ELSEVIER

Biochimica et Biophysica Acta 1505 (2001) 15–27

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Review

Sodium ion-translocating decarboxylases

Wolfgang Buckel *

Laboratorium für Mikrobiologie, Fachbereich Biologie, Philipps-Universität, D-35032 Marburg, Germany

Received 28 July 2000; received in revised form 1 November 2000; accepted 17 November 2000

Abstract

The review is concerned with three Na^+-dependent biotin-containing decarboxylases, which catalyse the substitution of CO_2 by H^+ with retention of configuration ($\Delta G^{\circ\prime} = -30$ kJ/mol): oxaloacetate decarboxylase from enterobacteria, methylmalonyl-CoA decarboxylase from *Veillonella parvula* and *Propiogenium modestum*, and glutaconyl-CoA decarboxylase from *Acidaminococcus fermentans*. The enzymes represent complexes of four functional domains or subunits, a carboxytransferase, a mobile alanine- and proline-rich biotin carrier, a 9–11 membrane-spanning helix-containing Na^+-dependent carboxybiotin decarboxylase and a membrane anchor. In the first catalytic step the carboxyl group of the substrate is converted to a kinetically activated carboxylate in *N*-carboxybiotin. After swing-over to the decarboxylase, an electrochemical Na^+ gradient is generated; the free energy of the decarboxylation is used to translocate 1–2 Na^+ from the inside to the outside, whereas the proton comes from the outside. At high [Na^+], however, the decarboxylases appear to catalyse a mere Na^+/Na^+ exchange. This finding has implications for the life of *P. modestum* in sea water, which relies on the synthesis of ATP via $\Delta\mu Na^+$ generated by decarboxylation. In many sequenced genomes from *Bacteria* and *Archaea* homologues of the carboxybiotin decarboxylase from *A. fermentans* with up to 80% sequence identity have been detected.

Keywords: Mechanism of decarboxylation; Sodium ion- and biotin-dependent decarboxylase; Oxaloacetate decarboxylase; Methylmalonyl-CoA decarboxylase; Glutaconyl-CoA decarboxylase; Sodium ion translocation; Bioenergetics

1. Introduction

This review is concerned with a unique class of biotin-containing membrane enzymes, which catalyse the decarboxylation of β-keto acids and analogues thereof. The enzymes are able to use the free energy of decarboxylation ($\Delta G^{0\prime}$ = ca. −30 kJ/mol) in order to translocate Na^+ from the cytoplasm to the outside of the cell and thus contribute to energy conservation. Decarboxylases of this type occur only in a certain number of *Bacteria* (and *Archaea*?) grown under anaerobic conditions. This subject has been reviewed by Peter Dimroth many times starting from his discovery in 1980 that the then long known oxaloacetate decarboxylase from *Klebsiella pneumoniae* [1] acts as primary Na^+ pump [2–4]. The present review focuses on the mechanisms of decarboxylations in general and on that of the Na^+-translocating decarboxylases in particular. In addition some recent developments on the structure of these unusual decarboxylases are presented, which appeared after the latest review in 1997.

* Fax: +49-6421-28-28979;
E-mail: buckel@mailer.uni-marburg.de

0005-2728/01/$ – see front matter
PII: S0005-2728(00)00273-5

Carboxylases and decarboxylases catalyse the formation and cleavage of carbon–carbon bonds, whereby one partner of the reaction is either bicarbonate or carbon dioxide. In 'Enzyme Nomenclature' (1992) 73 enzymes of this class are listed as 'carboxy lyases' (EC 4.1.1) [5]. In addition there are numerous carboxylases and decarboxylases with concomitant redox reactions, which are found under 'oxidoreductases', e.g. isocitrate dehydrogenases (EC 1.1.1.41 and 42) or pyruvate synthase (EC 1.2.7.1). Finally carboxylases, which are coupled to hydrolysis of ATP, form a distinct small group of five biotin-containing 'carbon–carbon ligases' (EC 6.4.1.). Hence, carboxylases and decarboxylases represent the major class of carbon–carbon bond forming and cleaving enzymes.

2. Different mechanisms of decarboxylations

In general, enzyme-catalysed decarboxylations, in which a carbon–carbon bond is cleaved, can be described as the conversion of a carboxylate anion attached to an sp^3 centre of an organic compound into CO_2 and an sp^2 centre. The different types of decarboxylations are distinguished by the stabilisation of the negative charge at the sp^2 centre, the further fate of the sp^2 centre and, if applicable, by the formation of the sp^3 centre preceding decarboxylation.

1. β-Eliminations: the negative charge is removed by the elimination of a leaving group in the β-position, e.g. decarboxylation of (*R*)-5-diphosphomevalonate-3-phosphate to inorganic phosphate and isopentenyl diphosphate (EC 4.1.1.33).
2. β-Keto acids: the negative charge is attracted by a carbonyl or thiol ester group in the β-position. The resulting enolate is subsequently stabilised by phosphorylation (phosphoenolpyruvate carboxykinase, EC 4.1.1.32/49) or protonated to the ketone or thiol ester. Hence, the most frequent substrates of the known decarboxylases are β-keto acids or malonyl mono thiol esters and derivatives thereof. In some enzymes the decarboxylation is facilitated by the condensation of the β-keto acid with a primary amine, e.g. a lysine residue of the enzyme, to form a Schiff's base or β-imino acid, which in the protonated form neutralises the developing negative charge. A well-studied enzyme of this type is acetoacetate decarboxylase which contains a specific lysine residue with the unusual low $pK = 5$ [6]. Instead of an amine, many decarboxylations of β-keto acids require divalent metal ions as are Mg^{2+} or Mn^{2+}, which co-ordinate to the carbonyl oxygen and act as electron withdrawing Lewis acids. In a recently discovered enzyme from *Escherichia coli* with methylmalonyl-coenzyme A (CoA) decarboxylase activity, the negative charge of the enolate is assumed to be stabilised by strong hydrogen bonds with two backbone NH groups like in the homologous crotonase (enoyl-CoA hydratase, EC 4.1.2.17) [7].
   The four sodium ion-translocating decarboxylases described in this review also belong to these group 2 enzymes. They differ, however, from most decarboxylases, since prior to the actual Na^+-dependent decarboxylation, the carboxylate is transferred to biotin in a Na^+-independent manner.
3. Conversions to β-keto acid-like structures: prior to decarboxylations, α-keto acids as well as oxalyl-CoA are converted to pseudo β-imino acids by reversible addition to thiamine diphosphate. Pyridoxal-5′-phosphate or a pyruvoyl residue of the protein transiently isomerise α-amino acids to β-imino acids, which decarboxylate to amines. β-Hydroxy acids are oxidised to β-keto acids; the consecutive decarboxylation leads to a ketone. Examples are the 'malic enzymes' (EC 1.1.1.38/39), which catalyse the oxidative decarboxylation of malate with NAD^+ to pyruvate. Alternatively, the ketone is reduced again by the same enzyme, which requires also NAD^+ as cofactor, e.g. UDP-D-glucuronate decarboxylase leading to UDP-D-xylose (EC 4.1.1.35).
4. Aromatic acids: carboxylic acids, in which the carboxyl group is attached to an sp^2 centre, e.g. to an aromatic ring, have to be protonated at this centre in order to obtain the required sp^3 centre. Therefore only aromatic carboxylic acids, which contain a hydroxyl group in the *ortho*- or *para*-position, are readily decarboxylated. An example is 6-methylsalicylate, which is able to tautomerise

to the β-keto acid 6-methylcyclohexa-3,5-diene-2-one-1-carboxylate. Rearomatisation by decarboxylation and protonation leads to *m*-cresol (EC 4.1.1.52).

