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Δψ and ΔpH are equivalent driving forces for proton transport through isolated F_0 complexes of ATP synthases

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ABSTRACT

The membrane-embedded F_0 part of ATP synthases is responsible for ion translocation during ATP synthesis and hydrolysis. Here, we describe an *in vitro* system for measuring proton fluxes through F_0 complexes by fluorescence changes of the entrapped fluorophore pyranine. Starting from purified enzyme, the F_0 part was incorporated unidirectionally into phospholipid vesicles. This allowed analysis of proton transport in either synthesis or hydrolysis direction with Δψ or ΔpH as driving forces. The system displayed a high signal-to-noise ratio and can be accurately quantified. In contrast to ATP synthesis in the *Escherichia coli* F_1F_0 holoenzyme, no significant difference was observed in the efficiency of ΔpH or Δψ as driving forces for H^+-transport through F_0. Transport rates showed linear dependency on the driving force. Proton transport in hydrolysis direction was about 2400 $H^+/(s\times F_0)$ at Δψ of 120 mV, which is approximately twice as fast as in synthesis direction. The chloroplast enzyme was faster and catalyzed H^+-transport at initial rates of 6300 $H^+/(s\times F_0)$ under similar conditions. The new method is an ideal tool for detailed kinetic investigations of the ion transport mechanism of ATP synthases from various organisms.

1. Introduction

F_1F_0 ATP synthases are complex molecular machines that reside in the inner membrane of mitochondria, the thylakoid membrane of chloroplasts or the cytoplasmic membrane of bacteria. They catalyze the synthesis of ATP from ADP and P_i with the energy stored in an electrochemical ion gradient across the membrane [1].

In fermenting bacteria, where respiratory enzymes are not active ATP synthases can work in reverse as ATPases to generate the membrane potential (Δψ) required for cell viability. Hence, both working modes of F_1F_0 ATP synthases are physiologically relevant.

The construction of the F_1F_0 ATP synthases is bipartite: The F_1 part with the subunits $\alpha_3\beta_3\gamma\delta\varepsilon$ harbors the catalytic sites for ATP synthesis and hydrolysis and protrudes into the aqueous compartment of the cell. The F_0 part in its simplest bacterial form consists of subunits ab_2c_{10-15}. It is membrane-embedded and catalyzes ion transport across the membrane. The number of c monomers in the oligomeric c-ring varies between species and concomitantly affects the ion/ATP ratio during ATP synthesis or hydrolysis [2].

ATP synthesis measurements with the purified enzymes of *Escherichia coli* and spinach chloroplasts have shown that electric potential and proton gradient are not equivalent driving forces [3]. It is unknown, however, if these discrepancies are F_0 intrinsic properties or if they are related to the holoenzyme.

To understand the principle of proton pumping in either direction and to investigate asymmetries between the synthesis and hydrolysis mode of F_1F_0 ATP synthases, a defined system is needed, in which unidirectional proton transport can be measured. In this work we developed a method to measure unidirectional proton transport through the F_0 part in synthesis and hydrolysis direction, energized selectively by either a pH difference (ΔpH) or an electric potential difference (Δψ) across the liposome membrane. The assay is based on the hydrophilic fluorophore pyranine which is entrapped inside proteoliposomes and allows proton transport to be quantified by monitoring fluorescence changes. The relative simplicity of the system accounts for a high reproducibility and straightforward determination of all parameters required for a quantification of H^+ transport rates. It is moreover generally applicable to ATP synthases from various origins and displays several advantages over described procedures.

2. Material and methods

Antibody against subunit b was a generous gift of Karl-Heinz Altendorf, University of Osnabrück, Germany. Chemicals were purchased from Fluka (Buchs, Switzerland) if not otherwise indicated.

