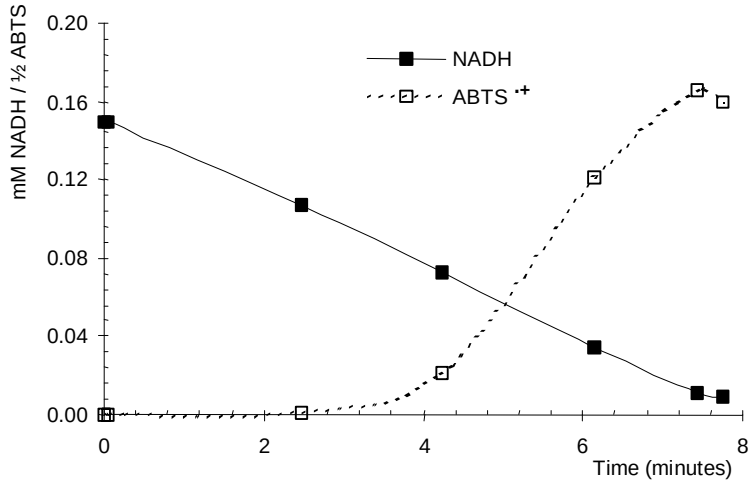


Fig. 14 First experiment on NADH oxidation and peroxide formation catalyzed by butyryl-CoA dehydrogenase-ETF.

In order to overcome the apparent deficiency in the peroxide detection within the first minutes of reaction, the butyryl-CoA dehydrogenase-ETF concentration was doubled in the second experiment with no remarkable difference in the results (Fig. 15).

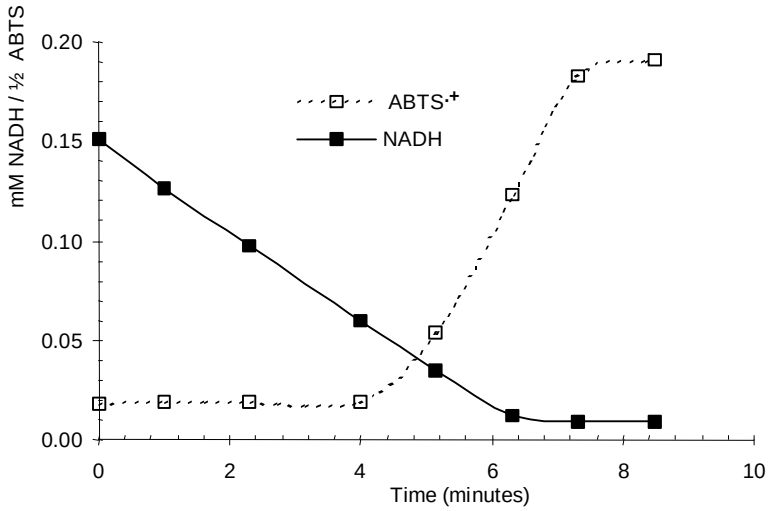


Fig. 15 Second experiment on NADH oxidation and peroxide formation catalyzed by butyryl-CoA dehydrogenase-ETF.

In the third experiment (Fig. 16), the same butyryl-CoA dehydrogenase-ETF concentration was used but the sample was kept over three days at 4°C. In this case, earlier peroxide formation was detected. The NADH consumption is less linear than in the first experiment. The NADH curve shows faster reaction at beginning of the assay ending with a long phase of slow oxidation after the maximal peroxide amount is formed. In this case the $ABTS^{\cdot+}$ radical formation was followed at $\lambda = 422$ nm ($\varepsilon_{422nm} = 4.6\ mM^{-1}\ cm^{-1}$).

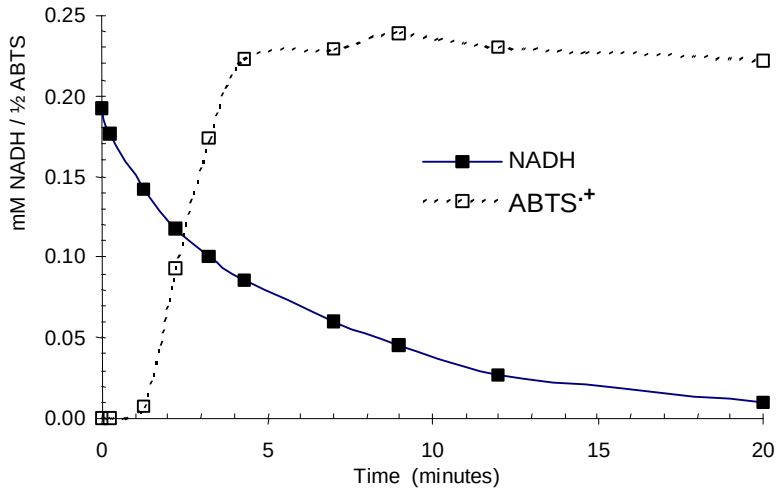


Fig. 16 Third experiment on NADH oxidation and peroxide formation catalyzed by butyryl-CoA dehydrogenase-ETF.

In these three experiments, the peroxide formation begins after a delay of 2 to 4 minutes from the start NADH oxidation reaction. A comparison of the results and the experimental conditions is resumed in table 16.

Table 16: NADH oxidation and peroxide formation in butyryl-CoA dehydrogenase-ETF. Comparison of three experiments.

	Experiment 1	Experiment 2	Experiment 3
Experimental conditions	Pox 50 mg/ml 25 µl sample (1) Fresh sample λ 412 nm	Pox 5 mg/ml 50 µl sample(1) Fresh sample λ 412 nm	Pox 5 mg/ml 50 µl sample(1) Old enzyme λ 422 nm
Results: NADH activity [ABTS] formation Time elapsed (2)	 0.12 U/ml 160 mM 7 min	 0.13 U/ml 190 mM 8 min	 0.24 U/ml 220 mM 4 min

Pox peroxidase from horseradish.

(1) sample volume from the NADH mix tested containing BCD-ETF assayed in the ABTS test for peroxide detection.

(2) Time elapsed until maximal $ABTS^{\cdot+}$ amount was formed

5.2 Anaerobic catalysis of the complex butyryl-CoA dehydrogenase-ETF

5.2.1 Methylviologen assay for crotonyl-CoA reductase activity

In this work methylviologen was useful as a dye indicator to measure the reduction of acrylyl-CoA or crotonyl-CoA by either acrylyl-CoA- or crotonyl-CoA reductase. The redox potential for one electron reduction of methylviologen is –0.446 mV at 25 °C and neutral pH (Mayhew, 1978). Thus methylviologen can substitute ETF mediating the flavin reduction in the referred enzymes (Fig 19). Before performing the assay it was necessary to produce stochiometric amounts of the methylviologen semiquinone by anaerobic titration of the assay mixtures with titanium citrate [Ti(III)Ci].

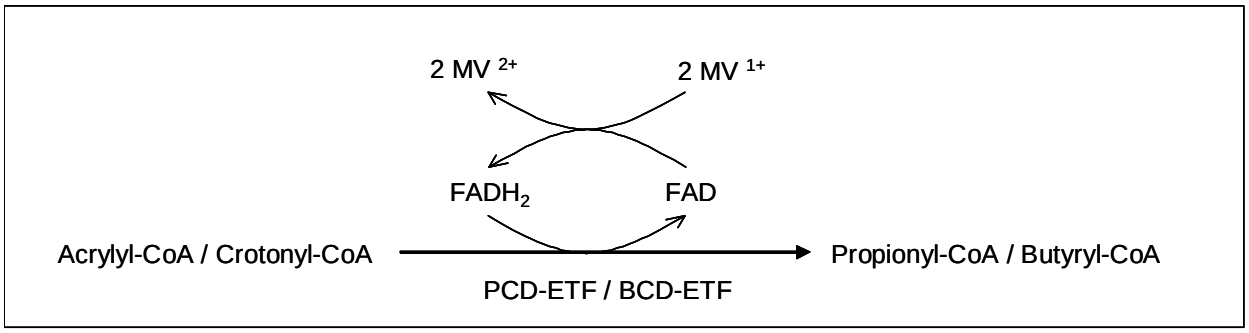


Fig. 18 Methylviologen assay. PCD-ETF propionyl-CoA dehydrogenase, BCD butyryl-CoA dehydrogenase, ETF electron transfer flavoprotein. MV Methylviologen.

The spectrophotometric methylviologen assay is appropiate to detect acrylyl-CoA reduction in presence of acrylyl-CoA reductase, showing a rapid linear decrease in the absorption at λ = 600 nm (ε = 27.2 mM^{-1} cm^{-1}). Methylviologen serves as electron donor during the hydrogenation reaction whereby two mol of the semiquinone are oxidised for every mol enoyl-CoA substrate that is reduced (Fig. 18). There was high acrylyl-CoA reductase activity (26 ± 1.7 U ml^{-1}) in cell-free extracts from *C. propionicum* on addition of Na-acrylate, propionyl-CoA and CoA transferase to the assay mixture. The partially purified acrylyl-CoA reductase from *C. propionicum* was used as a helper enzyme to measure β-alanyl-CoA ammonia lyase activity. Several trial to achieve similar results with free-extracts and pure fractions of lactyl-CoA dehydratase failed, observing only unstable changes in absorbance (ΔE_{600}/min) which cycled between positive and negative values.

The assay allows a rapid acrylyl-CoA clearance and therefore it was found appropriate for the detection of β-alanyl-CoA ammonia lyase activity even in the presence of high ammonium ion concentration (see results of β-alanyl-CoA ammonia lyase). The reduction of acrylyl-CoA

to propionyl-CoA is irreversible under physiological conditions, thus acrylyl-CoA could be readily consumed in the coupled reaction after the ammonia elimination from β-alanyl-CoA. The assay though not reproducible enough with different butyryl-CoA dehydrogenase-ETF samples (varying in flavin oxidation state and/or content), was useful to make comparisons between kinetic parameters obtained with other anaerobic crotonyl-CoA reductase assay methods. For kinetic calculation, the mean values of three experiments done under the same conditions were analysed by both the Michaelis-Menten and its double reciprocal, Lineweaver-Burk plots methods. The final K_m values were calculated using the mathematical technique of least-squares.

In the anaerobic methylviologen assay, butyryl-CoA dehydrogenase-ETF showed a crotonyl-CoA reductase activity in the absence of other cofactors. There was a considerable variability between the enzymes samples assayed, most probably due to differences in the content of activated flavins. For the following results the same fresh purified enzyme sample in its green form was used.

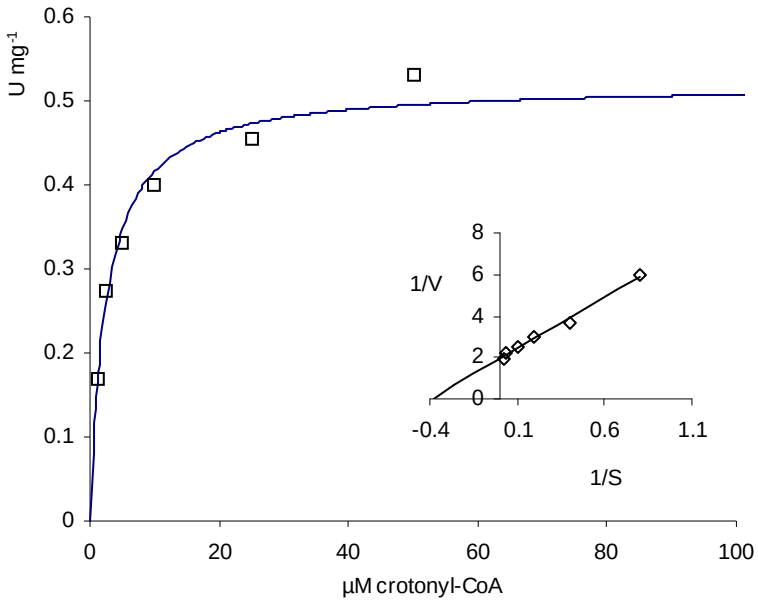


Fig. 19 Crotonyl-CoA reductase activity of butyryl-CoA dehydrogenase-ETF with methylviologen as electron donor. Lineweaver-Burk double reciproque in insert.

The K_m of 2.5 ± 0.2 µM for crotonyl-CoA was obtained at 1 mM methylviologen that was previously reduced to the semiquinone form with 7 µM Ti(III)citrate (Fig. 19).

5.2.2 NADH/FAD crotonyl-CoA reductase assay

The enzyme butyryl-CoA dehydrogenase-ETF has a high aerobic NADH oxidase activity, *i.e.* a diaphorase activity of $\geq$ 10 U mg^{-1} measured directly from NADH oxidation at 340 nm (see the experiment hydrogen peroxide formation in correlation with NADH oxidation). Under air, this activity is independent of crotonyl-CoA substrate addition. Conversely, anaerobic enzyme preparations are catalytically active towards crotonyl-CoA as long as the enzyme is saturated with flavin and has a constant NADH diaphorase activity less than 1 U mg^{-1} (Fig. 20).

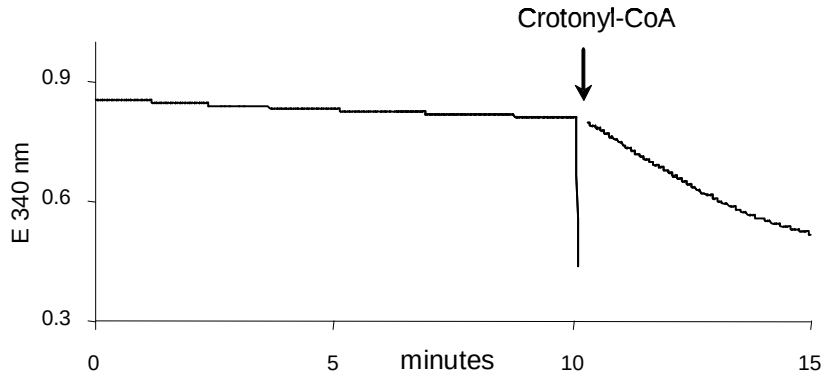


Fig. 20 Anaerobic NADH/FAD crotonyl-CoA reduction assay

To measure crotonyl-CoA reduction in the presence of NADH and FAD, the enzyme was pre-treated as follows in 3 consecutive preparations:

Solution I (1ml): enzyme [1 U mg^{-1}] pre incubation at 100 µM NADH and 6-fold excess FAD (300 µM). Solution I was incubated under anoxic conditions for 10 minutes before gel filtration.

