Na⁺ Transport by the A₁A₉-ATP Synthase Purified from Thermococcus onnurineus and Reconstituted into Liposomes*

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Background: The ATP synthase of many archaea is predicted to use Na⁺ as coupling ion.

Results: The enzyme from Thermococcus onnurineus reconstituted in proteoliposomes catalyzed ATP-driven Na⁺ transport.

Conclusion: The enzyme uses Na⁺ as coupling ion.

Significance: First direct proof of ion (Na⁺) transport by a reconstituted A-ATP synthase.

The ATP synthase of many archaea has the conserved sodium ion binding motif in its rotor subunit, implying that these A₁A₉-ATP synthases use Na⁺ as coupling ion. However, this has never been experimentally verified with a purified system. To experimentally address the nature of the coupling ion, we have purified the A₁A₉-ATP synthase from T. onnurineus. It contains nine subunits that are functionally coupled. The enzyme hydrolyzed ATP, CTP, GTP, UTP, and ITP with nearly identical activities of around 40 units/mg of protein and was active over a wide pH range with maximal activity at pH 7. Noteworthy was the temperature profile. ATP hydrolysis was maximal at 80 °C and still retained an activity of 2.5 units/mg of protein at 45 °C. The high activity of the enzyme at 45 °C opened, for the first time, a way to directly measure ion transport in an A₁A₉-ATP synthase. Therefore, the enzyme was reconstituted into liposomes generated from Escherichia coli lipids. These proteoliposomes were still active at 45 °C and coupled ATP hydrolysis to primary and electrogenic Na⁺ transport. This is the first proof of Na⁺ transport by an A₁A₉-ATP synthase and these findings are discussed in light of the distribution of the sodium ion binding motif in archaea and the role of Na⁺ in the bioenergetics of archaea.

Members of the third domain of life, Archaea (1), use the same principle mechanisms of energy conservation as bacteria or eukaryotes. Chemiosmotic energy conservation is widespread and seen as a mechanism to allow growth close to the thermodynamic limit, a situation that many archaea face (2, 3). Despite the differences in the mechanisms used to generate the electrochemical ion gradient, the ATP synthase that drives ATP synthesis at the expense of the electrochemical ion gradient is not only highly conserved but present in any archaeal species. Thus, it is the key enzyme in energy conservation not only in archaea but throughout all life forms (4).

Archaeal A₁A₉-ATP synthases are evolutionary more closely related to V₁V₉-ATPases found in organelles of eukarya, although these act as ATP-driven ion pumps and are therefore functionally different (4–7). All ATP synthases/ATPases evolved from a common ancestor and comprise two motors connected by a central stalk and peripheral stalk(s) (5). The membrane motor, A₉, is driven by downhill (inward) translocation of H⁺ or Na⁺ that leads to rotation of the c ring, made by multiple copies of the c subunit, against the stator subunit a. Rotational energy is transmitted via the central stalk to the soluble A₁ motor, that then drives the synthesis of ATP. The machine operates fully reversible and is also capable of ion transport driven by ATP hydrolysis (4).

In most A₁A₉-ATP synthases, subunit c has a single-hairpin topology as seen in F₁F₉-ATP synthases. In contrast, in eukaryotic V₁V₉-ATPases (8, 9), the c subunit underwent gene duplication, resulting in a protein with four transmembrane helices (10). Moreover, one ion-binding site was lost during the duplication event leading to a rotor with only half the number of ion-binding sites. These missing binding sites have been seen as the reason for the inability of V₁V₉-ATPases to act as ATP synthases (9). Instead, the rotor favors generation of large ion gradients, a function important for the cellular physiology of eukaryotes (5, 6).

The c subunit determines the ion specificity of the ATP synthases/ATPases. A few bacterial species use Na⁺ instead of H⁺ as coupling ion (11) and the Na⁺ binding site has been resolved in the high resolution x-ray structure of subunit c from F₁F₉-ATP synthases (12, 13). Some of these residues are highly conserved and build the conserved Na⁺ binding motif. This motif is also present in the c subunits from many A₁A₉-ATP synthases, among those are the methanogens and the pyrococci/thermo cocci (7) suggesting that they use Na⁺ as coupling ion. However, Na⁺ dependence was not observed for the enzyme from Methanosarcina mazei (14). Furthermore, A₁A₉-ATP synthase from Methanosarcina acetivorans has the same motif, but apparently translocates Na⁺ and H⁺ simultaneously (15). Thus
the presence of the motif is no evidence for the use of Na\(^+\) as coupling ion.