5. Unusual decarboxylations: anaerobic bacteria are able to decarboxylate 'difficult' substrates as are *p*-hydroxyphenylacetate to *p*-cresol [8], phenylacetate to toluene [9] and phthalate to benzoate (B. Schink, personal communication). All three reactions cannot be described by the mechanistic schemes described above. It has been proposed that the electron donating phenolate anion of *p*-hydroxyphenylacetate requires an 'Umpolung' by transient oxidation to an electron attracting phenoxy radical. Decarboxylation of this radical species yields the ketyl radical anion, which is protonated and reduced to the *p*-cresolate anion [10]. Finally it should be mentioned that a decarboxylase has been recognised as the most proficient enzyme known to date. Orotidine 5′-phosphate decarboxylase accelerates the rate of decarboxylation of the nucleotide by 10^{17}-fold, but the mechanism still remains elusive [11].

3. Na^+-translocating and biotin-containing decarboxylases

At least four enzymes or enzyme systems of this type are known

1. Oxaloacetate decarboxylases (EC 4.1.1.3) from enterobacteria: *K. pneumoniae* [2,12–14] and *Salmonella typhimurium* [15].
2. Malonate decarboxylase system (EC 4.1.1.-) from *Malonomonas rubra* [16].
3. Methylmalonyl-CoA decarboxylases (EC 4.1.1.41) from *Veillonella parvula* (clostridial cluster IX, [17]) [18,19], *Propionigenium modestum* (XIX) [20,21] and *Peptostreptococcus* sp. [22].
4. Glutaconyl-CoA decarboxylases (EC 4.1.1.70) from *Acidaminococcus fermentans* (IX) [23–25], *Fusobacterium nucleatum* (XIX) [26], *Peptostreptococcus asaccharolyticus* (XIII) [27], *Clostridium symbiosum* (XIVa) [27], *Pelospora glutarica* (VIII) [28] and *Synthrophus gentianae* (VIII) [29].
5. The decarboxylases deduced from the genomes of several organisms (Table 1).

Table 1
Sequence comparisons of the β-subunit of glutaconyl-CoA decarboxylase from *A. fermentans* with those of other characterised or deduced Na^+-dependent decarboxylases

Organism/accession number	Class/phylum[e]	Amino acids[f]	Identities (%)
A. fermentans[a] AF030576 [25]	Clostridia/BXIII	375–375/375	100
V. parvula[b] Z247754 [19]	Clostridia/BXIII	373–332/372	80
P. modestum[b] AJ002015 [21]	Fusobacteria/BXXI	395–226/392	57
Pyrococcus abyssi AJ248284	*Thermococci*/AII	400–234/395	59
Pyrococcus horikoshii AP000005 [72]	*Thermococci*/AII	400–237/398	59
Vibrio cholerae AE004141 [73]	Gammaproteobacteria/BXII	376–224/373	60
V. cholerae AE004164	Gammaproteobacteria/BXII	433–221/430	51
Archaeoglobus fulgidus AE000960 [74]	*Archaeoglobi*/AII	354–205/352	58
Treponema pallidum AE001190 [75]	Spirochetes/BXVII	469–198/362	54
S. typhimurium[c] M96434 [15]	Gammaproteobacteria/BXII	433–209/428	49
K. pneumoniae[c] [14]	Gammaproteobacteria/BXII	433–205/428	48
Haemophilus ducreyi AF200362	Gammaproteobacteria/BXII	435–186/300	62
Thermotoga maritima AE001753 [76]	*Thermotogae*/BII	384–182/369	49
M. rubra[d] U87980 [77]	Deltaproteobacteria/BXII	401–103/369	27

[a]Glutaconyl-CoA decarboxylase.
[b]Methylmalonyl-CoA decarboxylases.
[c]Oxaloacetate decarboxylases.
[d]Malonate decarboxylase; the other organisms contain putative decarboxylases.
[e]Bergey's manual of systematic bacteriology, 2nd edn.; A, *Archaea*; B, *Bacteria*.
[f]Total number of amino acids of the corresponding β-subunit−number of identical amino acids/number used for comparison.

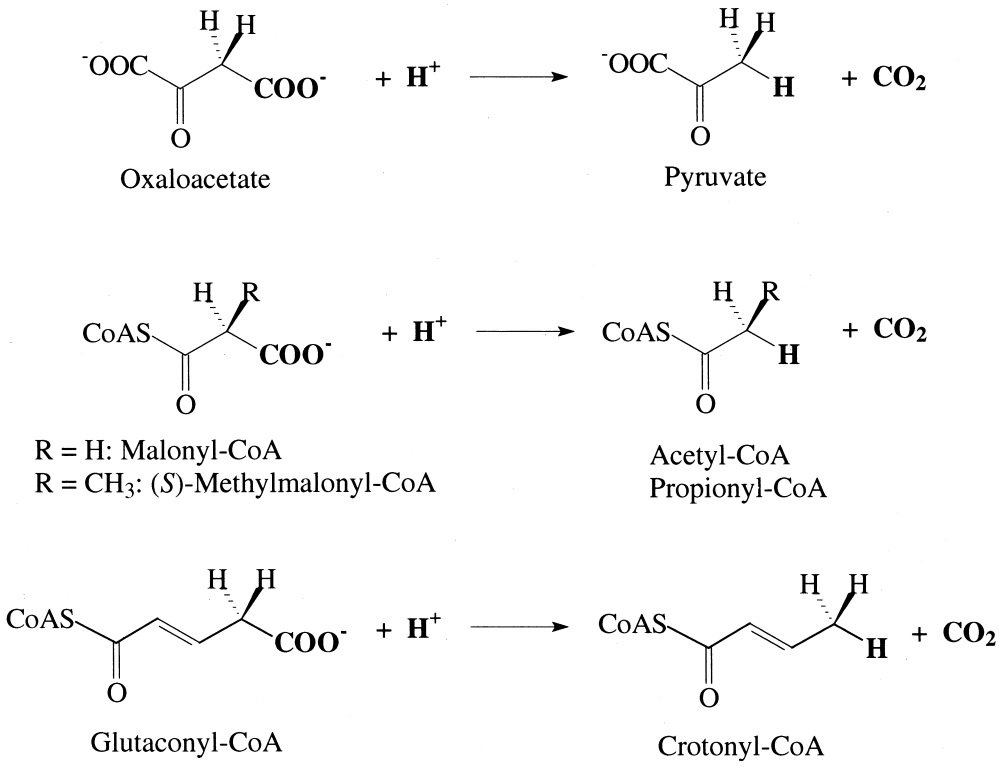


Fig. 1. Na^+-dependent decarboxylations.

In all these biotin-dependent decarboxylations, the carboxylate of an organic acid is replaced by a proton under retention of configuration [30–32] and carbon dioxide is released [33,34] (Fig. 1).