Solution II (1.2 ml): enzyme eluate from G25 SEC cartridges in 100 mM Kpp pH 7.5.

Solution III (0.2 ml): 20 µl of solution II in 500 µL 100 µM NADH, 50 µM FAD and 20 mM Kpp pH 7.5 (reaction buffer). This preparation was used for 10 measurements.

Solution IV (0.5 ml): enzyme assay starting with 20 µl of solution III in new 0.5 ml reaction buffer preparation (100 µM NADH, 50 µM FAD). The decrease in absorbance was followed at 340 nm for 10 minutes until NADH oxidation was stabilized at approximately 1 U mg^{-1} $(\Delta E_{340}/min)_1$; at this moment substrate was added (crotonyl-CoA) to record the second slope $(\Delta E_{340}/min)_2$ and the final activity was calculated subtracting $(\Delta E/min)_1$ from $(\Delta E/min)_2$ (Fig 21).

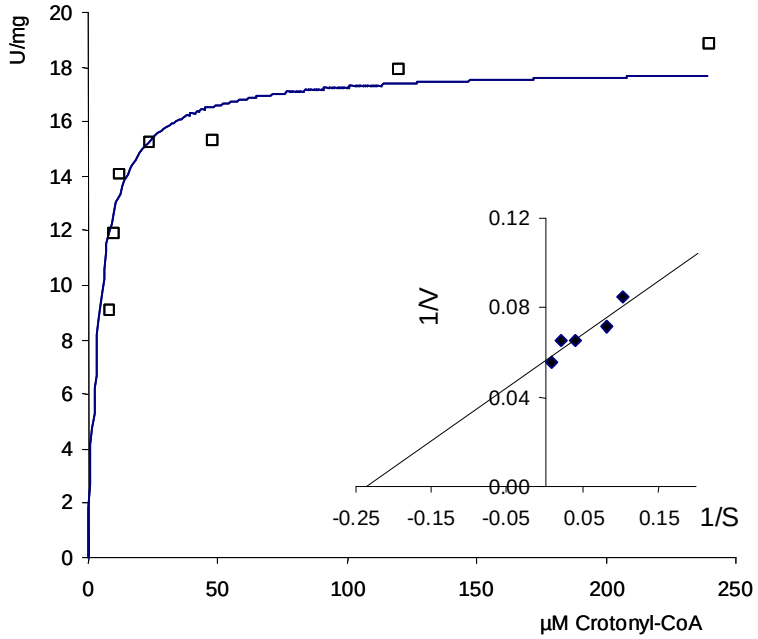


Fig. 21 Crotonyl-CoA reduction activity of butyryl-CoA dehydrogenase-ETF in presence of 50 µM FAD and 100 µM NADH

The enzyme activity measured at 340 nm at 50 µM FAD and 100 µM NADH follows a Michaelis-Menten kinetic comparable to the measurements made with methylviologen. The values: K_m = 4 µM for crotonyl-CoA (50 µM FAD, 100 µM NADH) and K_m = 18 µM for NADH (80 µM crotonyl-CoA, 80 µM FAD) were obtained between 12 and 18 U mg^{-1} maximal catalytic velocity.

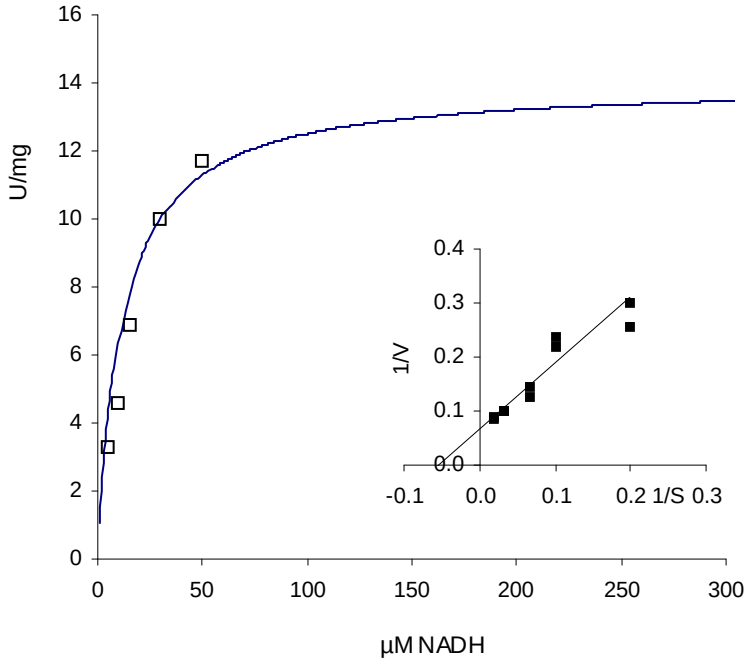


Fig. 22 NADH dependent crotonyl-CoA reduction activity of butyryl-CoA dehydrogenase-ETF in presence of 80 µM FAD and 80 µM crotonyl-CoA

Experiments were done comparing the enzyme activity with the final amount of FAD freshly incorporated into the complex. The protocol for the enzyme reconstitution was exactly the same as explained above, but in this case different incubation times (10, 30 and 60 min) were applied before continuing with gel filtration. Flavin content was inferred from the absorbance at 450 nm once the unbound cofactor was completely removed by gel filtration. The starting material (BCD-ETF at 0 min) had ≤ 1 mol FAD. After 30 to 60 min incubation time the total flavin amount increased until 2 molecules FAD. Crotonyl-CoA dependent NADH activity was 3-fold higher (from 7.3 U mg^{-1} to 22 U mg^{-1}) after incorporation of one extra FAD molecule (Fig 23). A similar observation was made for the reverse butyryl-CoA dehydrogenase reaction; the initial ferricenium activity (0.8 U mg^{-1}) was 3-fold higher (2.4 U mg^{-1}) in the same enzyme preparation when 2 mol FAD was present in the enyzme. Although the enzyme is saturated with FAD, it still requires additional oxidised flavin, whether FAD or FMN to reduce crotonyl-CoA during the NADH oxidation assay. Using FMN instead of FAD in the assay, almost 2-fold lower specific activity was found (14 U mg^{-1}) but the increment was 30 times higher when compared to the initial activity (0.4 U mg^{-1}) of the non-reconstituted starting enzyme material.

It could be debated whether there is an exchange of FAD or FMN moieties between the enzymes during catalysis. It is known that one of the two flavins is tightly bound in butyryl-CoA dehydrogenase from *M. elsdenii,* while the second molecule associates weakly within the enzyme and shows unusual spectral properties (Sato et al, 2003). Concerning the mechanism of the reduction reaction, there is evidence that butyryl-CoA dehydrogenase from *M. elsdenii* transfers the beta hydrogen to the flavin N-5 position as a hydride (Ghisla et al, 1984).

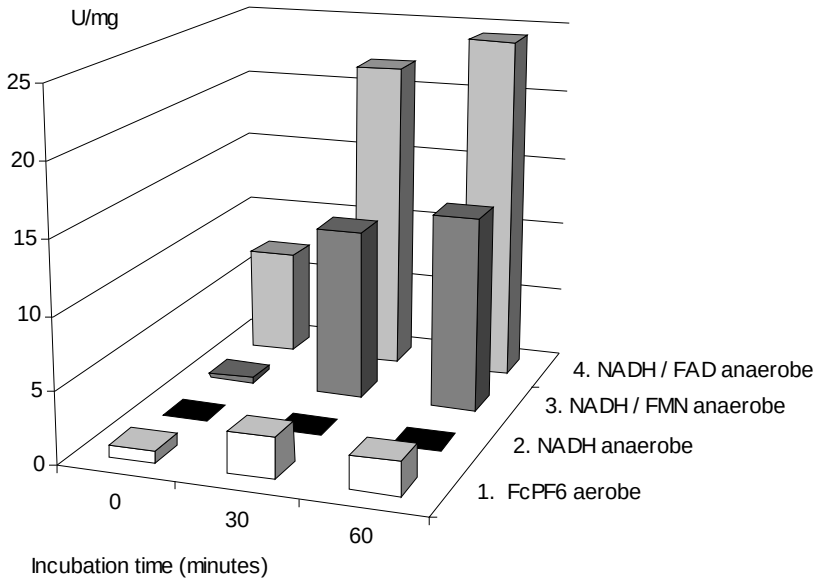


Fig. 23 Anaerobe NADH oxidation and ferricenium mediated dehydrogenase ($FcPF_6$) activity of the butyryl-CoA dehydrogenase-ETF complex. At time 0 of incubation in presence of excess of FAD there is one mol total FAD detected in the enzyme; up to 30 min there are 2 mol FAD per αβγ-heterotrimer.

FAD is rapidly reduced by the BCD-ETF complex as seen in absorption measurements done at 450 nm. The estimation K_m for FAD was determined using the already mentioned anaerobic NADH/FAD assay at 340 nm.

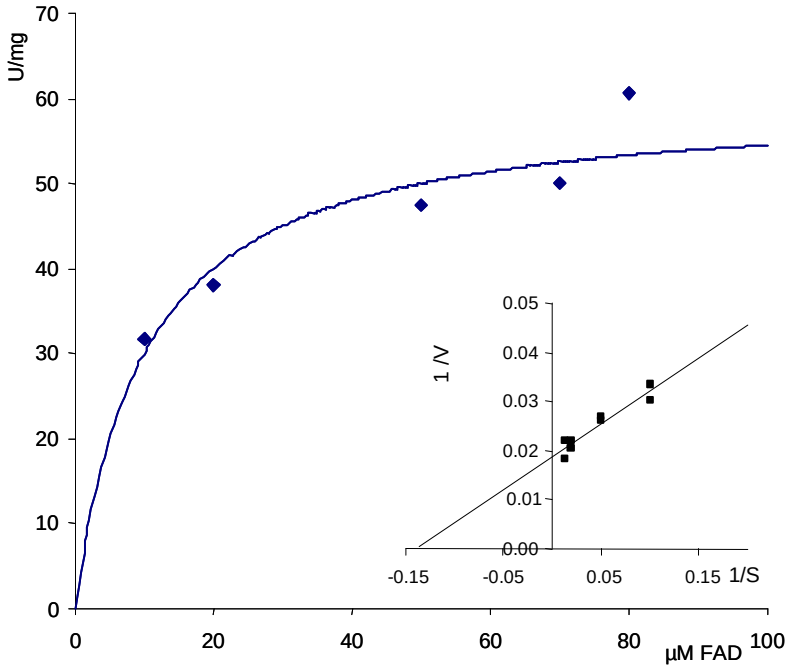


Fig. 24 FAD dependent crotonyl-CoA reduction activity of butyryl-CoA dehydrogenase-ETF in presence of 80 µM crotonyl-CoA and 100 µM NADH.

The enzyme was saturated with 300-fold excess of the cofactor and then tested in the reaction mix containing 80 µM crotonyl-CoA and 100 µM NADH. The K_m for FAD was deduced to be 10 ± 0.6 µM from the data presented in figure 24.

5.2.3 Butyryl-CoA dehydrogenase activity in membrane fractions

The investigation of butyryl-CoA dehydrogenase activity in soluble and membrane fractions from crude extracts of *C. tetanomorphum* was done under anaerobic conditions. The employment of lysozyme was in order to achieve a smooth separation of the membrane from the soluble fraction to distinguish the enzyme activity possibly associated with the membrane.

Using ferricenium hexafluorophosphate as electron acceptor without substrate, activity was found in membrane preparations after treatment with 1 % dodecylmaltoside in the range of 1.6 ± 0.1 U ml^{-1}. When 100 µM butyryl-CoA was added, the final activity was 2.0 ± 0.1 U ml^{-1}. This 20 % residual activity was repeatedly found after several trials in different membrane preparations (Table 17).

Table 17: Butyryl-CoA dehydrogenase-ETF activity associated to the membrane

	Soluble fraction	Wash	Membrane fraction I	Membrane fraction II	Membrane fraction III
			Lysozyme 0.3 mg/ml		
Units	16	2	4	16	5
U mg^{-1}	0.28	0.08	0.07	0.27	0.12
			Lysozyme 10 mg/ml		
Units	14	10	2.3	10	7
U mg^{-1}	0.13	0.22	0.05	0.13	0.11

Membrane fractions consisted of triturated pellets from one preparation, before detergent treatment (I), after incubation with 1% dodecylmaltoside (II) and in supernatant after centrifugation (III) of preparation II.

In Table 17 is a comparison of the activity found in the two cellular compartments of *C. tetanomorphum* cell lysates. The specific butyryl-CoA dehydrogenase activity increased 4-fold after processing the raw membrane extracts (fraction I) with the detergent

dodecylmaltoside. This dodecylmaltoside-treated membrane fraction (fraction II) exhibited the same total activity found in the soluble fractio, but it is reduced almost to half in the supernatant separated after centrifugation (fraction III). The loss of activity in the wash step is greater when applying lysozyme at 10 mg/ml than at 0.3 mg/ml concentration.

Little increase in the butyryl-CoA dehydrogenase specific activity was found in fractions collected after loading a Red Reactive^{TM} column with a membrane extract from *C. tetanomorphum*. Some particular interesting heavy bands (>97 kDa) and at the level of butyryl-CoA dehydrogenase (~ 40 kDa) were associated with green fractions that contained a specific activity of 0.05 U mg^{-1} (Fig 27).

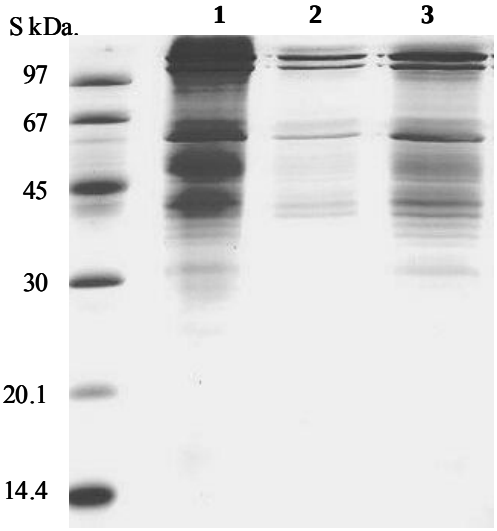


Fig. 25 SDS PAGE with proteins associated to the membrane of *C. tetanomorphum*. Lane 1, membrane extracts; lane 2 and 3, Red Reactive© fractions with butyryl-CoA dehydrogenase activity.