So far the identity of the coupling ion used by A\(_1\)A\(_O\)-ATP syntheses could only be addressed rather indirectly: by determining Na\(^+\) dependence of ATP hydrolysis, as shown for Pyrococcus furiosus (16), Methanobrevibacter ruminantium (17), and M. acetivorans (15), by the prevention of DCCD\(^2\) inhibition by Na\(^+\), as shown for P. furiosus (16, 18) and M. ruminantium (17), by showing Na\(^+\) dependence of ATP synthesis (17), or by determining ATP hydrolysis-driven \(^{22}\)Na\(^+\) transport in inverted membrane vesicles (15, 19). Direct proof would require an isolated enzyme reconstituted into liposomes. Although A\(_1\)A\(_O\)-ATP syntheses have been purified in the past, reconstitution into liposomes was not achieved for technical reasons. So far, intact A\(_1\)A\(_O\)-ATP syntheses could only be purified from hyperthermophilic archaeal species (16, 20, 21). These enzymes have a temperature optimum between 80 and 100 °C and are almost inactive at temperatures <80 °C. This would require a liposome system stable at temperatures >80 °C, but despite several efforts using different lipids this could, so far, not be obtained.

The recent discovery of a sodium ion current in the hyperthermophilic archaean Thermococcus onnurineus (19) prompted us to analyze the function of its A\(_1\)A\(_O\)-ATP synthase in more detail. ATP synthesis from formate oxidation could be detected in cell suspensions at 40 °C (3) suggesting a rather unusual temperature profile of the enzyme. Therefore, we purified the enzyme, which led to the discovery of a temperature profile enabling reconstitution of the enzyme into lipids from Escherichia coli. We describe here the first functional reconstitution of an A\(_1\)A\(_O\)-ATP synthase into liposomes and present direct proof for Na\(^+\) transport coupled to ATP hydrolysis.

**EXPERIMENTAL PROCEDURES**

Strain and Cultivation Condition—T. onnurineus NA1 (KCTCC 10859) was isolated from a deep-sea hydrothermal vent area in the PACMANUS field (22). The strain was cultivated anaerobically in a 100-liter fermentor at 80 °C in MM1 medium, which had the following composition (in grams/liter): yeast extract (1), sodium formate (10), NaCl (35), KCl (0.7), MgSO\(_4\) (3.9), CaCl\(_2\) × 2H\(_2\)O (0.4), NH\(_4\)Cl (0.3), Na\(_2\)HPO\(_4\) (0.15), Na\(_2\)SO\(_3\) (0.03), NaHCO\(_3\) (0.5), cystein × HCl (0.5), resazurin (0.001), as well as 1 ml/liter of Holdens trace elements/Fe-EDTA solution and 1 ml/liter of Balch’s vitamin solution (19). The medium was degassed with argon prior to inoculation. Cells were harvested and stored at –80 °C.

Membrane Preparation and Protein Determination—20 g of T. onnurineus cells (wet weight) were resuspended in buffer A (25 mM Tris, pH 7.5, 5 mM MgCl\(_2\) × 6H\(_2\)O, 0.1 mM PMSF) containing 0.1 mg of DNase I/ml. Cells were homogenized and disrupted by three passages through a French pressure cell (Aminco) at 1200 p.s.i. and a flow rate of 10 ml/min. Cell debris was removed by two centrifugation steps (Beckman Avanti J-25, JA 14 rotor, 7,500 and 7,900 rpm, each 20 min at 4 °C). Membranes were sedimented from the crude extract by centrifugation (Beckman Optima L90-K, 50.2 Ti rotor, 12,000 rpm, 16 h, 4 °C) and washed with buffer B (100 mM HEPES, pH 7.5, 5 mM MgCl\(_2\) × 6H\(_2\)O, 5% (v/v) glycerol, 100 mM NaCl, 0.1 mM PMSF) to remove peripheral membrane proteins. The washed membranes were collected by centrifugation (Beckman Optima L90-K, 50.2 Ti rotor, 16,000 rpm, 5 h, 4 °C), resuspended in buffer C (100 mM HEPES, pH 7.5, 5 mM MgCl\(_2\) × 6H\(_2\)O, 5% glycerol (v/v), 0.1 mM PMSF), and the protein concentration was determined as described (23).

