A Na⁺ A₁A₀ ATP synthase with a V-type c subunit in a mesophilic bacterium

Dennis Litty and Volker Müller

Molecular Microbiology & Bioenergetics, Institute of Molecular Biosciences, Johann Wolfgang Goethe University, Frankfurt/Main, Germany

Keywords
acetogen; bioenergetics; Eubacterium; Na⁺ transport

Correspondence
V. Müller, Molecular Microbiology & Bioenergetics, Institute of Molecular Biosciences, Johann Wolfgang Goethe University Frankfurt/Main, Max-von-Laue-Str. 9, Frankfurt 60438, Germany
Tel: +49 69 79829507
E-mail: vmueller@bio.uni-frankfurt.de

(Received 20 September 2019, revised 18 November 2019, accepted 22 December 2019)
doi:10.1111/febs.15193

Introduction

Despite all the differences in the reactions generating the electrochemical ion potential across the membrane of living cells, the ATP synthase is the universal enzyme present in every living cell to harvest electrochemical energy and transform it into biological useful fuel, ATP [1,2]. All known ATP synthases use the same principle mechanisms of energy conservation and they evolved from a common ancestor [3]. ATP synthases are rotary machines that are composed of two motor domains connected by one central stalk and at least one peripheral stalk. The membrane-embedded domain translocates ions (H⁺ or Na⁺) across the membrane leading to rotation of the c ring against the stator. The resulting rotational energy is transmitted via the central stalk to the A₁ motor which then drives ATP synthesis. This operation is fully reversible, resulting in ATP hydrolysis coupled to ion transport across the membrane [2,4].

Evolutionary, archaeal A₁A₀ ATP synthases are more closely related to V₁V_O ATPases, mainly present in vacuoles of eukarya, than to F₁F_O ATP synthases found in bacteria [2]. V₁V_O ATPases are designed by nature as ATP-driven ion pumps and are not able to synthesize ATP in vivo [2,5,6]. In most archaeal A₁A₀

Abbreviations
ΔμNa⁺, electrochemical sodium ion potential; ΔpNa, sodium ion potential; ΔΨ, membrane potential; DCCD, N,N'-dicyclohexylcarbodiimide; DDM, n-dodecyl-β-maltoside; ETH 2120, N,N,N',N'-tetra-cyclo-hexyl-1,2-phenylenedioxymidacetamide; MALDI, matrix-assisted laser desorption/ionization; TCS, 3,3',4',5-tetrachlorosalycilamide.
ATP synthases, the subunit c consists of two transmembrane helices with one ion-binding site as seen in F$_1$F$_0$ ATP synthases. In the course of evolution, the c subunit in eukaryotic V$_1$V$_0$ ATPases [7,8] underwent gene duplication, giving rise to a proteolipid with four transmembrane helices [9]. Additionally, one ion-binding site was lost resulting in a rotor missing half the number of ion-binding sites. This binding site composition is generally seen as the reason for the inability of V$_1$V$_0$ ATPases to synthesize ATP in vivo [7,10]. Notably, some A$_1$A$_O$ ATP synthases have a c subunit with four transmembrane helices but only one ion-binding site [6]. This structural feature would predict that the enzyme has lost its ATP synthesis capability. However, since no other ATP synthase genes are found in these archaea, one has to postulate that such an enzyme is able to synthesize ATP. This is indeed substantiated by measuring ATP synthesis in whole cells and membrane vesicles [11]. At this point in time, intact A$_1$A$_O$ ATP synthases could only be purified from hyperthermophilic archaea [12–16]. However, these hyperthermophilic ATP synthases are inactive at temperatures below 80 °C. Under these conditions, all commonly used liposome systems are inactive and suitable lipids for these conditions could not be obtained so far, despite all efforts.

Inspection of genome sequences from mesophilic bacteria and archaea revealed one gene cluster in *Eubacterium limosum* (*T*$_{opt}$ = 37 °C) potentially encoding an A$_1$A$_O$ ATP synthase with an unusual V-type c subunit [17]. We have purified the enzyme from membranes of *E. limosum* and will demonstrate that it is a Na$^+$-dependent A$_1$A$_O$ ATP synthase containing a V-type like c subunit capable of ATP synthesis.