These results suggest that butyryl-CoA dehydrogenase is not membrane bound.

6 Immunological methods applied to butyryl-CoA dehydrogenase-ETF

6.1 Antibodies for butyryl-CoA dehydrogenase-ETF

The antibody detection was developed by Western blot and chemiluminescence analysis.

In the Western blot technique, the protein under study is fixed to a vinyl membrane and is distinguished from other proteins when a peroxidase conjugated secondary antibody (Anti-rabbit IgG-HRP) binds to the primary antibody (against the protein in study ergo BCD-ETF) and reacts with a substrate that gets oxidised in the detection reaction giving a luminescent

final product. The detection reaction takes place when the HRP peroxidase, coupled to the mentioned secondary antibody, reacts with hydrogen peroxide in an alkaline mixture containing luminol. The product of this reaction is excited compound and emits light while it decomposes to a lower electronic ground state (Fig. 26). This light emission is known as chemiluminescence and can be detected on X-ray films causing strong signal amplification.

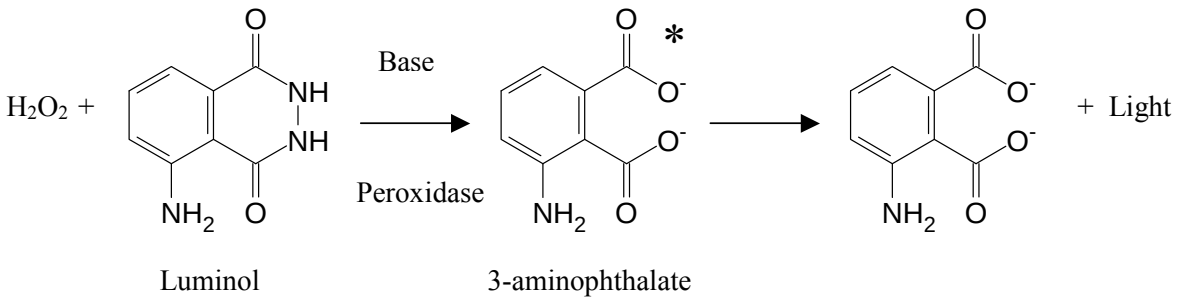


Fig. 26 Chemiluminescence reaction for antibody detection

Before immunization the serum collected from four rabbits were tested by Western blot analysis in order to prove the lack of positive signal at the corresponding sizes where the protein is expected to appear after immunization, that is between 28 and 36 kDa belonging to the three subunits of butyryl-CoA dehydrogenase-ETF. The immunization candidate, a rabbit with negative cross reaction against the protein under study (Silent Signal in Western Blot), was injected with an aliquot containing 30 µg of a sterile butyryl-CoA dehydrogenase-ETF purified fraction. At the initial phase of immunization, a non-specific stimulator of the immune response, an unspecific adjuvant was co-injected with the antigen. The animal's immune system reacted with a drastic increase of the humoral response producing high amounts of antibodies within the first weeks. Subsequent inoculation sustained the process leading to the accumulation of the desired antibodies.

Depending on the quality of the antiserum, the signal-to-noise ratio improves after incubating the blots with sera of longer immunization time. The sera dilution at which a clear signal of each of the three bands between 35 to 40 kDa appeared was adjudicated as the sample's titer. The detection was done against 100-200 ng pure butyryl-CoA dehydrogenase-ETF protein fixed to the membrane by Western blot. During the first stage of antibody production, the sera samples were examined in order to follow the increment of antibody titer. The maximal antibody titer of 1/4000 was achieved after 14 weeks of immunization, the same moment

when the final whole blood sample was collected. There is more information about the immunization schedule in table 18, also showing the titer results of the probes analysed during the whole procedure.

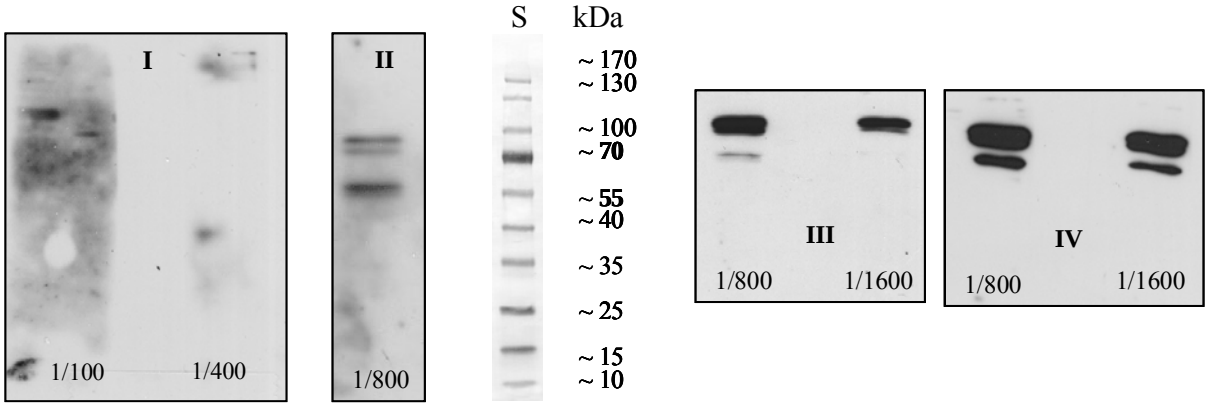


Fig. 27 Western blot of pre-immunesera at 1/100 and 1/400 dilution (I) and immunsera 0605 after 2 month rabbit inoculation with butyryl-CoA dehydrogenase-ETF at 1/800 dilution, against 100 ng pure protein (II) and at 1/800 and 1/1600 dilution against 200 ng protein (III). Immunsera 0705 after 3 month inoculation at 1/800 and 1/1600 dilution against 200 ng pure protein (IV), S for pre-stained marker used as reference standard.

Table 18: Schedule of immunization and sampling time of sera for antibody production

Immunesera	**Pre-Is 007**	**Is 0505**	**Is 0605**	**Is 0705**	**Is 0805**
Immunization time	None	1 Month	2 Month	3 Month	3 ½ Month
Titer	n.d.	n.d.	1:800	1:1600	1:4000
Sample volume	2 ml	2 ml	2 ml	2 ml	50 ml

n.d. not detected; immunization time from the moment of butyryl-CoA dehydrogenase-ETF inoculation; **pre-Is** pre-immunsera, **Is** Immunsera.

The second stage of antibody production consists of the further purification of specific butyryl-CoA dehydrogenase-ETF Immunoglobulin G (Ig G) from the total mix of antibodies. In figure 27, the Western blot made with *C. tetanomorphum* cell-free extracts incubated at the optimal selected titer dilution of final immunesera 0805 (table 18) shows additional positive signal at higher molecular size levels. Some of these unspecific signals are as strong as those expected for the selective detection of butyryl-CoA dehydrogenase-ETF.

During affinity purification the protein butyryl-CoA dehydrogenase-ETF is fixed to a matrix in order to bind selectively only antibodies against butyryl-CoA dehydrogenase-ETF to the self-made affinity column. All other unspecific IgG's normally present in the sera are removed in the flow through. After this process the resulting affinity purified immunesera contains a 1:1 ratio of the three distinct classes of antibodies raised against each of the three subunits of butyryl-CoA dehydrogenase-ETF complex, and shows an optimal titer of 1/3200 ready to be use as working solution (Fig. 28).

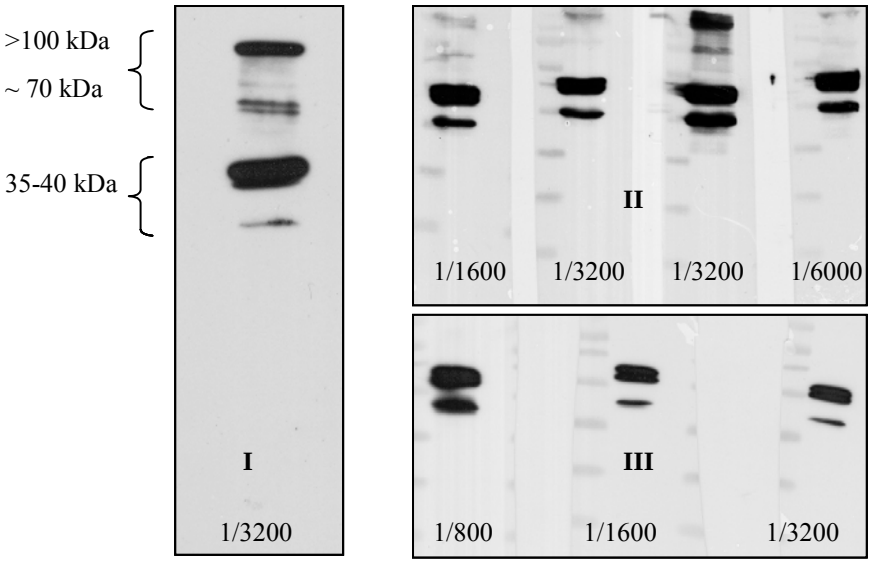


Fig. 28 Western blot incubated with the final immunsera 0805 tested against cell-free extract of *C. tetanomorphum*. Before affinity purification the immunsera at dilution 1/3200 present unspecific bands at 70 and > 100 kDa in blots containing 2 µg total proteins from cell-free extracts (I); same at different dilutions (II). After affinity purification only three bands reveal a specific recognition of the enzyme butyryl-CoA dehydrogenase-ETF at dilutions up to 1/3200 (III).

6.2 Immuno-gold labelling and electron microscopy techniques

The clean material obtained after affinity purification was used as antibody solution to localize butyryl-CoA dehydrogenase-ETF in the cellular compartments of *C. tetanomorphum*. The success of this *in situ* localization depended primarily on the efficient fixation of the cells, which are embedded in a polymerised resin before being cut in fine slices of less than 100 nm. The second antibody linked to a microscopic dense marker (colloidal gold conjugates) recognised the first antibody that targeted the protein situated inside or outside the cell. The photographs of figures 29 and 30 show cells of cylindrical and round forms resulting from transversal or longitudinal cuts. It is possible to distinguish a thick membrane from the homogenous cytoplasm with a high content of deposited granules that occupy approximately

60 to 70 % of the cell volume. The gold particles are randomly distributed within the cell and no marker is found in the surrounding, outside the bacteria. They are mainly found as single particles in the cytoplasma; some of them are inside the membrane. Out of 910 counts, 793 gold particles were found within the cytoplasm, 117 of them (10 %) inside the membrane.

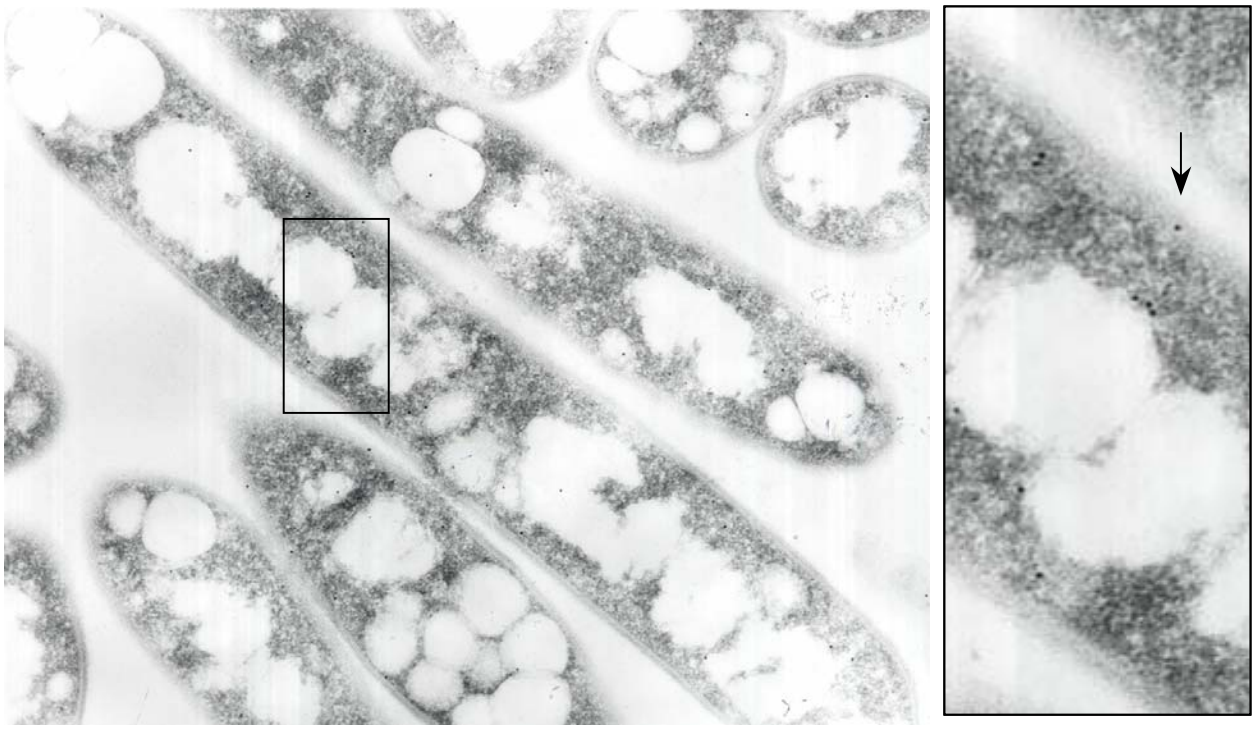


Fig. 29 Electron microscopy photographs of *C. tetanomorphum* cells. Dense black particle signals antigen antibody recognition involving butyryl-CoA dehydrogenase-ETF protein.
Note the abundant presence of storage vesicles, presumed to be poly-β-hydroxybutyrate.

