

Phylogenomic Analysis Identifies a Sodium-Translocating Decarboxylating Oxidoreductase in Thermotogae

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Abstract—Bacterial sodium-dependent decarboxylases were the first enzymes exemplifying sodium-dependent bioenergetics. These enzyme complexes couple decarboxylation of organic acids with the export of sodium ions via a special membrane subunit. In 711 representative prokaryotic genomes, we have analyzed genomic neighborhoods of the genes that code the membrane subunit of sodium decarboxylases. In representatives of Thermotogae, the operons with the gene of this subunit lack the genes of subunits that perform non-oxidative decarboxylation. Instead, these operons contain the genes of alpha- and delta-subunits of decarboxylating oxidoreductases of alpha-ketoacids. The genes of beta- and gamma-subunits of the decarboxylating oxidoreductases were found within the genomes of respective Thermotogae species as separate, two-gene operons. We suggest that the described two operons code together for sodium-translocating decarboxylating oxidoreductases capable of coupling oxidative decarboxylation of alpha-ketoacids with the export of sodium ions, which is a novel type of bioenergetic coupling.

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In the early 1980s, Vladimir Skulachev and coauthors proved that a gradient of sodium ions could serve, in several bacteria, as a membrane-associated energetic intermediate, the same as the proton gradient in most organisms [1-3]. In the following years, sodium-dependent bioenergetics was recognized as one of the fundamental mechanisms of biological energy conversion [4, 5]. Initially, sodium-dependent bioenergetics was considered an exotic adaptation of some anaerobic, alkaliphilic, and/or thermophilic prokaryotes to their unusual habitats. Later, however, it was shown that sodium-dependent bioenergetics is common for pathogenic prokaryotes [6] and, therefore, deserves detailed investigation. The reconstruction of the ancestral sequences of Na⁺- and H⁺-translocating rotary membrane ATP synthases showed that sodium-dependent bioenergetics was the original (ancestral) form of membrane bioenergetics and should have prevailed in anoxic conditions of the ancient Earth [7-10].

The key elements of sodium-dependent bioenergetics are primary sodium pumps capable of utilizing avail-

able energy sources for pumping sodium ions out of cells, which yields a transmembrane difference of electrochemical potentials of Na⁺ – the sodium potential; the latter can be used to satisfy energy needs of the cell, primarily to drive ATP synthesis. As discussed in detail previously [7-10], the function of exporting Na⁺ could precede the emergence of sodium-dependent bioenergetics proper. Many key cell systems and, most remarkably, the protein synthesis system require prevalence of K⁺ over Na⁺ in the cytoplasm, which is likely due to emergence of the first cells in a specific, potassium-rich environment [11-15]. Since both freshwater and marine reservoirs contain more sodium than potassium, the primordial cells could have inhabited them only after the emergence of Na⁺-tight cell membranes and systems for exporting Na⁺. The interaction of several such initially independent systems could eventually cause the emergence of sodium-dependent membrane bioenergetics, as discussed elsewhere [10].

Historically, the first primary sodium pump to be described was the oxaloacetate decarboxylase from *Klebsiella pneumoniae* [16], which couples decarboxylation of oxaloacetate with the translocation of Na⁺ across the cell membrane against the electrochemical gradient.

