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The ATPases of *Propionigenium modestum* and *Bacillus alcalophilus*. Strategies for ATP synthesis under low energy conditions

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In *Propionigenium modestum*, ATP synthesis is coupled via $\Delta\tilde{\mu}_{Na^+}$ to the decarboxylation of (*S*)-methylmalonyl-CoA. The low energy yield of this reaction implies that approx. 4 decarboxylation cycles are necessary to synthesize 1 molecule of ATP. Theoretical considerations in accord with experimental results suggest ATP synthesis in *P. modestum* at $\Delta\tilde{\mu}_{Na^+} = -110$ mV. Other anaerobic bacteria synthesize ATP at a $\Delta\tilde{\mu}_{H^+}$ of similar size and alkaliphilic bacteria at pH 10.3 have a $\Delta\tilde{\mu}_{H^+}$ of only -103 mV. In these cases, the $H^+(Na^+)$ to ATP stoichiometry must be at least 4.

Introduction

According to the chemiosmotic theory, ATP synthesis by bacteria, mitochondria or chloroplasts is accomplished at the expense of the free energy of an electrochemical gradient of protons ($\Delta\tilde{\mu}H^+$) established by a respiratory or photosynthetic electron transport chain [1]. The ATPases responsible for this type of ATP synthesis are multimeric enzyme complexes composed of an integral membrane-bound F_0 portion and the peripheral F_1 moiety. The F_0 portion catalyzes the conduction of protons through the membrane, thereby apparently altering the conformation of F_1 to allow this protein moiety to synthesize ATP from ADP and P_i. All ATPases which function physiologically as ATP synthases are of the F_1F_0 type and all are phylogenetically related, as shown by the similar subunit structure and sequence homologies among the corresponding subunits (for recent reviews see Refs. 2–4).

This general picture of ATP synthesizing F_1F_0 ATPases was extended recently by the discovery of the F_1F_0-ATPase of *Propionigenium modestum* which instead of $\Delta\tilde{\mu}_{H^+}$ uses $\Delta\tilde{\mu}_{Na^+}$ as the driving force for ATP synthesis [5–8]. Nevertheless, the *P. modestum* ATPase clearly is a member of the F_1F_0-ATPase family as shown by functional biochemical and sequencing studies. The percentage of identical amino-acid residues within the sequences of the ATPases of *P. modestum* and *E. coli* are, thus, 69% for the β-subunits, 18% for subunits a, 11% for subunits b, and 17% for subunits c [9–11]. In addition, the *P. modestum* ATPase shows the typical pattern of inhibition by a number of inhibitors of prokaryotic F_1F_0 ATPases. Most remarkable is the modification of a specific glutamate residue of subunit c on incubation of the enzyme with DCCD which leads to the complete destruction of ATPase activity [7,10].

These homologies in structure and function of the F_1F_0-ATPases point to a general mechanism of energy transfer within the enzyme from the electrochemical gradient of H^+ or Na^+ to the energy-rich phosphoric anhydride bond of ATP. However, there is great variation in the amount of energy supply, and especially certain bacterial species are confronted with the problem to synthesize ATP under low energy conditions. Two examples will be discussed in this report: *Propionigenium modestum*, a strict anaerobe, that from the fermentation of 1 succinate to 1 propionate and 1 CO_2 ($\Delta G^{o\prime} = -20.6$ kJ/mol) gains less energy than required to synthesize 1 ATP from 1 ADP and 1 P_i [5], and *Bacillus alcalophilus* which at high environmental pH is forced to a low $\Delta\tilde{\mu}_{H^+}$ by inversion of the ΔpH for the protection of cellular components (for review, see Ref. 12).

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Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; TPP^+, tetraphenylphosphonium ion.

ATP synthesis in *P. modestum*

P. modestum grows from the fermentation of succinate to propionate and CO_2 by the sequence of reactions shown in Fig. 1 [5]. The only step in this pathway that is sufficiently exergonic to be used for energy conservation is the decarboxylation of (*S*)-methylmalonyl-CoA. The decarboxylase is a membrane-bound Na^+-pump related to other Na^+ translocating decarboxylases of anaerobic bacteria (for review see Ref. 13). The $\Delta\tilde{\mu}_{Na^+}$ derived from the decarboxylation of methylmalonyl-CoA is used by a Na^+-translocating ATPase present in the membrane of *P. modestum* for ATP synthesis.

