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Energy conservation in the decarboxylation of dicarboxylic acids by fermenting bacteria

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Abstract Decarboxylation of dicarboxylic acids (oxalate, malonate, succinate, glutarate, and malate) can serve as the sole energy source for the growth of fermenting bacteria. Since the free energy change of a decarboxylation reaction is small (around –20 kJ per mol) and equivalent to only approximately one-third of the energy required for ATP synthesis from ADP and phosphate under physiological conditions, the decarboxylation energy cannot be conserved by substrate-level phosphorylation. It is either converted (in malonate, succinate, and glutarate fermentation) by membrane-bound primary decarboxylase sodium ion pumps into an electrochemical gradient of sodium ions across the membrane; or, alternatively, an electrochemical proton gradient can be established by the combined action of a soluble decarboxylase with a dicarboxylate/monocarboxylate antiporter (in oxalate and malate fermentation). The thus generated electrochemical Na^+ or H^+ gradients are then exploited for ATP synthesis by Na^+- or H^+-coupled F_1F_0 ATP synthases. This new type of energy conservation has been termed decarboxylation phosphorylation and is responsible entirely for ATP synthesis in several anaerobic bacteria.

Key words ATP synthase · Decarboxylation · Electrogenic substrate/product antiporter · Sodium ion pump · Malo-lactic fermentation · Oxalate · Malonate · Succinate · Glutarate

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Chemistry and energetics of decarboxylation reactions

A number of dicarboxylic acids are formed in nature as fermentation products (succinate), complexing agents (oxalate and malate), or secondary metabolites in plants (malonate and glutarate). They also originate from industrial production. Since some of these acids are produced in or distributed into anoxic environments, their degradation by anaerobic bacteria is of interest. Furthermore, the decrease of acidity of natural fruit products (e.g., wine) by the decarboxylation of dicarboxylic acids in a bacterial process (malo-lactic fermentation) is also of major economic importance in the food industry.

Bacteria that grow anaerobically on saturated dicarboxylic acids are confronted with two major problems, a bioenergetic one and a chemical one. Fermentative growth has to be redox-balanced; therefore, the dicarboxylic acids cannot be completely oxidized to CO_2. Nonoxidative decarboxylation reactions provide a means for a redox-neutral catabolism of these compounds, and bacteria that grow from the decarboxylation of a dicarboxylic to the corresponding monocarboxylic acid have in fact been isolated. This type of fermentation has been discovered for *Propionigenium modestum* (Schink and Pfennig 1982), which grows from the catabolism of succinate to propionate and CO_2. From a bioenergetic point of view, growth based on this decarboxylation reaction is puzzling. The free energy change of a decarboxylation reaction is in the range of –17 to –25 kJ per mol and thus makes up only a fraction of the amount needed for synthesis of ATP from ADP and inorganic phosphate (70–75 kJ per mol under the conditions within a living cell; Thauer et al. 1977; Schink 1990). As a consequence, the stoichiometric formation of energy-rich precursors with a group transfer potential at least as high as that of the phosphoric anhydride bond of the ATP molecule itself is thermodynamically not feasible in these catabolic pathways; therefore, chemiosmotic processes are mandatory for energy conservation. The small energy gain of the decarboxylation reaction increases by approximately 8 kJ per mol if gaseous rather than aqueous CO_2 is assumed as the product (Thauer et al. 1977). Since the

Table 1 Decarboxylation reactions supporting growth of fermenting bacteria

Reaction	Bacteria	References
$Oxalate^{2-} + H^+ \rightarrow Formate^- + CO_2$	*Oxalobacter formigenes*	Allison et al. (1985)
	Oxalobacter vibrioformis	Dehning and Schink (1989a)
	Clostridium oxalicum	Dehning and Schink (1989a)
$Malonate^{2-} + H^+ \rightarrow Acetate^- + CO_2$	*Sporomusa termitida*	Breznak et al. (1988)
	Malonomonas rubra	Dehning and Schink (1989b)
	Sporomusa malonica	Dehning et al. (1989)
	Klebsiella oxytoca	Dehning and Schink (1994)
	Citrobacter diversus	Janssen and Harfoot (1990)
$Succinate^{2-} + H^+ \rightarrow Propionate^- + CO_2$	*Propionigenium modestum*	Schink and Pfennig (1982)
	Propionigenium maris	Janssen and Liesack (1995)
	Peptostreptococcus sp.	Janssen et al. (1996)
	Veillonella parvula[a]	Denger and Schink (1992), Janssen (1992)
$Glutarate^{2-} + H^+ \rightarrow 0.5\ Butyrate^- + 0.5\ Isobutyrate^- + CO_2$	Strain WoG13	Matthies and Schink (1992a)
$Malate^{2-} + H^+ \rightarrow Lactate^- + CO_2$	*Lactobacillus plantarum*	Kolb et al. (1992)
	Lactobacillus sake	Kolb et al. (1992)

[a] Succinate-dependent growth only in the presence of a co-substrate

overall reaction consumes one proton per reaction run, the reaction becomes more exergonic at lower pH, which may be of interest especially in acidic environments such as in the wine must (see malo-lactic fermentation below).