Abbreviations: DCCD, dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TBT, tributlytin

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2.1. Enzyme purification and reconstitution into liposomes

For expression of the *E. coli* F_1F_0 ATPase plasmid pBWU13 [4] encoding the entire *atp* operon and an N-terminal His_{10}-tag on the β-subunit was used (ATGCACCATCACCATCACCATCACCATCACCAT was introduced by PCR in front of the starting ATG). Mutant plasmid containing the cAsp61Asn was generated by PCR using overlapping primers. The presence of the mutant codons was confirmed by automated sequencing of the cloned DNA at Microsynth AG (Balgach, Switzerland).

The plasmid was transformed and expressed in *E. coli* strain DK8 which lacks the entire ATP operon (ΔuncBEFHAGDC) [5]. Cells were grown overnight at 30 °C in LB medium supplemented with 100 μg/l ampicillin and 20 μg/l tetracyclin.

F_1F_0 ATPase was isolated and purified following a slightly modified procedure of Ishmukhametov et al. [6]. Briefly, 5 g cells were resuspended in 20 ml lysis buffer (200 mM Tris–Cl pH 7.8, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM K_2-EDTA and 2.5% glycerol) and subsequently passed twice through a French press cell at 100 MPa. Unbroken cells were removed by centrifugation for 5 min at 8000 *g*. Membranes were collected by centrifugation of the supernatant for 30 min at 200,000 *g*.

For solubilization, membranes were suspended in 10 ml extraction buffer (50 mM Tris–Cl pH 7.5, 100 mM KCl, 250 mM sucrose, 40 mM ε-aminocaproic acid, 15 mM *p*-aminobenzamidine, 5 mM $MgCl_2$, 0.1 mM K_2-EDTA, 0.2 mM DTT, 0.8% phosphatidylcholine, 1.5% octylglucopyranoside, 0.5% sodium deoxycholate, 0.5% sodium cholate, 2.5% glycerol, 30 mM imidazole) and gently stirred for 1 h at 4 °C. Unsolubilized material was removed by centrifugation for 45 min at 200,000 *g* and the supernatant passed through a 0.22 μm filter (Millipore AG, Zug, Switzerland). The filtrate was loaded on a Ni-IDA column (GE Healthcare, Glattbrugg, Switzerland) and washed with 10 column volumes of extraction buffer. The F_1F_0 ATPase was eluted with extraction buffer containing 400 mM imidazole. The elution fractions were analyzed on SDS-PAGE and stored in liquid nitrogen. Routinely, 5–10 mg purified ATP synthase was obtained per liter of culture.

Chloroplast F_1F_0 ATP synthase was isolated from spinach leaves as described [7]. Briefly, 1.5 to 2 kg of spinach leaves was homogenized in a sucrose-containing buffer with a blender. Subsequent filtration and centrifugation steps were performed to obtain a thylakoid membrane preparation (chlorophyll concentration of 5 mg/ml) and the samples were stored at −20 °C.

The ATP synthase was solubilized from the membranes, fractionated by ammonium sulphate precipitation and subsequent sucrose density gradient centrifugation as described [7]. The purified protein samples (5 mg protein/ml) were stored in liquid nitrogen.

2.2. Purification of *E. coli* F_1 complex

E. coli F_1 complex was purified as described previously [8].

2.3. Reconstitution of F_1F_0 ATP synthase from *E. coli* and chloroplasts

The F_1F_0 ATPase from *E. coli* and chloroplasts was reconstituted following a slightly modified procedure as described [6]. Soybean phosphatidylcholine (Sigma-Aldrich, Buchs, Switzerland) was dissolved at a concentration of 30 mg/ml in buffer A (10 mM Tricine–NaOH pH 8.0, 2.5 mM $MgCl_2$, 0.1 mM Na_2-EDTA, 0.2 mM DTT) and sonicated at 7.5 μ for 2×30 s on ice using a tip sonicator (Sanyo MSE Soniprep, München, Germany) to form unilamellar liposomes.