The stoichiometric relations are of special interest in this mechanism of decarboxylation phosphorylation: 2 mol Na^+ ions are pumped out of the cell at maximum (see below) per decarboxylation of 1 mol methylmalonyl-CoA [14,15]. At this stoichiometry the free energy of the decarboxylation reaction ($\Delta G^{o\prime} = -20.6$ kJ/mol) will be in equilibrium with $\Delta\tilde{\mu}_{Na^+}$ of -114 mV. This is the maximum driving force that can be obtained over the membrane of a *P. modestum* cell.

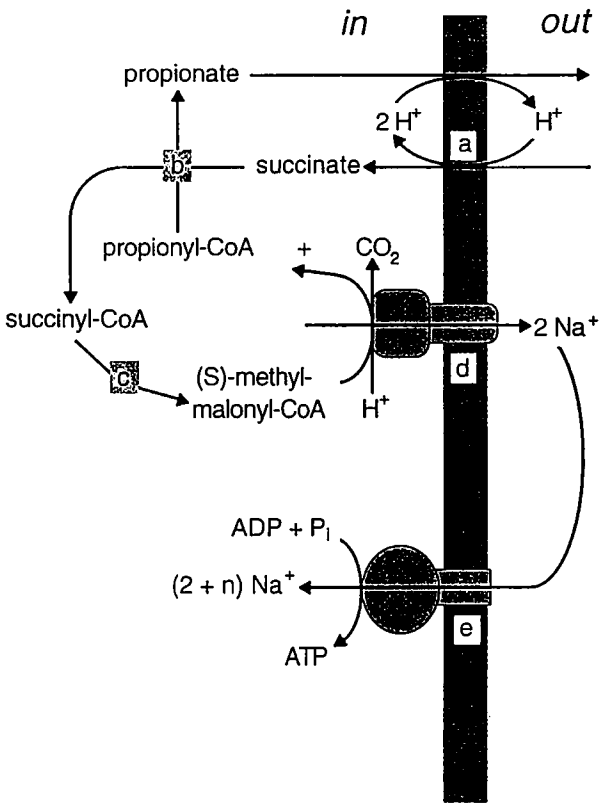

Fig. 1. Energy metabolism of *Propionigenium modestum* with a Na^+ cycle coupling the exergonic decarboxylation of (*S*)-methylmalonyl-CoA to endergonic ATP synthesis. A hypothetical proton circuit could couple succinate uptake with the extrusion of propionate and CO_2. (a) Succinate uptake system; (b) succinate propionyl-CoA: CoA transferase; (c) methylmalonyl-CoA mutase and methylmalonyl-CoA epimerase; (d) methylmalonyl-CoA decarboxylase; (e) ATPase.

The value is also in accord with a $\Delta\tilde{\mu}_{Na^+}$ of -110 mV measured in the steady state with methylmalonyl-CoA decarboxylase-containing proteoliposomes [15]. A $\Delta\tilde{\mu}_{Na^+}$ of this size must, therefore, suffice to energize ATP synthesis in *P. modestum*. Assuming that ATP synthesis occurs energetically close to equilibrium of $\Delta\tilde{\mu}_{Na^+}$ of -110 mV and a phosphorylation potential of 50 kJ/mol, one can calculate a Na^+/ATP stoichiometry of 4. While a H^+/ATP stoichiometry of 3 is more commonly encountered for F_1F_0-ATPases, such enzymes may have to transduct more cations (H^+ or Na^+) per ATP synthesized if the electrochemical cation gradient ($\Delta\tilde{\mu}_{H^+}$ or $\Delta\tilde{\mu}_{Na^+}$) is low ($< \sim -150$ mV). Such a situation also applies for other anaerobic bacteria, e.g., *Methanobacterium thermoautotrophicum*, for which $\Delta\tilde{\mu}_{H^+}$ of -120 mV was determined [16], for *Methylophilus methylotrophus*, where $\Delta\tilde{\mu}_{H^+}$ varies between -109 and -165 mV [17], and for sulfate reducing bacteria with $\Delta\tilde{\mu}_{H^+}$ between -110 and -155 mV (H. Cypionka, personal communication).