4. Subunit composition and amino acid sequences of the Na^+-translocating decarboxylases

This paragraph is mainly concerned with a comparison of oxaloacetate, methylmalonyl-CoA and glutaconyl-CoA decarboxylases. For the malonate decarboxylase system the reader is referred to P. Dimroth's recent review on this subject [35]. The three enzymes are readily purified from the membrane fractions of the corresponding organisms by solubilisation with Triton X-100 followed by affinity chromatography on Sepharose to which monomeric avidin or streptavidin is covalently bound. The biotin-containing decarboxylases remain on the column, from which they are eluted with free biotin in buffers with the desired detergent, e.g. the chemical homogenous dodecylmaltoside instead of the less well-defined and UV-absorbing Triton X-100. Other detergents except some members of the Tween and Brij series dissociate the enzyme complex into subunits. Analysis of the purified decarboxylase preparations by SDS–PAGE and staining with Coomassie brilliant blue reveals a strong band around 65 kDa for each enzyme (α-subunit), whereas the other subunits are difficult to detect. They either have to be stained with silver, are only stainable via their biotin content, are very small and escape the gel or give artefacts [36–38]. Hence, the complete picture of the subunit compositions had to await gene cloning and expression which started in 1988 [12] and was almost completed in 1999 [25]. Surprisingly, the molecular mass of none of these enzymes in the non-denatured state has been reported yet. With glutaconyl-CoA decarboxylase various size exclusion columns were tested, but in no case the enzyme could be recovered (S. Rospert and W. Buckel, unpublished observations).

Each of the three enzymes contains a substrate specific hydrophilic subunit, designated as α, which shares sequence similarities with the corresponding ATP- and biotin-dependent carboxylase. The α-sub-

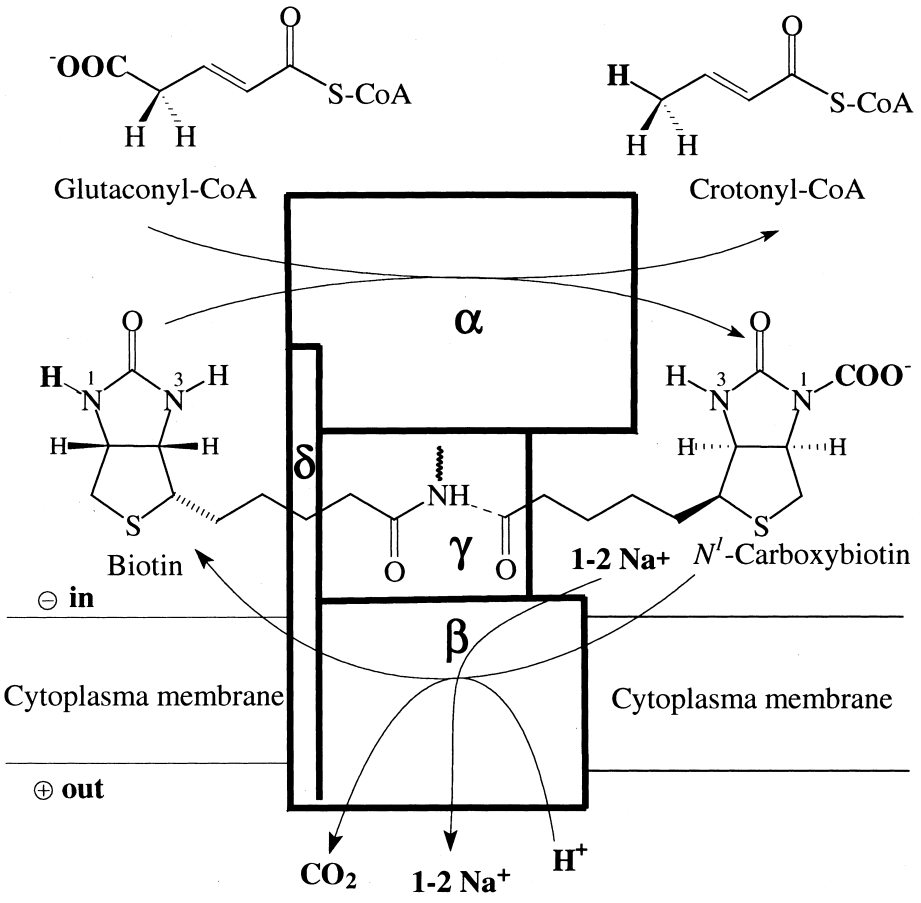
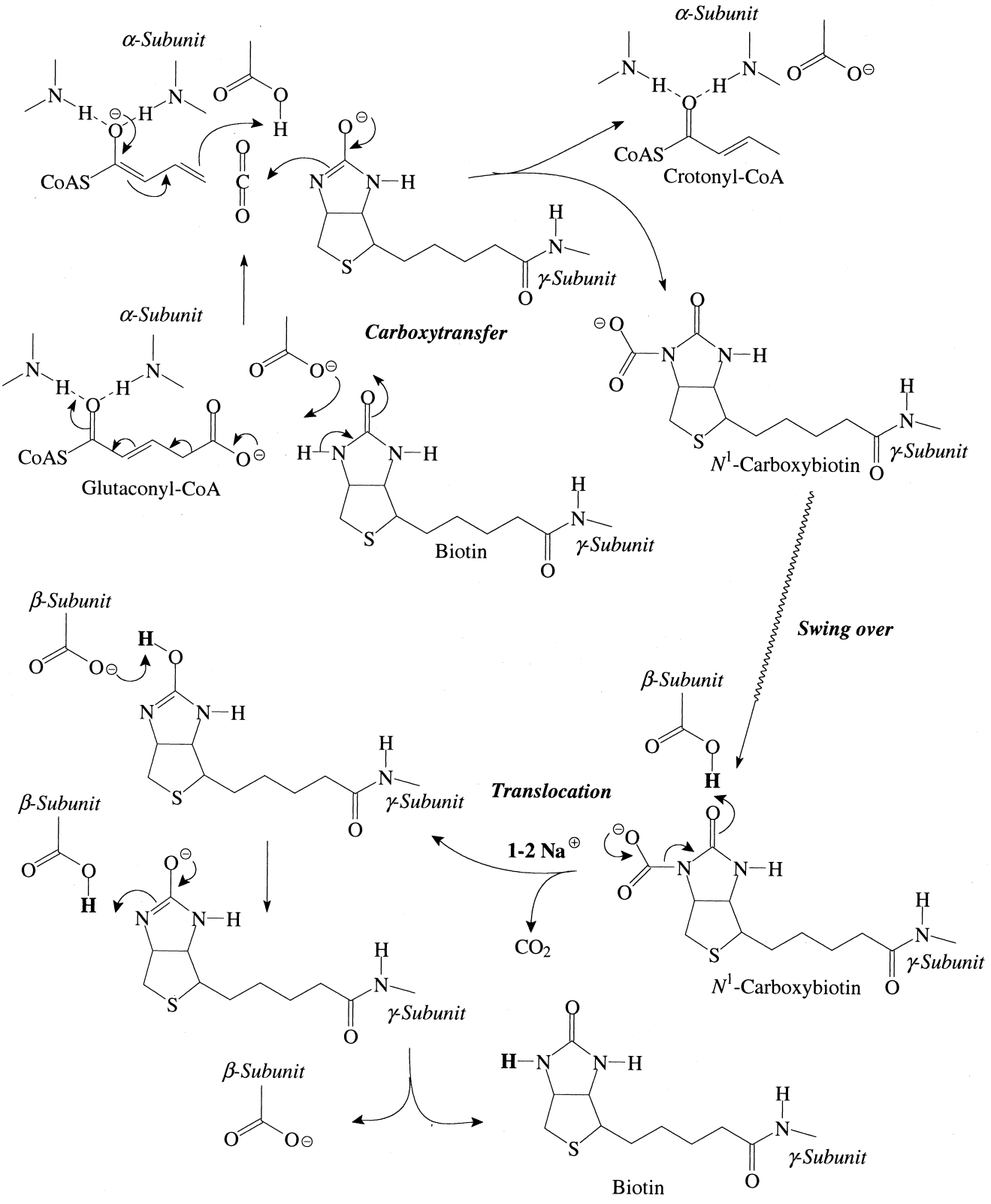


Fig. 2. Model showing the postulated function and interaction of the α-, β-, γ-, and δ-subunits of glutaconyl-CoA decarboxylase. Slightly modified version of [25].

unit of oxaloacetate decarboxylase from *K. pneumoniae* is related to pyruvate carboxylase and that of methylmalonyl-CoA decarboxylase from *V. parvula* to various propionyl-CoA carboxylases, to transcarboxylase [19] and to 3-methylcrotonyl-CoA carboxylase from *Arabidopsis thaliana* [39]. The α-subunit of glutaconyl-CoA decarboxylase from *A. fermentans* (Fig. 2) shows lower sequence identities to methylmalonyl-CoA decarboxylase and to 3-methylcrotonyl-CoA carboxylase. By overexpression of the *gdcA* gene from *A. fermentans* in *E. coli*, the α-subunit could be obtained as a homogenous protein. Despite its presence in the soluble fraction, it required dodecylmaltoside in order to prevent aggregation at protein concentrations above 0.8 mg/ml. The protein eluted from gel filtration columns in the presence of this detergent with an apparent molecular mass of 200 kDa, probably as a homotrimer [25].