**Results**

**Genome organization, genes, and subunits of the A$_1$A$_O$ ATP synthase from *E. limosum***

The genes *Eli*$_{2184}$–2192 encode an enzyme with highest similarity to an archaeal A$_1$A$_O$ ATP synthase (Fig. 1A). Upstream of the A$_1$A$_O$ ATP synthase operon on the reverse strand *Eli*$_{2183}$ (117 bp) encodes for a hypothetical protein with 41% identity to dynein protein 3 of *Saccharomyces cerevisiae* (Table 1). *Eli*$_{2184}$ is 309 bp long and encodes for subunit H with a molecular mass of 11.4 kDa (Table 1). The encoded protein is 53% similar to subunit H of the A$_1$A$_O$ ATP synthase from *Methanothermobacter marburgensis*. The next gene of the operon (*Eli*$_{2185}$) has a 3 bp overlap with *Eli*$_{2184}$. It is 1875 bp long and encodes for subunit a of the hydrophobic A$_O$ domain with a molecular mass of 69.8 kDa. The deduced gene product is 31% and 26% identical to the corresponding subunit from *Methanocaldococcus jannaschii* [14] and *Methanosarcina mazei* Görl [18], respectively. Most interestingly, 14 bp downstream of *Eli*$_{2185}$ is the 474-bp-long gene encoding the rotor subunit c (*Eli*$_{2186}$) with a predicted mass of a 16 kDa and four transmembrane helices (Fig. 1B). The similarity/identity to subunit c of *Pyrococcus furiosus* is 51/35%. Recently, we have shown that the c subunit from *P. furiosus* has indeed four transmembrane helices but only one ion (Na$^+$)-binding site (Q...ET) [19] which is also conserved in *Thermococcus onnurineus* and in subunit c of *E. limosum*. As in *P. furiosus*, there is no second proton or sodium ion-binding site conserved. *Eli*$_{2187}$ (606 bp) is located 36 bp downstream of *Eli*$_{2186}$, and the deduced protein (22.6 kDa) is annotated as subunit B with a molecular mass of 66.8 kDa. The deduced gene product is 59% identical to the corresponding subunit from *M. jannaschii* and *T. onnurineus*. *Eli*$_{2191}$ (1410 bp) encodes for subunit C with a molecular mass of 52.3 kDa. 71, 67, and 30% of the residues are similar/identical to subunit F of the A$_1$A$_O$ ATPase from *M. barkeri* [20]. *Eli*$_{2190}$ (1806 bp) is located 26 bp downstream of *Eli*$_{2189}$ and encodes for subunit A with a molecular mass of 66.8 kDa. The deduced gene product is 59% identical to the corresponding subunit from *M. jannaschii* and *T. onnurineus*. *Eli*$_{2191}$ is *Eli*$_{2192}$ with a length of 636 bp. The protein has a molecular mass of 29.4 kDa. Sequence alignments showed 42% and 39% identity to subunit D of *M. jannaschii* and *T. onnurineus*, respectively. The next gene downstream of the putative ATP synthase operon *Eli*$_{2193}$ (138 bp) is located in 3’→5’ direction. For this gene, no homologues were found.

**Purification of the A$_1$A$_O$ ATP synthase from *E. limosum***

To determine the effect of the growth substrate on ATPase activity, *E. limosum* KIST612 was grown on complex medium with 50 mm glucose, 50 mm...
methanol, 50 mM formate, 101 kPa H₂ + CO₂, or 101 kPa CO as sole carbon and energy source. Cells were harvested during exponential growth, washed membranes were prepared, and ATP hydrolysis was determined. Washed membranes of H₂ + CO₂-grown cells had the highest ATPase activity of 68.3 mU mg⁻¹. The activity of washed membranes from methanol-, formate-, and CO-grown cells was similar and slightly below the latter one (54, 41.3, and 50.7 mU mg⁻¹). Membranes from glucose-grown cells showed the lowest activity with 24 mU mg⁻¹. Due to the low yield of cell mass from H₂ + CO₂-grown cultures (final OD₆₀₀ = 0.4), methanol was selected as growth substrate (final OD₆₀₀ = 1.2) for large-scale cultivation. For purification of the A₁A₀ ATP synthase, washed membranes of around 15 g cells (wet mass) were prepared and solubilized with 1 mg n-dodecyl-β-maltoside (DDM) per mg membrane protein. The ATP synthase was further purified by a sucrose density gradient (20–65%) and the subsequent purification by an anion exchange chromatography step (DEAE) as well as a size-exclusion chromatography (Superose 6 column) resulted in 23.6-fold enrichment.

Table 1. Properties of the A₁A₀ ATP synthase subunits

| Gene    | Subunit | Residues | M_r | pI  |
|---------|---------|----------|-----|-----|
| Eli_2184| H       | 102      | 11 406 | 4.81 |
| Eli_2185| a       | 624      | 69 757 | 4.8 |
| Eli_2186| c       | 157      | 15 720 | 5.64 |
| Eli_2187| E       | 201      | 22 574 | 4.83 |
| Eli_2188| C       | 323      | 36 377 | 5.19 |
| Eli_2189| F       | 105      | 11 283 | 4.89 |
| Eli_2190| A       | 601      | 66 841 | 4.78 |
| Eli_2191| B       | 469      | 52 296 | 4.85 |
| Eli_2192| D       | 211      | 23 986 | 7.73 |

**Fig. 1.** Genetic organization of the A₁A₀ ATP synthase operon from *Eubacterium limosum* and alignment of subunit c. The A₁A₀ ATP synthase operon of *E. limosum* comprises nine genes. The genes of the ATP synthase operon are highlighted in red (A). The c subunit of the putative A₁A₀ ATP synthase has a conserved Na⁺-binding motif. The amino acid sequence of the c-subunit of the putative A₁A₀ ATP synthase from *E. limosum* (accession number: ESGN4) was aligned with the sequences of subunit c of *Pyrococcus furiosus* (accession number: Q8U4B0) and *Thermococcus onnurineus* (accession number: BBYV10). The Na⁺-binding motif (Q...ET) is highlighted in yellow. The sequence alignment was performed by using CLUSTAL OMEGA (Dublin, Ireland) (B).
of the activity with a specific activity of 1277 mU·mg⁻¹ and a yield of 12.2% (Table 2).