Chemically, decarboxylation reactions often involve a heterolytic cleavage by which the electron pair remains at the organic residue, which subsequently becomes protonated:

$$\begin{array}{l} R\text{–}COO^- \rightarrow R^- + CO_2 \\ \quad\quad H^+ \downarrow \\ \quad\quad\ RH \end{array}$$

Electron-withdrawing substituents on R thus stabilize the intermediate R^- and facilitate the decarboxylation reaction. Such electron-withdrawing substituents are introduced in many different ways into the various dicarboxylic acids to make their decarboxylation chemically feasible under physiological conditions. A carbonyl group in β-position to the carboxylate acts as an electron-withdrawing substituent, and β-oxo acids such as oxaloacetate are therefore classical substrates for decarboxylases. Oxaloacetate is generated as an intermediate in the fermentation of citrate in *Klebsiella pneumoniae* or tartrate in *Salmonella typhimurium*, where it serves as the substrate for an oxaloacetate decarboxylase Na^+ pump [for a review, see Dimroth (1997)]. Via this enzyme, in fact, the principle of conserving decarboxylation energy by conversion into a Na^+ gradient across the membrane was discovered.

An alternative strategy to activate a dicarboxylic acid for its decarboxylation is to introduce a thioester group in β-position to the carboxylate. This is exemplified in the catabolism of succinate by *P. modestum* (Hilpert et al. 1984). Succinate is first converted to succinyl-CoA, and the carbon skeleton is subsequently rearranged to methylmalonyl-CoA in order to bring the thioester in β-position to the carboxylate. The same principle is used for the activation of malonate and glutarate. Malonate is converted into its thioester derivative with a CoA-like prosthetic group of an acyl carrier protein (Berg et al. 1996), and glutarate is probably converted to glutaconyl-CoA, which is the vinylogue of malonyl-CoA. In *Oxalobacter formigenes*, oxalate is converted to oxalyl-CoA and, this, is subsequently decarboxylated by a soluble decarboxylase to formyl-CoA (Baetz and Allison 1989). Oxalyl-CoA has the thioester substituent in α-position to the carboxylate. The decarboxylase therefore contains a thiamine pyrophosphate prosthetic group to which the oxalyl moiety probably becomes transiently attached for proper activation of the decarboxylation reaction.

Several different strains of fermenting bacteria able to grow by decarboxylation of dicarboxylic acids have been isolated and described in the last two decades (Table 1). Biochemical studies intended to determine the underlying mechanism of ATP synthesis in these bacteria have revealed two different concepts: either the decarboxylation energy is converted directly into an energy-rich electrochemical gradient of Na^+ ions over the membrane [for a review see Dimroth (1997)] or an electrochemical H^+ gradient is created by an electrogenic dicarboxylate/monocarboxylate antiporter operating together with a soluble decarboxylase [for reviews, see Maloney et al. (1990) and Poolman and Konings (1993)]. The present review intends to summarize our present knowledge of various of these systems and to compare the two different concepts realized for conversion of decarboxylation energy into ATP synthesis.

Energy conservation by primary sodium ion pumps

Discovery of the oxaloacetate decarboxylase Na^+ pump

The new concept to conserve decarboxylation energy emerged from biochemical studies with the oxaloacetate

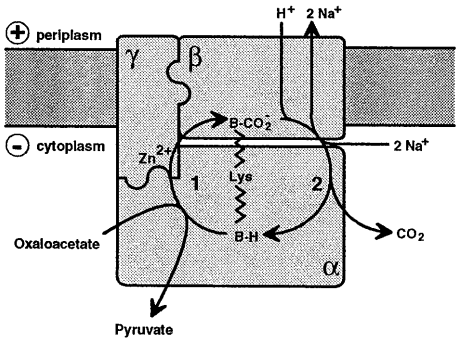

Fig. 1 Model of oxaloacetate decarboxylase showing the location, interaction, and function of the α-, β-, and γ-subunits. (*B-H* biotin, *B-CO₂* carboxybiotin, *Lys* biotin-binding lysine residue, *1* carboxyltransferase reaction, and *2* decarboxylase reaction