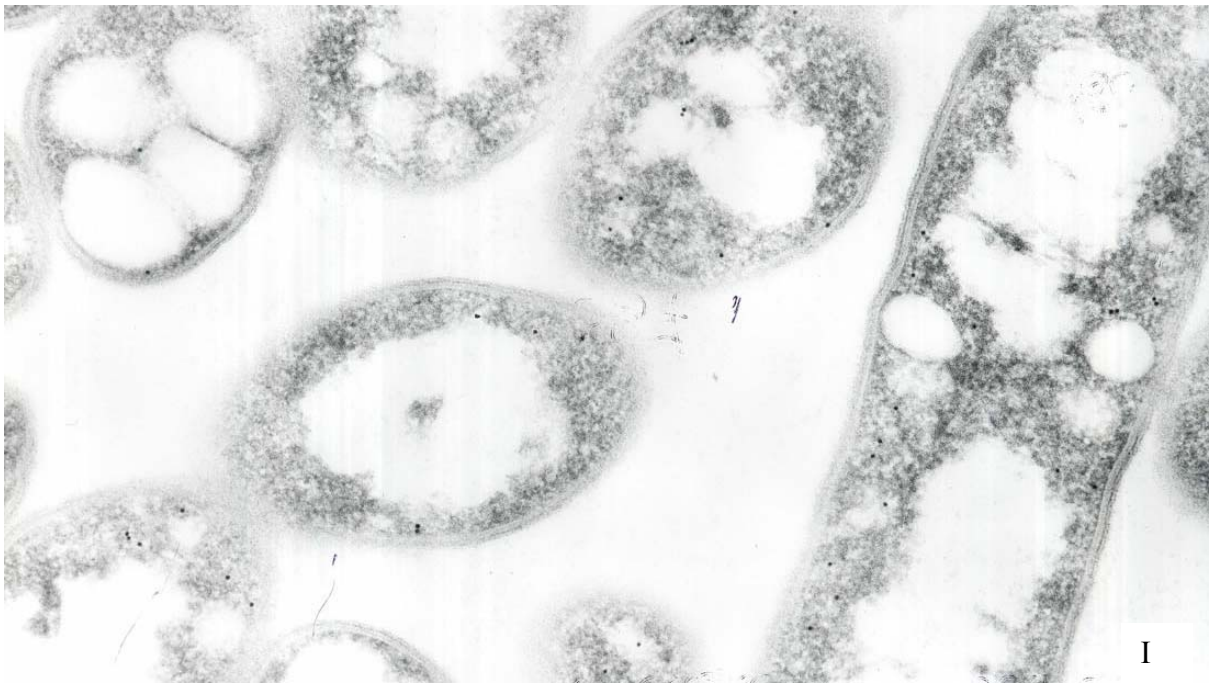
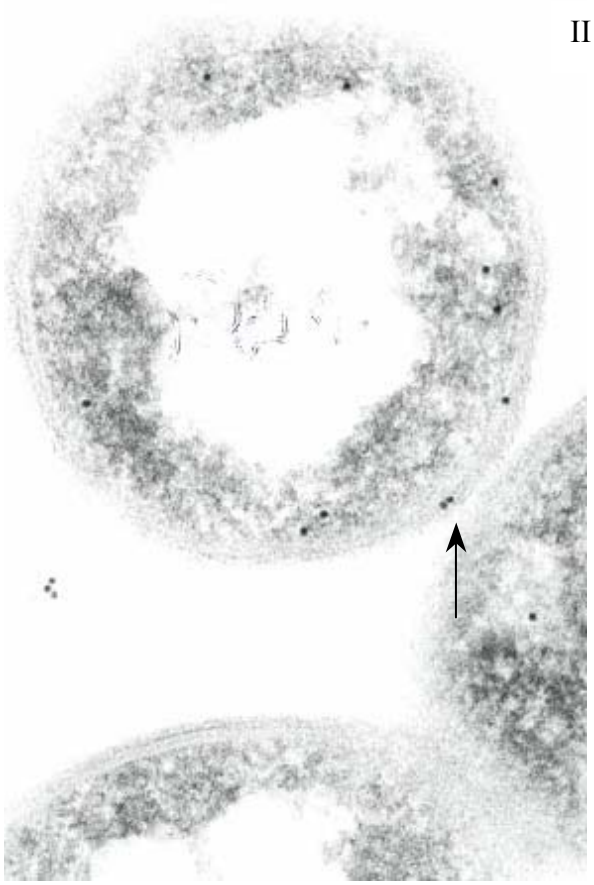


Fig. 30 Electron microscopy photographs of *C. tetanomorphum* cells with gold particles conjugates binding to butyryl-CoA dehydrogenase-ETF (I) and (II).

II



7 Cultures of *C. tetanomorphum* on glutamate and crotonate

Growth of *C. tetanomorphum* in standard glutamate medium (1 % yeast extract/0.2 M glutamate) is fast and copious, reaching $OD_{578} \geq 3$ in a time lapse of 15 hours. The doubling time in the referred standard media conditions is 40 minutes.

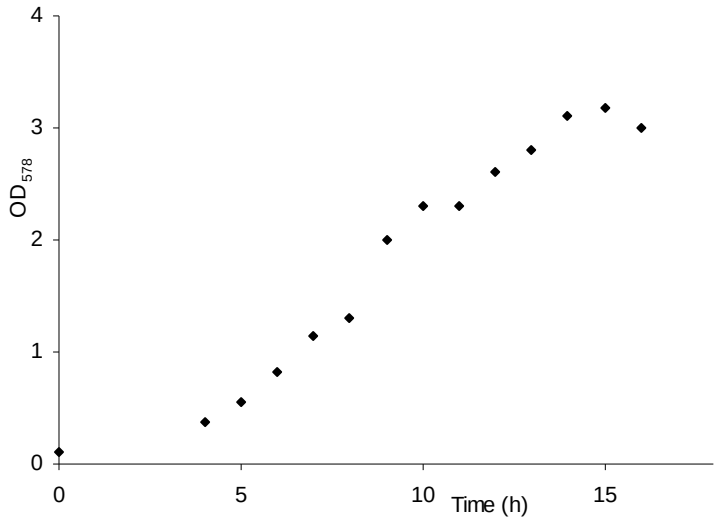


Fig. 31 *C. tetanomorphum* growth on glutamate standard medium

The growth of *C. tetanomorphum* was affected when crotonate was supplemented into the medium with low to moderately low nutritional components, a fast increment of growth response in short time and a longer stationary phase was observed. Growth experiments in presence of crotonate were done comparing the cultures without and with additional crotonate, analysing the consecutive passage of cultures from:

I. Poor-to-poor media passage: 0.1 % yeast extract (YE) / 0.02 M glutamate to
0.1 % YE / 0.02 M glutamate (Fig. 32).

II. Poor-to-rich media passage 0.1 % yeast extract (YE) / 0.02 M glutamate to
1 % YE / 0.2 M glutamate (Fig. 33).

The letter **A** refers to control cultures in absence of crotonate; letter **B** refers to cultures with added crotonate

All control cultures prepared under the same conditions with crotonate as the sole carbon source did not grow so far and there was little and unsustainable growth in cultures prepared with only yeast extract.

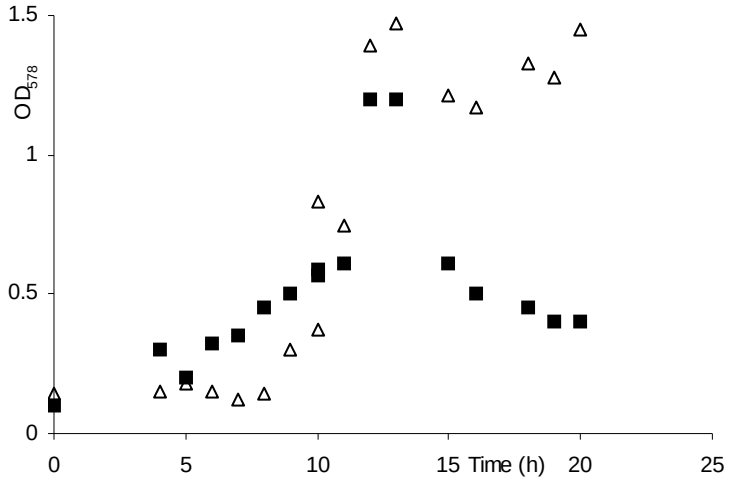


Fig. 32 Graph I: Poor-to-poor media passage of glutamate/crotonate cultures from *C. tetanomorphum*. Passage from 0.1 % YE/0.02M glutamate to 0.1 % YE/0.02M glutamate medium in presence (I B, hollow triangles) and absence (I A, bold squares) of 50 mM crotonate.

Distinctive growth patterns were found for both different media passage situations, in presence and absence of crotonate. Culture A and B were followed in parallel in order to observe only the effect of crotonate and not the changes in nutrient concentrations. In poor to poor media passage (Fig. 32), the lag phase of culture B with crotonate is longer than in the control (IA, Fig. 32) and growth continued long after the cells in control culture A entered into starvation phase. After 15 hours from begging of growth (In the same graph 32), culture B is able to resist longer time in the stationary phase than culture A lacking crotonate.

Those crotonate cultures resulting from a second passage, from low nutrient conditions to rich standard media (II B, Fig. 33), showed an exponential growth that lasts 5 hours longer than in their control cultures (II A, Fig. 33) comparable to the growth results from poor-to-poor media passage (I B, Fig. 32). The final OD value of culture B is higher (OD_{578nm} = 5.0) than in the control ($OD_{578\ nm}$ = 4.3) and the exponential phase is characteristic biphasic, becoming more steep in the middle of growth.

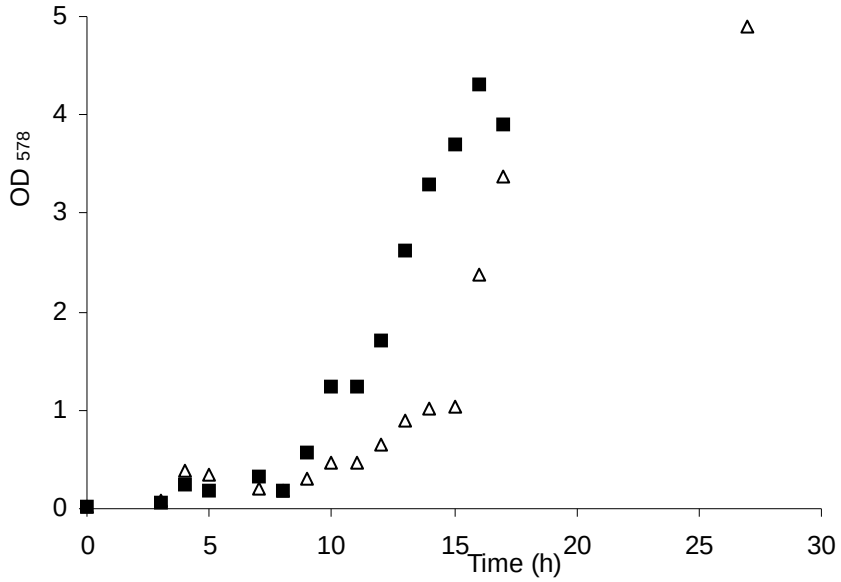


Fig. 33 Graph II: **Poor-to-rich media passage glutamate/crotonate cultures from *C. tetanomorphum*.** Passage from poor cultures (0.1 % yeast extract; 0.02 M glutamate) to rich standard media (1% yeast extract; 0.2 M glutamate), in presence (**II B**, hollow triangle) and abscence (**II A**, bold squares) of 50 mM crotonate.

The aim of this experiment was to show the increased growth rate of the bacteria when the shift of passage from low nutrients to richer media also included crotonate. Even though *C. tetanomorphum* could not be grown with crotonate as the sole carbon source, a considerably improvement in the growth behaviour has been noted when this carboxylic acid was present. Under light microscope there were abundant vesicles found inside the cells of crotonate growing cultures. These vesicles probably contain storage compounds like poly-β-hydroxybutyrate. Crotonate consumption had been previously correlated with proton production in *C. kluyveri* (Thauer et al, 1968a). In addition, acetate CoA-transferase is predicted to be involved in crotonate fermentation after the genome analysis of the referred micro-organism (Seedorf et al, 2008).

II. Lactyl-CoA dehydratase and β-Alanyl-CoA ammonia lyase from *Clostridium propionicum*

1 Cultures of *C. propionicum*

1.1 Growth on alanine and threonine

In the following experiment growth response of *C. propionicum,* an anaerobic bacteria able to ferment three carbons amino-acids with acrylyl-CoA as intermediate, is shown.

C. propionicum grows in yeast extract supplemented media containing either alanine or threonine as main substrate for fermentation processes. Both cultures present the same initial growth phase including a lag phase of about 3 hours followed by an exponential phase that is about one hour longer in the case of alanine fermentation. The doubling time in alanine cultures, as well as in threonine cultures supplemented with 0.5 % yeast extract is 1 ½ hour.

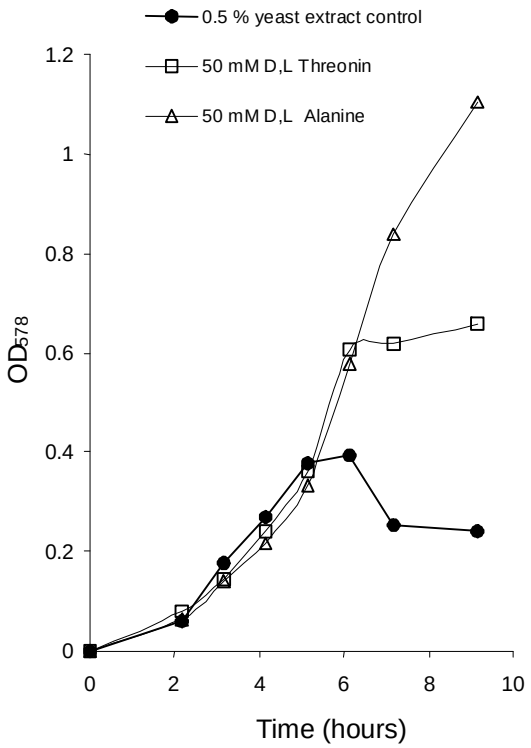


Fig. 34 L-alanine and D,L-threonine *C. propionicum* growth. The control refers growth on 0.5 % (w/v) yeast extract

Cultures containing 50 mM D,L-alanine grow better than those containing equal concentrations of D,L-threonine (Fig. 34). In medium with only 0.5 % yeast extract, cells enter into starvation conditions at around 5 hours after growth; compared to the control D,L-threonine growth is extended to an additional hour and remains at constant OD values in an apparently long stationary phase. No growth was detected in cultures containing L-threonine so *C. propionicum* is suppose to use only D-threonine as fermentable substrate. The increased growth on D,L-alanine can be explained by the presence of an alanine specific racemase that would allow the micro-organism to convert D-alanine into L-alanine; this can not be assumed for threonine because there is no growth on L-threonine alone.