**Subunit composition of the A₁A₀ ATP synthase from *E. limosum***

The preparation contained 13 proteins (Fig. 2) with apparent molecular masses of 67 kDa (subunit A), 58 kDa (subunit B), 39 kDa (subunit C), 26 kDa (subunit D), 25 kDa (subunit E), 11 kDa (subunit F), 12 kDa (subunit H), and 14 kDa (subunit c). As seen before with A₁A₀ ATP synthases from hyperthermophilic archaebacteria, there is a protein above subunit A which could be a SDS-resistant ac subcomplex [12,15]. Single subunit a was not detected. The identity of the subunits was proven by MALDI-TOF-MS (Fig. 3) and immunological with antibodies generated and specific against the purified subunits. The 14 kDa protein was identified as subunit c by peptide mass fingerprinting. Notably, the loops connecting TMH1 and 2 as well as TMH3 and 4 were detected, demonstrating that subunit c indeed contains four transmembrane helices (Fig. 4).

**Basic biochemical properties of ATP hydrolysis**

ATPase activity was detected over a pH range between 5.0 and 9.0, with optimal activity between pH 7.0 and 7.5 (Fig. 5A). pH values lower than 6.0 and higher than 8.0 decreased the activity very strongly (9.5% at pH 5.0, 10% activity at pH 9.0). ATP hydrolysis activity was optimal between 30 °C and 40 °C (Fig. 5B). After 45 °C, the activity decreased sharply. Methanol stimulated ATP hydrolysis activity as described before [21] (Fig. 5C). Optimal activity was determined with 20% methanol.

**Na⁺ dependence of ATP hydrolysis**

ATP hydrolysis was strictly dependent on the Na⁺ concentration of the assay buffer (Fig. 6). At the contaminating amount of 71 µM Na⁺, ATP hydrolysis was only 326 mU·mg⁻¹ but activity was stimulated fourfold by the addition of Na⁺. The Na⁺ dependence followed a classical Michaelis–Menten kinetics; full activity was observed at 0.6 mM and half maximal was at 0.3 mM. The addition of Li⁺ also stimulated ATP hydrolysis; full activity was observed at 2 mM and half maximal activity was at 1 mM. K⁺ did not stimulate ATP hydrolysis.

**DCCD and Na⁺ compete for binding to subunit c**

DCCD is a common inhibitor for ATP synthases that covalently binds to the highly conserved protonated carboxylate (E138 in helix 4) in subunit c, thereby inhibiting proton or Na⁺ binding and transport [22,23]. In all known Na⁺ ATP synthases, the access of DCCD is blocked in the presence of Na⁺, thus reducing the inhibitory effect of DCCD on the enzyme [12,19,24–26]. DCCD inhibited the A₁A₀ ATP synthase from *E. limosum* with half maximal inhibition at 80 µM DCCD (Fig 7). When the enzyme was preincubated with Na⁺, DCCD inhibition was relieved. NCD-4 is a fluorescent analogue of DCCD and bound to subunit c (Fig. 8). Preincubation with Na⁺ prevented NCD-4 binding. These data are consistent with the proposal that Na⁺ is the coupling ion of the A₁A₀ ATP synthase from *E. limosum*.

**Na⁺ transport and ATP synthesis in proteoliposomes**

In order to unequivocally proof Na⁺ transport by the A₁A₀ ATP synthase, the enzyme was reconstituted into liposomes consisting of phosphatidylcholine type II S from soybeans. Liposomes were generated by sonification [27]. Upon addition of ATP, ²²Na⁺ was transported into the lumen of the vesicles with a rate of 0.6 nmol Na⁺·min⁻¹·mg⁻¹ protein, leading to a final accumulation of 9.2 nmol Na⁺·mg⁻¹·protein (Fig. 9). ²²Na⁺ transport was not observed in the absence of ATP or in the presence of the sodium ionophore *N*,*N*,*N*′,*N*″-tetra-cyclo-hexyl-1,2-phenylenedioxydiamide (ETH 2120). The protonophore 3,3′,4′,5-tetrachlorosalicylanilide (TCS) slightly stimulated ²²Na⁺ transport.

---

**Table 2. Purification of the Na⁺ A₁A₀-ATP synthase from *Eubacterium limosum* KIST612**

| Step                | Total protein (mg) | Volume (mL) | Total activity (U) | Specific activity (mU·mg⁻¹) | Purification (x-fold) | Yield (%) |
|---------------------|--------------------|-------------|--------------------|-----------------------------|-----------------------|-----------|
| Washed membranes    | 272                | 14          | 14.69              | 54                          | 1                     | 100       |
| Solubilize          | 124                | 8           | 11.9               | 96.2                        | 1.8                   | 81        |
| Sucrose gradient    | 23                 | 28          | 7.08               | 308                         | 5.7                   | 48.2      |
| DEAE                | 4.1                | 20          | 2.89               | 703                         | 13                    | 19.7      |
| Gel filtration      | 1.4                | 1           | 1.79               | 1277                        | 23.6                  | 12.2      |
These data are consistent with a primary and electrogenic Na⁺ transport catalyzed by the A₁Aₐ ATP synthase.

Next, we investigated whether the enzyme is capable of ATP synthesis despite its V-type like c subunit. Therefore, the enzyme was again reconstituted into liposomes and an electrochemical Na⁺ potential (ΔµNa⁺) of around 270 mV was applied. ΔµNa⁺ was generated by a sodium ion potential (ΔpNa) of 75 mV combined with a K⁺/valinomycin diffusion potential of around 195 mV. Upon addition of 2 μM valinomycin, ATP was synthesized with a rate of about 54 nmol·min⁻¹·mg⁻¹ protein (Fig. 10). The data demonstrate that the A₁Aₐ ATP synthase with its V-type like c subunit from E. limosum is capable of ATP synthesis.