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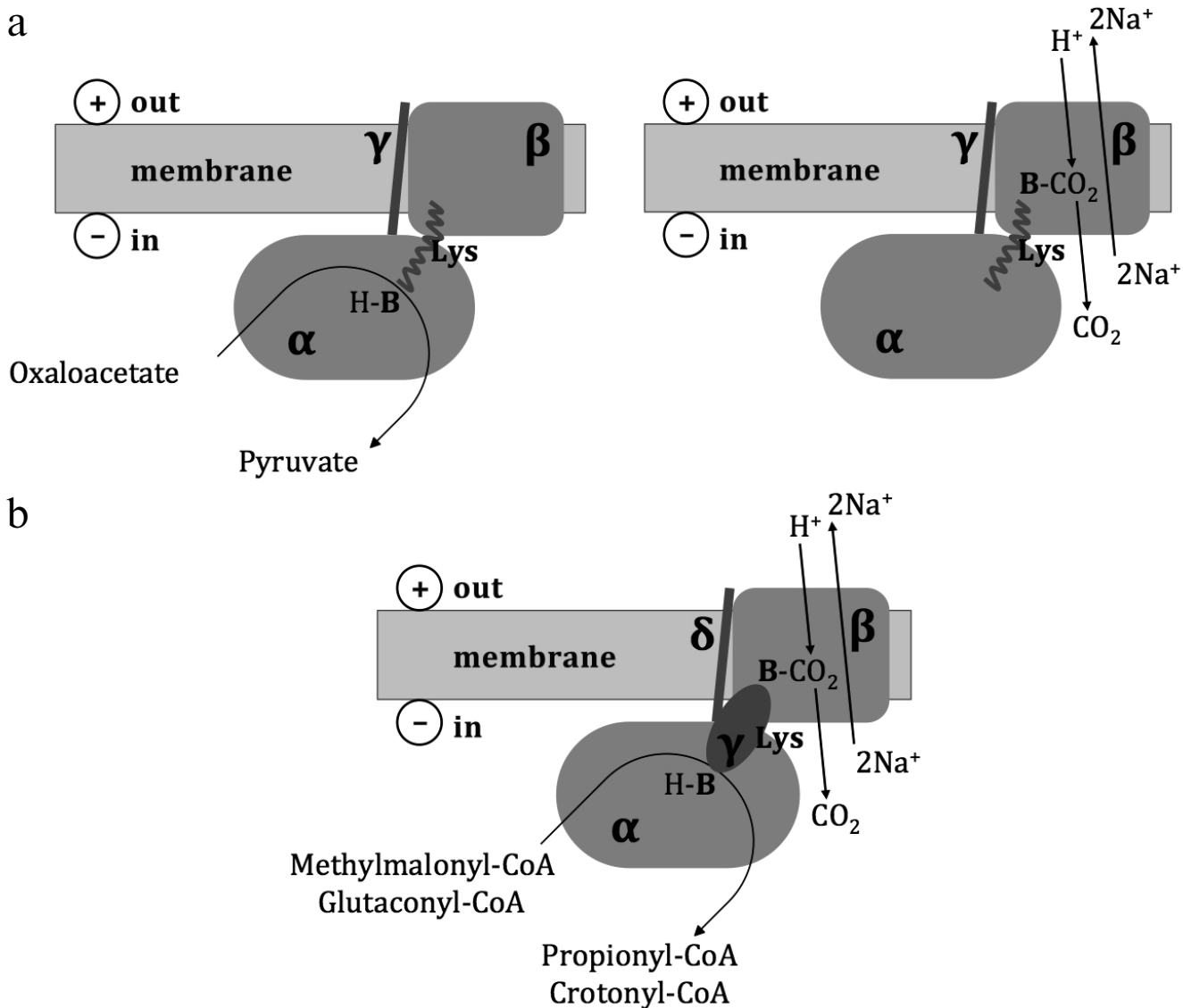


Fig. 1. Structural schemes and putative catalytic mechanisms of oxaloacetate, methylmalonyl-CoA, and glutaconyl-CoA decarboxylases (from Buckel [17]). Lys – a biotin-binding lysine residue; H-B – biotin; B-CO₂ – carboxybiotin. a) Oxaloacetate decarboxylase; b) methylmalonyl-CoA and glutaconyl-CoA decarboxylases.

Several similar enzymes were later described in different anaerobic microorganisms [17]; some of them, in particular the methylmalonyl-CoA decarboxylase from *Veillonella parvula* [18], the glutaconyl-CoA decarboxylase from *Acidaminococcus fermentans* [19], and the malonate decarboxylase system from *Malonomonas rubra* [20] were experimentally studied. It was shown that the oxaloacetate decarboxylase consists of α -, β -, and γ -subunits; the methylmalonyl-CoA and glutaconyl-CoA decarboxylases consist of α -, β -, γ -, and δ -subunits, wherein the δ -subunit of the oxaloacetate decarboxylase is related to the γ -subunit of the methylmalonyl-CoA and glutaconyl-CoA decarboxylases, and the γ -subunit of the methylmalonyl-CoA and glutaconyl-CoA decarboxylases

is functionally equivalent to the C-terminal biotin-binding domain of the α -subunit of the oxaloacetate decarboxylase [17]. As depicted in Fig. 1, the α -subunit of these enzymes is localized in the cytoplasm and catalyzes carboxyl group transfer from a carboxylic acid to biotin. The β -subunit is localized within the cell membrane and couples decarboxylation of carboxybiotin in the cytoplasmic α -subunit with the translocation of Na⁺ out of the cell. The γ/δ -subunit serves as a membrane anchor for the α -subunit [17]. The malonate decarboxylase system is more complicated, being encoded by an operon of 14 genes [21] whose functions are not fully understood.