1.2 Growth on cyclopropane carboxylic acid

In the radical mechanism proposed for the dehydration reaction of lactyl-CoA to acrylyl-CoA catalysed by the (*R*)-lactyl-CoA dehydratase (Buckel, 1996) the effect of cyclopropane carboxylic acid (CPC) was investigated in L-alanine cultures of *C. propionicum*. Cyclopropane carboxylic acids have been used as molecular stopwatches for timing radical reactions due to ring opening of the cyclopropylcarbinyl radical (Liu et al, 1993). The hypothesis was that a CPC-CoA derivate could act as a radical substrate causing inhibition of growth in the bacteria.

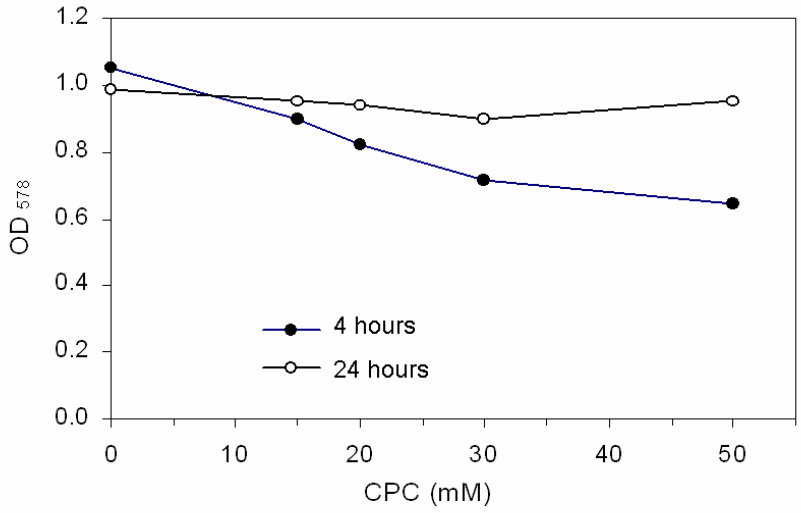


Fig. 35 Gowth of *C. propionicum* in presence of cyclopropane carboxylic acid (CPC). Medium contains 0.5 % (w/v) yeast extract and 28 mM L-alanine. Endpoint OD_{578nm} measured at 4 and 24 hours of growth. Control contains 0 M CPC.

C. propionicum cultures reached a maximal OD_{578nm} of 1 at already 4 hours of growth in 0.5 % yeast extract and 28 mM D,L-alanine. The OD values of all cultures in presence of cyclopropane carboxylic acid (CPC) at end of growth time (24 hours) present no difference in opposite to those values reached in the first 4 hour of growth. Up to 20 mM CPC concentration the growth is apparently slowred, presenting lower OD values than the control, but later they adjust to comparable final optical densities (Fig. 35).

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

The anaerobic enzyme lactyl-CoA dehydratase (LCD) composed of component D (dehydratase active site) and component A (activator) was purified under strict reducing conditions (1 mM DTT, 1mM dithionite) from cell extracts of *C. propionicum*. Repurification of component D could be effectively achieved immediately after the Q-Sepharose ion exchange column (Fig 36). The activator or component A was present in two dark green-brown fractions: F28 and F29 at 0.28 molar salt concentrations. Component D came later, at 0.43 molar salt concentration, in pale green fractions spread out between fraction numbers F44 and F46.

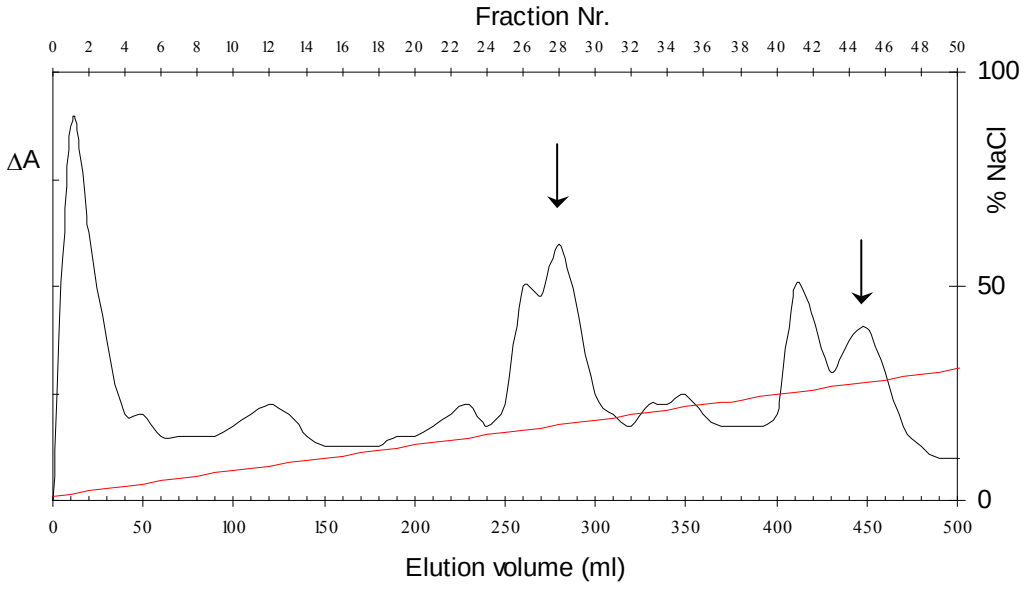


Fig. 36 Q-sepharose chromatogram after loaded with *C. propionicum* crude extracts. Elution of lactyl-CoA dehydratase is indicated with arrows (first elution of Component A fractions, second elution of Component D fractions).

The purification of lactyl-CoA dehydratase (LCD) was followed using a qualitative assay based on MALDI mass spectrometry to identify substrates and product of the assay reaction. In this sense, the lactyl-CoA dehydratase activity was tested in different directions depending on the purification stage. At the begining and at the end of the purification, equilibrium I (Fig. 37) starting with LCD specific substrate lactyl-CoA (reaction I) was assayed detecting activity in the high diluted free extract of *C. propionicum* as well as in mixtures of pure protein components of the LCD system. In order to visualize the reaction in the direction of lactyl-CoA formation, the incubation time of equilibrium II was prolonged 5 min during the purification process.

Propionyl-CoA is rapidly formed due to the co-existence of acrylyl-CoA reductase in crude extracts of *C. propionicum*, leading the equilibrium of dehydration to the following reduction reaction step. To discriminate activity of both enzymes substrates were alternatively added into the assay.

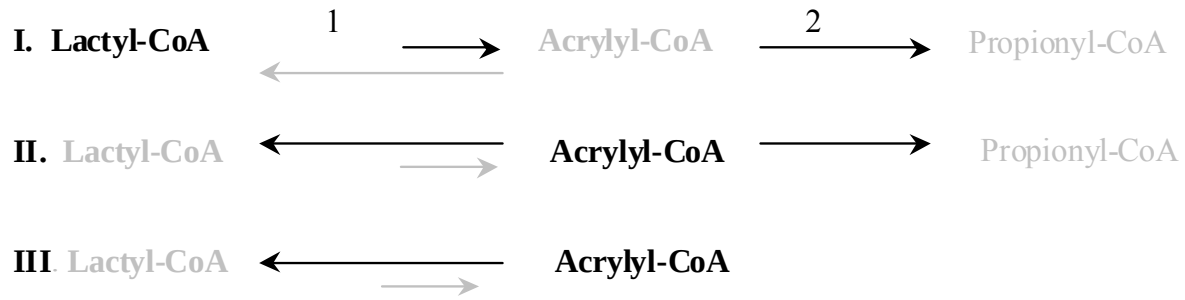


Fig. 37 Lactyl-CoA dehydratase equilibrium of the reactions assayed during purification in the anaerobic MALDI assay. Substrates are in bold, products in grey colour. Enzymes: **1** lactyl-CoA dehydratase, **2** acrylyl-CoA reductase. Reactions **I** and **II** after cell-free extract addition; reaction **III** involves only the purified lactyl-CoA dehydratase.

As purification advanced, the products of the assayed reaction in the MALDI test were mixed, that is, beside lactyl-CoA, propionyl-CoA was also formed due to the additional reaction of the enzyme acrylyl-CoA reductase when present as impurity in the enzyme pool. At the end of the purification, pure LCD protein samples no longer required addition of free extract into the assay, exhibiting only one product formation: lactyl-CoA. Equilibrium III, being rapid and the most sensitive of all, was selected as the standard assay for further analysis of the purified enzyme.

To summarize LCD purification, the two principal pools were collected: fractions 28-29 (pool 1) and fractions 44-46 (pool 2) from the first Q-sepharose column, were cross mixed in order to have a 1:1 ratio of component A to component D of the lactyl-CoA dehydratase complex in order to measure enzyme activity. Lactyl-CoA dehydratase component D in the pool fractions 44-46 (pool 2) was finally purified through a second Phenyl Sepharose column. Active fractions were tested in the same anaerobic MALDI assay with acrylyl-CoA as substrate and at this point no further cell-free extract was needed to obtain the hydration product lactyl-CoA (Fig. 38).

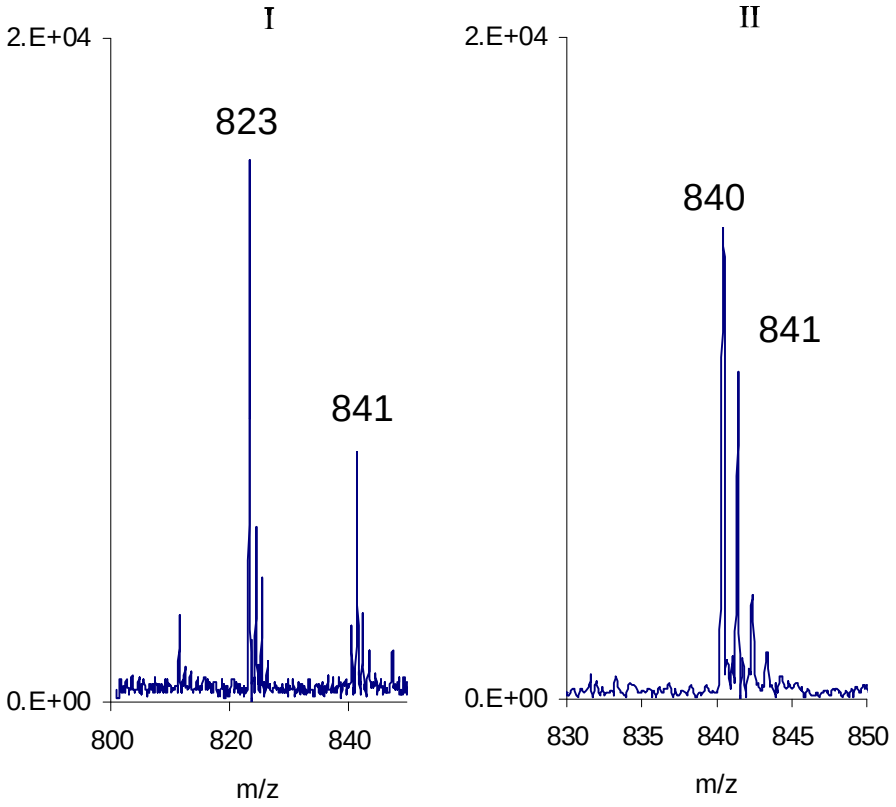


Fig. 38 MALDI spectra from the reaction products of lactyl-CoA dehydratase and β-alanyl-CoA ammonia lyase activity. Assays were started with the common substrate acrylyl-coA (m/z = 823). In presence of high ammonium sulphate concentration both enzymes react in the crude extract until formation of the respective products: lactyl-CoA (m/z = 841) and β-alanyl-CoA (m/z = 840) (**II**), whereas lactyl-CoA is the unique product in the reaction mixture containing purified lactyl-CoA dehydratase components A and D (**I**).

In conclusion the activity of lactyl-CoA dehydratase could be measured by identifying lactyl-CoA and propionyl-CoA by MALDI-TOF mass spectrometry. The direct dehydration product acrylyl-CoA could not be identified directly, but indirectly by reduction to propionyl-CoA or by addition of ammonia to β-alanyl-CoA. Cyclopropane dicarboxylate could not be converted to the CoA thioester using propionyl-CoA transferase and acetyl-CoA as detected by MALDI.

3 Purification and molecular characterization of β-alanyl-CoA ammonia lyase

In the process of lactyl-CoA dehydratase purification, an unexpected mass peak of only one mass difference appeared beside lactyl-CoA in the MALDI spectra. It was found that instead of a water molecule (18 Da) as expected for lactyl-CoA (m/z = 841), ammonia (17 Da) was added to acrylyl-CoA, forming β-alanyl-CoA (m/z = 840), shown in figure 38. The enzyme catalysing the ammonification of acrylyl-CoA was investigated in different cultures. A 300-fold higher activity (112 U mg^{-1}) was found in cell-free extracts from β–alanine grown cells in contrast to the low activity (0.38 U mg^{-1}) observed in the same cells grown on standard L-alanine cultures. The high activity present in free extracts from β-alanine grown cells made possible a one step purification recovering almost 50 % of the protein purified until homogeneity after running one Source 15-Q column. As seen in SDS-PAGE on figure 39, only one band bellowed the 20 kDa band was obtained after loading the final pool of purified β-alanyl-CoA ammonia lyase with a specific activity of 1033 U mg^{-1}. In table 19 is schematized the high yield purification process to obtain final 10 mg protein from only 2 g *C. propionicum* wet cells.

Table 19: β-alanyl-CoA ammonia lyase purification from 2 g *C. propionicum* wet cells grown on β-alanine

Material	Total protein mg	Total activity U	Specific activity U mg^{-1}	Yield %
CFE	183	22,661	124	100
Source 15-Q	10	10,208	1,033	45

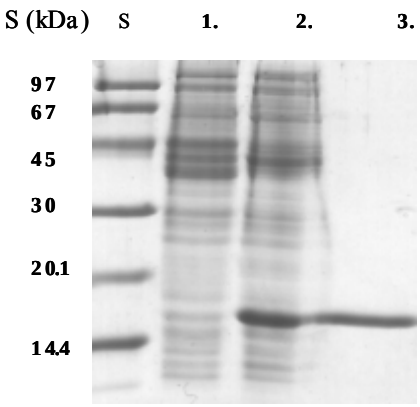


Fig. 39 SDS-PAGE: Extract of *C. propionicum* cells grown on L-alanine (**1**) and β-alanine (**2**). Purified β-alanyl-CoA ammonia lyase after Source 15-Q (**3**). **S** protein standard.

