Electronic Supplementary Information

Monitoring ATPase induced pH changes in single proteoliposomes with the lipid-coupled fluorophore Oregon Green 488

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1. Expression and purification of TF O F 1 -ATPase

TF O F 1 -ATPase was expressed in E. coli DK8 harboring the plasmid pTR19-ASDS, encoding for the ATPase of the thermophilic Bacillus sp. PS3.1 Expression and purification was carried according to the protocol of Schenck and coworkers with some minor changes.2 Briefly, the cells were grown in 2 L terrific broth medium with 100 µg/mL ampicillin at 37 °C to an OD 600 of 1.7. After centrifugation, the cell pellets were resuspended in 50 mM Tris, 0.5 mM EDTA, 1 mg/mL lysozyme, pH 8.0 and incubated for 30 min at 37 °C. MgCl 2 (5 mM) and DNase I buffer (1:1000 (v/v), 5 mg/mL DNase I in 50 % glycerol (w/v), 20 mM Tris, 1 mM MgCl 2, pH 7.5) was added and stirred for 30 min at room temperature. Subsequently, the suspension was pumped three times through a microfluidizer (Microfluidizer LM10, Microfluidics, Westwood, MA, USA). 250 mM Na 2 SO 4 and 0.7 % sodium cholate (w/v) were added and after stirring for 20 min at room temperature, the sample was centrifuged at 20,000 × g at 4 °C for 30 min. The supernatant was discarded and the pellet was resuspended in 20 mM imidazole, 100 mM KCl, 5 mM MgCl 2 and 1 % n-dodecyl-β-D-maltoside (DDM) (w/v), pH 7.6 and stirred for 45 min at room temperature. The supernatant resulting from centrifugation at 20,000 × g at 4 °C (30 min) was incubated with TALON metal affinity resin (Clontech, Takara Bio Europe, Saint-Germain-en-Laye, France) for 2 h at 4 °C. After washing with 10 column volumes of 20 mM imidazole, 100 mM KCl, 5 mM MgCl 2, 0.08 % DDM (w/v), pH 7.6, the protein was eluted with 250 mM imidazole, 50 mM KCl, 5 mM MgCl 2, 0.05% DDM (w/v). After dialysis against 20 mM HEPES, 20 mM NaCl, 5 mM MgCl 2, pH 7.5, the sample’s concentration was increased by performing an anion exchange chromatography (MonoQ 5/50, ÄKTA Purifier 10, GE Healthcare Europe, Freiburg, Germany) starting with 20 mM
HEPES, 20 mM NaCl, 5 mM MgCl₂, 0.05 % DDM (w/v), pH 7.5 and increasing the NaCl concentration to 1 M. The protein concentration was determined with the Pierce BCA protein assay kit. The ATPase was stored at 4 °C for several weeks. The elution profile of the anion exchange chromatography is shown in Fig. S1 and the SDS-PAGE gel of the peak fractions is shown in Fig. S2.

**Fig. S1** Elution profile of TFoF₁-ATPase (Abs. 280 nm) and the conductivity of the NaCl-gradient, ranging from 20 mM to 1.0 M. The anion exchange chromatography (Mono Q 5/50 column, ÄKTA Purifier, GE Healthcare Life Science) increased the protein concentration to 2.2 µM in a volume of 1.2 mL. The course of the conductance indicates an elution at 30 mS/cm corresponding to a fraction of 30 % buffer B (314 mM NaCl). Buffer A: 20 mM NaCl, 20 mM HEPES, 5 mM MgCl₂, 1 mM DDM, pH 7.5, buffer B: 1 M NaCl, 20 mM HEPES, 5 mM MgCl₂, 1 mM DDM, pH 7.5.

**Fig. S2** (A) SDS-PAGE gel showing the bands of the eight single units of the TFoF₁-ATPase in the elution fractions F9 to F13 of the anion exchange chromatography (Veletion = 6-7.2 mL). (B) Assignment of the TFoF₁-ATPase subunits to the SDS-PAGE gel bands by Suzuki et al.
2. Characterization of the reconstitution of TF₀F₁-ATPase into preformed vesicles

**Fig. S3** The Nycodenz density gradient (0 % to 40 % (w/v)) was prepared in ATPase-buffer (0.5 mM MOPS, 100 mM KCl, 2 mM MgCl₂, pH 7.3) and divided into seven 20 µL fractions for SDS-PAGE analysis. The SDS-PAGE gel from the reconstitution sample with a nominal protein/lipid ratio of 1:40,000 shows the strongest protein band intensities for the fractions of low density, F₁–F₃, representing reconstituted TF₀F₁-ATPase into vesicles of DOPC/POPE/POPS/cholesterol (50:20:10:20). The gel lane corresponding to the fraction of highest density, F₇, shows that some amount of protein was not reconstituted into liposomes. S₁ to S₄ represent the standard samples of 1 mg, 0.5 mg, 0.25 mg and 0.1 mg used for the calibration. For quantification, gray values of the α, β, and γ-bands were read out. A calibration curve obtained from the standard S₁-S₄ allowed assigning the intensities of F₁-F₃ to the protein mass reconstituted into vesicles.