Biotin is covalently attached either to the C-terminal domain of the α-subunit of the oxaloacetate decarboxylases [12] or to separate small subunits (15–18 kDa), designated as γ, in the other two enzymes [19,25]. The conserved lysine, to which biotin forms an amide bond, is located 34 amino acids before the C-terminus flanked on both sides by two methionine residues, which are conserved in every biotin-dependent enzyme. A remarkable feature of these biotin carriers is the flexible region upstream of that lysine consisting mainly of alanines and prolines. In the extreme case of glutaconyl-CoA decarboxylase from *A. fermentans*, the polypeptide contains in the middle of the total 146 amino acids a stretch of 50 amino acids (residues 26–75) comprising 34 alanines, 14 prolines, one valine and one lysine, whereas the lysine residue to which biotin is bound has position 112 [25]. It has been known from ^{1}H-nuclear mag-


α-Subunit
CoAS
Crotonyl-CoA
γ-Subunit
Carboxytransfer
Glutaconyl-CoA
Biotin
N1-Carboxybiotin
Swing over
β-Subunit
Translocation
1-2 Na⊕
CO2

netic resonance (NMR) spectroscopy that in proteins such alanine- and proline-rich regions are highly mobile [40]. Furthermore, the whole glutaconyl-CoA decarboxylase (*m* ca. 200 kDa) labelled with [2-^{13}C]biotin gives a ^{13}C-NMR spectrum with a relatively sharp carbonyl signal, which again demonstrates the mobility of the prosthetic group [41].

All three enzymes contain a very hydrophobic β-subunit (39–45 kDa), whose sequences share 50–80% identity. By standard hydropathic plots up to 11 transmembrane helices were predicted [14,19,25]. A careful topological analysis of the β-subunit of oxaloacetate decarboxylase, however, revealed a somewhat different picture (see also the review by P. Dimroth in this issue), which most likely can also be applied to the CoA-ester decarboxylases [42]. The results of fusions with alkaline phosphatase and β-galactosidase confirmed three of the predicted α-helices at the N-terminal part and six at the C-terminal part. Modification of genetically engineered cysteine residues by the membrane-impermeable fluorescent reagent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate corroborated the new model with the nine α-helices. The part between the α-helices III and IV, which was predicted to fold into the two remaining α-helices, gave ambiguous results. The authors propose that this part (IIIa) folds into four short α-helices of 9–11 amino acids each, which reach from the periplasm only to the middle of the membrane. Similar short α-helices have been detected in the pore helices of the K^+ channel from *Streptomyces lividans* whose crystal structure has been determined [43].

Treatment of purified glutaconyl-CoA decarboxylase from *A. fermentans* in Triton X-100 with 200 mM 1-butanol precipitates the β-subunit. This selective denaturation can be completely prevented by the presence of 100 mM NaCl, whereas KCl has no effect [44]. Similarly, this β-subunit [27] and also that of the oxaloacetate decarboxylase from *K. pneumoniae* [45] is protected by NaCl from tryptic digestion. Furthermore, the periplasmic loop between the predicted α-helices V and VI contains the conserved sequence motive ESGV. Only in glutaconyl-CoA decarboxylase the valine (V301 in oxaloacetate decarboxylase) is replaced by cysteine (C243), which can be alkylated by *N*-ethyl[2,3-^{14}C]maleimide. Again this alkylation is specifically inhibited by 200 mM NaCl [25]. This does not necessarily imply that the cysteine residue C243 or the peptide bonds susceptible to proteolysis by trypsin participate directly in low affinity Na^+ binding. These data suggest, however, that the β-subunits have a second low affinity Na^+ binding site, which, when occupied, keeps the protein in a more resistant and tightly packed conformation. The first Na^+ binding site has certainly a much higher affinity as shown by the low apparent $K_m = 1$ mM for Na^+ in the decarboxylation assay. It should be noted that the affinity of a substrate or cofactor to an enzyme is expressed by the thermodynamic dissociation constant $K_s = k_2/k_1$ rather than by the kinetic constant $K_m = (k_2+k_3)/k_1$. As pointed out by W.P. Jencks, K_s often is even much smaller than K_m, since many enzymes such as CoA-transferases use binding energy for rate enhancement by increasing k_3 [46,47]. Therefore, the first binding site of Na^+ at the β-subunit should have an affinity of $K_s \leq 1$ mM Na^+, whereas the affinity of the second is in the range of $K_s = 20$–50 mM Na^+.

All three decarboxylases contain an additional small subunit (10–15 kDa) designated as δ in the CoA-ester decarboxylases and unfortunately for historic reasons as γ in the oxaloacetate decarboxylases. The δ/γ-subunits contain one transmembrane α-helix and bind to the corresponding α-subunits. Therefore it has been proposed that these small subunits may serve as membrane anchors of the α-subunits. Apparently, only the γ-subunits of the oxaloacetate decarboxylases contain about one Zn^{2+}, which has been suggested to act as a Lewis acid facilitating the decarboxylation [48] most likely by stabilisation of the keto form oxaloacetate bound to the α-subunit. A distinct feature of the methylmalonyl-CoA decarboxylase from *V. parvula* is the presence of an additional very small ε-subunit (55 amino acids) [19],

←

Fig. 3. Postulated mechanisms of the chemical events during decarboxylation of glutaconyl-CoA to crotonyl-CoA. The binding of the thiol esters to the carboxyltransferase was taken from that postulated for the crotonase superfamily [7]. It is assumed that the protons are transferred by yet unknown carboxylates in the α- and the β-subunits. The ^{13}C-NMR signal of glutaconyl-CoA decarboxylase-containing [2-^{13}C]biotin indicated that already in the resting enzyme part of the biotin exhibits the enol form [41].

which is absent in all other sequenced decarboxylases. Deletion of this subunit gave an active enzyme albeit with lower stability [49]. A model of glutaconyl-CoA decarboxylase showing the proposed interaction of the four subunits α, β, γ and δ is depicted in Fig. 2.