According to X-ray diffraction data, the α -subunits of the oxaloacetate decarboxylase form a homodimer

[22], and the α -subunits of the methylmalonyl-CoA decarboxylase form a homotrimer [23], whereas the α -subunits of the glutaconyl-CoA decarboxylase form either a homodimer [24] or a homotetramer [25]. A monomer of the α -subunit of the methylmalonyl-CoA and glutaconyl-CoA decarboxylases contains one carboxyltransferase domain (the Pfam protein domain database [26] identifier: PF01039), whereas the monomer of the α -subunit of the oxaloacetate decarboxylase usually consists of three other Pfam domains that are not related to the carboxyltransferase domain. The 3D structure of the membrane part of Na⁺-translocating decarboxylases has not been reported yet.

Experimentally studied sodium-dependent decarboxylases translocate Na⁺ against the electrochemical gradient by using the free energy that is released upon cleavage of a chemical bond (decarboxylation). In this report, by analyzing the structure of prokaryotic operons, we predict a new class of primary sodium pumps, namely the Na⁺-translocating, decarboxylating oxidoreductases.

MATERIALS AND METHODS

As initial data, we used the set of 711 complete prokaryotic genomes whose protein-coding genes were attributed to the Clusters of Orthologous Groups (COGs) [27]. Sequences of 180 membrane β -subunits of Na⁺-translocating decarboxylases (these proteins form COG1883) were found in 141 complete genomes. Sequences of 255 cytoplasmic α -subunits of oxaloacetate decarboxylases that belong to COG5016 and consist of two Pfam domains, HMGL-like (PF00682) and PYC_OADA (PF02436), were found in 216 complete genomes, and these proteins are designated below as α_1 -subunits. In particular, 170 of the α_1 -subunits contain an additional biotin-binding Pfam domain (PF00364 or PF13533). Sequences of 876 proteins that belong to COG4799 and contain one carboxyltransferase Pfam domain (PF01039) were found in 413 complete genomes; these proteins include the cytoplasmic α -subunits of methylmalonyl-CoA and glutaconyl-CoA decarboxylases and are designated below as α_2 -subunits.

Multiple alignment of the amino acid sequences of the β -subunits (180 proteins) was done using the MUSCLE program [28]. Conserved blocks (alignment regions in which all participating sequences are reliably aligned), were manually selected and contained 367 positions in total. A phylogenetic tree of the β -subunits was constructed by the Neighbor-Joining algorithm using the MEGA 5.2 program [29]. The branch support values supporting branches were calculated from 100 iterations. Genomic neighborhoods of the β -subunit genes (180 proteins) were analyzed using the OLESA program [30]. Operon boundaries were determined according to the following criteria: (i) the genes are located on the same

DNA strand, (ii) the distance between the genes is small, (iii) the promoter elements (Shine–Dalgarno sequence and Pribnow box) could be detected, and (iv) the termination elements [31] could be detected (Fig. S1 in the Supplement to this paper on the site of this journal <http://protein.bio.msu.ru/biokhimiya> and Springer site Link.springer.com).

RESULTS AND DISCUSSION

First, we examined the co-occurrence of the genes of subunits α_1 , α_2 , and β of Na⁺-translocating decarboxylases in 711 complete prokaryotic genomes (Table S1 in Supplement). The α -subunit genes were often found in prokaryotic genomes in the absence of the β -subunit genes. In 382 complete genomes from the 711 examined, the α -subunit genes (α_1 and/or α_2) were not accompanied by β -subunit genes. The genes of both types of subunits (α and β) were found in 141 complete genomes. The β -subunit genes were found in only six archaeal genomes of 83 examined complete archaeal genomes, namely in all representatives of Thermococci (*Thermococcus kodakarensis* KOD1, *Pyrococcus furiosus* DSM 3638, *Pyrococcus abyssi* GE5), as well as in *Archaeoglobus fulgidus* DSM 4304, *Aciduliprofundum boonei* T469, and *Staphylothermus hellenicus* DSM 12710. The β -subunit genes were also found in all representatives of Thermotogae and Synergistetes, all representatives of Bacteroidae, many representatives of Chlorobi, Chloroflexi, Firmicutes, Spirochaetes, and Verrucomicrobia, many representatives of Desulfobacterales and Syntrophobacterales, diverse representatives of γ - and ϵ -proteobacteria, as well as in some other bacteria (Table S1 in Supplement).