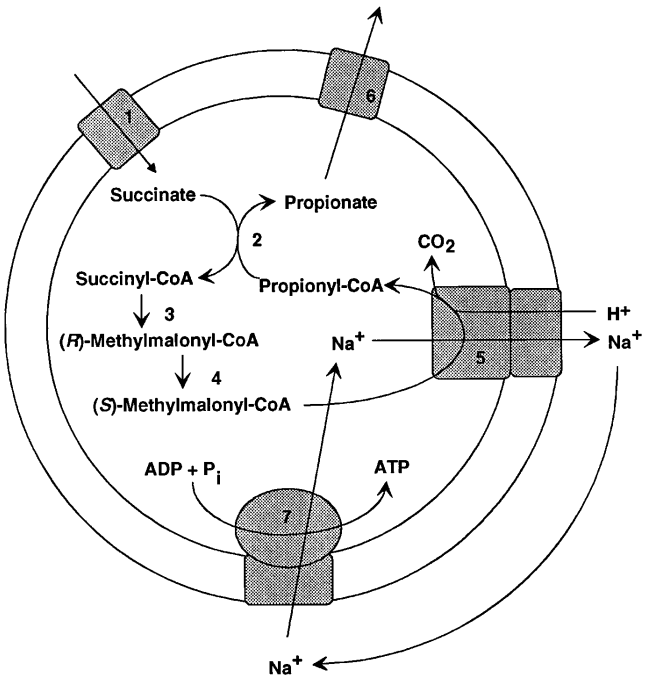

Fig. 2 Energy metabolism of *Propionigenium modestum* with a Na^+ cycle coupling the exergonic decarboxylation of (*S*)-methylmalonyl-CoA to endergonic ATP synthesis. *1* Hypothetical succinate uptake system, *2* succinate propionyl-CoA:CoA transferase, *3* methylmalonyl-CoA mutase, *4* methylmalonyl-CoA isomerase, *5* methylmalonyl-CoA decarboxylase Na^+ pump, *6* hypothetical propionate export system, and *7* Na^+-translocating ATP synthase

decarboxylase from *K. pneumoniae*. The enzyme was found to act as an electrogenic Na^+ pump and is now the paradigm of Na^+ transport decarboxylases (Dimroth 1982), a protein family that includes methylmalonyl-CoA decarboxylase, glutaconyl-CoA decarboxylase, and malonate decarboxylase from *Malonomonas rubra* [for a review, see Dimroth (1997)]. Oxaloacetate decarboxylase consists of the water-soluble α-subunit and the two membrane-bound subunits β and γ. The α-subunit is linked via the γ-subunit to the β-subunit, as shown schematically in Fig. 1 (Di Berardino and Dimroth 1995). The catalytic reaction cycle starts with the carboxyl transfer from oxaloacetate to the prosthetic biotin group that is attached to the C-terminal part of the α-subunit. This reaction is catalyzed at a low rate by the α-subunit alone. In the additional presence of the Zn^{2+}-containing γ-subunit, the rate increases approximately 1,000-fold, probably because the metal ion polarizes the carbonyl oxygen bond of oxaloacetate, thereby facilitating the carboxyl transfer to biotin.

The carboxybiotin now moves from the carboxyltransfer site at the α-subunit to the decarboxylase site at the β-subunit. The mobility of the biotin residue is facilitated by an extended alanine/proline linker in the interdomain region between the carboxyltransferase and the biotin domain. In the course of the subsequent decarboxylation reaction, one to two Na^+ ions are pumped across the membrane into the periplasm, and 1 H^+ traverses the membrane in the opposite direction. This proton is consumed during the release of CO_2 from the biotin carboxylate (Di Berardino and Dimroth 1996; Dimroth 1997). The β-subunit contains eleven putative membrane-spanning α-helical segments. Site-directed mutagenesis of a conserved aspartate residue (D203) in helix five to asparagine or glutamate completely abolished Na^+ transport and decarboxylase activities, whereas the carboxyltransferase activity was retained (Di Berardino and Dimroth 1996). Based on these and other results, a direct coupling mechanism has been proposed in which D203 plays an essential role in both the vectorial and the chemical reaction. In the proposed mechanism, the carboxybiotin binds together with a Na^+ ion close to D203 of the β-subunit. Subsequently, the Na^+ ion is envisaged to switch to D203, and simultaneously the proton originally bound to this residue moves to the biotin carboxylate, where it catalyzes the immediate decarboxylation of this acid-labile compound. This exergonic decarboxylation is coupled to the release of the bound Na^+ ion to the positive side of the membrane.