**Purification of the A\(_1\)A\(_O\)-ATP Synthase from T. onnurineus**—Membranes that were washed and resuspended in buffer C were used for membrane protein solubilization. Oxidized Triton X-100 was added to a concentration of 3% (v/v) (1 g of Triton X-100, 1 g of membrane protein) and membranes were incubated under shaking for 2 h at 40 °C and then overnight at room temperature. Membranes were sedimented by ultracentrifugation (Beckman Optima L90-K, VTi50 rotor, 43,000 rpm, 4 °C) and contaminating proteins in the supernatant were precipitated with PEG 6000 (4.1%, w/w) for 30 min at 4 °C. Precipitated proteins were removed by centrifugation (Beckman Optima L90-K, VTi50 rotor, 38,000 rpm, 2 h, 4 °C) and the supernatant was loaded onto a sucrose gradient (20–66%) and centrifuged for 19 h in a vertical rotor (Beckman Optima L90-K, VTi50 rotor, 43,000 rpm, 4 °C). Afterward each sucrose gradient fraction was tested for ATP hydrolysis activity as described (16). The fractions of sucrose gradient with the highest ATPase activity were pooled and applied to anion exchange chromatography. Therefore DEAE-Sepharose CL-6B (GE Healthcare) was equilibrated with buffer D (50 mM Tris, pH 7.5, 5 mM MgCl\(_2\) × 6H\(_2\)O, 10% (v/v) glycerol, 0.05 mM PMSF, 0.1% (v/v) reduced Triton X-100). For elution, a salt gradient (0–1 m NaCl) in buffer D at a flow rate of 0.5 ml/min was used. Fractions with the highest ATPase activity were pooled again, concentrated (MWCO 100 kDa), and applied to a Superose 6 column (10/300 GL, GE Healthcare). Gel filtration was performed in buffer D at a flow rate of 0.2 ml/min. Again, fractions with the highest ATP hydrolysis activity were pooled.

**Gel Electrophoresis with the Purified A\(_1\)A\(_O\)-ATP Synthase from T. onnurineus**—To estimate the molecular mass of the whole native protein complex high resolution clear native electrophoresis was performed with the purified A\(_1\)A\(_O\)-ATP synthase according to standard protocols (24). 12.5% SDS-PAGE was performed to analyze the subunit composition of the A\(_1\)A\(_O\)-ATP synthase (25). To visualize proteins in native or SDS gels, staining with Coomassie Brilliant Blue (26) or with silver (27) was performed according to standard protocols.

**Biochemical Characterization of the A\(_1\)A\(_O\)-ATP Synthase from T. onnurineus**—The ATPase activity was measured in an assay mixture containing 100 mM Tris, 10 mM MgCl\(_2\) × 6H\(_2\)O, 60 mM NaHSO\(_4\) (pH 7.0), and enzyme solution. After incubation for 3 min at 80 °C, the reaction was started by the addition of 2.5 mM Na\(_2\)ATP. ATPase activity was determined by following the ATP-dependent formation of inorganic phosphate as described (28). To characterize the A\(_1\)A\(_O\)-ATP synthase of T. onnurineus biochemically, different parameters such as temperature (20–100 °C), pH (5.5–10), or compounds of the assay mixture as divalent cations (Mg\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\), Ca\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\), Fe\(^{2+}\), and Zn\(^{2+}\)), NaHSO\(_4\) (0–100 mM), and NTPs (ATP,
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CTP, GTP, UTP, and ITP) were varied. Also organic acids could have an effect on the ATPase activity as described before (29). To test this NaHSO₃ was substituted by acetate, carbonate, fumarate, maleate, or succinate (100 mM each). For determination of the pH optimum of the enzyme the assay mixture was modified to 100 mM Tris, 100 mM MES, 100 mM CHES, 10 mM MgCl₂ × 6H₂O, 60 mM NaHSO₃.