Our analysis of genomic neighborhoods of the genes of β -subunits has shown that the genes of β -subunits often, but not always, form putative operons with the α -subunit gene(s) (α_1 and/or α_2), as well as additional subunits γ and δ . Sometimes such putative operons include genes of other proteins or, alternatively, the β -subunit genes seem not to form operons; further analysis is required to explain such evolutionary events.

Although the β -subunit genes were not found in prokaryotic genomes in the absence of the genes of α -subunits (α_1 and/or α_2), analysis of genomic neighborhoods indicates that the β -subunits are not always functionally associated with the α -subunits. In particular, we have noticed that operons with genes of the β -subunit homologs do not include the genes of β -subunits (neither α_1 , nor α_2) in all representatives of Thermotogae; these operons are depicted in Fig. 2. The respective clade, which contains the α -subunit homologs from all representatives of Thermotogae, is separated from the rest of the phylogenetic tree and supported by a reliable bootstrap value (100%). These Thermotogae genomes also contain the genes of α_2 -subunits; however, these genes are

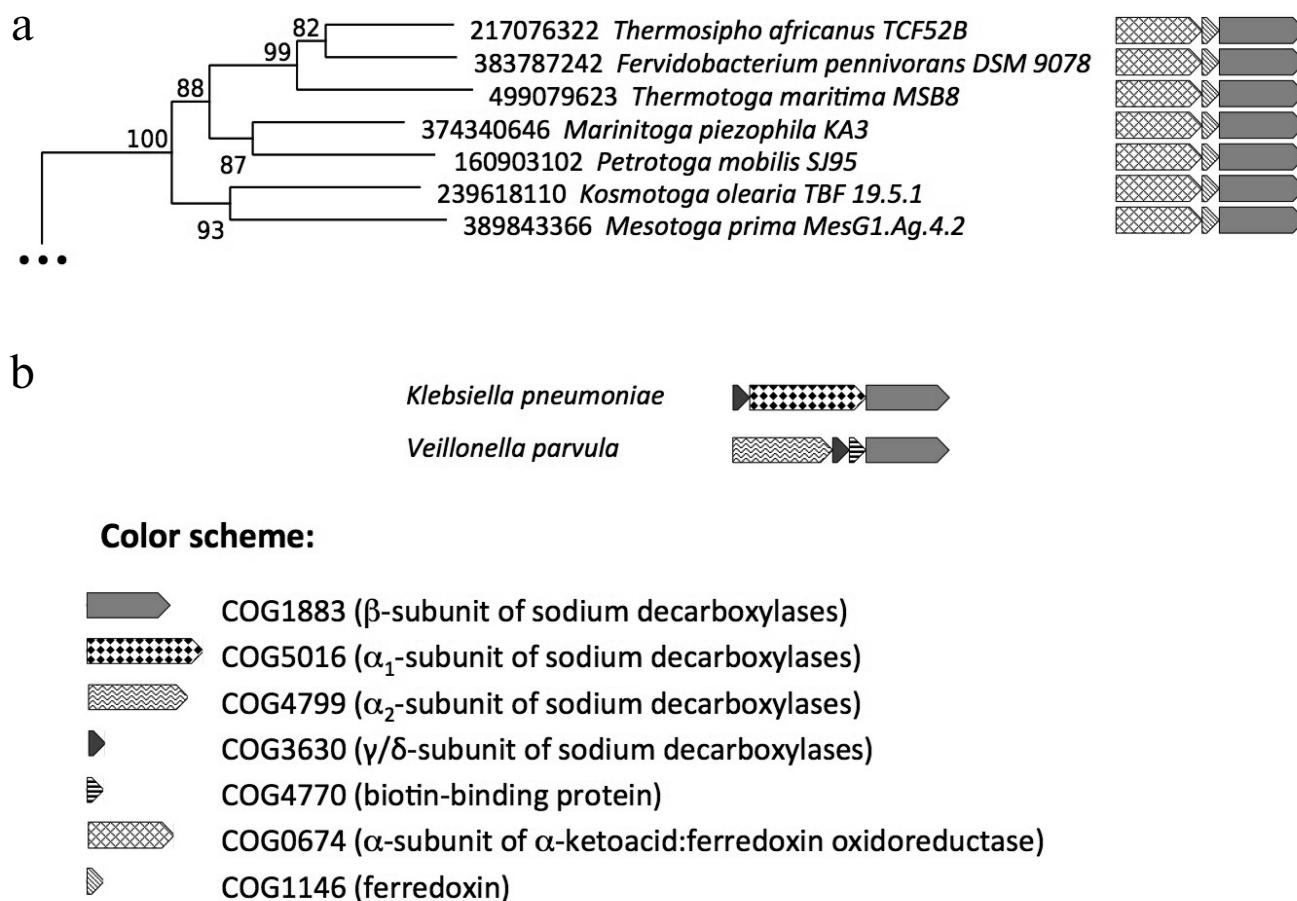


Fig. 2. Homologs of membrane β -subunits of Na^+ -translocating decarboxylases. a) Part of the phylogenetic tree of the membrane β -subunits of Na^+ -translocating decarboxylases with *Thermotoga* genes. Genomic neighborhoods of the genes are depicted to the right of the tree. The genes are marked with different shadings in accordance with the COGs they belong to. b) Operon structures typical for experimentally studied oxaloacetate, methylmalonyl-CoA, and glutacyonyl-CoA decarboxylases are given for comparison.

located away from the genes of the β -subunit homologs, being involved in other conservative operon structures (Fig. S2 in Supplement).

This separate *Thermotoga* clade of β -subunit homologs not associated with the α -subunits is characterized by a special operon structure. In *Thermotoga maritima* MSB8, the putative operon consists of genes of proteins with GI-identifiers 499079621, 499079622, and 499079623 (corresponding loci: Tmari_0880, Tmari_0881, and Tmari_0882). The protein with GI-identifier 499079623 belongs to the COG1883 that unites the Na^+ -pumping membrane β -subunits of Na^+ -translocating decarboxylases (comparison with the β -subunit of the sodium decarboxylase from *K. pneumoniae* yields an e-value of $3 \cdot 10^{-106}$). The proteins with GI-identifiers 499079621 and 499079622 belong to COG0674 and COG1146, respectively. The functions of these *Thermotoga* proteins are not experimentally identified, but the database search revealed that the protein with GI-identifier 499079622 belongs to ferredoxins and contains two [4Fe-4S]-clusters (comparison with ferredoxin from *Pyrococcus furiosus*