Like in other cells, ATP synthesis in *P. modestum* cannot be accomplished close to thermodynamic equilibrium with the exergonic reaction, i.e., without energy loss. It has been found that growing bacteria require about 70–75 kJ to synthesize 1 mol of ATP [18]. With this value and the free energy of succinate decarboxylation (-20.6 kJ/mol) it can be calculated that 3–4 mol succinate must be degraded to synthesize 1 mol of ATP. This figure is in reasonable agreement with the cell yield (2.0–2.5 g/mol succinate), indicating that 4–5 decarboxylations are necessary to allow synthesis of one ATP. A likely target for the energy loss in the overall reaction is the generation of $\Delta\tilde{\mu}_{Na^+}$ by methylmalonyl-CoA decarboxylase. We could recently show with reconstituted proteoliposomes that a Na^+/methylmalonyl-CoA stoichiometry of 2 is observed only in the beginning of $\Delta\tilde{\mu}_{Na^+}$ generation while later on decarboxylation proceeds unchanged but with contributing all the less to the $\Delta\tilde{\mu}_{Na^+}$ the more the $\Delta\tilde{\mu}_{Na^+}$ has increased [15]. This apparent change of the Na^+ to methylmalonyl-CoA stoichiometry to values < 2 at increasing $\Delta\tilde{\mu}_{Na^+}$ is not due to Na^+ leaks in the membrane but to a turnover of Na^+ ions by the pump itself. In the first part of the reaction cycle 2 Na^+ ions are always transported against its electrochemical gradient and in the second half 0–2 Na^+ ions cross the membrane in the opposite direction, depending on the magnitude of $\Delta\tilde{\mu}_{Na^+}$ [15]. The consequence of this mechanism may be partial uncoupling of the pump leading to a loss of energy and an apparent Na^+/methylmalonyl-CoA stoichiometry < 2 under physiological conditions.

If the Na^+/ATP stoichiometry is 4, the above results indicate a Na^+/methylmalonyl-CoA stoichiometry of about 1 under the physiological conditions of growing cells. These considerations have shed some

light on the minimum quantum of energy that must be acquired by an organism per mol of substrate metabolized. It is not an equivalent sufficient to synthesize 1 mol of ATP but only the fraction of the electrochemical cation gradient required for ATP synthesis that is obtained per mol substrate degradation (an equivalent of 0.2–0.3 ATP in this example). *P. modestum* is, thus, a paradigm for ATP synthesis by anaerobic bacteria growing by the degradation of substrates with a free energy change that is not sufficient for the synthesis of one ATP molecule.

ATP synthesis in *Bacillus alcalophilus*

An other interesting object for studying ATP synthesis under low energy conditions is the alkaliphilic bacteria, e.g., *Bacillus alcalophilus*. These are aerobic cells with a very active respiratory chain and, therefore, have no difficulties, in principle, in the supply of metabolic energy. The problem arises during growth at alkaline pH because these conditions demand an acidification of the cytoplasm relative to the environment in order to protect cellular components from denaturation. A reversed ΔpH thus results depending in size on the pH of the medium that may reduce the total proton motive force ($\Delta\tilde{\mu}_{H^+}$) to critically low levels [12].

The bioenergetic implications of ATP synthesis in alkaliphilic bacteria have in the past been investigated especially by T. Krulwich and colleagues [12]. We became interested in this problem when our discovery of the Na^+-translocating ATPase in *P. modestum* offered an elegant solution [5–8]. If the alkaliphiles contained a similar ATPase using Na^+ instead of H^+ as coupling ion, the ATP synthesis would be driven by $\Delta\tilde{\mu}_{Na^+}$, which is much higher than $\Delta\tilde{\mu}_{H^+}$, because the electrical membrane potential ($\Delta\psi$) and the chemical gradient of Na^+ (ΔpNa^+) are of the same sign, whereas $\Delta\psi$ and ΔpH are of opposite sign [12,19].

In order to test this hypothesis we purified the ATPase of *B. alcalophilus* [20]. Inspection of the purified enzyme by SDS-gel electrophoresis revealed the typical pattern of an F_1F_0-ATPase composed from 8 different subunits. The α- and β- subunits of the *B. alcalophilus* ATPase reacted with antibodies raised against the corresponding subunits of the *E. coli* ATPase. Evidence for the presence of subunit c was obtained by specific labeling of this polypeptide with [^{14}C]-DCCD as in other F_1F_0-ATPases [20].

A peculiar property of the *B. alcalophilus* ATPase is the very low ATPase activity with the physiological substrate Mg^{2+}-ATP. The enzyme catalyses substantial ATP hydrolysis either by substituting the Mg^{2+} by Ca^{2+} or after the addition of methanol. With 25% methanol the hydrolysis of Mg^{2+}-ATP increased about 100-fold, indicating that the high potential of this enzyme to act as an ATP hydrolase is essentially blocked under physiological conditions [20]. The rationale for this property of the ATPase may be to protect the cells from ATP hydrolysis and concomitant proton extrusion at some transient drop of the $\Delta\tilde{\mu}_{H^+}$ that would be detrimental at high environmental pH. The kinetics of ATP hydrolysis with Ca^{2+}-ATP revealed the cooperativity of three catalytic ATP binding sites [20]. These results are in accord with a model of the *E. coli* ATPase with three cooperative catalytic sites [2–4].