Decarboxylation of succinate

P. modestum was the first isolated anaerobic bacterium for which conservation of decarboxylation energy was proven to support growth (Schink and Pfennig 1982). The type strain was isolated from a marine sediment in saltwater medium; enrichment cultures from freshwater sources grew only weakly and were not studied in detail. Actually, the enhancing effect of saltwater medium on the growth of these bacteria was a hint that sodium ions could be involved in the energy conservation and, therefore, that a mechanism similar to that described above for the oxaloacetate decarboxylase was applicable. The cell yield per mol succinate decarboxylated was in the range of 2.1–2.4 g dry matter. This low yield indicates that less than one ATP is formed per reaction run: on the basis of comparisons of many metabolic types of bacteria, Stouthamer (1979) calculated that Y_{ATP}, the cell yield per mol ATP metabolized, should be in the range of 5–10 g dry cell matter depending on the type of assimilated sub-

strate. The energy metabolism in *P. modestum* involves a CoA transfer from propionyl-CoA to succinate, rearrangement of succinyl-CoA to (*S*)-methylmalonyl-CoA via (*R*)-methylmalonyl-CoA, decarboxylation to propionyl-CoA, and release of propionate. All enzymes involved have been demonstrated in cell-free extracts (Hilpert et al. 1984), but the mechanism of succinate import and propionate export is still open (Fig. 2).

The key enzyme for energy conservation in this bacterium is the membrane-bound methylmalonyl-CoA decarboxylase, which acts as a primary Na^+ pump by a mechanism similar to that described above for oxaloacetate decarboxylase. The Na^+ ion gradient established by the decarboxylase is employed to drive ATP synthesis by a Na^+-translocating ATP synthase (Hilpert et al. 1984; Laubinger and Dimroth 1988). *P. modestum* thus employs a Na^+ cycle across the membrane and not the usual proton cycle as a direct link between an exergonic chemical reaction and endergonic ATP synthesis. *P. modestum* was the first organism for which this new type of ATP synthesis was described. The other unusual feature of this ATP synthesis is the utilization of decarboxylation energy rather than of electron transport reactions. Therefore, this ATP synthesis mechanism has been termed decarboxylation phosphorylation. The energy-linking reactions are shown schematically in Fig. 2.

The methylmalonyl-CoA decarboxylase of *P. modestum* has recently been purified and biochemically characterized (Bott et al. 1997). The enzyme consists of four different subunits: α (MmdA, 56.1 kDa) with the function of carboxyltransferase, β (MmdB, 41.2 kDa) with the function of a carboxybiotin-carrier protein decarboxylase, γ (MmdC, 13.1 kDa) with the function of biotin carrier protein, and δ (MmdD, 14.2 kDa) with a likely function in the assembly of the complex. The four genes encoding these proteins are clustered on the chromosome in the order *mmdADCB*. The gene cluster encoding the related methylmalonyl-CoA decarboxylase of *Veillonella parvula* (*mmdADECB*) contains a fifth gene (*mmdE*) located within the *mmd* gene cluster (Huder and Dimroth 1993). *mmdE* encodes a protein with 55 amino acid residues that is 47% identical to the C-terminal region of the δ-subunit of the same enzyme. A gene duplication event may thus be responsible for the existence of *mmdE* in *V. parvula*. The MmdE protein is part of the methylmalonyl-CoA decarboxylase complex of *V. parvula*. It has no catalytic function but increases the stability of the complex, as has been shown by comparing the stability of the five-subunit and the four-subunit complex after production from appropriate plasmids in *E. coli* (Huder and Dimroth 1995). These data are consistent with the fact that the four-subunit methylmalonyl-CoA decarboxylase of *P. modestum* is functional but exhibits poor complex stability.

The F_1F_0 ATP synthase of *P. modestum* was the first enzyme of this kind for which an extension of the coupling ion specificity from exclusively H^+ to alternatively H^+, Li^+, or Na^+ could be demonstrated and in which Na^+ acts as the physiological coupling ion (Laubinger and Dimroth 1988, 1989). This remarkable property of the *P. modestum* ATP synthase has proven to be an excellent tool for investigating important aspects of the enzyme mechanism, particularly the coupling between ion translocation across the F_0 moiety and the synthesis of ATP from ADP and phosphate at the F_1 subcomplex of the enzyme. For these results, the reader is referred to consult a recent review (Dimroth 1997).