The molecular mass of the enzyme β-alanyl-CoA ammonia lyase was determined by Superdex 200 size exclusion chromatography (Fig. 40).

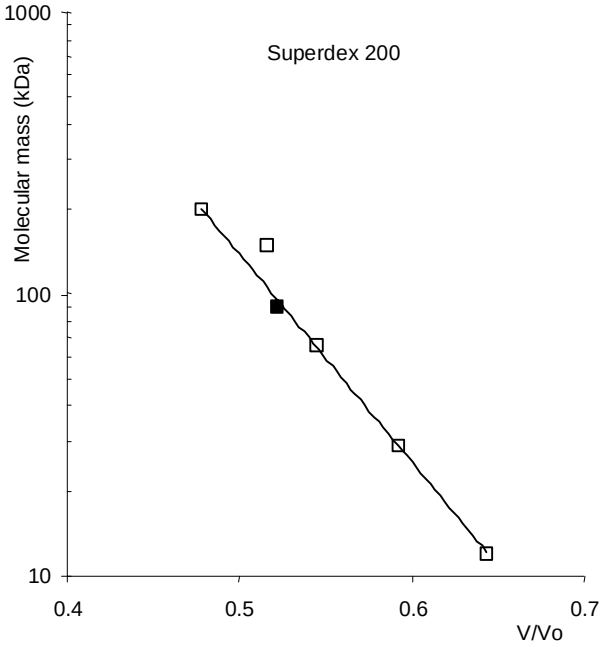


Fig. 40 Size exclusion chromatography of the enzyme β-alanyl-CoA ammonia lyase. The followed standards are depicted in hollow squares: cytochrome *c* (12 kDa), carbonic anhydrase (29 kDa), bovine serum albumine (66 kDa) and β-amylase (200 kDa), alcohol dehydrogenase (150 kDa). The enzyme β-Alanyl-CoA ammonia lyase is depicted in bold square.

The elution of the enzyme from the column at 16 ml was calibrated with protein standards of masses between 12 and 200 kDa giving a final value of 95 ± 5 kDa. The monomeric enzyme has a subunit molecular mass of 16 kDa as seen in SDS-PAGE and may exist in a homohexameric α_6-structure (95 kDa molecular size). Recent studies on the ACL crystal structure confirme this observation (Heine & Reuter, In preparation).

4 β-Alanyl-CoA ammonia lyase activity

The addition of ammonia to the double bond of acrylyl-CoA is easily detected following the decrease of absorbance at 259 nm; on the contrary, the reverse reaction was not. In order to measure the elimination of ammonia from β-alanyl-CoA it was necessary to perform an anaerobic assay using reduced methylviologen as a redox indicator for the second helping enzyme. By coupling the reaction with acrylyl-coA reductase the ammonia lyase dependent conversion of β-alanyl-CoA to propionyl-CoA could be detected at 600 nm.

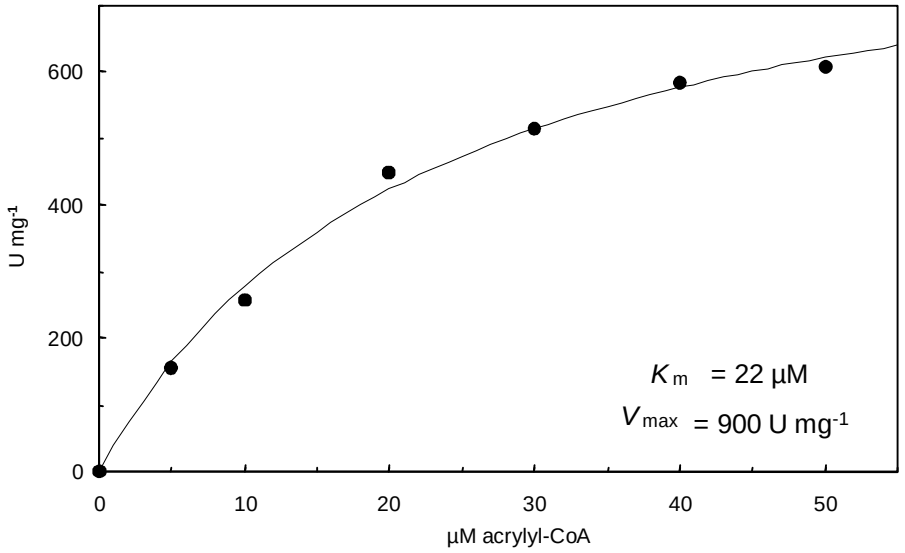


Fig. 41 Michaelis-Menten kinetics of β-alanyl-CoA ammonia lyase activity with the substrate acrylyl-CoA in presence of 100 mM ammonia.

In presence of ammonia (100 mM) the acrylyl-CoA concentration at which the enzyme reacts at half of its maximal velocity V_{max} = 900 ± 43 U mg^{-1}, is K_m= 22 ± 3.5 µM. Nearly identical was the K_m at 50 mM ammonium chloride of 26 ± 4 µM. These values were established for acrylyl-CoA concentrations between 5 and 50 µM (Fig 41).

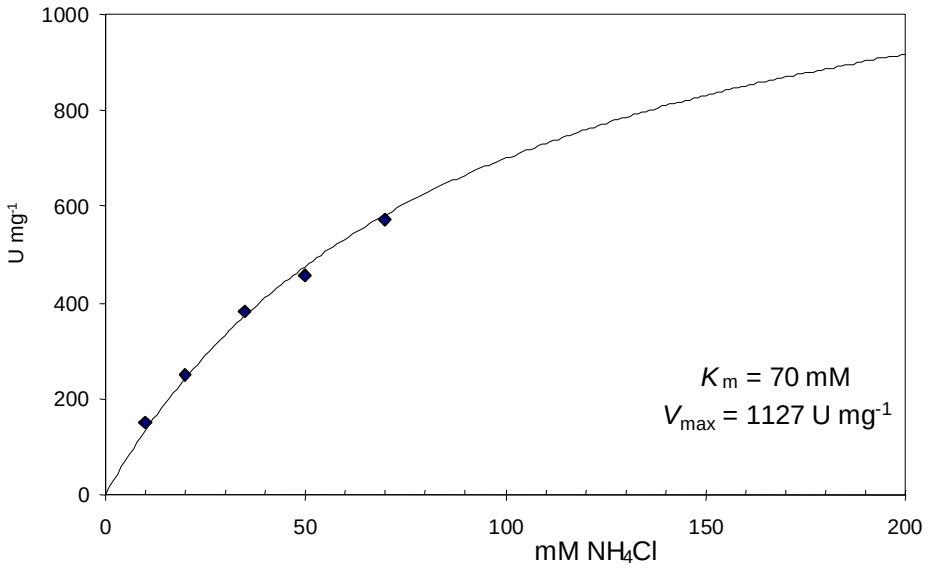


Fig. 42 Michaelis-Menten kinetics of β-alanyl-CoA ammonia lyase activity with the substrate ammonia in presence of 150 µM acrylyl-CoA.

To measure the K_m of ammonia at non-limiting second substrate concentrations (acrylyl-CoA 150 µM) the same assay was performed at 280 nm, where the double bond of acrylyl-CoA exhibits maximal absorbance. In this case the extinction coefficient of the difference acrylyl-CoA/β-alanyl-CoA used for activity calculations was $\Delta\varepsilon = 3.5\ mM^{-1}\ cm^{-1}$. The result shown in figure 42 demonstrate that β-alanyl-CoA ammonia lyase has a K_m of 70 ± 5 mM for ammonia at 150 µM acrylyl-CoA

In the reverse reaction, the enzyme β-alanyl-CoA ammonia lyase splits 3-aminopropionyl-CoA into the end products acrylyl-CoA and ammonia. This was made possible in a coupled assay with acrylyl-CoA reductase and methylviologen as redox indicator. The K_m value for β-alanyl-CoA as the sole substrate was assayed at different ammonium chloride concentrations. At zero ammonia concentration the K_m for β-alanyl-CoA was determined to be 0.2 ± 0.06 mM at a V_{max} of 94 ± 11.2 U mg^{-1} (Fig. 43). The data was obtained from measurements of 8 different β-alanyl-CoA concentrations ranging from 30 to 600 µM. In the presence of 10 and 100 mM ammonium chloride the respective K_m values were 0.23 ± 0.029 mM and 0.165 ± 0.066 mM, with a V_{max} of 81 ± 4.2 U mg^{-1} at 10 mM and 27 ± 3.4 U mg^{-1} at 100 mM ammonia. A summary of these results is shown in table 20.

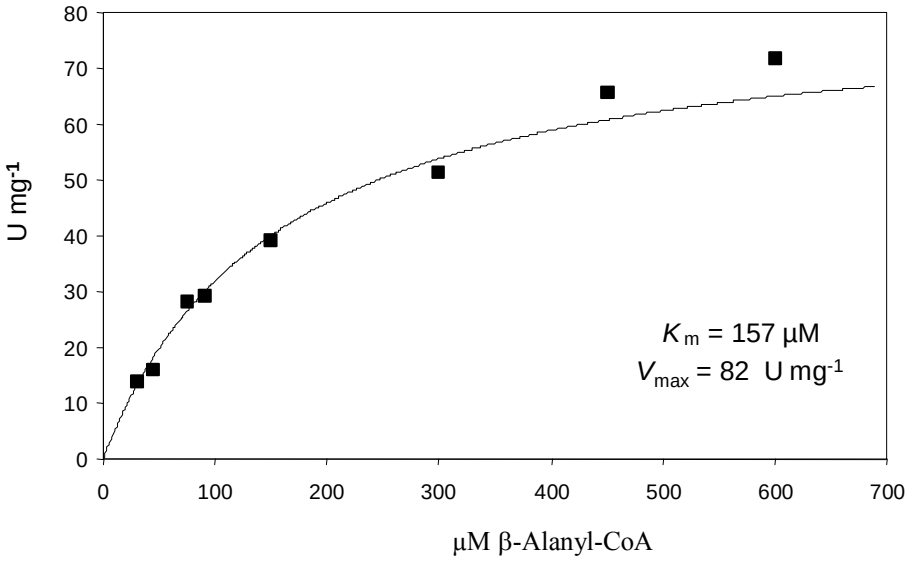


Fig. 43 Michaelis-Menten kinetics of β-alanyl-CoA ammonia lyase activity with the substrate β-alanyl-CoA in absence of ammonia.

Table 20: Summary of the kinetic parameters of the enzyme β-alanyl-CoA ammonia lyase

Substrate	Co-substrate/Inhibitor	app. K_m	V_{max}	K_{cat}	K_{cat}/K_m
		mM	U mg^{-1}	s^{-1}	μM^{-1} s^{-1}
Acrylyl-CoA	50 mM NH_4Cl	0.023 ± 0.04	730	195	85
Acrylyl-CoA	100 mM NH_4Cl	0.026 ± 0.04	956	255	98
NH_4Cl	150 µM Acrylyl-CoA	70 ± 5	1127	301	0.0043
β-Alanyl-CoA	-	0.21 ± 0.03	95	25	0.012
β-Alanyl-CoA	10 mM NH_4Cl	0.23 ± 0.03	81	-	-
β-Alanyl-CoA	100 mM NH_4Cl	0.16 ± 0.07	27	-	-

The Lineweaver-Burk plot of figure 44 displays the kinetic behaviour of β-alanyl-CoA ammonia lyase in presence of low and high ammonia salt concentrations. There is a change in the velocity of the reaction: the 1/V values are different while the intersection point in the abscissa coordinate remains the same. Ammonia may produce a non-competitive inhibitory effect in the catalytic activity of ACL leading to acrylyl-CoA formation.

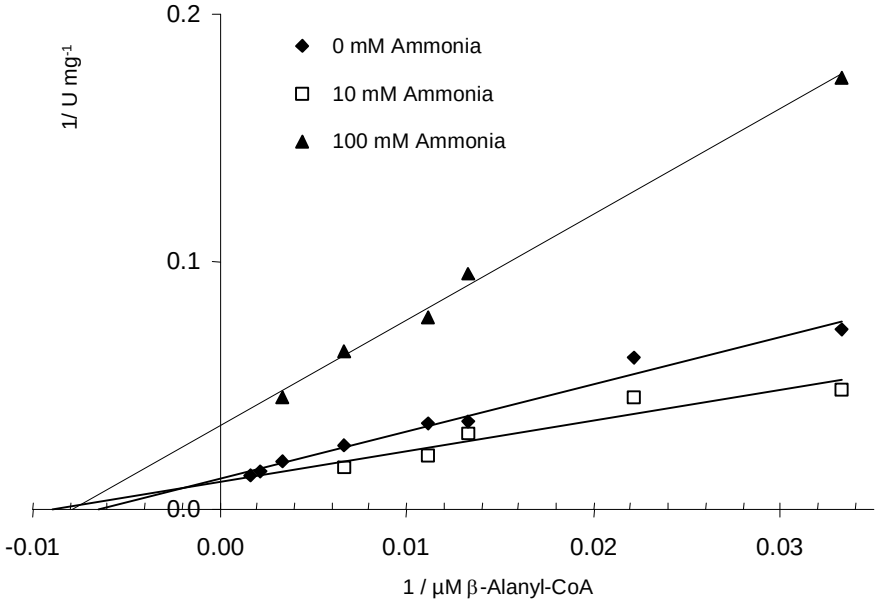


Fig. 44 Inhibitory effect of ammonia in β-alanyl-CoA ammonia lyase

Testing different ammonia donors for β-alanyl-CoA ammonia lyase, the enzyme reacted only with methylamine, except for ammonium chloride which was the positive control. After one minute, the products of the reaction analysed by MALDI presented signals of m/z = 840 for β-alanyl-CoA and m/z = 854 Da (840 + 14) for (β-methylamino-) propionyl-CoA, and none for glycine and hydroxylamine.

Discussion

1 Lactyl-CoA dehydratase and β-alanyl-CoA ammonia lyase from *Clostridium propionicum.*

L-Alanine is degraded by *C. propionicum* in a fermentation process where 2 molecules of propionate are formed in the reductive branch for every molecule of pyruvate that is oxidized to acetate in order to conserve 1 molecule of ATP. All enzymes are identified and the whole process is described to completion; however the high free energy released by the fermentation of L-alanine ($\Delta G°' = -138$ kJ (mol acetate)$^{-1}$) indicates that there is still a potential use of energy to produce up to 2 ATP molecules (Cardon & Barker, 1947; Schweiger & Buckel 1984; Hetzel et al, 2003).