**Discussion**

Many archaea like methanogens, *Thermococcus*, or *Pyrococcus* live under extreme conditions: at high temperatures of > 80 °C, sometimes paired with another extreme, a metabolism that allows for the production of only a small fraction of an ATP per mol of substrate oxidized [28–30]. This life at the thermodynamic edge requires adaptions in the ATP-generating mechanisms. ATP is synthesized exclusively or in addition by a chemiosmotic mechanism that involves unusual respiratory enzyme such as membrane-bound, ion-translocating hydrogenases (progenitors of complex I) or a formate dehydrogenase [30,31]. Often, the free energy change is so small that it allows for the translocation of only one ion per mol of substrate oxidized, sometimes, as in the case of *T. onnurineus*, even less than that [31]. Special adaptions in the ATP synthase also contribute to this lifestyle. One adaption is the use of Na⁺ instead of H⁺ as coupling ion, the other a c ring with a reduced number of ion translocation sites [28,29,32]. This would allow the enzyme to synthesize ATP at lower costs; however, this would only work if the phosphorylation potential in these cells is also lower [33].

In F₁F₀ ATP synthases, the minimal ion/ATP ratio is 2.7, restricted by the minimal number of 8 c subunits with two transmembrane helices to form a ring structure. However, a doubling of the size of the individual c subunits forming the ring increases the stability of the ring structure due to its longer size and possibly allows a further reduction of the ion/ATP ratio. Indeed, the c ring of the A₁Aₐ ATP synthase of *P. furiosus* is proposed to have 10 [13] or 9 [34] subunits.

Measurements of energetic parameter such as phosphorylation potential, membrane potential, and ion transport are scarce in these hyperthermophilic archaea, due to their difficult cultivation and inherent problems of measuring these values at temperatures at 80–100 °C. A solution to this dilemma comes with acetogenic bacteria such as *Acetobacterium woodii* or, here, *E. limosum*. Both are mesophiles but like most other acetogenic bacteria live at the thermodynamic edge of life. *A. woodii* grows heterotrophically but also autotrophically on H₂ + CO₂ [35]. The phosphorylation potential in these cells was measured to be much below the ‘textbook-knowledge’ value of 60 kJ·mol⁻¹·C₆H₁₂O₆ 37.9 ± 1.3 kJ·mol⁻¹ for fructose, 32.1 ± 0.3 for H₂ + CO₂, and 30.2 ± 0.9 for CO [36]. This would allow ATP synthesis with only a fraction of ions. Indeed, the c ring of the Na⁺ F₁F₀ ATP synthase from *A. woodii* is unique since it contains one V-type c subunit along with nine F-type c subunits, the first and so far, only F/V-hybrid motor found in nature [37].
ATP synthase of *E. limosum* has a V-type like c subunit, but, as shown here, is able to synthesize ATP. With this mesophilic enzyme in hand, the road is paved to analyze the dependence of ATP synthesis on energetic parameters in these unusual organisms whose metabolism is considered to be maybe the first metabolic scenario on early Earth.

### Materials and methods

#### Cultivation of cells and preparation of washed membranes

*Eubacterium limosum* KIST612 was cultivated in anoxic phosphate-buffered basal medium (PBBM) or carbonate-buffered...
basal medium (CBBM) at 37 °C as described previously [38]. Glucose (50 mM), methanol (50 mM), formate (50 mM), \( \text{H}_2 + \text{CO}_2 \) \( [101 \text{kPa}, 80 : 20 (\text{v/v})] \), or CO (101 kPa) was used as growth substrates. The cells were harvested in midexponential growth phase by centrifugation (Beckman Avanti J25, JA10 rotor, Beckmann Coulter, Krefeld, Germany). For the preparation of washed membranes, around 1 g (wet mass) of cells grown on different substrates was suspended in 5 mL of buffer A (50 mM Tris/HCl, 10 mM MgCl\(_2\), 420 mM sucrose, pH 7.5). Lysozyme (20 mg g\(^{-1}\) cells) was added, and the suspension was incubated for 1 h at 37 °C. All subsequent steps were performed at 4 °C unless indicated otherwise. After addition of PMSF (final concentration: 0.5 mM) and a few crystals of DNase I, the resulting protoplasts were disrupted by passing three times through a French pressure cell press at 110 MPa (SLM Aminco, SLM Instruments, Urbana, IL, USA). After dilution with four volumes of buffer A, cell debris was removed by centrifugation (Beckman Optima L90-K, 70.2 Ti rotor, 149 000 g, 45 min, Beckmann)

Fig. 5. Basic biochemical properties of the A\(_1\)A\(_0\)-ATP synthase from Eubacterium limosum. The buffer used for the pH optima determination contained 50 mM MES, 50 mM Tris, 50 mM HEPES, 50 mM CHES, 10 mM MgCl\(_2\), 20% methanol with a pH as indicated and 10 \( \mu \)g enzyme. The reaction temperature was set to 37 °C (A). The temperature optimum was determined with a buffer containing 100 mM Tris, 100 mM maleic acid, 10 mM MgCl\(_2\), 20% methanol (v/v), pH 7.3, and 10 \( \mu \)g enzyme. After incubating the enzyme for 3 min at the temperature as indicated, the reaction was started by addition of a final concentration of 2.5 mM Na\(_2\)-ATP (B). The dependence on methanol was measured with 10 \( \mu \)g enzyme in the same buffer at 37 °C. The enzyme was incubated for 3 min at 37 °C, and the reaction was started by addition of ATP to a final concentration of 2.5 mM (C). The data represent the mean and standard deviation of three independent experiments (\( n = 3 \)), each performed in triplicate.