(PORD_PYRFU) yields an e-value of $3 \cdot 10^{-13}$), whereas the protein with GI-identifier 499079621 can bind α -keto acids and contains thiamine pyrophosphate as a coenzyme (comparison with subunit KorA of the α -ketoglutarate synthase from *Methanobacterium thermoautotrophicum* (KORA_METTM) yields an e-value of $4 \cdot 10^{-117}$). Corresponding pairwise alignments of amino acid sequences are depicted in Figs. S3-S5 in Supplement.

The two cytoplasmic subunits, whose genes form an operon with homologs of the sodium decarboxylase β -subunit gene in *Thermotoga*, are otherwise parts of oxidoreductase complexes that perform oxidative decarboxylation of α -keto acids in anaerobic prokaryotes [32-42]. This complex is functionally equivalent, but not homologous, to the mitochondrial α -ketoglutarate dehydrogenase of aerobic organisms that is involved in the Krebs cycle [43]. The functional unit of a bacterial α -ketoacid:ferredoxin oxidoreductase consists of four cytoplasmic subunits (α , β , γ , and δ), which can be represented either by separate polypeptides or by products of fused genes. For example, the enzyme from *Desulfovibrio*



Fig. 3. Fragments of the multiple sequence alignment of homologs of the membrane β -subunits of Na^+ -translocating decarboxylases, as found in Thermotogae, with the membrane β -subunit of the oxaloacetate decarboxylase from *K. pneumoniae*. The topology of helices is given in accordance with Wilde et al. [44]. The amino acid residues presumably responsible for the binding of Na^+ are marked with asterisks, and other functionally important residues are marked with "+" [44, 45]. Amino acid residues are shaded in accordance with their physicochemical properties: charged polar residues (D, E, H, K, R) are shaded black, uncharged polar residues (N, Q, S, T, Y) are shaded gray, and nonpolar residues are not shaded.

africanus, the first with a deciphered 3D structure, has a fused $\alpha + \gamma + \delta + \beta$ structure [39, 40]. Another enzyme, whose 3D structure was deciphered recently, belongs to the bacterium *Moorella thermoacetica* and consists of three subunits: α , β , and an elongated ferredoxin subunit having fused $\gamma + \delta$ structure [41, 42].