5. The genes encoding the Na^+-translocating decarboxylases

The three genes, *oadG*, *oadA* and *oadB*, which encode the γ-, α- and β-subunits of oxaloacetate decarboxylase from *K. pneumoniae*, respectively, are arranged in an operon in this order together with *citA* and *citB* (for a two-component regulatory system) [50]. In a similar way, an operon is formed by the genes *mmdADCB*, which encode the corresponding α-, δ-, γ-, and β-subunits of methylmalonyl-CoA decarboxylase from *P. modestum* [21], respectively, whereas in *V. parvula* the additional *mmdE* gene (for the ε-subunit) is inserted after *mmdD* [19]. The initial cloning efforts with *A. fermentans* led only to the identification of *gcdA*, the gene encoding the α-subunit of glutaconyl-CoA decarboxylase [51], which is located in the middle of the 'hydroxyglutarate operon'. The gene is flanked upstream by *gctA* and *B* encoding glutaconate CoA-transferase, and downstream by *hgdC*, *A* and *B* encoding the 2-hydroxyglutaryl-CoA dehydratase system [52]. Together with glutaconyl-CoA decarboxylase, these enzymes belong to the pathway of glutamate fermentation to ammonia, CO_2, acetate, butyrate and molecular hydrogen [53]. Later a separate operon with the genes *gcdD*, *C* and *B* encoding the general δ-, γ-, and β-subunits has been detected [25]. Surprisingly, the cloned DNA contained upstream of *gcdD* the fragment of an open reading frame (*mmdA*), whose deduced amino acid sequence is homologous to the C-terminal part of the α-subunits of the methylmalonyl-CoA decarboxylases from *V. parvula* and *P. modestum*. Hence, *A. fermentans* should be able to synthesise a complete methylmalonyl-CoA mutase from genes arranged in a manner almost identical to that of *P. modestum*. Northern blots showed, however, that only the *gcdD*, *C* and *B* genes were transcribed, most likely under the control of the promoter between the putative *mmdA* gene fragment and *gcdD*. In addition, no growth condition was found, under which *A. fermentans* exhibited methylmalonyl-CoA decarboxylase activity. In summary, the synthesis of glutaconyl-CoA decarboxylase in *A. fermentans* requires genes from two separate operons [25]. In *C. symbiosum* there is a different situation; a homologue of *gcdA* encoding the α-subunit of glutaconyl-CoA decarboxylase is missing upstream of *hdgCAB* encoding 2-hydroxyglutaryl-CoA dehydratase [54].

6. On the mechanism of the carboxyltransferase step

The biotin-dependent decarboxylases catalyse the reaction in two steps, the Na^+-independent transfer of the carboxyl group to biotin followed by the Na^+-dependent decarboxylation of carboxybiotin. The only exception is malonate decarboxylase. This enzyme system contains an additional transferase, in order to form the malonyl thiol ester with 2′-(5″-phosphoribosyl)-3′-dephospho CoA, which is attached via a phosphodiester to a serine residue of an acyl carrier protein [55]. The two-step mechanism corroborates with the structure of these enzymes. The carboxylation of biotin is catalysed by the substrate specific α-subunits and the subsequent decarboxylation of carboxybiotin is catalysed by the hydrophobic β-subunits (Fig. 2). The question arises, why biotin is required as mediator between the two processes. There is a topological answer, since the specific lysine residue, which forms an amide bond with the carboxyl group of biotin, is part of the highly mobile γ-subunit (see above). Biotin can therefore be loaded with CO_2 at the transferase site and then swings over to the decarboxylase site.

In contrast to the other Na^+-dependent decarboxylases, the isolated α-subunit of glutaconyl-CoA decarboxylase is able to catalyse the carboxylation of free biotin with glutaconyl-CoA. Using [1,5-$^{13}C_2$]glutaconyl-CoA and [1-$^{15}N_1$]biotin, the formation of the ^{13}C–^{15}N bond concomitant with the release of [1-^{13}C]crotonyl-CoA and $^{13}CO_2/H^{13}CO_3^-$ could be followed directly by ^{13}C-NMR spectroscopy. Hence this experiment shows that the transfer of the carboxylate occurs directly to the nitrogen of biotin without any intermediate such as carboxylation at the carbonyl oxygen followed by rearrangement [56]. The transfer is facilitated by partial for-

mation of the enolate of biotin as revealed by ^{13}C-NMR spectroscopy of [carbonyl-^{13}C]biotin-containing glutaconyl-CoA decarboxylase [41]. The binding of glutaconyl-CoA to the enzyme in a similar manner as in enoyl-CoA hydratase (see Section 1 [7]) would enhance the cleavage of the C–COO^- bond by stabilisation of the resulting dienolate. Thus CO_2 is removed from the equilibrium between glutaconyl-CoA^- and CO_2+dienolate$^-$ by trapping with the enolate of biotin (Fig. 3). The subsequent decarboxylation of carboxybiotin at the β-subunit coupled to Na^+ translocation is very facile. Just protonation of the 2-carbonyl oxygen immediately releases CO_2. Since the overall equilibrium of the transfer step is not far from $K_{eq} = 1$ as revealed by the NMR experiment, carboxybiotin can therefore be regarded as a 'kinetically activated' rather than as an 'energy-rich' carboxylate. It is concluded that biotin has three functions in this class of decarboxylases: (i) facilitation of the decarboxylation of the carbon substrate by transfer to biotin, (ii) swing over of the carboxyl group from the α- to the β-subunit, and (iii) fast decarboxylation of carboxybiotin necessary for efficient Na^+ translocation (Fig. 3).

7. The Na^+ translocation step

This vectorial step is either measured with inverted membrane vesicles from the corresponding organism or with the purified enzymes after incorporation into artificial phospholipid vesicles. Only those enzyme molecules, which are accessible by the substrate at their cytoplasmic side facing to the outside, are able to participate in the experiment. In order to detect Na^+ incorporation, the vesicles are separated from free Na^+ by ion-exchange chromatography on Dowex 50 (K^+ form) [36,38]. The Na^+ content of the vesicles is determined with the γ-ray emitting isotope $^{22}Na^+$ or by atomic absorption spectroscopy [57].

Numerous experiments with oxaloacetate, methylmalonyl-CoA and glutaconyl-CoA decarboxylases have demonstrated an initial strict coupling between decarboxylation and Na^+ transport into the vesicles. The initial uptake stoichiometry has been determined as ≤ 2 Na^+/CO_2 formed. The transport is completely abolished by μM concentrations of the Na^+/H^+ exchanging antibiotic monensin, whereas valinomycin/K^+ approximately doubles the initial rate and extent of Na^+ uptake. The effect of the latter, K^+ specific antibiotic indicates the electrogenic nature of this process. The influx of Na^+ builds up a counteracting membrane potential $\Delta\Psi$ which is abolished by the valinomycin-induced efflux of K^+. The net uptake of Na^+, however, ceases soon, regardless whether valinomycin/K^+ is absent or present, whereas the decarboxylation proceeds further. Apparently the vesicles are 'full' and the decarboxylation is now uncoupled. By subsequent addition of monensin, the vesicles are immediately emptied, whereas the rate of decarboxylation is almost doubled [3,27,38,58].

Experiments with oxaloacetate decarboxylase shed some light on these bewildering results. If the radioactive tracer $^{22}Na^+$ is added after the vesicles have been loaded with unlabelled Na^+, the uptake kinetics are indistinguishable from those in which the tracer has been added before start of the decarboxylation. Hence, decarboxylation of oxaloacetate not only drives the net uptake of Na^+, but also the $^{22}Na^+/^{23}Na^+$ exchange between outside and inside. Also the effect of monensin on the rate of decarboxylation is the same, regardless whether the vesicles are 'empty' or 'full' [59]. Furthermore, the concomitant uptake of [^{14}C]acetate and Na^+ shows that the interior of the vesicles becomes alkaline during decarboxylation. Only acetic acid can permeate the membrane and is trapped inside the vesicle by conversion to acetate anion. This indicates that the proton required to initiate decarboxylation comes from the inside of the vesicle [60]. Therefore oxaloacetate decarboxylase catalyses an electroneutral Na^+/H^+ exchange in addition to the uptake of a second 'electrogenic' Na^+. It seems likely that by increasing the interior Na^+ concentration concomitant with the pH, the proton is gradually displaced by Na^+ and the initial 1–2 Na^+/1 H^+ exchange is converted to a 1–2 Na^+/1 Na^+ exchange, whereby the proton has to come from the outside of the inverted vesicle. This means for the intact bacterial cell that at high external Na^+ concentrations (ca. >100 mM) the proton should come from the cytoplasm rather than from the periplasm (see also Section 8).