Fig. 6. \( \text{Na}^+ \) dependence of ATP hydrolysis by the A\(_1\)A\(_0\)-ATP synthase from Eubacterium limosum. ATPase activity was measured at 37 °C. The buffer contained 100 mM Tris, 100 mM maleic acid, 10 mM MgCl\(_2\), 20% methanol, pH 7.3, and 20 \( \mu \)g enzyme. The concentration of \( \text{Na}^+ \) in the sodium-free buffer was determined to be 71 \( \mu \)M by using a \( \text{Na}^+ \)-selective electrode. \( \text{NaCl} \) (black, circles), LiCl (blue, triangles up), or KCl (red, triangles down) was added as indicated. The reaction was started by addition of 2.5 mM Tris-ATP (final concentration). The data represent the mean and standard deviation of three independent experiments (\( n = 3 \)), each performed in triplicate.

Fig. 7. Inhibition of ATP hydrolysis by DCCD and its relieve by \( \text{Na}^+ \). The buffer contained 100 mM Tris, 100 mM maleic acid, 10 mM MgCl\(_2\), 20% methanol, pH 6.3, and 20 \( \mu \)g enzyme. After preincubation of the enzyme with increasing concentrations of DCCD (0–500 \( \mu \)M, solved in ethanol) in the presence (red, triangles up) or absence of \( \text{Na}^+ \) (blue, circles) for 25 min at room temperature and incubation for 3 min at 37 °C, the reaction was started by addition of 2.5 mM Tris-ATP (final concentration). The data represent the mean and standard deviation of three independent experiments (\( n = 3 \)), each performed in triplicate.
Coulter Krefeld, Germany) and washed twice with buffer B (50 mM Tris/HCl, 10 mM MgCl₂, 20 mM NaCl, pH 7.5).

**Purification of the A₁A₀ ATP synthase from E. limosum**

For purification of the ATP synthase, cells of *E. limosum* KIST 612 were grown in CBBM with 50 mM methanol as substrate at 37 °C under anoxic conditions in two 20-L carboys (Glasgerätebau Ochs, Bovenden-Lenglern, Germany). All purification steps were performed at 4 °C under oxic conditions. Methanol-grown cells were harvested by centrifugation (Heraeus centrifuge, Hanau, Germany; HCF 22.300 rotor), and washed membranes of around 15 g cells (wet mass) suspended in 30 mL buffer A were prepared as described above. The washed membranes were resuspended in 8 mL buffer B. To solubilize the ATP synthase, 1 mg n-dodecyl-β-D-maltoside (DDM) (Carl Roth, Karlsruhe, Germany) per milligram of membrane protein was added and incubated under shaking for 2.5 h. The remaining membranes were removed by ultracentrifugation (180 000 g, 30 min), and the solubilizate was loaded onto a sucrose gradient (20–65%) and centrifuged for 19 h in a vertical rotor (Beckman Optima L90-K, VTi50 rotor, 152 624 g). Each sucrose gradient fraction was tested for ATPase activity. The fractions of sucrose gradient with the highest ATPase activity were pooled and applied to anion exchange chromatography column. Therefore, DEAE Sepharose Fast Flow (GE Healthcare, Uppsala, Sweden) was equilibrated with buffer C [100 mM HEPES, 5 mM MgCl₂, 10% (v/v) glycerol, pH 7.3] and a salt gradient (0.15–0.4 M NaCl) in buffer C at a flow rate of 1 mL/min was used for elution. The ATP synthase eluted at around 0.25 M NaCl. Fractions containing the ATP synthase were pooled and concentrated to a volume of 1 mL (Vivaspin 20 columns, 100 kDa MWCO; Sartorius, Goettingen, Germany). To further purify the sample, an additional size-exclusion chromatography was performed. In aliquots of 0.5 mL, the protein was applied onto a Superose 6 column (10/300 GL; GE Healthcare, Uppsala, Sweden) that was equilibrated with buffer D [100 mM HEPES, 250 mM NaCl, 5 mM MgCl₂, 10% (v/v) glycerol, 0.02% (w/v) DDM, pH 7.3]. The purified ATP synthase eluted with buffer D with a flow rate of 0.5 mL/min⁻¹. For experiments under Na⁺-free conditions, the protein was eluted with buffer D without addition of NaCl. The concentration of Na⁺ was determined by using a Na⁺-selective electrode (ORION™ 8411BN ROSS; Thermo Fisher Scientific).
An A1A0 ATP synthase in *E. limosum* D. Litty and V. Müller