Lactyl-CoA dehydratase is the enzyme that catalyses the elimination of a water molecule from (*R*)-lactyl-CoA in the alanine fermentation pathway. The catalytic mechanism of this extremely oxygen sensitive enzyme has been explained by mean of radical intermediates (Buckel, 2001; Buckel et al, 2005). Catalysis starts with an electron transfer followed by the α-hydroxyl group abstraction of the substrate (*R*)-2-hydroxypropionyl-CoA [(*R*)-lactyl-CoA] and H^+ elimination of the β-carbon from the molecule. This dehydration reaction is difficult because the hydrogen of the methyl group at the 3 position is not acidic enough to get released; thus the formation of an enoxy radical lowers the pK_a of the inactive *β*-carbon allowing a *facile* de-protonation (Smith et al, 2003; Kim et al, 2008). There are different enzymes catalyzing a similar dehydration reaction of 2-hydroxyacid via their CoA-derivates in anaerobic bacteria *e.g.* the 2-hydroxyglutaryl-CoA dehydratases from *C. symbiosum*, *A. fermentans* and *F. nucleatum* (Hans et al, 1999; Schweiger et al, 1987; Klees et al, 1992), the (*R*)-phenyllactate dehydratase from *C. sporogenes* (Dickert et al, 2002) and the 2-hydroxyisocaproyl-CoA dehydratase from *C. difficile* (Kim et al, 2004; Kim et al, 2008).

Lactyl-CoA dehydratase is a complex composed of an activator, also named component A, and a dehydratase or component D, where the active centre of the enzyme lies. The component D of the (*R*)-lactyl-CoA dehydratase from *C. propionicum* is a heterodimer with similar constitution like that of 2-hydroxyglutaryl-CoA deydratase from *C. symbiosum*, containing each of them two $[4Fe\text{-}4S]^{2+}$ clusters, FMN and riboflavin. The best characterized component A is that from *A. fermentans*, an homodimeric protein having one $[4Fe\text{-}4S]^{2+}$

cluster (Buckel, 2001; Kuchta & Abeles, 1985; Kuchta et al, 1986). Dehydratase activity requires the presence of both components A and D, $MgCl_2$, ATP and a reducing agent (DTT or Dithionite). Only catalytic amounts of ATP are necessary to initiate the reaction with the activation of component D by component A. The electron involved in the catalysis is recycled for many turnovers upon activation and the reaction becomes self-sustaining (Buckel, 2001; Kim et al, 2004; Buckel et al, 2004).

The (*R*)-lactyl-CoA dehydratase enzyme from *C. propionicum* is still difficult to purify mainly because of its lability in the presence of oxygen. Different trials to assay the spectrophotometric activity of the combined reactions of (*R*)-lactyl-CoA dehydratase and acrylyl-CoA reductase were unsuccessful because the dithionite required for the activation of the dehydration directly reduces acrylyl-CoA without using the indicator NADH. Though it was not possible to follow the dehydration-and-reduction conversion of lactyl-CoA to propionyl-CoA in a continuous spectrophotometric assay, the products of the same coupled reaction were successfully identified by MALDI mass spectrometry. In the MALDI assay testing (*R*)-lactyl-CoA dehydratase alone, the dehydration product acrylyl-CoA was never found. On the other hand, using acrylyl-CoA as a substrate, (*R*)-lactyl-CoA could be detected if the enzyme preparation was devoid of acrylyl-CoA reductase. The dehydration of (*R*)-lactyl-CoA is a reversible process that strongly tends to the physiological reverse reaction, *i.e.* to lactyl-CoA formation. In order to pull the catalysis to the dehydration product, acrylyl-CoA reductase must be present; thus *C. propionicum* cell-free extract containing this helper enzyme was added during the purification process to visualize propionyl-CoA formation, the indirect product of the coupled reaction.

In L-alanine fermentation the intermediate acrylyl-CoA is rapidly reduced by acrylyl-CoA reductase; but acrylyl-CoA is also substrate of another very efficient enzyme: β-alanyl-CoA ammonia lyase, which facilitates a nucleophilic attack of ammonia to the double bond of the referred enoyl-CoA (eq. 9).

$$\text{Acrylyl-CoA} + NH_4^+ \rightarrow \beta\text{-Alanyl-CoA} \quad \text{(eq. 9)}$$

The enzyme β-alanyl-CoA ammonia lyase (ACL) was first described in *C. propionicum* by Vagelos and Stadtmann in 1958 (Vagelos et al, 1958). The author reported the protein purification from α-alanine grown cells in a four-step procedure using alternate ammonium sulphate treatments at different saturation amounts. With almost 30 % recovery, ACL was

purified with a final activity of 58 U mg^{-1} starting from 0.75 U mg^{-1} in the sonic extract; but the purification from β-alanine grown cells following the same procedure only resulted in a 3-fold increased specific activity (400 U mg^{-1}). In the current work, an 8-fold purification factor was achieved starting from β-alanine grown cells with a final yield above 50 %. There is another report for an analogous enzyme that has been purified from a lysine fermenting *Clostridium*; high level of L-3-aminobutyryl-CoA deaminase (EC 4.3.1.14) activity (370 U mg^{-1}) was described catalyzing crotonyl-CoA formation (Jeng and Barker, 1974).

Interestingly, β-alanyl-CoA ammonia lyase (ACL) is the only example of an enzyme allowing an amino acid like β-alanine to be almost immediately converted to a highly reactive intermediate like acrylyl-CoA. Therefore it is important to reconsider this reaction as a new shuttle of CoA intermediates in the alanine fermentation of *C. propionicum*. β-Alanine can be fermented prior activation to an acyl-CoA derivate by the action of a CoA-transferase (β-alanyl-CoA transferase, (Selmer T., unpublished results). The fermentation would yield up to 2 ATP judging from the free energy release from the whole process ($\Delta G^{\circ\prime}$ = -155 kJ mol^{-1}). ACL activity is present in β-alanine as well as in D,L-alanine grown cells, but only the shift of the carbon source to β-alanine in cultures induces a 300-fold increment of its activity. There were 2 genes identified encoding for ACL: *acl*1, which is responsible for the basal activity in presence of L-alanine (0.44 U mg^{-1}) and *acl*2, which is induced during β-alanine fermentation (143 U mg^{-1}). The enzyme was easily purified from β-alanine *C. propionicum* grown cells using only one anion exchange column (15Q-source) utilizing the rapid decrease in acrylyl-CoA absorbance was followed at 259 nm. A native size of 95 ± 5 kDa was determined by Superdex 200; inferred from SDS PAGE, the enzyme is composed of 16 kDa subunits in a α_6-homohexameric structure. The specific ACL activity was characterized with a K_m of 23 µM for acrylyl-CoA and a k_{cat}/K_m of 10^7 M^{-1} S^{-1} (Herrmann et al, 2005). This latter parameter (k_{cat}/K_m) shows the enzyme's high efficiency to remove acrylyl-CoA from solution. The question on its physiological role arises, but the answer may require a deeper study of protein synthesis and gene expression during fermentation. One of the most accepted reasons is the rapid cell clearance of free acrylyl-CoA in order to avoid cross reaction with other cellular compounds. Acrylamide has shown to be toxic because of the highly reactive double bound near the carbonyl group (Tareke et al, 2000; Ötles, 2004). The analogue compound acrylyl-CoA is supposed to react even more strongly with nucleophiles due to a greater electron carbonyl character of the thiol ester as compared to an amide.

ACL is considered of particular interest for the manufacturing of 3-hydroxypropionic acids (3-HP). Upon hydrogenation, 3-HP results into acrylic acid. Acrylates and metacrylates (salt and esters of acrylic acids) are the building blocks of a great variety of polymers like for example the plastic poly(methyl methyacrylate), commercially known as Plexiglas®. Lactyl-CoA dehydratase has also been considered for the synthesis of 3-hydroxypropionic acids but because of its oxygen lability, alternative pathways for 3-HP production are continuously being investigated. Progress on this research field has been achieved in collaboration with Cargill Incorporated.

As show in figure 45, *β*-alanine can readily isomerise to L-alanine by action of an alanine 2,3-aminomutase. It can also be isomerised by an ω-amino acid:pyruvate aminotransferase (EC 2.6.1.18). By bio-engineered transformed cells, it is possible to convert β-alanine to 3-hydroxypropionate (3-HP) through a malonate semialdehyde intermediate, whereby β-alanyl-CoA ammonia lyase offers an alternative path reaction to skip the oxygen sensitive step of lactyl-CoA dehydratase reaction (Fig. 45).

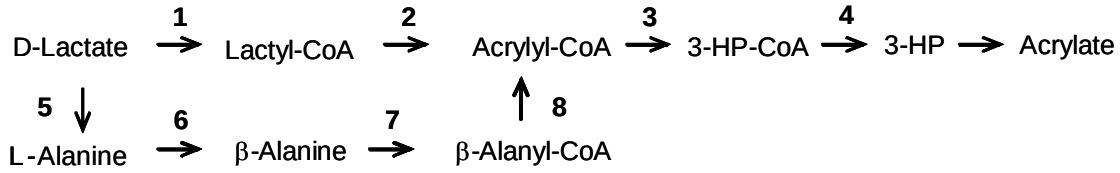


Fig. 45 Synthesis of 3-hydroxypropionic acid (3-HP). The enzymes catalyzing the reactions are: **1** lactate CoA-transferase, **2** (*R*)-lactyl-CoA dehydratase **3** 3-hydroxypropionyl-CoA dehydratase, **4** 3-hydroxypropionyl-CoA hydrolase and CoA transferase, **5** D-lactate dehydrogenase and alanine aminotransferase, **6** alanine 2,3-aminomutase, **7** β-alanyl-CoA transferase, **8** β-alanyl-CoA ammonia lyase.

2 Butyryl-CoA dehydrogenase-ETF from *Clostridium tetanomorphum*

In the 3-methylaspartate glutamate fermentation pathway of *C. tetanomorphum* (eq. 10), only 3 ATP molecules are conserved via SLP, although thermodynamic calculations attest a greater potential of energy that can be additionally conserved (Buckel, 2001; Thauer et al, 1977; Herrmann et al, 2008).

5 glutamate$^-$ + 6 H_2O + 2 H^+ → 5 NH_4^+ + 5 CO_2 + 6 acetate$^-$ + 2 butyrate$^-$ + H_2; (eq. 10)

$\Delta G^{\circ\prime}$ = - 63.5 kJ mol^{-1} glutamate; 106 kJ mol^{-1} ATP; $\Delta G^{\circ\prime}$ = - 317 kJ reaction^{-1}

NADH is the electron carrier providing the reducing equivalents in the reductive branch of the process and reduced ferredoxin must be reoxidised in order to maintain the fermentation equilibrium. These facts supported the suggestion on the existence of a NADH:ferredoxin oxidoreductase in *C. tetanomorphum* that may conserve energy in form of an electrochemical gradient. In this sense, at beginning of this work, two proteins were investigated to be possibly involved with a membrane dependent NADH activity: butyryl-CoA dehydrogenase and a novel NADH dehydrogenase. The latter enzyme could be successfully purified and was identified as an Rnf-type protein in membrane extracts of *C. tetanomorphum*. The Rnf complex originally described in *Rhodobacter capsulatus* is proposed to function as an oxidoreductase that reduces ferredoxin at the expense of the membrane potential generated from electron donors like NADH (Kumagai et al, 1997; Jouanneau et al, 1998; Boiangiu et al, 2005). Nevertheless, butyryl-CoA dehydrogenase (crotonyl-CoA reductase) had also been suggested to participate in energy conservation, similar to anaerobic respiration, based on the high redox potential difference observed between the redox couple crotonyl-CoA/butyryl-CoA ($E^{0\prime}$ = -10 mV) and the NAD^+/NADH ($E^{0\prime}$ = -320 mV) pair. The enzyme described in this work was purified under oxic conditions following only the oxidation of butyryl-CoA with ferricenium hexafluorophosphate, which is an electron acceptor generally used to screen acyl-CoA dehydrogenase activity (Lehman & Thorpe, 1990). The N-terminal sequence determination and molecular composition of the native enzyme shows a great similarity of the enzyme to the propionyl-CoA dehydrogenase isolated from *C. propionicum* (Hetzel et al, 2003). Both enzymes have the same subunit composition: α-subunit (propionyl-CoA or butyryl-CoA dehydrogenase), a large ETF β-subunit (36 kDa) and a small ETF γ-subunit (28 kDa), in a possible $\alpha_2\beta\gamma$ structure. A closely related enzyme of butyryl-CoA dehydrogenase with identical characteristics was also purified from *C. kluyveri* (Li et al 2008) and from *C. pascui* (Mai, 2007) using different purification procedures. The protein purified from *C. tetanomorphum* has a molecular mass of 360 kDa and contains less than one mol FAD as

cofactor. One of the most important features is that butyryl-CoA and propionyl-CoA dehydrogenases are isolated in a complex form with electron transfer flavoprotein (ETF), a very versatile electron carrier that has been described as participating in one electron and in two electron transfer reactions. The electron transfer flavoprotein (ETF) is in tight interaction with some dehydrogenase enzymes and can be reduced with very low potential electron donors (Pace & Stankovich, 1987). Its reduction potential can vary considerably depending on the flavin surrounding within the three-dimensional protein structure. In *M. methylotrophus* (sp. W_3A_1) ETF the highly positive midpoint reduction potential of the flavin oxidized/semiquinone couple ($E'1$ = + 153 mV) is able to stabilize the anionic flavin semiquinone, which is far away from the detected midpoint potential for the semiquinone/dihydroquinone pair ($E'2$ = < - 250) (Talfournier et al, 2001). In contast, ETF flavin redox species of *P. denitrificans* and human ETF have narrower differences. Under aerobic conditions *P. denitrificans* develops an electron-transport system comparable to the eukaryotic mitochondrial electron transport chain (Watmough et al, 1992). While these are related to mitochondrial dehydrogenases, *M. methylotroph* ETF only accepts electrons from trimethylamine dehydrogenase, an iron sulphur protein found in methylotrophic bacteria (Huang et al, 1995). *P. denitrificans* is also a methylotroph, but is unable to grow on trimethylamine (Davidson et al, 1986). The ETF from *Megasphaera elsdenii* has been well characterized but differs from the human ETF protein in its cofactor composition, containing 2 FAD molecules and no AMP. In contrast to *C. tetanomorphum*, *M. elsdenii* enzyme has been purified in an uncomplexed form from its butyryl-CoA dehydrogenase (Sato et al, 2003).