The mechanism postulated by Di Berardino and Dimroth [60] can only explain, although very elegantly, the electroneutral Na^+/H^+ exchange. It is assumed that Na^+ is attracted by the anion of carboxy-

biotin from the cytoplasmic side into the membrane spanning β-subunit and the proton is delivered by the conserved essential aspartate residue (β-203 in oxaloacetate decarboxylase). Exchange of both cations induces decarboxylation and such a conformational change that the Na^+ is released to the periplasmic side. For the second Na^+, however, the proposal shows no driving force. Extensive site-directed mutagenesis of the β-subunit of oxaloacetate decarboxylase leads to a new and different proposal of the mechanism of Na^+ translocation [45,61], which is put forward in Peter Dimroth's review in this volume. A major draw-back, however, is the lack of a three-dimensional structure, which is required to answer the urgent question whether all the identified essential residues indeed participate in Na^+ channel forming. A very first step in this direction is the successful crystallisation of the α-subunit of glutaconyl-CoA decarboxylase from *A. fermentans*, which diffracts to 2.2 Å resolution (K. Wendt and U. Jacob, Max-Planck-Institut für Biochemie, Martinsried, Germany, personal communication).

8. Energy conservation by decarboxylation

The standard free energy of the decarboxylations mentioned in this review, $\Delta G^{0\prime}$ = ca. −30 kJ/mol, has been calculated with carbon dioxide being formed as gas ($CO_{2,gas}$). The free energy is considerably less negative if carbon dioxide remains in aqueous solution ($CO_{2,aq}$) or is converted to hydrogencarbonate (HCO_3^-) [62].

$$CO_{2,gas}(+H_2O) = CO_{2,aq};\ \Delta G^{0\prime} = +8.34\ \text{kJ/mol}$$

$$CO_{2,gas} + H_2O = HCO_3^- + H^+;\ \Delta G^{0\prime} = +4.82\ \text{kJ/mol}$$

Since CO_2 is the product of decarboxylases and gaseous CO_2 is formed in most fermentations, for the sake of simplicity, in this review $\Delta G^{0\prime} = -30$ kJ/mol is taken as free energy of decarboxylation. In fatty acid biosynthesis, this energy is used to drive the elongation step by decarboxylation of each malonyl-CoA building unit. In gluconeogenesis, the very energy-rich compound phosphoenolpyruvate ($\Delta G^{0\prime}{}_{\text{hydrolysis}} = -60$ kJ/mol) is formed by combined action of decarboxylation together with ATP- or GTP-dependent phosphorylation. For coupling of decarboxylation with ATP synthesis, however, the free energy of one decarboxylation is not sufficient. Under the irreversible growth conditions, at least 70 kJ is required for the synthesis of 1 mol ATP [62]. The only way to synthesise ATP from decarboxylation is to accumulate the energy from at least three decarboxylations via an electrochemical ion gradient, $\Delta\mu H^+$ or $\Delta\mu Na^+$. There are two anaerobic organisms which are able to conserve their whole energy via decarboxylation, *Oxalobacter formigenes* [63] and *P. modestum* [64]. A comparison of the catabolism of both bacteria could give an answer why coupling by $\Delta\mu H^+$ is used by the former and $\Delta\mu Na^+$ by the latter organism. The oxalyl-CoA decarboxylase from *O. formigenes* has been characterised as a soluble, thiamine diphosphate-dependent enzyme, which is devoid of biotin and does not require Na^+ for activity [65]. The driving force for ATP synthesis, $\Delta\mu H^+$, is derived from ΔpH generated by the consumption of H^+ inside the cell and from $\Delta\Psi$ generated by the electrogenic exchange of oxalate^{2-} dianion versus formate$^-$ monoanion catalysed by a specific antiporter [66]. In principle the same mechanism could be applied for *P. modestum*, which thrives from the decarboxylation of succinate to propionate [20]. In contrast to formate and oxalate, propionate with the high $pK = 4.9$ and also succinate monoanion (succinate: $pK_1 = 4.2$, $pK_2 = 5.6$) can dissipate ΔpH [67]. At pH 7, about 1% of propionate is present as membrane-permeable propionic acid, whereas the much stronger and more hydrophilic formic acid ($pK = 3.8$; $< 0.1\%$ acid at pH 7) and oxalic acid ($pK_1 = 1.2$; $pK_2 = 4.2$) are much less impermeable, but see [68]. This might be the reason why *P. modestum* uses the membrane-bound methylmalonyl-CoA decarboxylase, which generates $\Delta\mu Na^+$ for ATP synthesis by a Na^+-dependent F_1F_0-ATPase [4]. This process apparently does not require an electrogenic succinate^{2-}/propionate$^-$ antiporter.

P. modestum has been isolated from marine sediments in the Canale Grande of Venice which contains about 0.5 M Na^+ [64]. It has been shown in vitro for oxaloacetate decarboxylase that at this concentration there is no net Na^+ uptake (see above) and the enzyme is inhibited [60]. Therefore it remains to be elucidated how in vivo under these conditions methylmalonyl-CoA decarboxylase can act as an

electrogenic Na^+ pump. The comparison of the molar growth yields of *O. formigenes* on oxalate (1–1.4 g dry cells) and *P. modestum* on succinate (2.4–3.5 g) suggests that the $\Delta\mu Na^+$ generating system apparently is more efficient in energy conservation. It has to be considered, however, that *O. formigenes* uses for its anabolism oxalate, CO_2 and additional acetate rather than succinate [69]. For the synthesis of 1 mol succinate from 2 mol acetate about 4 mol ATP is consumed. This extra ATP reduces the standard value of about 10 g dry cells/ATP to about 5 g. Hence, the growth yields of both organisms are almost equal.

The other Na^+-translocating decarboxylase-containing organisms conserve the major part of their energy via substrate level phosphorylation. The additional energy contributed by $\Delta\mu Na^+$ is used for citrate transport in *K. pneumoniae* [70] and probably for glutamate transport in clostridia. Experiments with washed cells of *A. fermentans* revealed an influence of the external Na^+ concentration on the energy metabolism [53]. The fermentation of glutamate required Na^+; the maximum rate was reached already at 1 mM Na^+, at the apparent K_m of glutaconyl-CoA decarboxylase. According to Eq. 1, acetate and butyrate were produced at the ratio 1:2; no hydrogen was formed. By increasing the Na^+ concentration to >100 mM, the fermentation rate decreased slightly (80% of the rate at 1 mM Na^+) but hydrogen was formed and the acetate:butyrate ratio increased to 3 (Eq. 2).

$$1\ \text{mM}\ Na^+:\ \text{Glutamate}^- + 0.5\ H^+ + H_2O = NH_4^+ + CO_2 + 1.0\ \text{acetate}^- + 0.5\ \text{butyrate}^-;\ 0.5\ \text{mol ATP/mol glutamate};\quad (1)$$

$$\geq 100\ \text{mM}\ Na^+:\ \text{Glutamate}^- + 0.4\ H^+ + 1.2\ H_2O = NH_4^+ + CO_2 + 0.2\ H_2 + 1.2\ \text{acetate}^- + 0.4\ \text{butyrate}^-;\ 0.6\ \text{mol ATP/mol glutamate};\quad (2)$$