**Fig. 10.** ΔψNa⁺-driven ATP synthesis by the purified A1A0 ATP synthase. Proteoliposomes containing 250 µg of the reconstituted enzyme (30 : 1) were generated in liposome buffer B (100 mM Tris, 100 mM maleic acid, 5 mM MgCl₂, 200 mM NaCl, pH 7.3) resulting in a concentration of 200 mM NaCl on the inside. The buffer on the outside was exchanged after centrifugation of the liposomes. The liposomes were resuspended in ATP synthesis buffer (100 mM Tris, 100 mM maleic acid, 5 mM MgCl₂, 10 mM NaCl, 200 mM KCl, 5 mM KH₂PO₄, pH 7.3) resulting in an outside concentration of 10 mM NaCl, thus creating a sodium ion potential (ΔpNa⁺). Upon addition of 2 mM valinomycin and 5 mM ADP, a membrane potential (ΔΨ) was induced and the reaction was started. ATP synthase was measured at 37 °C, and ATP was determined by luciferin–luciferase assay (blue, circles). In the red plot (triangles up), no ADP was added. Each value represents the mean and standard deviation of three independent experiments (n = 3).

**Antibody generation and western blot**

For expression of genes encoding for the single subunits A, B, C, D, and E, the amplified fragment was cloned into *pXc99a* and each corresponding plasmid was transformed in *E. coli* B, C, D, and E, the amplified fragment was cloned into pTrc99a and each corresponding plasmid was transformed in *Escherichia coli*. Antibody generation and western blot was performed as described previously [41]. The subunits cut from a Coomassie-stained SDS gel were analyzed by SDS/PAGE, using the buffer system of Schagger and von Jagow [39] and visualized by staining with Coomassie brilliant blue G250 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). All preparations were routinely analyzed by SDS/PAGE, using the buffer system of Schagger and von Jagow [39]. Proteins were visualized by staining with Coomassie brilliant blue G250 (Sigma-Aldrich Chemie GmbH) [40].

**Determination of ATPase activity**

The ATPase activity of washed membranes isolated from cells grown on different substrates was measured in a buffer containing 100 mM Tris, 100 mM maleic acid, 10 mM MgCl₂, 20% methanol (v/v), pH 7.3. After incubation for 3 min at 37 °C, the reaction was started by the addition of 2.5 mM Na₂-ATP. The ATPase activity was determined by a discontinuous assay following the ATP-dependent formation of inorganic phosphate, according to the method of Heinonen and Lahti [42], as previously described [43]. To determine the effect of pH and temperature, the enzyme was preincubated for 3 min at the pH (5–9) or temperature (20–50 °C) indicated, respectively. The reaction temperature for the determination of the pH optimum was set to 37 °C. The buffer used for the pH optima determination was 50 mM MES, 50 mM Tris, 50 mM HEPES, 50 mM CHES, 10 mM MgCl₂, 20% methanol. For inhibitor studies with N’,N’-dicyclohexyl carbodiimide (DCCD), the reaction mixture was preincubated at room temperature for 30 min before the reaction was started by addition of Tris-ATP. For determination of ATPase activity in the absence of sodium ions, the activity was determined in 100 mM Tris/HCl, 5 mM MgCl₂, and 20% methanol as described above.

**Labeling with NCD-4**

Purified ATP synthase was labeled with NCD-4 (solved in ethanol) by incubation with 60 or 200 nmol of NCD-4 overnight at room temperature in the presence or absence of 200 nM NaCl. After incubation, the protein was separated by SDS/PAGE (12.5%) and fluorescence was detected after excitation with UV light.

**Reconstitution of the A1A0 ATP synthase in proteoliposomes**

The reconstitution of the A1A0 ATP synthase from *E. limosum* in unilamellar vesicles of phosphatidylcholine type II S from soybeans (Sigma-Aldrich Chemie GmbH) was carried out as for the ATP synthase from *A. woodii*, according to Ref. [27] with slight modifications. In order to solve phosphatidylcholine type II S from soybeans, either liposome buffer A (50 mM Tris/HCl, 10 mM MgCl₂, pH 7.5) or, for the determination of ΔΨNa⁺-driven ATP synthesis, liposome buffer B (100 mM Tris, 100 mM maleic acid, 5 mM MgCl₂, 200 mM NaCl, pH 7.3) was used.

**Measurement of Na⁺ translocation**

Na⁺ translocation experiments in proteoliposomes were performed according to Ref. [27] in a buffer composed of 50 mM Tris/HCl, 10 mM MgCl₂, pH 7.3. The sodium ionophore ETH 2120 and the protonophore TCS were added from ethanolic stock solutions and controls received the...
solvent only [final concentration: 0.3% (v/v)]. The proteoliposomes, the buffer, supplements, and 22NaCl (carrier-free, final activity 0.5 µCi·mL⁻¹) were combined in a reaction tube to a final Na⁺ concentration of 4.5 mM and incubated at 37 °C for 30 min to assure equilibration of 22Na⁺, before the reaction was started by addition of 2.5 mM Tris-ATP (final concentration). Eighty microlitre samples were withdrawn and passed over a column (0.5 × 3.2 cm) of Dowex 50-WX8 (100–200-mesh) [43]. The proteoliposomes were collected by washing the column with 1 mL of 420 mM sucrose. The radioactivity in the eluate was determined by liquid scintillation counting.