The suspension was adjusted to 1% sodium cholate from a 10% stock solution and mixed with F_1F_0 ATPase (lipid:protein ratio (w/w) 1:100). The mixture was incubated for 20 min on ice and subsequently 1 ml was loaded on a PD-10 column (GE Healthcare, Glattbrugg, Switzerland), preequilibrated with buffer A. Turbid fractions were pooled and the proteoliposomes collected by centrifugation for 45 min at 200,000 *g* at 4 °C.

2.4. Preparation of F_0 liposomes

F_1F_0 proteoliposomes prepared as described above were dialyzed overnight against 1000 volumes of stripping buffer (0.5 mM Tricine pH 8.5, 0.5 mM Na_2-EDTA) at 4 °C to remove the F_1 part. The sample was diluted with stripping buffer and F_0 liposomes were collected by centrifugation for 45 min at 200,000 *g* at 4 °C.

The liposomes were resuspended at 60 mg/ml in buffer B (2 mM MOPS–NaOH pH 7.2, 2.5 mM $MgCl_2$, 50 mM Na_2SO_4 or 50 mM K_2SO_4) including 1 mM pyranine and frozen in liquid nitrogen for 5 min, thawed in cold water and sonicated twice for 10 s in a water bath sonicator. The freeze/thaw/sonication procedure was repeated once.

To remove external pyranine, the liposomes were loaded on a PD-10 column equilibrated with buffer B containing either Na_2SO_4 or K_2SO_4. The turbid yellowish fraction was collected and concentrated by centrifugation (30 min at 200,000 *g* at 4 °C). The liposomes were resuspended at a lipid concentration of 120 mg/ml in the same buffer and stored at 4 °C. No loss in activity was observed within 7 days. Depending on the inner salt, they were denoted Na^+- or K^+-liposomes.

For equilibration at the desired pH, liposomes were diluted 20-fold in the respective buffer and incubated for 16–72 h at 4 °C.

2.5. Measurement of ATP hydrolysis activity

ATP hydrolysis measurements were performed using a coupled enzyme assay as described [9] with the following modifications. Instead of potassium phosphate, 50 mM Tris–Cl, pH 7.5 was used and Triton X-100 was omitted in the experiments with intact proteoliposomes.

2.6. Determination of buffer capacity of liposomes

The buffer capacity of the lipid headgroups was determined as described [10] with the following modification. Liposomes containing Na^+ or K^+ were prepared as described above in a buffer containing 50 mM Na_2SO_4, 2.5 mM $MgCl_2$ and 50 mM K_2SO_4, 2.5 mM $MgCl_2$, respectively, diluted to 10 mg lipid/ml and adjusted to pH 6 with 5 mM H_2SO_4. Aliquots of a 10 mM KOH solution were added and the pH change was recorded with a glass electrode. The buffering capacity at a certain pH value was calculated using the linear regression derived from three values in proximity of the desired pH (to obtain the amount of KOH used to change the pH value by one unit) and the weight of lipid used and was expressed as μM H^+/(g lipid × pH unit).

2.7. Proportion of empty and F_0-containing liposomes

The fraction of empty liposomes was determined as described [11]. Briefly, $\Delta\psi$-driven H^+-transport through F_0 was initiated by the addition of 8 nM valinomycin and followed until completion of the reaction. Then, 2 μl of a 2 mM stock solution of CCCP was added to allow H^+-transport into empty liposomes. The raw data were then corrected for the external pyranine and converted into pH traces as described in the manuscript. The pH change observed before CCCP addition (F_0 liposomes) was divided by the total pH change (F_0 and empty liposomes) to obtain the fraction of liposomes containing an F_0 part as lined out in Fig. 1D.

2.8. Fluorescence measurements

Pyranine fluorescence was measured with a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, USA). The excitation wavelengths were set to 405 and 460 nm and their emission at 510 nm was recorded with 2 Hz. The slits were set to 20 nm and the photomultiplier voltage was set to 550 V. During measurements, the fluorescence spectrophotometer can remain open, which facilitates *in situ* addition of chemicals.