Isolation of Subunit c and DCCD Labeling Experiments—Subunit c of the A1A0-ATP synthase from T. onnurineus was isolated by chloroform/methanol (2:1, v/v) extraction using the purified enzyme. Therefore, 9 μg of purified A1A0-ATP synthase in buffer D (pH 6) was bound to the C₄ matrix (bed volume, 0.6 μl) of a 10-μl Zip Tip to remove salts, which could interfere with the mass spectrometry measurements, as described earlier (18). A1A0-ATP synthase was then eluted from the C₄ matrix with 10 μl of 90% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid. The eluted protein was mixed with 10 μl of chloroform/methanol (2:1, v/v) to disintegrate the ATP synthase and the ring of T. onnurineus into c monomers. The solution containing c monomers was dried by vacuum evaporation for 1.5 h at room temperature. The dried protein pellet was mixed with 1 μl of 2,5-dihydroxyacetophenone matrix and applied to MALDI-TOF-MS as described below. For labeling experiments with N,N'-dicyclohexylcarbodiimide (DCCD, dissolved in ethanol) also purified A1A0-ATP synthase from T. onnurineus was used. Therefore 9 μg of protein was incubated with 250 μM DCCD at pH 6.0 for 60 min at room temperature. For competition experiments between DCCD and NaCl or KCl the salts were added in concentrations of 1.5, 2.5, 5, 10, or 25 mM to the ATP synthase solution directly before labeling. After labeling with DCCD, ATP synthase was purified using C₄ Zip Tips to remove excessive DCCD and salts, as described before. Again, disintegration of the ATP synthase and the ring into c monomers was performed with chloroform/methanol extraction as described prior and the molecular mass of the labeled c subunits was measured with MALDI-TOF-MS.

MALDI-TOF-MS Measurements—For protein molecular mass determination, chloroform/methanol extracts were mixed in a 1:1 (v/v) ratio with 2,5-dihydroxyacetophenone (15 mg/ml of 2,5-dihydroxyacetophenone in 75% ethanol in 20 mM sodium citrate, Bruker Daltonics) and were spotted on a ground steel target plate (Bruker Daltonics). Then MALDI mass spectra were recorded in the mass range of 5–20 kDa using a Bruker Autoflex III Smartbeam mass spectrometer. Detection was optimized for m/z values between 5 and 20 kDa and calibrated using calibration standards (protein molecular mass calibration standard 1, Bruker Daltonics).

Protein Identification Using Mass Spectrometry (Peptide Mass Fingerprinting)—For protein identification purified A1A0-ATP synthase from T. onnurineus was submitted to 12.5% SDS-PAGE (25) and stained with silver, suitable for mass spectrometry (30). Bands of interest were excised, reduced, alkylated, and digested using trypsin, chymotrypsin, or both proteases according to standard mass spectrometry protocols (31). Proteolytic digests were applied to reverse phase columns using a nano-HPLC (Proxeon easy-nLC), eluted in gradients of water and acetonitrile according to Ref. 18. Eluted peptides were ionized using a Bruker Apollo ESI source with a nanoSprayer emitter and analyzed in a quadrupole time-of-flight mass spectrometer (Bruker maxis). Proteins were identified by matching the mass lists on a Mascot server (version 2.2.2, Matrix Science, United Kingdom) against the NCBI nr database.

Preparation of Liposomes from E. coli Total Lipid Extract—Total lipid extract (0.5 g) from E. coli solved in chloroform was mixed drop by drop with 75 ml of cold acetone. The mixture was gassed with N₂ to prevent oxidation of the lipids and stirred overnight at 4 °C. The mixture was centrifuged (JA-25.50 rotor, 6,000 rpm, 10 min, 4 °C) and the pellet was dried with a N₂ stream. Afterward the dried pellet was solved in 75 ml of diethyl ether and centrifuged (JA-25.50 rotor, 6,000 rpm, 10 min, 4 °C) again. The supernatant was transferred to a round bottom flask and the solvent was removed using a rotary evaporator. The lipid film was solved in chloroform, evaporated, washed with 2 ml of ethanol, and evaporated again using the rotary evaporator. The dried lipid film was solved in 50 mM potassium phosphate buffer (pH 7) to a concentration of 20 mg/ml and aliquoted to 1 ml in cryo tubes. Finally, liposomes were formed by three freeze and thaw cycles in liquid nitrogen.