The reconstitution efficiency was determined for nominal p/l of 1:40,000, 1:20,000 and 1:8,000 by means of a density centrifugation with Nycodenz (Fig. S3).

<table>
<thead>
<tr>
<th>Nominal protein/lipid</th>
<th>final protein/lipid</th>
<th>Reconstitution efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:40,000</td>
<td>1:29,000</td>
<td>138 %</td>
</tr>
<tr>
<td>1:20,000</td>
<td>1:21,000</td>
<td>95 %</td>
</tr>
<tr>
<td>1:8,000</td>
<td>1:19,000</td>
<td>42 %</td>
</tr>
</tbody>
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**Table S1** Characterization of ATPase reconstitution efficiency, starting from different nominal protein/lipid ratios. With increasing nominal p/l of 1:40,000, 1:20,000 and 1:8,000 a saturation of reconstituted protein occurred, resulting in final p/l of 1:29,000, 1:21,000 and 1:19,000.
3. Determination of the size distribution of ATPase-liposomes

Dynamic light scattering (DLS) experiments were performed with a Malvern Zetasizer.

Fig. S4 Intensity dependent size distribution of ATPase-containing liposomes (DOPC/POPE/POPS/cholesterol (50:20:10:20), nominal p/l = 1:20,000, ATPase-buffer: 0.5 mM MOPS, 100 mM KCl, 2 mM MgCl₂, pH 7.3) measured by dynamic light scattering (DLS). The proteoliposomes showed a diameter distribution of 35-450 nm, with an intensity-weighted mean diameter of 95 nm (FWHM: 51-191 nm).

4. Calculation of ATPase molecules per vesicle

To estimate the average number of TF₉O₁₅₁-ATPase molecules per vesicle \( N_{\text{ATPase}} \), the intensity-weighted mean diameter (95 nm, determined by DLS) and the protein/lipid ratio (p/l = 1:20,000, determined by Nycodenz density gradient centrifugation and quantification of lipid concentration) are needed. Furthermore, a bilayer thickness of \( h_{\text{bilayer}} = 4.5 \) nm, an area of a single ATPase of \( a_{\text{protein}} = 20 \) nm² were assumed. The average lipid area \( a_{\text{lipid}} = 0.62 \) nm² of the used lipid mixture was calculated from the values of the CHARMM-GUI Membrane builder (see Table S2).

Table S2 The average area per lipid of the lipid mixture used was calculated from the individual areas per lipid, which were obtained from the CHARMM-GUI Membrane builder.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Area/lipid</th>
</tr>
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<tbody>
<tr>
<td>DOPC (50%)</td>
<td>69.7 Å²</td>
</tr>
<tr>
<td>POPE (20%)</td>
<td>58.8 Å²</td>
</tr>
<tr>
<td>POPS (10%)</td>
<td>60.4 Å²</td>
</tr>
<tr>
<td>Cholesterol (20%)</td>
<td>40.0 Å²</td>
</tr>
<tr>
<td>Average</td>
<td>61.6 Å²</td>
</tr>
</tbody>
</table>

\[
N_{\text{ATPase}} = N_{\text{lipid}} \cdot \frac{p}{l} \tag{S1}
\]

The number of lipids per vesicle \( N_{\text{lipid}} \) is calculated with Eq. (S2), with \( A_{\text{lipids}} \) being the total area of lipids in a vesicle and \( a_{\text{lipid}} \) being the average area of a single lipid. For calculation of \( A_{\text{lipids}} \), the
total protein area \( A_{\text{proteins}} \) is subtracted from the sum of the outer \( A_{\text{outer surface}} \) and the inner \( A_{\text{inner surface}} \) vesicle surface area:

\[
N_{\text{l lipid}} = \frac{A_{\text{l lipids}}}{a_{\text{l lipid}}} = \frac{A_{\text{outer surface}} + A_{\text{inner surface}} - A_{\text{proteins}}}{a_{\text{l lipid}}}. \tag{S2}
\]

The outer vesicles surface is defined by \( A_{\text{outer surface}} = \pi \cdot d_{\text{LUV}}^2 \) and the inner surface by \( A_{\text{inner surface}} = \pi \cdot (d_{\text{LUV}} - 2h_{\text{bilayer}})^2 \), with \( d_{\text{LUV}} \) being the diameter of the vesicle and \( h_{\text{bilayer}} \) the bilayer thickness.