Determination of ATP synthesis

To generate a ΔΔNa⁺, the purified ATP synthase was first reconstituted with liposome buffer B (100 mM Tris, 100 mM maleic acid, 5 mM MgCl₂, 200 mM NaCl, pH 7.3) as described above. The loaded proteoliposomes were collected by extract (Beckman Optima L90-K, 70.2 Ti rotor, 150 000 g, 30 min), washed once with ATP synthesis buffer (100 mM Tris, 100 mM maleic acid, 5 mM MgCl₂, 10 mM NaCl, 200 mM KCl, 5 mM KH₂PO₄, pH 7.3 (30 min, 150 000 g, 30 min), and resolved in the same buffer. ATP synthesis of the reconstituted ATP synthase was started by addition of 2 µM valinomycin (Sigma-Aldrich) to induce a ΔΨ, 5 µM ADP (final concentration each) and measured by a standard luciferin/luciferase assay (Sigma-Aldrich) at 37 °C, monitoring the emitted light with a chemiluminometer (Berthold Technologies, Bad Wildbad, Germany) as described by Ref. [44].

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft via SFB807 is gratefully acknowledged.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

VM designed the experiments. DL performed the experiments. VM and DL analyzed the data. VM and DL wrote the paper.

References

1 Senior AE (1988) ATP synthesis by oxidative phosphorylation. *Physiol Rev* 68, 177–231.
2 Müller V & Gruber G (2003) ATP synthases: structure, function and evolution of unique energy converters. *Cell Mol Life Sci* 60, 474–494.
3 Cross RL & Müller V (2004) The evolution of A-, F-, and V-type ATP synthases and ATPases: reversals in function and changes in the H⁺/ATP stoichiometry. *FEBS Lett* 576, 1–4.
4 Stock D, Gibbons C, Arechaga I, Leslie AG & Walker JE (2000) The rotary mechanism of ATP synthase. *Curr Opin Struct Biol* 10, 672–679.
5 Furgac M (2007) Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. *Nat Rev Mol Cell Biol* 8, 917–929.
6 Grüber G, Manimekalai MS, Mayer F & Müller V (2014) ATP synthases from archaea: the beauty of a molecular motor. *Biochim Biophys Acta* 1837, 940–952.
7 Müller V (2004) An exceptional variability in the motor of archaeal A₁A₀ ATPases from multicentric to monomeric rotors comprising 6–13 ion binding sites. *J Bioenerg Biomembr* 36, 115–125.
8 Müller V, Lemker T, Lingl A, Weidner C, Coskun Ü & Grüber G (2005) Bioenergetics of archaea: ATP synthesis under harsh environmental conditions. *J Mol Microbiol Biotechnol* 10, 167–180.
9 Mandel M, Moriyama Y, Hulmes JD, Pan Y-CE, Nelson H & Nelson N (1988) cDNA sequence encoding the 16-kDa proteolipid of chromaffin granules implies gene duplication in the evolution of H⁺-ATPases. *Proc Natl Acad Sci USA* 85, 5521–5524.
10 Nelson N (1992) Evolution of organelar proton-ATPases. *Biochim Biophys Acta* 1100, 109–124.
11 Lim JK, Mayer F, Kang SG & Müller V (2014) Energy conservation by oxidation of formate to carbon dioxide and hydrogen via a sodium ion current in a hyperthermophilic archaeon. *Proc Natl Acad Sci USA* 111, 11497–11502.
12 Pisa KY, Huber H, Thomm M & Müller V (2007) A sodium ion-dependent A₁A₀ ATP synthase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *FEBS J* 274, 3928–3938.
13 Vonck J, Pisa KY, Morgner N, Brutschy B & Müller V (2009) Three-dimensional structure of A₁A₀ ATP synthase from the hyperthermophilic archaeon *Pyrococcus furiosus* by electron microscopy. *J Biol Chem* 284, 10110–10119.
14 Lingl A, Huber H, Stetter KO, Mayer F, Kellermann J & Müller V (2003) Isolation of a complete A₁A₀ ATP synthase comprising nine subunits from the hyperthermophile *Methanococcus jannaschii*. *Extremophiles* 7, 249–257.
15 Mayer F, Lim JK, Langer JD, Kang SG & Müller V (2015) Na⁺ transport by the A₁A₀-ATP synthase purified from *Thermococcus onnurineus* and reconstituted into liposomes. *J Biol Chem* 290, 6994–7002.
16 Kreuter LJ, Weinfurtner A, Ziegler A, Weigl J, Hoffmann J, Morgner N, Müller V & Huber H (2019) Purification of a crenarchaeal ATP synthase in...
the light of the unique bioenergetics of Ignicoccus species. *J Bacteriol* **201**, 510–518.

17. Jeong J, Bertsch J, Hess V, Choi S, Choi IG, Chang IS & Müller V (2015) Energy conservation model based on genomic and experimental analyses of a carbon monoxide-utilizing, butyrate-forming acetogen, *Eubacterium limosum* KIST612. *Appl Environ Microbiol* **81**, 4782–4790.

18. Wilms R, Freiberg C, Wegerle E, Meier I, Mayer F & Müller V (1996) Subunit structure and organization of the genes of the A1A0 ATPase from the archaeon *Methanosarcina mazei* Göl. *J Biol Chem* **271**, 18843–18852.

19. Mayer F, Leone V, Langer JD, Faraldo-Gómez JD & Müller V (2012) A c subunit with four transmembrane helices and one ion (Na+) binding site in an archaeal ATP synthase: implications for c ring function and structure. *J Biol Chem* **287**, 39327–39337.