Reconstitution of the A1A0-ATP Synthase from T. onnurineus into E. coli Liposomes—Liposomes (diameter of 400 nm) were formed by extrusion with a “LipoFast” Extruder and a polycarbonate filter (diameter 19 mm, Avestin Europe GmbH, Mannheim). Then, the liposomes were diluted to a concentration of 4 mg/ml with buffer E (100 mM Tris, 10 mM MgCl₂ × 6H₂O, 40 mM KH₂PO₄, 5 mM NaCl, pH 7). The liposomes were destabilized with oxidized Triton X-100 until an A₅₄₀ between 0.3 and 0.4 was achieved. At that point the liposomes are 50% solubilized and 50% saturated. Purified A1A0-ATP synthase was added to the destabilized liposomes in a protein to lipid ratio of 1:100 (w/w). The reconstitution mixture was incubated for 30 min at room temperature under gently shaking. The detergent was removed using Bio-Beads (Bio-Rad Laboratories). Bio-Beads were added (25 mg/ml) followed by an incubation at room temperature for 30 min. With the addition of more Bio-Beads the Bio-Beads concentration was increased to 40 mg/ml and the mixture was incubated under gentle movement for 1 h at 4 °C. Then again the Bio-Beads concentration was increased to 59 mg/ml and the mixture was incubated for 12 h at 4 °C. Afterward a final amount of Bio-Beads was added to increase to 88 mg/ml and the mixture was incubated for 2 h at 4 °C. Subsequently the Bio-Beads were removed by filtering the mixture through a polypropylene column (35 ml) with a polyethylene filter (Machery-Nagel, Düren). The flow-through was centrifuged (50.2 Ti, 50,000 rpm, 45 min, 4 °C) to sediment the proteoliposomes. Then the proteoliposomes were resuspended in and washed with buffer E. Again proteoliposomes were sedimented by ultracentrifugation as mentioned before, finally resuspended in 1 ml of buffer E, and were used for the ²²Na⁺ translocation experiments.

Measurement of ²²Na⁺ Translocation—Measurements of the ²²Na⁺ translocation coupled to ATP hydrolysis were performed in buffer E (100 mM Tris, 10 mM MgCl₂ × 6H₂O, 40 mM KH₂PO₄, 5 mM NaCl, pH 7) at 45 °C in a water bath. Ionophores ETH 2120 and SF 6847 as well as ATPase inhibitors DCCD and diethylstilbestrol (DES) were added from ethanolic stock solutions. The protein and sodium ion concentrations used as well
as inhibitor and ionophore concentrations are as indicated. In a 3.5-ml glass vial, the proteoliposomes (150 µl, protein concentration, 0.15 mg/ml), buffer E (850 µl), and if necessary, supplements (500 µM DCCD, 750 µM DES, 40 µM ETH 2120, or 40 µM SF 6847) were mixed and incubated for 20 min at room temperature. Afterward 8 µl of 22NaCl (carrier-free, final activity 0.5 µCi/ml) was added and incubated for 30 min at room temperature to ensure equilibration of 22Na+ (concentration of NaCl was 5 mM). Next, the mixture was tempered at 45 °C (the temperature where the A1AO-ATP synthase still showed ATP hydrolysis activity) and finally the reaction was started with 5 mM K2ATP. 80-µl samples were taken and passed over a column (0.5 × 3.2 cm) of Dowex 50-WX8 (100–200 mesh) according to standard protocols (32). The proteoliposomes were collected by washing the column with 1 ml of 420 mM sucrose. Subsequently, the radioactivity of the elution fractions was determined using liquid scintillation counting.

RESULTS

Purification of the A1AO-ATP Synthase from T. onnurineus—Proteins from membranes of T. onnurineus grown on MM1 medium at 80 °C were solubilized with oxidized Triton X-100, separated from non-solubilized membranes by ultracentrifugation, and precipitated by PEG 6000. At 4.1% (w/w) PEG 6000 containing proteins were precipitated, whereas the A1AO-ATP synthase stayed in the supernatant. Subsequent purification by density gradient centrifugation, anion exchange chromatography, and gel filtration resulted in a 7.7-fold enrichment of the activity to a specific activity at around 40 units/mg of protein. This is by far the highest activity ever observed for an A1AO-ATP synthase.

When analyzed on a native gel, one major protein complex with an apparent molecular mass of around 670 kDa was visible (Fig. 1A). The preparation contained proteins of apparent molecular masses of 66, 55, 40, 27, 23, 13, and 11 kDa that correspond to subunits A, B, C, D, E, F, and H, as determined by peptide mass fingerprinting (Fig. 1B). Interestingly, as seen before for the enzyme from P. furiosus (20), the preparation contained a SDS-resistant ac subcomplex of an apparent molecular mass of 65 kDa.