Unlike *P. modestum*, *V. parvula* is not capable of growth with succinate as the sole energy source. The biochemical basis for this discrepancy is probably the absence of an appropriate ATP synthase. Tests performed to demonstrate the presence of an ATP synthase of the F_1F_0 type on the enzyme level or by hybridization with appropriate probes on the DNA level failed (Laubinger et al. 1990; W. Laubinger and P. Dimroth, unpublished results). However, if succinate is supplied as an additional substrate together with malate or lactate, the growth yield increases in the range of 2.4–3.5 g per mol succinate in relation to the amount of succinate provided; this is the same as the molar growth yield of *P. modestum* growing with succinate only (Denger and Schink 1992; Janssen 1992). Thus, the sodium ion gradient established during succinate decarboxylation saves metabolic energy (e.g., for substrate transport) that would otherwise need to be supplied by ATP hydrolysis.

Decarboxylation of malonate

Malonate decarboxylation was used as a diagnostic reaction in the identification of enteric bacteria that carry out this reaction cometabolically in the presence of other substrates. Decarboxylation of malonate as the sole source of energy for (anaerobic) bacterial growth was first documented with *M. rubra* and *Sporomusa malonica* (Dehning and Schink 1989b; Dehning et al. 1989). Growth by malonate decarboxylation was later also proven for *Sporomusa termitida* isolated from termite guts (Breznak et al. 1988). *M. rubra* was isolated from marine sediments with saltwater medium containing malonate as the sole source of organic carbon and energy; *S. malonica* was obtained from a freshwater sediment. It was later shown that also *Klebsiella oxytoca*, *K. pneumoniae*, and *Citrobacter diversus* can gain substantial amounts of metabolic energy from malonate decarboxylation in the presence of limiting amounts of other fermentable substrates (Janssen and Harfoot 1992). It should be emphasized that assimilation of acetate into cell material requires partial oxidation of acetyl-CoA to CO_2 in order to provide electrons for reductive carboxylation of acetyl-CoA to pyruvate and further reduction to triose phosphates. *M. rubra* has a complete tricarboxylic acid cycle, and *S. malonica* can use the carbon monoxide dehydrogenase (Wood) pathway for the same purpose, but typical fermenting bacteria have no means of acetate oxidation and would therefore not be able to grow with malonate as the sole source of organic carbon.

At neutral pH, both carboxylic groups of malonate are deprotonated, and in this form malonate cannot be decarboxylated. Malonyl-CoA, on the other hand, is suffi-

ciently activated for enzymic decarboxylation, and malonyl-CoA decarboxylases have in fact been described (Kolattokudy et al. 1981). Initially, therefore, the decarboxylation of malonate was believed to proceed via malonyl coenzyme A (Dehning and Schink 1994). Detailed work on the malonate decarboxylating system of *M. rubra* has revealed that this is not the case: malonate is actually activated for decarboxylation by forming a thioester with a protein-bound thiol cofactor [Hilbi et al. 1992; for a review, see Dimroth and Hilbi (1997)]. The malonate decarboxylase enzyme system involves several proteins that catalyze the individual reactions leading to malonate decarboxylation as outlined in Fig. 3. The inactive SH-form of the enzyme is converted into the catalytically active acetyl-S enzyme by post-translational acetylation of its acyl carrier protein (ACP) subunit with a specific ligase, and ATP and acetate as substrates (Hilbi et al. 1992; Berg et al. 1996). The acetyl-S-ACP is then converted to malonyl-S-ACP by an acyl carrier protein transferase. Subsequently, the free carboxyl group of the malonyl thioester with the enzyme is transferred to a small biotin protein, thereby regenerating the acetyl-S-ACP. The carboxybiotin protein is believed to diffuse to the membrane, where it is decarboxylated by an integral membrane protein that couples this exergonic reaction to Na^+ pumping across the membrane (Hilbi and Dimroth 1994).

Several of the proteins catalyzing the reaction sequence of malonate decarboxylation have been isolated and characterized. Of special interest is the acyl carrier protein with the unusual thiol cofactor 2′-(5′′-phosphoribosyl)-3′-dephospho-CoA to which the acyl residues are bound during catalysis. The only other known enzymes harboring this cofactor are malonate decarboxylase from *K. pneumoniae*, citrate lyase, and citramalate lyase, which all activate their specific substrate for cleavage by conversion into the thioester with the thiol cofactor [for a review, see Dimroth and Hilbi (1997)].

The individual proteins participating in the decarboxylation of malonate are encoded by the *mad* gene cluster, which comprises 14 genes. It could be identified by sequence comparison with proteins of known function that most of these genes encode a specific protein of the malonate decarboxylase system. The functions of these gene products are indicated in Fig. 3. Other genes of the cluster could participate in the biosynthesis of the prosthetic group or could have other, still unknown functions in the catalysis of malonate decarboxylation (Berg et al. 1997).