In β-oxidation acyl-CoA dehydrogenase transfers electrons to ETF, from which they pass via ETF:ubiquinone oxidoreductase to ubiquinone. Hence ETF links the oxidation of soluble substrates to membrane-bound respiratory chains in mitochondria and bacteria. Even though respiratory chains may be linked to β-oxidation, no evidence of oxidative phosphorylation had yet been obtained (Parker & Engel, 2000).

The oxidation catalyzed by acyl-CoA dehydrogenases is analogous to succinate dehydrogenation also present in the citric cycle. The latter enzyme is bound to the inner membrane; it introduces a double bond in the carboxylic acid between the α and β carbons, and transfers electrons to ubiquinone and ultimately to the respiratory chain. A convenient comparison of butyryl-CoA dehydrogenase from *C. tetanomorphum* would be fumarate reductase, which is membrane bound. This hypothesis could not be supported since the results

obtained from the gold labelling localization, as observed under the electron microscope by using specific antibodies, have demonstrated that the enzyme is mainly found in the soluble fraction within the bacterial cell. This result is in contrast to the clear evidence found in *A. fermentans*, where glutaconyl-CoA decarboxylase could be effectively located in the membrane using the same targeting procedure (Rohde et al, 1988). In *C. tetanomorphum* the BCD-ETF marked particles were found singly, in pairs or in groups of three in the cytoplasma and also near the storage vesicles. Only a non-statistical amount of particles (~ 10 %) were found within the membrane, maybe due to the fortuitous positioning of non-washed particles left during preparation.

In the beginning of the current work, the reduction reaction of crotonyl-CoA was incomplete since there was an unknown molecule or system interacting with the protein under study. Under certain conditions, crotonyl-CoA reduction was anaerobically detected, namly, only after the enzyme reconstitution with up to 1 mol added FAD and in presence of low diaphorase activity ($\leq$ 1 U mg^{-1}). Thus, it was possible to measure a crotonyl-CoA dependent NADH oxidation at 340nm with additional free FAD as electron acceptor. Therefore it was suggested, a second endergonic reaction drives the reduction of crotonyl-CoA by NADH. Interestingly, a crotonyl-CoA carboxylase/reductase enzyme catalyzing the carboxylation of crotonyl-CoA to ethylmalonyl-CoA in *R. sphaeroides* was recently published. This reaction is significant because it demonstrates how the reduction of crotonyl-CoA can be coupled to a high demanding energy process without an investment of ATP (Erb et al, 2007). Experiments done with BCD-ETD protein from *C. kluyveri* were decisive to find this missing link in the crotonyl-CoA reduction. Li and co-workers purified the enzyme under anoxic conditions and found an absolute ferredoxin dependent oxidation of NADH during crotonyl-CoA reduction (Li et al, 2008). As represented in equation 11, 2 NADH are oxidised to 2 NAD^+ with the concomitant reduction of one mol ferredoxin, during crotonyl-CoA reduction to butyryl-CoA. Based on these findings, the hypothesis presented on the accompanying publication postulates the bifurcation of electrons from NADH to substrate and cofactor (Herrmann et al, 2008). One electron is transferred to the most positive electron acceptor that is crotonyl-CoA ($E°'$= -10 mV) and the other is proposed to proceed in a reverse electron transfer to the most negative electron acceptor which is ferredoxin ($E°'$= - 0, 420 V) in two consecutives steps to finally form butyryl-CoA and reduced ferredoxin (ferredoxin^{2-}) from two molecules of NADH (eq. 11). This would be mechanistically explained if NADH interacts with the enzyme's prostethic group FAD to form the hydroquinone.

Since additional FAD is needed to drive the NADH dependent crotonyl-CoA reduction, it is assumed that FAD can substitute ferredoxin. It would be interesting to investigate if flavodoxin can act as a possible partner of the BCD-ETF system.

$$\text{Crotonyl-CoA} + 2\ \text{NADH} + \text{Fd}^{ox} \rightarrow \text{Butyryl-CoA} + 2\ \text{NAD}^{+} + \text{Fd}^{2-} \quad \text{(eq. 11)}$$

Considering the whole fermentation process, the major part of reduced ferredoxin molecules (4/5) is re-oxidised during the NADH:oxidoreductase reaction of the Rnf complex which generates a proton gradient for energy conservation; the rest of the reduced ferredoxin (1/5) molecules are reoxidised by the [Fe-Fe] hydrogenase to H_2 formation (Fig 46). The reduction of ferredoxin ($E°'$= - 0, 420 V) by NADH ($E°'$= - 0, 320 V) coupled to butyrate synthesis would increase hydrogen formation and leave more crotonyl-CoA for β-oxidation to acetyl-CoA, resulting in more ATP formation via SLP. This is only possible assuming reverse electron transfer to raise the energy of one of the two electrons during the simultaneous reaction of the exergonic crotonyl-CoA reduction to butyryl-CoA and the endergonic ferredoxin reduction at the expenses of NADH. The high NADH dehydrogenase activity observed with BCD-ETF in the presence of FAD can now be readily explained. FAD just substitutes ferredoxin, as can be concluded from the results shown in results section 5.2.2.

Another example of ATP synthesis by a chemiosmotic mechanism is the H_2-dependent caffeate reduction in the acetogenic bacterium *Acetobacterium woodii*. The electron donor for the cytosolic caffeate reduction is NADH and caffeate must be first activated to caffeyl-CoA prior reduction. In this report, the authors propose ETF as the central electron shuttle intermediate receiving electrons from different donors for the reduction reaction. An energy conserving membrane bound ferredoxin:NAD oxidorreductase is postulated to link the electron transfer from ferredoxin to ETF (Imkamp et al, 2007).

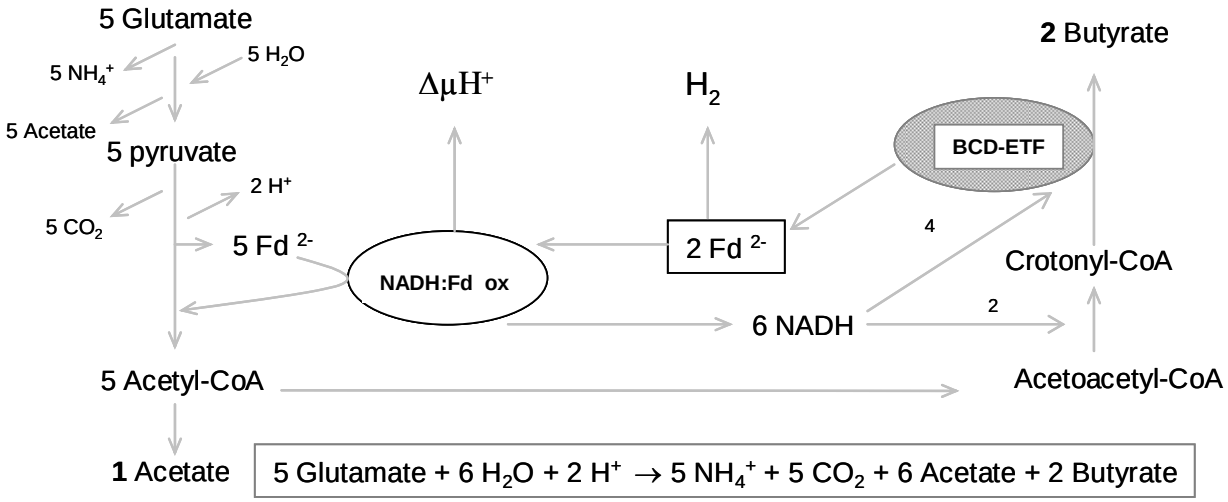


Fig. 46 General scheme for electron cycling in the glutamate fermentation pathway from *C. tetanomorphum* (Fd^{2-} reduced ferredoxin).

The BCD-ETF role in energy conservation implies the generation of an extra molecule of reduced ferredoxin. In figure 6 a general scheme for the cycling of electrons during glutamate fermentation via 3-methylaspartate is shown; in principle 5 molecules reduced ferredoxin (Fd^{2-}) derived from pyruvate decarboxylation plus two molecules (Fd^{2-}) resulting from the BCD-ETF reaction make 6 NADH by the Rnf protein with concomitant production of H_2 and a membrane potential ($\Delta\mu H^+$). Nevertheless, latest experiments attest a NADH:ferredoxin ratio of 3 to 2 during crotonyl-CoA reduction (Kölzer, 2008). This would mean that 8 NADH are then regenerated in the whole system and BCD-ETF complex produces now 4 reduced ferredoxin molecules (Fd^{2-}) from 6 NADH consumed during crotonyl-CoA reduction. The stochiometry and molecular composition of the whole BCD-ETF system is still under study and thus remains subject to change.

The hypothesis of electron bifurcation solves the question of energy conservation in *Clostridium kluyveri* (Seedorf et al, 2008). Hydrogen is also formed in the ethanol fermentation of *C. kluyveri*, producing 1 ATP for 2 H_2 as shown in equation 12:

6 Ethanol + 3 $acetate^-$ → 3 $butyrate^-$ + $caproate^-$ + H^+ + $2H_2$ + 4 H_2O (eq. 12)

($\Delta G°' = -184$ kJ/mol) (Thauer et al, 1969; Jungermann et al, 1969)

Each ethanol molecule is oxidised by 2 NAD^+ via acetaldehyde to acetyl-CoA. It was assumed that the two NADH formed here are linked to hydrogen formation (Thauer et al, 1968a). H_2 formation was also proven to be ferredoxin and acetyl-CoA dependent (Thauer et al, 1969). Then, hydrogenase and ferredoxin were thought to be involved in an exergonic reaction where 2 acetyl-CoA are converted by two NADH to butyryl-CoA ($\Delta G°' = -57$

kJ/mol). Now it has been proven that the ferredoxin reduction was not only acetyl-CoA but also crotonyl-CoA dependent. This latter activity could be finally successfully associated to butyryl-CoA dehydrogenase-ETF complex (Li et al, 2008; Herrmann et al, 2008).

3 Butyryl-CoA dehydrogenase-ETF complex as a module for metabolic shift

Crotonyl-CoA is a crossover point in diverse metabolic pathways; besides its role as an electron sink intermediate to butyrate formation, crotonyl-CoA is a precursor of β-polyhydroxybutyrate (PHB).

Polyhydroxybutyrate is a type of polyhydroxyalkanoate (PHA), a polymer of the polyesters class that are produced as reserve compounds in many bacteria when other carbon source and final electron acceptors like oxygen are not available (Anderson & Dawes, 1990). Further on, PHA had been previously considered as a sink of reducing power and as a cell redox regulator (Senior & Dawes 1971). The most common PHA, poly-3-hydroxybutyrate (P3HB) is synthetized from 3-hydroxybutyrate via dimerization of acetyl-CoA catalyzed by β-ketothiolase (EC 2.3.1.9), and subsequent (*R*)-specific reduction catalyzed by a NADPH-acetoacetyl-CoA reductase (hydroxybutyryl-CoA dehydrogenase EC 1.1.1.36) and poly(3-hydroxybutyrate) synthase. The resultant 3-HB-CoA molecules are then polymerized by the action of a PHB synthase (Ritchie et al, 1971; Anderson & Dawes, 1990).

In most fatty acid synthesis pathways the (*S*)-isomer of 3-hydroxybutyryl-CoA is formed; thus an additional reaction step to convert the (*S*)-isomer into the (*R*)-isomer is required for PHB synthesis. In *Rhodospirillum rubrum*, the stereospecific conversion of L-(+)-3-hydroxybutyryl-CoA to D-(-)-3-hydroxybutyryl-CoA is catalyzed by the reaction of two stereospecific enoyl-CoA hydratases via crotonyl-CoA (Moskowitz & Merrick, 1969; Fukui et al, 1998; Anderson & Dawes, 1990). Two other possible ways to produced (*R*)-3-hydroxybutyryl-CoA is via the (*R*)-specific reduction of acetoacetyl-CoA reductase or by epimerization of (*S*)-hydroxyacyl-CoA.

The PHB synthesis in *C. tetanomorphum* is expected to affect fatty acid synthesis since both systems are based on the same CoA derivatives as intermediates. It is interesting to find out if butyryl-CoA dehydrogenase-ETF may play a broader role in this process.

Electron microscope photographs of *in situ* immunochemical localization (present work) indicate that BCD-ETF complex is probably associated to PHB granules. A similar report was made in *Rhodoccocus ruber* where a 14 kDa granule associated protein was analyzed in close relation with PHB synthesis (Pieper-Fürst et al, 1994; Pieper-Fürst et al, 1995). Even though there are a great variety of different unknown proteins associated to these vesicles, some of the purified proteins from *R. ruber* granules separated by SDS-PAGE exhibite the same range of masses like those BCD-ETF related polypeptides (Pieper-Fürst, 1995).

Experiments based on growth cultures of *C. kluyveri* show its ability to use vinylacetate. The CoA transferase of this bacterium transfers the CoA moiety from propionyl-CoA not only to acetate, but also to vinylacetate; in addition the BCD-ETF system can readily isomerise vinylacetyl-CoA to crotonyl-CoA; however crotonate has not been proved yet to participate as a direct intermediate (Li et al, 2008). Even though *C. tetanomorphum* did not use crotonate as a co-substrate, there was a remarkable improvement of growth when the referred bacteria was cultured in its presence. Previous reports remark on the use of crotonate as a sole substrate in *C. kluyveri* and in axenic cultures of syntrophic bacteria (Thauer et al, 1968b; Dale & McInerney, 1991).

Hitherto, the reductive branch of fermentation has been mostly considered merely as an electron sink process. Beside glutaconyl-CoA decarboxylase from *A. fermentans* and the membrane bound NADH:ferredoxin oxidoreductase from *C. tetanomorphum*, results presented in this work on butyryl-CoA dehydrogenase-ETF may show new ways of energy conservation during the regeneration of oxidants like NAD^+.

To conclude, the third energy conservation process may involve ferredoxin as an intermediate of flavin containing enzymes responsible for the transfer of electrons between different redox systems; a dual electron transfer is proposed here where electrons are recycled between oxidation and reduction processes.

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Erklärung

Ich versichere, dass ich meine Dissertation:

Enzymes of two clostridial amino-acid fermentation pathways

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

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

_____ _____

(Ort, Datum) (Unterschrift)

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Under-degree study

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