The purified ATPase was reconstituted into proteoliposomes and the specificity for the coupling ion investigated with transport studies [21]. The proteoliposomes were unable to catalyze Na^+ translocation to the inside under various conditions, but readily performed proton pumping, as shown by the ACMA fluorescence quenching assay. The transport was strictly dependent on Mg^{2+}-ATP, not Ca^{2+}-ATP, in accord with the supposition that Mg^{2+} is the physiological metal ion of the ATPase, although the ATP hydrolysis activity in its presence is very low (see above). The proteoliposomes catalyzed an ATP/[^{32}P]phosphate exchange that was insensitive to monensin, but was completely abolished by CCCP. These results thus led to the conclusion that that ATPase of *B. alcalophilus* uses H^+ and not Na^+ as the coupling ion [21]. The same conclusion has been drawn for the F_1F_0 ATPase of *Bacillus firmus* OF4 [22].

As alkaliphilic bacilli have to synthesize ATP by a proton-coupled mechanism (see above), they encounter a severe bioenergetic problem, if the $\Delta\tilde{\mu}_{H^+}$ is as low as

TABLE I

Bioenergetic parameters of two different B. alcalophilus strains (DSM 485 and ATCC 27647) at alkaline pH [19]

The values were obtained from harvested cells which were resuspended at high density in fresh medium of pH 10.8 (strain ATCC 27647) or pH 10.5 (strain DSM 485). The phosphorylation potential (ΔG_p) was converted from kJ/mol into mV by division through Faraday's constant ($F = 96.5\ kJ\,V^{-1}\,mol^{-1}$).

Strain	pH_{out}	ΔpH	$Z\Delta$pH (mV)	$\Delta\psi$	$\Delta\tilde{\mu}_{H^+}$	ATP/ADP	ΔG_p (kJ/mol) (mV)
ATCC	10.3	1.9	+110	−213	−103	3.9	−43.7 (−453)
DSM	10.1	1.7	+97	−206	−109	4.5	−44.1 (−457)

~ -50 mV at high pH, as has been reported in the literature [12]. While the magnitude of $\Delta\tilde{\mu}_{H^+}$ is decisive for potential ATP synthesis mechanisms, there is a number of possible pitfalls in its accurate determination, especially in that of the $\Delta\psi$ component [23]. We have, therefore, reinvestigated the bioenergetic parameters of *B. alcalophilus* cells growing at high pH. Values resulting from these studies are listed in Table I [19]. While the ΔpH values are in the range of those reported previously [12], our $\Delta\psi$ and, therefore, also $\Delta\tilde{\mu}_{H^+}$ was considerably larger. Erroneously low $\Delta\psi$ determined in previous investigations probably results from the high TPP + concentrations (1–4 μM) used in these studies.

The protonmotive force of -103 mV resulting from these measurements is certainly on the lower edge of the bioenergetic scale, but could still account for ATP synthesis by conventional chemiosmosis at reasonable H^+/ATP stoichiometries. For *B. alcalophilus* strain ATCC 27647, the ratio of H^+/ATP at pH 10.3 can be calculated from the phosphorylation potential of 43 kJ/mol (453 mV) and $\Delta\tilde{\mu}_{H^+}$ of -103 mV to be 4.4.

Krulwich and colleagues have proposed ATP synthesis in the alkaliphiles by a localized proton translocation pathway within the membrane between respiratory chain components and the ATPase at high pH, but conventional chemiosmosis at pH 8–9, assuming a pH-regulated gate that causes the switch between these two mechanisms [12]. If this hypothesis was correct, the membrane potential should drop at more akaline pH, when the gate opens and the protons are short circuited through an internal membrane pathway. In addition, the ATP synthesis should not be significantly affected by protonophorous ion channels traversing the membrane. Our experimental evidence is not in accord with this prediction: the membrane potential increases with increasing pH and gramicidin abolishes growth and ATP synthesis at pH > 10.0 [9].

In summary, these results suggest a unifying mechanism of ATP synthesis by F_1F_0-ATPases by conventional chemiosmosis. This mechanism explains ATP synthesis even at the lower edge of $\Delta\tilde{\mu}_{H^+}$ ($\Delta\tilde{\mu}_{Na^+}$) found for growing bacteria in the range of -100 to -120 mV. At these low driving forces an H^+ (Na^+) to ATP stoichiometry of at least 4 has to be postulated.

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