The Thermotogae operons shown in Fig. 2, in addition to the homologs of the membrane β -subunit gene, comprise only the genes of subunits α (long) and δ (ferredoxin, short). However, the genes of subunits β and γ could be found in all examined complete Thermotogae genomes, where they form two-gene separate operons (the gene loci of β - and γ -subunits from *Thermotoga maritima*: Tmari_0402 and Tmari_0403, respectively; Fig. S6 in Supplement). Genomic neighborhoods of the genes of β - and γ -subunits seem to be random in Thermotogae (Fig. S6 in Supplement); therefore, the β - and γ -subunits might form functional complexes with the α - and δ -subunits.

We tested whether the homologs of the sodium decarboxylase β -subunit, as found in Thermotogae, contain amino acid residues presumably involved in Na^+ transport in sodium decarboxylases [44, 45]. As can be seen in Fig. 3, all of the sodium binding residues and most of the other functionally important residues are retained in the Thermotogae subunits. Interestingly, the methionine residue (M386 in the subunit from *K. pneumoniae*), which is presumably involved in the Na^+ -translocation [44, 45], is replaced in Thermotogae by aspartate or threonine, which can coordinate Na^+ [9, 46] (see also Fig. 4). The conservation of the key residues suggests that these homologs of the sodium decarboxylase β -subunit are likely to be functional and capable of transferring Na^+ across the cell membrane.

The type of bioenergetics of each organism is determined by the ion specificity of its ATP synthase, which in turn entirely depends on the ion specificity of its membrane rotor [8, 9, 47]. Figure 4 shows that the membrane c -subunits of the ATP synthase of the same Thermotogae contain complete sets of known Na^+ -binding ligands [9, 46], which indicates sodium-dependent bioenergetics in these organisms.

Based on these results, we suggest that Thermotogae possess a Na^+ -translocating membrane enzyme of a type not previously described, which pumps Na^+ out of a bacterial cell on account of oxidative decarboxylation, wherein the transfer of two electrons from an organic α -keto acid to ferredoxin is coupled with removal of a carboxyl group. A hypothetical structure of the enzyme is depicted in Fig. 5, where the oxidoreductase, as formed by four cytoplasmic subunits, performs oxidative decarboxylation of α -keto acids, which is coupled to Na^+ -translocation by the membrane subunit m_{Na} .

From the high sequence similarity with experimentally characterized decarboxylating oxidoreductases of α -keto acids, we suggest that α -ketoglutarate or pyruvate could serve as substrates of the described enzyme complex; to accurately identify the substrate, experimental studies are necessary. The standard redox potential of α -ketoglutarate and pyruvate at pH 7.0 (E_0') is approximately -0.7 V [48]. Since the standard redox potential of ferredoxins is usually higher than -0.5 V [49], the released free energy should be sufficient to transfer at least one Na^+ out of the cell.

As already mentioned, a plethora of data indicates that bioenergetics based on the transmembrane potential of Na^+ is not something specific only for dwellers of unusu-