The data show that increasing Na^+ concentrations stimulate H_2 formation, whereby the yield of ATP/glutamate via substrate level phosphorylation increases by 20%. In *A. fermentans*, the NADH generated by the oxidation of 3-hydroxybutyryl-CoA to acetoacetyl-CoA ($E_0' = -240$ mV) is the only available source of hydrogen ($E' = -380$ mV). Therefore hydrogen production requires energy, which could be contributed by the increasing Na^+ gradient. However, the apparent K_m (ca. 20 mM) of this stimulation by Na^+ is as high as that of the low affinity Na^+ binding to the β-subunit (see Section 4). Furthermore 100 mM Na^+ should be high enough to switch from Na^+/H^+ exchange to mere Na^+/Na^+ exchange (see Section 6). Thus the proton for decarboxylation should come from the cytoplasm rather than from the outside of the cell, which would lead to $\Delta\mu H^+$ rather than to $\Delta\mu Na^+$. It has been proposed therefore that hydrogen formation might be driven by $\Delta\mu H^+$ [53]. An NADH dehydrogenase suitable for this purpose has been detected in the cytoplasmic membrane from *A. fermentans* (J. Bresser, A.J. Pierik and W. Buckel, unpublished results).

9. Distribution of Na^+-dependent and -independent decarboxylases

Mammalian mitochondria and many proteobacteria contain a soluble glutaryl-CoA dehydrogenase, which catalyses the oxidation of glutaryl-CoA to glutaconyl-CoA followed by the Na^+-independent decarboxylation of the enzyme-bound or of free glutaconyl-CoA to crotonyl-CoA. The enzyme is involved in aerobic lysine and tryptophan oxidation as well as in anaerobic benzoate oxidation with nitrate as electron acceptor [71]. On the other hand, the Na^+-dependent membrane-bound glutaconyl-CoA decarboxylase is found in glutamate fermenting clostridia, glutarate fermenters and synthrophic benzoate oxidisers (see Section 3). Since the latter organisms gain much less ATP from their catabolic pathways, it appears reasonable that glutaconyl-CoA decarboxylase conserves extra energy. However, the opposite seems to be the case with oxaloacetate decarboxylase. Proteobacteria contain the well-characterised Na^+-translocating enzyme, whereas *A. fermentans* [53] and other clostridia (P. Dimroth, personal communication) use a soluble Mg^{2+}-dependent decarboxylase.

In all Na^+-dependent decarboxylases the β-subunit catalyses the identical reaction, the translocation of Na^+ driven by decarboxylation of carboxy biotin. Among the characterised decarboxylases the sequen-

ces of this subunit are highly conserved as compared to the other subunits. Furthermore no significant sequence similarities could be detected to proteins with different functions. A search of the recently available genomes revealed several deduced proteins with high sequence identities to the β-subunits of glutaconyl-CoA decarboxylase, methylmalonyl-CoA decarboxylase and oxaloacetate decarboxylase (Table 1). Apparently there is a random distribution among the various phyla of the *Archaea* and *Bacteria*. The substrates of these new putative Na^+-translocating decarboxylases remain to be established.

Acknowledgements

The author thanks Professor Duilio Arigoni (ETH Zürich, Switzerland) and Professor Bernard T. Golding (University of Newcastle upon Tyne, UK) for helpful discussions on the mechanisms. The work was supported by the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie and the European Commission.

References

[1] J.R. Stern, Biochemistry 6 (1967) 3545–3551.
[2] P. Dimroth, FEBS Lett. 122 (1980) 234–236.
[3] P. Dimroth, Microbiol. Rev. 51 (1987) 320–340.
[4] P. Dimroth, Biochim. Biophys. Acta 1318 (1997) 11–51.
[5] E.C. Webb, Enzyme Nomenclature, Academic Press, San Diego, CA, 1992.
[6] D.E. Schmidt Jr., F.H. Westheimer, Biochemistry 10 (1971) 1249–1253.
[7] T. Haller, T. Buckel, J. Rétey, J.A. Gerlt, Biochemistry 39 (2000) 4622–4629.
[8] L. D'Ari, H.A. Barker, Arch. Microbiol. 143 (1985) 311–312.
[9] J.L. Pons, A. Rimbault, J.C. Darbord, G. Leluan, Ann. Microbiol. (Paris) 135B (1984) 219–222.
[10] W. Buckel, B.T. Golding, FEMS Microbiol. Rev. 22 (1999) 523–541.
[11] A. Radzicka, R. Wolfenden, Science 267 (1995) 90–93.
[12] E. Schwarz, D. Oesterhelt, H. Reinke, K. Beyreuther, P. Dimroth, J. Biol. Chem. 263 (1988) 9640–9645.
[13] E. Laussermair, E. Schwarz, D. Oesterhelt, H. Reinke, K. Beyreuther, P. Dimroth, J. Biol. Chem. 264 (1989) 14710–14715.
[14] G. Woehlke, E. Laussermair, E. Schwarz, D. Oesterhelt, H. Reinke, K. Beyreuther, P. Dimroth, J. Biol. Chem. 267 (1992) 22804–22805.
[15] G. Woehlke, K. Wifling, P. Dimroth, J. Biol. Chem. 267 (1992) 22798–22803.
[16] H. Hilbi, I. Dehning, B. Schink, P. Dimroth, Eur. J. Biochem. 207 (1992) 117–123.
[17] M.D. Collins, P.A. Lawson, A. Willems, J.J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, J.A.E. Farrow, Int. J. Bacteriol. 44 (1994) 812–826.
[18] W. Hilpert, P. Dimroth, Nature 296 (1982) 584–585.
[19] J.B. Huder, P. Dimroth, J. Biol. Chem. 268 (1993) 24564–24571.
[20] W. Hilpert, B. Schink, P. Dimroth, EMBO J. 3 (1984) 1665–1670.
[21] M. Bott, K. Pfister, P. Burda, O. Kalbermatter, G. Woehlke, P. Dimroth, Eur. J. Biochem. 250 (1997) 590–599.
[22] P.H. Janssen, W. Liesack, C. Kluge, S. Seeliger, B. Schink, C.G. Harfoot, Antonie Van Leeuwenhoek 70 (1996) 11–20.
[23] W. Buckel, Eur. J. Biochem. 106 (1980) 439–447.
[24] W. Buckel, R. Semmler, FEBS Lett. 148 (1982) 35–38.
[25] A. Braune, K. Bendrat, S. Rospert, W. Buckel, Mol. Microbiol. 31 (1999) 473–487.
[26] B. Beatrix, K. Bendrat, S. Rospert, W. Buckel, Arch. Microbiol. 154 (1990) 362–369.
[27] W. Buckel, R. Semmler, Eur. J. Biochem. 136 (1983) 427–434.
[28] C. Matthies, N. Springer, W. Ludwig, B. Schink, Int. J. Syst. Evol. Microbiol. 50 (2000) 645–648.
[29] L. Schröcke, B. Schink, Arch. Microbiol. 171 (1999) 331–337.
[30] P. Dimroth, Eur. J. Biochem. 115 (1981) 353–358.
[31] W. Buckel, Eur. J. Biochem. 156 (1986) 259–263.
[32] A. Hoffmann, P. Dimroth, FEBS Lett. 220 (1987) 121–125.
[33] P. Dimroth, A. Thomer, Eur. J. Biochem. 137 (1983) 107–112.
[34] W. Hilpert, P. Dimroth, Eur. J. Biochem. 132 (1983) 579–587.
[35] P. Dimroth, H. Hilbi, Mol. Microbiol. 25 (1997) 3–10.
[36] P. Dimroth, Methods Enzymol. 125 (1986) 530–540.
[37] P. Dimroth, Methods Enzymol. 125 (1986) 540–546.
[38] W. Buckel, Methods Enzymol. 125 (1986) 547–558.
[39] A.L. McKean, J. Ke, J. Song, P. Che, S. Achenbach, B.J. Nikolau, E.S. Würtele, J. Biol. Chem. 275 (2000) 5582–5590.
[40] P.E. Stephens, M.G. Darlison, H.M. Lewis, J.R. Guest, Eur. J. Biochem. 133 (1983) 155–162.
[41] K. Bendrat, S. Berger, W. Buckel, W.A. Etzel, K.H. Röhm, FEBS Lett. 277 (1990) 156–158.
[42] P. Jockel, M. Di Berardino, P. Dimroth, Biochemistry 38 (1999) 13461–13472.
[43] D.A. Doyle, J. Morais Cabral, R.A. Pfuetzner, A. Kuo, J.M. Gulbis, S.L. Cohen, B.T. Chait, R. MacKinnon, Science 280 (1998) 69–77.
[44] W. Buckel, H. Liedtke, Eur. J. Biochem. 156 (1986) 251–257.
[45] P. Jockel, M. Schmid, J. Steuber, P. Dimroth, Biochemistry 39 (2000) 2307–2315.
[46] H. White, W.P. Jencks, J. Biol. Chem. 251 (1976) 1688–1699.