Basic Biochemical Properties of the A1AO-ATP Synthase from T. onnurineus—In contrast to many other ATP synthases, the enzyme hydrolyzed ATP, CTP, GTP, UTP, and ITP with nearly identical activities of around 40 units/mg of protein. The enzyme required divalent cations for activity, the highest stimulation was with Mn2+ followed by Mg2+ and Co2+ (10 mM each, added as chloride salt) (Fig. 2A). Almost no ATPase activity was observed in the presence of CuCl2 or NiCl2. This effect could be due to the formation of disulfide bridges between cysteines in subunit A. T. onnurineus has two cysteins in the catalytic A subunit and it was already shown before for A1AO-ATP synthases and V1V0-ATPases that Cu2+ lead to the formation of disulfide bridges in subunit A and to an inhibition of the ATPase activity (33–37). Furthermore, the enzyme was stimulated by NaHSO3 (Fig. 2B) that could not be substituted by acetate, carbonate, formate, fumarate, maleate, or succinate. The enzyme was active over a wide pH range with maximal activity at pH 7 (Fig. 2C). Noteworthy was also the temperature profile. In contrast to the enzyme from P. furiosus, the enzyme from Thermococcus had a clear optimum at 80 °C (not 100 °C as for P. furiosus). Moreover, whereas the P. furiosus enzyme is nearly inactive at 45 °C, the T. onnurineus enzyme still retained an activity of 2.5 units/mg of protein (Fig. 2D) under optimized buffer conditions (100 mM Tris, 10 mM MgCl2 × 6H2O, 60 mM NaHSO3, pH 7.0).

DCCD and Na+ Compete for Binding to Subunit c—Inspection of the amino acid sequence of subunit c as deduced from the DNA data suggest a 16-kDa c subunit with four transmembrane helices (Fig. 3). The similarity/identity to subunit c of P. furiosus is 91/79%. Recently, we have demonstrated that the c subunit from P. furiosus indeed has four transmembrane helices but only one ion binding site (18). Na+ is bound via the conserved Na+ binding motif Q...E T that is also conserved in T. onnurineus with Glu (in helix 3), Glu (in helix 4), and Thr (in helix 2). In contrast, there is neither a second proton nor a second Na+ -binding motif conserved in T. onnurineus (Val in helix 1, Met in helix 2, and Thr in helix 4). To analyze the molecular mass of the mature protein it was extracted from the purified enzyme by chloroform/methanol and subjected to MALDI-TOF-MS. As expected from the DNA sequence, the mature protein has a molecular mass of 16,061 Da corresponding to a c subunit with four transmembrane helices (Fig. 4A).

DCCD is a common inhibitor for ATP synthases/ATPases and known to bind to the protonated carboxylate mediating proton transport or involved in Na+ binding (Glu in helix 4). In Na+ -ATP synthases, Na+ blocks access of DCCD to this carboxylate thus rendering the enzyme insensitive to DCCD in the presence of Na+ (16–18, 38, 39). DCCD inhibited the A1AO-ATP synthase from T. onnurineus with half-maximal inhibition at 100 µM DCCD. When the enzyme was incubated with DCCD prior to extraction and MALDI-TOF-MS analyses, an addi-
tional molecular mass of 16,269 was observed (Fig. 4B) that corresponds to one molecule of DCCD bound to one c subunit. This experiment is not only consistent with only one ion (Na\(^+\)/H\(^+\)) binding site per c subunit, but also proves covalent binding of DCCD to subunit c. The enzyme was then preincubated with increasing concentrations of Na\(^+\)/H\(^+\), whereas the DCCD concentration stayed constant. As seen in Fig. 5, the presence of Na\(^+\) prevented binding of DCCD to the A\(_1\)A\(_0\)-ATP synthase in a concentration-dependent manner. This is consistent with Na\(^+\) being the coupling ion of the A\(_1\)A\(_0\)-ATP synthase from T. onnurineus.

**Functional Reconstitution of the A\(_1\)A\(_0\)-ATP Synthase in Proteoliposomes and Direct Demonstration of Na\(^+\) Transport—**The surprising result that the A\(_1\)A\(_0\)-ATP synthase from a hyperthermophile is still active at a low temperature (45 °C) prompted us.
to reconstitute the enzyme into liposomes. Therefore we used the total lipid extract from *E. coli* and formed liposomes by three freeze and thaw cycles (40–42). The *E. coli* liposomes, which had a defined diameter of 400 nm, were reconstituted with purified *A1AO-ATP synthase* from *T. onnurineus* in a protein to lipid ratio of 1:100. The proteoliposomes were incubated at 45 °C in the presence of 5 mM Na^+ and the final activity of

**FIGURE 4.** DCCD labeling of subunit c from *T. onnurineus*. Purified *A1AO-ATP synthase* (9 μg of protein in 20 μl) from *T. onnurineus* was incubated with 250 μM DCCD at pH 6 for 60 min at room temperature. Subunit c was extracted by chloroform/methanol from unlabeled (A) and DCCD-labeled (B) ATP synthase/ATPase. The molecular mass of both c subunits was determined by MALDI-TOF-MS.