20. Inatomi KI, Eya S, Maeda M & Futai M (1989) Amino acid sequence of the alpha and beta subunits of *Methanosarcina Barkeri* ATPase deduced from cloned genes. *J Biol Chem* **264**, 10954–10959.

21. Anthon GE & Jagendorf AT (1983) Effect of methanol on spinach thylakoid ATPase. *Biochim Biophys Acta* **723**, 358–363.

22. Hermolin J & Fillingame RH (1989) H+–ATPase activity of *Escherichia coli* F1F0 is blocked after reaction of dicyclohexylcarbodiimide with a single proteolipid (subunit c) of the F0 complex. *J Biol Chem* **264**, 3896–3903.

23. Al tendor K (1977) Purification of the DCCD-reactive protein of the energy-transducing adenosine triphosphatase complex from *Escherichia coli*. *FEBS Lett* **73**, 271–275.

24. McMillan DGG, Ferguson SA, Dey D, Schröder K, Aung HL, Carbone GT, Attwood GT, Ronimos RS, Meier T, Janssen PH et al. (2011) A1A0-ATP synthase of *Methanobrevibacter ruminantium* couples sodium ions for ATP synthesis under physiological conditions. *J Biol Chem* **286**, 39882–39892.

25. Laubinger W & Dimroth P (1988) Characterization of the ATP synthase of *Propionigenium modestum* as a primary sodium pump. *Biochemistry* **27**, 7531–7537.

26. Spruth M, Reidlinger J & Müller V (1995) Sodium ion dependence of inhibition of the Na+–translocating F1F0–ATPase from *Acetobacterium woodii*. Probing the site(s) involved in ion transport. *Biochim Biophys Acta* **1229**, 96–102.

27. Brandt K, Müller DB, Hoffmann J, Hübert C, Brutschy B, Decker-Hebestreet G & Müller V (2013) Functional production of the Na+–F1F0 ATP synthase from *Acetobacterium woodii* in *Escherichia coli* requires the native Atpl. *J Bioenerg Biomembr* **45**, 15–23.

28. Deppenmeier U & Müller V (2008) Life close to the thermodynamic limit: how methanogenic archaea conserve energy. *Results Probl Cell Differ* **45**, 123–152.

29. Thauer RK, Kaster AK, Seedorf H, Buckel W & Hedderich R (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat Rev Microbiol* **6**, 579–591.

30. Mayer F & Müller V (2013) Adaptations of anaerobic archaea to life under extreme energy limitation. *FEBS Microbiol Rev* **38**, 449–472.

31. Kim YJ, Lee HS, Kim ES, Bae SS, Lim JK, Matsumi R, Lebedinsky AV, Sokolova TG, Kozhevnikova DA, Cha SS et al. (2010) Formate-driven growth coupled with H2 production. *Nature* **467**, 352–355.

32. Schlegel K & Müller V (2013) Evolution of Na+ and H+ bioenergetics in methanogenic archaea. *Biochim Biophys Acta* **241**, 421–426.

33. Müller V & Hess V (2017) The minimum biological energy quantum. *Front Microbiol* **8**, 2019.

34. Cossio P, Allegretti M, Mayer F, Muller V, Vonck J & Hummer G (2018) Bayesian inference of rotor ring stoichiometry from electron microscopy images of archaeal ATP synthase. *Microscopy* **67**, 266–273.

35. Balch WE, Schoberth S, Tanner RS & Wolfe RS (1977) *Acetobacterium*, a new genus of hydrogen-oxidizing, carbon dioxide-reducing, anaerobic bacteria. *Int J Syst Bact* **27**, 355–361.

36. Spahn S, Brandt K & Müller V (2015) A low phosphorylation potential in the acetogen *Acetobacterium woodii* reflects its lifestyle at the thermodynamic edge of life. *Arch Microbiol* **197**, 745–751.

37. Matthies D, Zhou W, Klyszeko AL, Anselmi C, Yildiz O, Brandt K, Müller V, Faraldo-Gómez JD & Meier T (2014) High-resolution structure and mechanism of an F/V-hybrid rotor ring in a Na+–coupled ATP synthase. *Nat Commun* **5**, 5286.

38. Chang IS, Kim BH, Kim DH, Lovitt RW & Sung HC (1999) Formulation of defined media for carbon monoxide fermentation by *Escherichia coli* and the growth characteristics of the bacterium. *J Biosoc Bioeng* **88**, 682–685.

39. Schägger H & von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* **166**, 369–379.

40. Weber K & Osborne M (1969) The reliability of the molecular weight determination by dodecyl sulfate polyacrylamide gel electrophoresis. *J Biol Chem* **244**, 4406–4412.

41. Towbin H, Staehelin T & Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**, 4350–4354.

42. Heinonen JE & Lahti RJ (1981) A new and convenient colorimetric determination of inorganic orthophosphate
and its application to the assay of inorganic pyrophosphatase. *Anal Biochem* **113**, 313–317.

43 Heise R, Müller V & Gottschalk G (1992) Presence of a sodium-translocating ATPase in membrane vesicles of the homoacetogenic bacterium *Acetobacterium woodii*. *Eur J Biochem* **206**, 553–557.

44 Fritz M & Müller V (2007) An intermediate step in the evolution of ATPases – the F1Fo-ATPase from *Acetobacterium woodii* contains F-type and V-type rotor subunits and is capable of ATP synthesis. *FEBS J* **274**, 3421–3428.