The enzymic and genetic basis of malonate decarboxylation has also been investigated in *K. pneumoniae* (Schmid et al. 1996; Hoenke et al. 1997). Free malonate is the substrate of this enzyme that is activated for the decarboxylation by forming a thioester with the thiol cofactor of an ACP subunit, which is similar to the activation mechanism described above for malonate decarboxylase of *M. rubra*. An important difference of the *K. pneumoniae* decarboxylase is the direct liberation of CO_2 from malonyl-S-ACP without transfer to a biotin protein and without the participation of a membrane-bound decarboxylase Na^+ pump. The direct conversion of decarboxylation energy into an electrochemical Na^+ gradient is, therefore, not possible with the *K. pneumoniae* malonate decarboxylase.

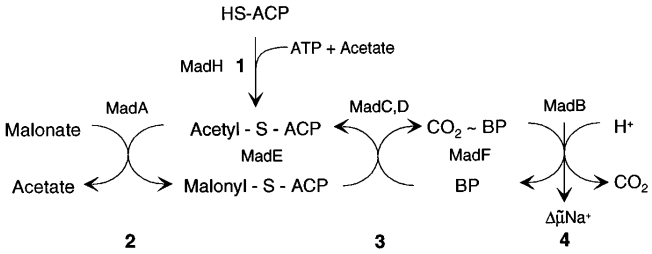

Fig. 3 Reaction mechanism proposed for malonate decarboxylase of *Malonomonas rubra* including the anticipated functions of the *mad* gene products. *1* Deacetyl acyl carrier protein:acetate ligase (MadH), *2* acetyl-S-acyl carrier protein malonate acyl carrier protein-SH transferase (MadA), *3* carboxyltransferase (MadC,D), and *4* carboxybiotin decarboxylase (MadB). *ACP* refers to the acyl carrier protein (MadE), and *BP* to the biotin carrier protein (MadF)

Decarboxylation of glutarate

Enrichment cultures in mineral media with glutarate as the sole organic substrate yielded fermenting cultures from which strain WoGl3 was isolated (Matthies and Schink 1992a). The molar growth yield was small (0.5–0.9 g cell dry mass per mol glutarate), and the substrate was fermented to approximately equal amounts of butyrate and isobutyrate. Glutarate is activated in these bacteria by exchange with acetyl-CoA and is dehydrogenated by an NAD^+-dependent enzyme to glutaconyl-CoA, which is decarboxylated to crotonyl-CoA. This is reduced to butyryl-CoA, which undergoes subsequent B_{12}-dependent isomerization to isobutyryl-CoA [for the reaction mechanism, see Moore et al. (1995)]. Both acyl CoAs exchange with acetate through a CoA transferase to form acetyl-CoA and to close the reaction cycle. The physiological function of the isomerization of butyryl-CoA to an isobutyryl-CoA residue is still unknown. However, this reaction plays a key role in fermentative degradation of isobutyrate via butyrate to methane and CO_2 (Matthies and Schink 1992b). The key enzyme of the energy metabolism of this bacterium is the membrane-bound glutaconyl-CoA decarboxylase (Matthies and Schink 1992c). This enzyme appears to be a member of the Na^+ transport decarboxylase family similar to the glutaconyl-CoA decarboxylase from *Acidaminococcus fermentans* (Buckel and Semmler 1983) because the activity was Na^+-dependent and avidin-sensitive. The glutaconyl-CoA decarboxylase from this source and from other glutamate-fermenting bacteria has been well-characterized [Bendrat and Buckel (1993) and references therein]. The enzyme from *A. fermentans* consists of four subunits: GcdA, the carboxyltransferase (65 kDa); GcdB, the carboxylase (36 kDa); GcdC, the biotin carrier (24 kDa); and GcdD (142 kDa), a protein of unknown function. The GcdA gene has been cloned, sequenced, and expressed in *Escherichia*

coli. Unlike the carboxyltransferases of the related Na^+-translocating decarboxylases, GcdA catalyzed the carboxyl-transfer reaction not only with the native substrate (the biotin carrier protein) but also with free biotin. In the case of *Peptostreptococcus asaccharolyticus*, it has been shown that the sodium ion pump contributes to energy conservation (Wohlfarth and Buckel 1985). More recently, it has been demonstrated that *A. fermentans* cells produce hydrogen in a Na^+-dependent manner (Härtel and Buckel 1996). The hydrogenase is located in the cell membrane, and H_2 production is inhibited by $\Delta\mu$-dissipating ionophores. Hence, the glutaconyl-CoA decarboxylase Na^+ pump may play an essential role in energizing H_2 formation with NADH as the electron donor.