**FIGURE 5.** DCCD labeling of subunit c of the *A1AO-ATP synthase* from *T. onnurineus* is prevented by the presence of Na^+. The purified *A1AO-ATP synthase* (9 μg of protein in 20 μl) of *T. onnurineus* was incubated with different concentrations of NaCl or KCl and labeled with 250 μM DCCD at room temperature and at pH 6 for 60 min. Then subunit c was extracted by chloroform/methanol and the molecular mass was determined by MALDI-TOF-MS.

**FIGURE 6.** 22Na^+ transport coupled to ATP hydrolysis catalyzed by the *A1AO-ATP synthase*. Proteoliposomes containing the reconstituted *A1AO-ATP synthase* from *T. onnurineus* (protein concentration 0.15 mg/ml) in Tris buffer (pH 7) with 10 mM MgCl₂, 5 mM NaCl, and 40 mM KHSO₃ showed 22Na^+ transport upon the addition of 5 mM K₂ATP (◼). A control did not receive ATP (▲). ATP hydrolysis in the reconstituted system was 1.5 units/mg of protein.

22Na^+ was 0.5 μCi/ml. Upon addition of K₂ATP, 22Na^+ was indeed transported into the lumen of the proteoliposomes due to ATP hydrolysis (Fig. 6). ATP hydrolysis in the reconstituted system was 1.5 units/mg of protein. A control without ATP addition showed no Na^+ translocation. In a further experiment the proteoliposomes were preincubated with DCCD or DES, known inhibitors of archaeal ATP synthases (16, 34). The ATP synthase from *T. onnurineus* had its highest activity at pH 7. Therefore, DCCD inhibition was tested at pH 7. As mentioned,
DCCD only binds to the protonated carboxylate and, therefore, 500 μM DCCD inhibited ATP hydrolysis of the purified enzyme only to 30% at that pH. Furthermore, when incubating the proteoliposomes at the same pH with the same amount of DCCD an inhibition of Na\(^+\)/H\(^+\) translocation of 30% is also seen (Fig. 7A).

Diethylstilbestrol is another specific inhibitor of A\(_1\)AO-ATP synthases that acts on the A\(_1\) domain and inhibition is thus not prevented by Na\(^+\)/H\(^+\) or is not pH-dependent. The A\(_1\)AO-ATP synthase from *T. onnurineus* was inhibited by 750 μM DES almost completely. Correspondingly, ATP-driven Na\(^+\) transport in proteoliposomes was also inhibited completely (Fig. 7B). Transport of Na\(^+\) was slightly stimulated by the protonophore SF 6847, indicating that Na\(^+\) transport is electrogenic, but completely inhibited by the Na\(^+\) ionophore ETH 2120 (Fig. 8A). Moreover, addition of ETH 2120 to the assay during active transport of Na\(^+\) immediately stopped further Na\(^+\) translocation into the lumen of the proteoliposomes (Fig. 8B). These data clearly demonstrate primary Na\(^+\) transport coupled to ATP hydrolysis catalyzed by the A\(_1\)AO-ATP synthase of *T. onnurineus*.

**DISCUSSION**

Many c subunits of A\(_1\)AO-ATP synthases contain the Na\(^+\) binding motif found in c subunits of F\(_{1}\)F\(_{0}\)-ATP synthases. Whether or not the presence of this motif goes along with sodium ion transport by the A\(_1\)AO-ATP synthases remains to be established for each enzyme. So far, three strategies have been followed to experimentally address the ion specificity: first, by analyzing whether Na\(^+\) stimulates ATP hydrolysis (16). This proved to be the case for the A\(_1\)AO-ATP synthases from *P. furiosus* (16) and *M. ruminantium* (17). However, if negative, these experiments can be difficult to interpret because the contaminating amounts of Na\(^+\) in the buffer may already be sufficient. A second strategy is to use the known competition of Na\(^+\) and the inhibitor DCCD for their common binding site: DCCD does not bind and thus does not inhibit in the presence of Na\(^+\) (16–18). But again, if negative, contaminating amounts of Na\(^+\) in the buffer may already be sufficient. Therefore, the third and final strategy would be to directly measure ion transport coupled to ATP hydrolysis. This was always hampered by the fact that the A\(_1\)AO-ATP synthase that could be isolated so far had temperature optima >80 °C. Liposomes withstand ing this high temperature are not available yet, despite many efforts. Therefore, the unusual temperature profile and outstanding high activity of the A\(_1\)AO-ATP synthase from *T. onnurineus* allowed A\(_1\)AO-ATP synthase to overcome
the hurdle that existed for decades. The enzyme is still more active at 45°C and the liposomes prepared from E. coli were stable as well at this temperature. This allowed the first functional reconstitution of an A1A0-ATP synthase into liposomes. Moreover, the studies clearly demonstrated Na\(^+\) transport driven by ATP hydrolysis, and H\(^+\) transport could not be obtained. Although the lipids found in Thermococcus or archaea in general are quite different from the ones found in bacteria (43), the enzyme is as active in E. coli lipids as in detergents or as in the membrane of its native host.