The total protein area \( A_{\text{proteins}} \) is calculated from the area fraction of protein \( \chi \) for a protein/lipid ratio of 1:20,000 and the sum of the outer and inner membrane area \( (A_{\text{outer surface}} + A_{\text{inner surface}}) \):

\[
\chi = \frac{a_{\text{protein}}}{a_{\text{protein}} + 20,000 \cdot a_{\text{l lipid}}}, \tag{S3}
\]

\[
A_{\text{proteins}} = \chi \cdot \left( \pi \cdot d_{\text{LUV}}^2 + \pi \cdot (d_{\text{LUV}} - 2h_{\text{bilayer}})^2 \right). \tag{S4}
\]

**Table S3** Results of the calculation of TFOF1-ATPase molecules per 95 nm diameter vesicle. Four ATPase molecules are reconstituted into an average-sized vesicle.

| \( A_{\text{outer surface}} \) / nm\(^2\) | 28,450 |
| \( A_{\text{inner surface}} \) / nm\(^2\) | 23,240 |
| \( \chi \) | 0.16 % |
| \( A_{\text{proteins}} \) / nm\(^2\) | 83.1 |
| \( A_{\text{l lipids}} \) / nm\(^2\) | 51,510 |
| \( N_{\text{l lipid}} \) | 83,070 |
| \( N_{\text{protein}} \) | 4 |

### 5. ATPase proton pumping activity monitored with the ACMA assay

The ACMA (9-amino-6-chloro-2-methoxyacridine) based ATPase assay was performed on a Jasco FP-6500 spectrofluorometer at 37 °C. To ATPase buffer (0.5 mM MOPS, 100 mM KCl, 2 mM MgCl\(_2\), pH 7.3, 860 µL) 0.9 µM ACMA (3.5 µL of a 0.2 mM stock solution in DMSO) and 100 µL proteoliposomes (\( c_{\text{phospholipid}} = 64 \mu M \)) were added. The \( K^+ \)-ionophore valinomycin (5.0 nM, 4.4 µL of a 0.9 µM stock solution in DMSO) was supplemented to allow for charge equilibration. ATP addition (1.2 mM, 7.8 µL of a 120 mM stock solution in ATPase buffer, adjusted to pH 7.3) induced proton pumping leading to fluorescence quenching. To collapse the proton gradient, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (0.4 µM, 1.6 µL of a 0.2 mM stock solution in DMSO) was added. ACMA was excited at \( \lambda_{\text{ex}} = 410 \) nm and emission was recorded at \( \lambda_{\text{em}} = 490 \) nm. The fluorescence intensity was normalized to the intensity obtained immediately after ATP addition \( (t = 0 \text{ s}) \). A characteristic result of an ACMA assay clearly demonstrating the activity of reconstituted ATPase is shown in Fig. S5.
ACMA-based activity measurement of ATPase-containing liposomes (DOPC/POPE/POPS/cholesterol (50:20:10:20), nominal p/l = 1:20,000) at 37 °C. ATPase buffer (0.5 mM MOPS, 100 mM KCl, 2 mM MgCl$_2$, pH 7.3) was supplemented with 0.9 µM ACMA and 5 nM valinomycin. ATP addition (1.2 mM, t = 0 s) led to a significant quenching of fluorescence intensity. CCCP addition (0.4 µM) collapsed the proton gradient, resulting in a restoration of fluorescence intensity to the initial value. $\lambda_{ex/em} = 410/490$ nm.

6. Calculated outer membrane area fraction as a function of vesicle diameter

Calculated outer membrane area fraction as a function of vesicle diameter, assuming a bilayer thickness of 4.5 nm.$^{3,6}$ The relative amount of fluorescence intensity emitted from the OG488-DHPE of the outer vesicles membrane leaflet, correlates with the area fraction of the outer vesicle surface. For a 100 nm diameter vesicle the outer membrane area is approx. 55 % of the total area, which is in good agreement with the calibration curve's minimum of 59 % remaining intensity.
7. Measurement of proton leakage of vesicles

Fig. S1. (A) To assess proton permeability, the fluorescence intensities of OG488 DHPE-doped vesicles with luminal pH-values of 7.3 (black), 6.3 (red), 5.3 (blue) and 4.3 (green) were observed in an external buffer of pH 7.3. All intensities were normalized to the fluorescence intensity at \( t = 0 \) s of the vesicles with an internal pH of 7.3. The small intensity decrease of the sample with luminal pH of 7.3 is a result of fluorescence bleaching. (B) Intensity ratios measured at 150 s \( I/I_{0} \) normalized to the initial intensity at \( t = 0 \) s \( I_{0} \). No significant intensity changes are observed within this time window of observation supporting that proton leakage can be excluded.

References