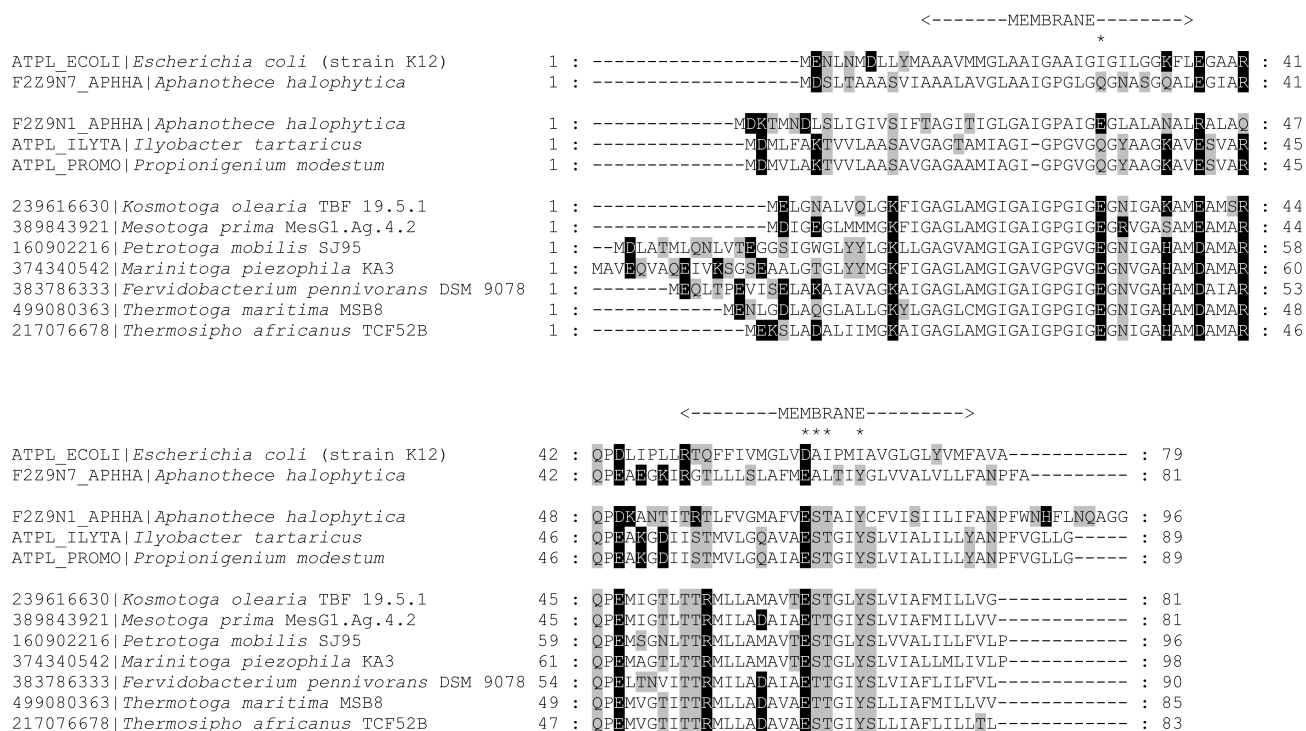


Fig. 4. Multiple sequence alignment of Thermotogae ATPase rotor *c*-subunits, which are responsible for ion specificity, with corresponding subunits of experimentally studied Na⁺-translocating rotary ATPases (F-type ATPases from *Ilyobacter tartaricus* and *Propionigenium modestum*, N-type ATPase from *Aphanothece halophytica* [56, 57]) and H⁺-translocating rotary ATPases (F-type ATPase from *Escherichia coli* and a second copy of ATPase from *Aphanothece halophytica*). Transmembrane regions are shown in accordance with the structure of the ATPL_ILYTA protein from *Ilyobacter tartaricus* (identifier in PDB database: 2WGM [46]). Amino acid residues forming the Na⁺-binding site in the Na⁺-translocating enzymes are marked with asterisks [9, 46]. Amino acid residues are shaded in accordance with their physicochemical properties: charged polar residues (D, E, H, K, R) are shaded black, uncharged polar residues (N, Q, S, T, Y) are shaded gray, and non-polar residues are not shaded.

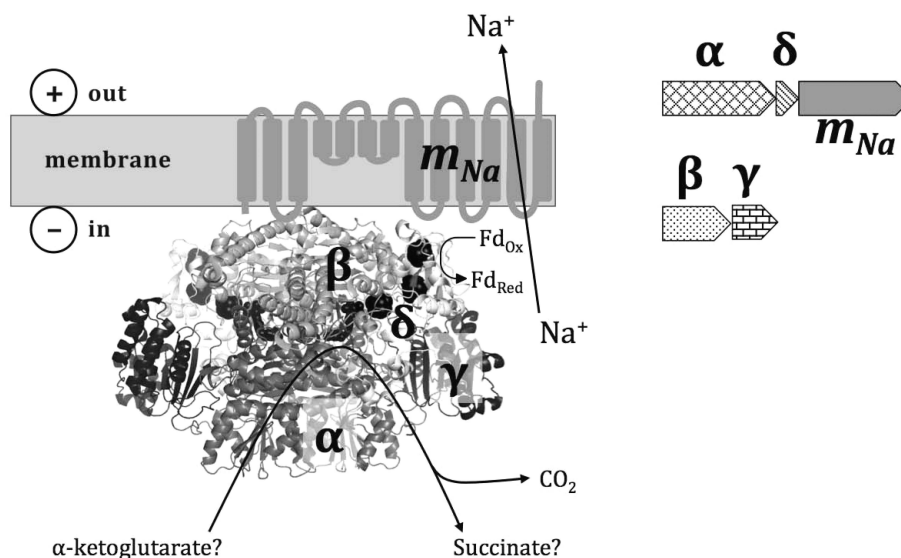


Fig. 5. Tentative structure of a sodium-translocating decarboxylating oxidoreductase. The topology of the membrane *m*_{Na}-subunit homologous to the membrane β-subunit of sodium decarboxylases is given in accordance with Wild et al. [44] and Schmid et al. [45]. The 3D structure of the pyruvate:ferredoxin oxidoreductase from *Desulfovibrio africanus* [39], PDB-identifier 4C42, was used for visualization of the cytoplasmic subunits α, β, γ, and δ. The cytoplasmic part of the enzyme presumably forms a homodimer. Three iron–sulfur clusters are shown as black circles; thiamine pyrophosphate is shown as a wireframe model. Two operons that together seem to encode the complex are depicted to the right.