The A1A0-ATP synthase from T. onnurineus apparently contains the conserved Na\(^+\) binding motif (Q...ET) in subunit c and was shown here experimentally to pump Na\(^+\). However, it is important to note and keep in mind that the presence of the Na\(^+\) binding motif of subunit c of F\(_{1}\)F\(_{0}\)-ATP synthases and subunit c of A1A0-ATP synthases is of course no proof that the enzyme uses Na\(^+\). The A1A0-ATP synthase from M. acetivorans has the conserved motif (E...ET) but apparently uses Na\(^+\) and H\(^+\) simultaneously (15). The c subunit of the enzyme from M. mazei also has the motif (E...ET), but, so far, sodium ion dependence or transport could not be detected (14). Apparently, more than this motif is required to determine ion selectivity. For example, earlier work on the ion specificity of the Na\(^+\) F\(_{1}\)F\(_{0}\)-ATP synthase of Propionigenium modestum revealed mutations in subunit a that influenced ion specificity (44). Because the overall sequence identity of subunits a from F\(_{1}\)F\(_{0}\)- and A1A0-ATP synthases is low, one cannot speculate about the role of these residues in ion transport.

What could be the advantage of using Na\(^+\)? Many archaea live at the thermodynamic limit of life (2). Methanogenesis from H\(_{2}\) + CO\(_{2}\) (\(\Delta G_0^* = -131\) kJ/mol) or acetate (\(\Delta G_0^* = -36\) kJ/mol) is close to what is considered to be the minimum biological energy quantum (\(\Delta G_0^* \approx -20\) kJ/mol) (45). Values of \(-20\) kJ/mol exclude substrate level phosphorylation and only allow for chemiosmotic energy conservation. A value of \(-20\) kJ/mol was considered minimal (46) because this amount of energy is required to pump at least one ion across the cytoplasmic membrane. That this value can be even smaller was discovered recently (3, 47). Formate-dependent hydrogen production under anaerobic conditions as catalyzed by T. onnurineus goes along with a \(\Delta G \approx -10\) kJ/mol and it was hypothesized that this amount of energy is sufficient for ion translocation by the concerted action of a proton-translocating membrane-bound hydrogenase in concert with a Na\(^+\)/H\(^+\) antiporter (19). If both ion extruders work with different stoichiometries, any value below \(-20\) kJ/mol would be sufficient to energize the membrane.

Life at the thermodynamic limit requires specific adaptations of the energy metabolism. This is even more important at temperatures close to the boiling point of water. Membranes have to be tight enough to hold an electrochemical ion potential and, in general, membranes can maintain a sodium ion gradient more efficiently than a proton gradient (48). At higher temperatures membranes are much leakier for protons than for sodium ions (49). This may be the reason that T. onnurineus or methanogens like Methanocaldococcus jannaschii exclusively use a sodium ion bioenergetics (2, 17, 18). This may be true for P. furiosus as well (16, 19). In the latter, oxidation of formate to CO\(_{2}\) and H\(_{2}\) is coupled to the generation of an electrochemical sodium ion gradient across the membrane that then drives the synthesis of ATP (19).

A second reason for Na\(^+\) being superior over H\(^+\) may be the resistant to protonophores such as weak acids. All organisms described so far rely on a Na\(^+\) current for ATP synthesis are anaerobic and most are energy-limited. They live in ecosystems that contain fermentation end products such as formate, acetate, or others. These are able to cross the cytoplasmic membrane in their protonated form by diffusion as thus act as “proton ferries” (protonophores). This “uncoupling” in energy-limited environments by weak organic acids may have also have selected for primary Na\(^+\) bioenergetics.

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