[47] T. Selmer, W. Buckel, J. Biol. Chem. 274 (1999) 20772–20778.
[48] M. Di Berardino, P. Dimroth, Eur. J. Biochem. 231 (1995) 790–801.
[49] J.B. Huder, P. Dimroth, J. Bacteriol. 177 (1995) 3623–3630.
[50] M. Bott, M. Meyer, P. Dimroth, Mol. Microbiol. 18 (1995) 533–546.
[51] K. Bendrat, W. Buckel, Eur. J. Biochem. 211 (1993) 697–702.
[52] M. Mack, K. Bendrat, O. Zelder, E. Eckel, D. Linder, W. Buckel, Eur. J. Biochem. 226 (1994) 41–51.
[53] U. Härtel, W. Buckel, Arch. Microbiol. 166 (1996) 350–356.
[54] M. Hans, J. Sievers, U. Müller, E. Bill, J.A. Vorholt, D. Linder, W. Buckel, Eur. J. Biochem. 265 (1999) 404–414.
[55] M. Berg, H. Hilbi, P. Dimroth, Biochemistry 35 (1996) 4689–4696.
[56] S. Berger, A. Braune, W. Buckel, U. Härtel, M.L. Lee, Angew. Chem. Int. Ed. Engl. 35 (1996) 2132–2133.
[57] J. Steuber, C. Schmid, M. Rufibach, P. Dimroth, Mol. Microbiol. 35 (2000) 428–434.
[58] W. Hilpert, P. Dimroth, Eur. J. Biochem. 138 (1984) 579–583.
[59] P. Dimroth, A. Thomer, Biochemistry 32 (1993) 1734–1739.
[60] M. Di Berardino, P. Dimroth, EMBO J. 15 (1996) 1842–1849.
[61] P. Jockel, M. Schmid, T. Choinowski, P. Dimroth, Biochemistry 39 (2000) 4320–4326.
[62] R.K. Thauer, K. Jungermann, K. Decker, Bacteriol. Rev. 41 (1977) 100–180.
[63] M.J. Allison, K.A. Dawson, W.R. Mayberry, J.G. Foss, Arch. Microbiol. 141 (1985) 1–7.
[64] B. Schink, N. Pfennig, Arch. Microbiol. 133 (1982) 209–216.
[65] A.L. Baetz, M.J. Allison, J. Bacteriol. 171 (1989) 2605–2608.
[66] V. Anantharam, M.J. Allison, P.C. Maloney, J. Biol. Chem. 264 (1989) 7244–7250.
[67] G. Kaim, P. Dimroth, EMBO J. 18 (1999) 4118–4127.
[68] P.C. Maloney, V. Anantharam, M.J. Allison, J. Biol. Chem. 267 (1992) 10531–10536.
[69] N.A. Cornick, M.J. Allison, Can. J. Microbiol. 42 (1996) 1081–1086.
[70] P. Dimroth, A. Thomer, Biol. Chem. Hoppe Seyler 367 (1986) 813–823.
[71] U. Härtel, E. Eckel, J. Koch, G. Fuchs, D. Linder, W. Buckel, Arch. Microbiol. 159 (1993) 174–181.
[72] Y. Kawarabayasi, M. Sawada, H. Horikawa, Y. Haikawa, Y. Hino, S. Yamamoto, M. Sekine, S. Baba, H. Kosugi, A. Hosoyama, Y. Nagai, M. Sakai, K. Ogura, R. Otsuka, H. Nakazawa, M. Takamiya, Y. Ohfuku, T. Funahashi, T. Tanaka, Y. Kudoh, J. Yamazaki, N. Kushida, A. Oguchi, K. Aoki, H. Kikuchi, DNA Res. 5 (1998) 55–76.
[73] J.F. Heidelberg, J.A. Eisen, W.C. Nelson, R.A. Clayton, M.L. Gwinn, R.J. Dodson, D.H. Haft, E.K. Hickey, J.D. Peterson, L. Umayam, S.R. Gill, K.E. Nelson, T.D. Read, H. Tettelin, D. Richardson, M.D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R.D. Fleishmann, W.C. Nierman, O. White, Nature 406 (2000) 477–483.
[74] H.P. Klenk, R.A. Clayton, J.F. Tomb, O. White, K.E. Nelson, K.A. Ketchum, R.J. Dodson, M. Gwinn, E.K. Hickey, J.D. Peterson, D.L. Richardson, A.R. Kerlavage, D.E. Graham, N.C. Kyrpides, R.D. Fleischmann, J. Quackenbush, N.H. Lee, G.G. Sutton, S. Gill, E.F. Kirkness, B.A. Dougherty, K. McKenney, M.D. Adams, B. Loftus, J.C. Venter et al., Nature 390 (1997) 364–370.
[75] C.M. Fraser, S.J. Norris, G.M. Weinstock, O. White, G.G. Sutton, R. Dodson, M. Gwinn, E.K. Hickey, R. Clayton, K.A. Ketchum, E. Sodergren, J.M. Hardham, M.P. McLeod, S. Salzberg, J. Peterson, H. Khalak, D. Richardson, J.K. Howell, M. Chidambaram, T. Utterback, L. McDonald, P. Artiach, C. Bowman, M.D. Cotton, J.C. Venter et al., Science 281 (1998) 375–388.
[76] K.E. Nelson, R.A. Clayton, S.R. Gill, M.L. Gwinn, R.J. Dodson, D.H. Haft, E.K. Hickey, J.D. Peterson, W.C. Nelson, K.A. Ketchum, L. McDonald, T.R. Utterback, J.A. Malek, K.D. Linher, M.M. Garrett, A.M. Stewart, M.D. Cotton, M.S. Pratt, C.A. Phillips, D. Richardson, J. Heidelberg, G.G. Sutton, R.D. Fleischmann, J.A. Eisen, C.M. Fraser et al., Nature 399 (1999) 323–329.
[77] M. Berg, H. Hilbi, P. Dimroth, Eur. J. Biochem. 245 (1997) 103–115.