Energy conservation by combining secondary transport systems with a soluble decarboxylase

Decarboxylation of oxalate

Bacteria able to grow by decarboxylation of oxalate to formate were first isolated from rumen contents and later described as a new species, *O. formigenes* (Allison et al. 1985). Further oxalate-decarboxylating fermenting bacteria were isolated from freshwater sediments (Smith et al. 1985; Dehning and Schink 1989a). The molar growth yields obtained and reported to date are in the range of 1.0–1.4 g cell dry matter per mol oxalate degraded. All strains depend on acetate as a cosubstrate for cell matter synthesis. The biochemistry employed for energy conservation by these bacteria is basically different from those described above (Fig. 4): oxalate is activated to oxalyl-CoA by CoA transfer from formyl-CoA. The water-soluble (cytoplasmic) decarboxylase is a homotetramer of 260 kDa and requires thiamine pyrophosphate as a cofactor (Baetz and Allison 1989). This decarboxylase alone is obviously unable to perform an energy conservation. A second key element for energy conservation in these bacteria is a secondary $oxalate^{2-}$:$formate^{1-}$ antiporter (Anantharam et al. 1989). Due to the obligate exchange of twofold negatively charged oxalate for onefold negatively charged formate, an electrical potential is generated across the membrane of the cells. Since the equilibrium of the exergonic decarboxylation of oxalyl-CoA to formyl-CoA is far to the side of the products and the equilibrium constant for the CoA transferase is probably close to 1, the intracellular concentration of formate will be up to 1,000-fold higher than that of oxalate. As a result, a large formate gradient is established across the membrane from the inside to the outside and an oxalate gradient is established in the opposite direction, both of which provide the driving force for $\Delta\psi$ generation by the electrogenic antiporter. The combination of a soluble decarboxylase with an electrogenic substrate/product antiporter is thus another means to conserve decarboxylation energy. The decarboxylation of oxalyl-CoA to formyl-CoA and CO_2 consumes a proton inside the cell, which creates a pH gradient (inside alkaline) since CO_2 rapidly equilibrates over

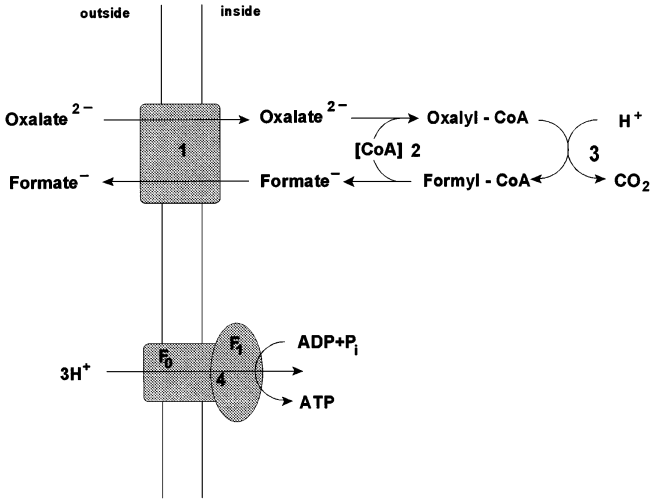

Fig. 4 Reactions involved in oxalate fermentation by *Oxalobacter formigenes*. *1* Oxalate/formate antiporter, *2* formyl CoA:oxalate CoA transferase, *3* oxalyl CoA decarboxylase, and *4* H^+-translocating ATP synthase

the membrane and is diluted out in the large extracellular fluid. The membrane potential ($\Delta\psi$) and the pH gradient (ΔpH) generated by this mechanism together make up the proton motive force ($\Delta\mu_{H^+}$) that can be employed to drive endergonic membrane-associated reactions, especially ATP synthesis.

Decarboxylation of malate (malo-lactic fermentation)

The fermentative conversion of L-malate to L-lactate is known as an important process during the secondary wine fermentation in which mainly acids are degraded. Since malic acid makes up approximately half of the total acid content of the must, malo-lactic fermentation contributes significantly to the deacidification of wine, and lactic acid bacteria have been found to be responsible for this important reaction. The question whether malate conversion to lactate provides any advantage to the bacteria carrying out this reaction has been discussed over the years with contradictory conclusions. The small energy gain possible from such a reaction has mostly been overlooked because very rich media with high backgrounds, for example, of fermentable glucose were used [see Kandler et al. (1973)]. Definitive proof of energy conservation from malate decarboxylation to lactate was obtained with *Lactobacillus plantarum* and *Lactobacillus sake* (Kolb et al. 1992). These strains exhibited a clear stoichiometric conversion of malate to lactate and CO_2 in a defined mineral medium with only 0.1% yeast extract as the background substrate. The growth yields varied between 2.0 and 3.7 g dry matter per mol malate decarboxylated. Malate-dependent growth occurred in batch cultures primarily during the early period of substrate transformation, when the malate concentration was still high and the lactate concentration low. In continuous culture, the malate-dependent cell yield

decreased by 25% and could be diminished even further by the addition of external lactate (Kolb et al. 1992).