Classification of energy converting H^+ and/or Na^+ -translocating enzymes. Indicated are energy sources, the life domains where particular enzymes have been found, and those studies in which these enzymes were first described

Na^+ -translocating enzymes	H^+ -translocating enzymes
Pyrophosphatases	
H^+ or Na^+ -translocating pyrophosphatases [50, 51]; bacteria, archaea	
ATPases	
H^+ or Na^+ -translocating rotary F-type ATPases [52, 53]; bacteria, archaea, eukaryotes	
H^+ or Na^+ -translocating rotary A/V-type ATPases [54, 55]; bacteria, archaea, eukaryotes	
Na^+ -translocating N-ATPases [56, 57]; bacteria, archaea ^a	not shown
ABC-type Na^+ -transporters NatAB [58]; bacteria, archaea	not shown
Methyltransferases	
Na^+ -translocating N ⁵ -methyltetrahydromethanopterin:coenzyme M-methyltransferases (MtrA-H) [59]; archaea	not shown
Decarboxylases^b	
Na^+ -translocating oxaloacetate decarboxylases (OAD) [16]; bacteria, archaea	not shown
Na^+ -translocating methylmalonyl-CoA decarboxylases (MMD) [18]; bacteria	not shown
Decarboxylating oxidoreductases? (this paper)	
Na^+ -translocating α -ketoacid:ferredoxin decarboxylating oxidoreductases; bacteria (Thermotogae)	not shown
Oxidoreductases	
H^+ and/or Na^+ -translocating type I NADH:quinone oxidoreductases (NDH1) [60-62]; bacteria, archaea, eukaryotes	
H^+ or Na^+ -translocating ferredoxin:NAD ⁺ oxidoreductases (RNF) [63, 64]; bacteria, archaea	
H^+ or Na^+ -translocating electrogenic hydrogenases [65, 66]; bacteria, archaea	
Na^+ -translocating NADH:quinone oxidoreductases (NQR) [67]; bacteria	not shown
Not shown	H^+ -translocating H_2 :heterosulfide oxidoreductases (VhoACG/HdrDE) [68]; archaea
Not shown	H^+ -translocating quinol:cytochrome <i>c</i> -oxidoreductases (cytochrome <i>bc</i> -complexes) [69]; bacteria, archaea, eukaryotes
H^+ or Na^+ (?)-translocating, copper- and heme-containing cytochrome and ubiquinol oxidases [70, 71]; bacteria, archaea, eukaryotes	
Not shown	H^+ -translocating terminal <i>bd</i> -type oxidases [72]; bacteria, archaea
Light-dependent ion pumps	
H^+ or Na^+ (or Cl^-)-translocating rhodopsins [73-79]	

^a Operons of N-ATPases, as discovered in genomes of two archaea, *Methanosarcina acetivorans* and *Methanosarcina barkeri*, are likely to have originated in bacteria and been obtained by these archaea as a result of horizontal gene transfer [56].

^b This protein family also includes the malonate decarboxylase system and glutaconyl-CoA decarboxylase [80, 81].

al habitats, but is the original form of membrane bioenergetics, traces of which are preserved in most modern organisms as various sodium-transferring membrane enzymes [7, 10].

The current data on the studied energy converting membrane enzymes are summarized in the table. Many of these enzymes are described as having both Na⁺- and H⁺-translocating forms. Redox-dependent proton transporters seem to be more diverse among organisms that rely on proton-dependent bioenergetics, as compared to organisms with sodium-dependent bioenergetics, which may be due to the ability of proton-transporting systems to particularly benefit from high-potential electron acceptors [7, 10]. The dwellers of the “sodium world”, however, are capable of coupling the transfer of Na⁺ with quite diverse exergonic reactions not described for organisms with the proton-dependent bioenergetics. Thus, in general, the variety of coupling mechanisms is greater in Na⁺-dependent energy converting systems, which may reflect the evolutionary primacy of sodium-dependent bioenergetics, which could have emerged under the anoxic conditions of the ancient Earth [7, 9].

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