In *L. plantarum* and *L. sake*, malate decarboxylation was catalyzed by the malo-lactic enzyme (Schütz and Radler 1973; Caspritz and Radler 1983) that is localized exclusively in the cytoplasmic fraction (Kolb et al. 1992). This enzyme catalyzes both a redox reaction with a structure-bound NAD cofactor and the decarboxylation without releasing an oxidized intermediate.

Obviously the observed energy conservation in malo-lactic fermentation is not accomplished by a primary decarboxylation-dependent pumping reaction, but by a secondary transport system. The nature of this system has been best characterized in *Lactococcus lactis*: there, the electrogenic malate/lactate exchanger operates together with the malo-lactic enzyme to conserve metabolic energy (Poolman et al. 1991). Indications of an electrogenic L-malate transporter were obtained with *L. plantarum* (Olsen et al. 1991) and with *Leuconostoc oenos* (Loubiere et al. 1992), and electrogenic lactate export has been hypothesized for *Streptococcus cremoris* (Otto et al. 1980). Other examples for energy conservation by this principle are the histidine/histamine antiport system of *Lactobacillus buchneri* (Molenaar et al. 1993) or the aspartate/alanine exchanger of *Lactobacillus* sp. M3 (Abe et al. 1996) operating in combination with the appropriate amino acid decarboxylase.

Primary versus secondary transport systems

The two different concepts of energy conservation during decarboxylation-dependent growth of fermenting bacteria outlined above have advantages and disadvantages. Decarboxylation by a membrane-bound primary pump as realized in succinate, malonate, and glutarate fermentation generates a sodium ion gradient across the membrane and is largely independent of the substrate and product concentrations. The sodium ion gradient can be exploited by an ATP synthase, by Na^+-dependent solute uptake systems, or by Na^+-dependent flagellar motors, or it can be converted into a proton gradient through a Na^+/H^+ antiporter [for a review, see Dimroth (1997)]. At first glance, the immediate conversion of the free energy of the sodium ion gradient into ATP synthesis as realized in *P. modestum* might be regarded as the most efficient energy conservation of this type. However, coupling of a primary generated Na^+ gradient to a H^+-translocating ATP synthase or of a primary generated H^+ gradient to Na^+-translocating ATP synthase via a Na^+/H^+ exchange system is also possible. The first mechanism may be exploited by *M. rubra* (H. Hilbi and P. Dimroth, unpublished results) and the second is used by *E. coli* strain PEF42, which expresses a Na^+-translocating *E. coli*/*P. modestum* F_1F_0 ATPase hybrid (Kaim and Dimroth 1993). Aerobic growth of this hybrid on succinate, i.e., under conditions where ATP synthesis occurs entirely through the chemiosmotic mechanism, was not significantly different from that of *E. coli* wild-type cells.

To conserve decarboxylation energy with a primary Na^+ transport system is restricted to a few substrates for which such a primary Na^+ pump is available. These multisubunit biotin enzymes combine a carboxyltransferase and a biotin carrier of clear evolutionary relationship to biotin-dependent carboxylases with membrane-bound subunits functioning as Na^+-pumping carboxybiotin decarboxylases [for a review, see Dimroth (1997)]. However, there is a much greater repertoire of soluble, not biotin-containing decarboxylases. The potential to conserve decarboxylation energy could, therefore, be significantly increased by a mechanism that combines the function of a soluble decarboxylase with that of an appropriate substrate/product antiporter. As outlined above, decarboxylation energy can be conserved by these systems in the form of $\Delta\mu_{H^+}$ which can be utilized to drive ATP synthesis. It is obvious that substrate import gradients find their limits with the affinity of the metabolic enzymes inside. Oxalyl-CoA decarboxylase of *O. formigenes* has a K_m for oxalyl-CoA of 0.24 mM; the K_m of malo-lactic enzymes for malate is approximately 10 mM (Lonvand-Funel and Strasser de Saad 1982; Caspritz and Radler 1983). If these enzymes operate with substrate concentrations in the range of their half-saturation constant, the establishment of an energy-yielding substrate import gradient would require substrate concentrations that may be reached only exceptionally, e.g., with oxalate in the rumen or with malate in the must. For energy conservation by secondary transport systems, the outward-oriented product gradient rather than the substrate gradient can therefore act as the main driving force also for an antiporter – especially if partner bacteria consume the reaction products – since this is typical of microbial life in natural habitats.

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