Electronic supplementary information

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

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Content

1. Expression and purification of TF_OF_1-ATPase

TF_OF_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 resuspendend 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_2SO_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 $MgCl_2$, 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.

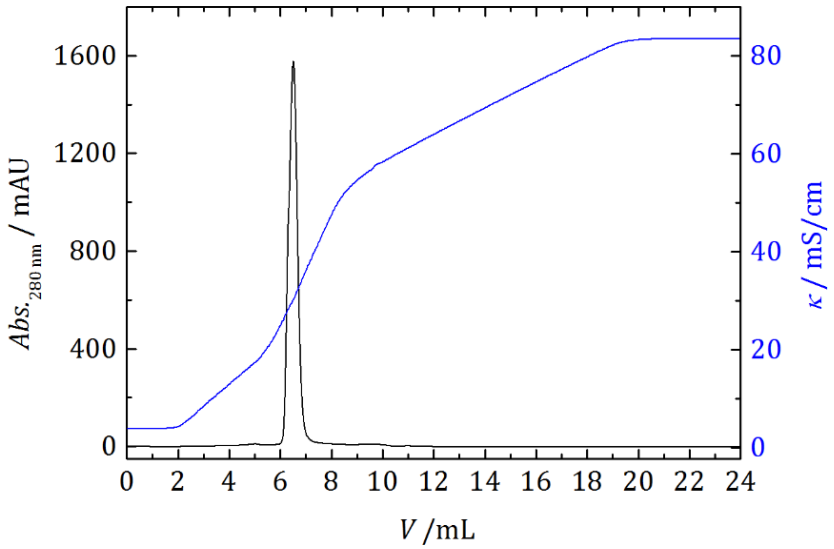

Fig. S1 Elution profile of TF_OF_1-ATPase ($Abs._{280\,nm}$) and the conductivity of the NaCl-gradient, ranging from 20 mM to 1.0 M. The anion exchange chromatography (Mono Q 5/50 column, ÄKTA Purifier, GE Healthcare Life Science) increased the protein concentration to 2.2 µM in a volume of 1.2 mL. The 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 $MgCl_2$, 1 mM DDM, pH 7.5, buffer B: 1 M NaCl, 20 mM HEPES, 5 mM $MgCl_2$, 1 mM DDM, pH 7.5.

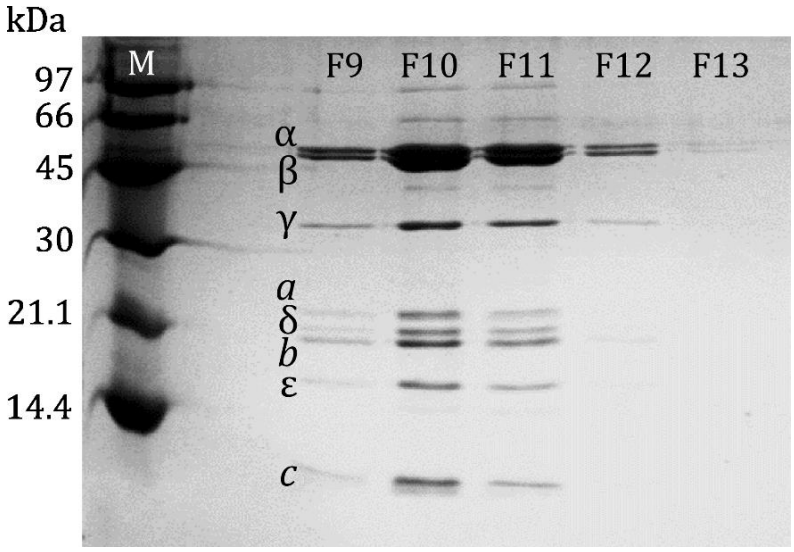

Fig. S2 (**A**) SDS-PAGE gel showing the bands of the eight single units of the TF_OF_1-ATPase in the elution fractions F9 to F13 of the anion exchange chromatography ($V_{elution}$ = 6-7.2 mL). (**B**) Assignment of the TF_OF_1-ATPase subunits to the SDS-PAGE gel bands by Suzuki *et al*.

2. Characterization of the reconstitution of TF_OF_1-ATPase into preformed vesicles

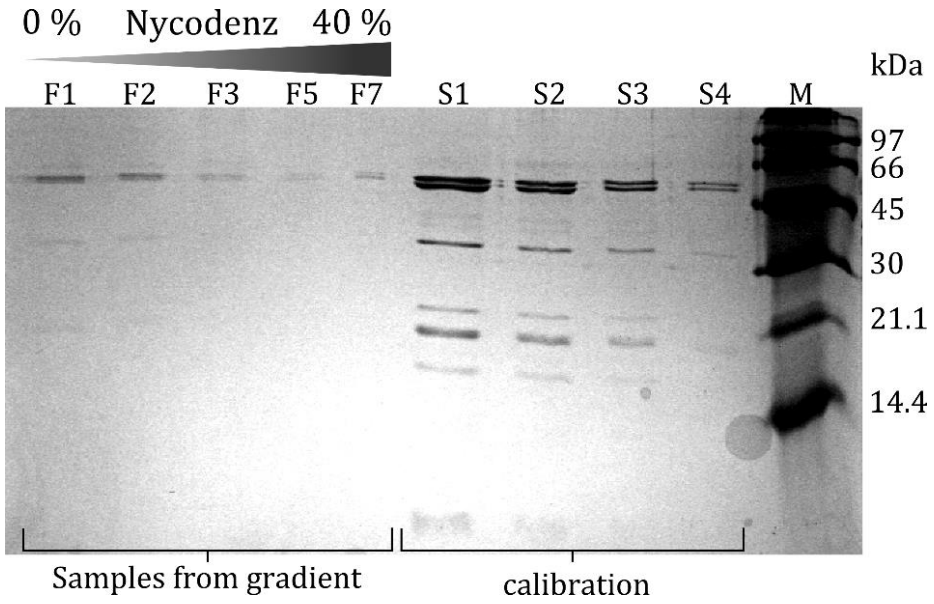

Fig. S3 The Nycodenz density gradient (0 % to 40 % (*w*/*v*)) was prepared in ATPase-buffer (0.5 mM MOPS, 100 mM KCl, 2 mM $MgCl_2$, pH 7.3) and divided into seven 20 µL fractions for SDS-PAGE analysis. The SDS-PAGE gel from the reconstitution sample with a nominal protein/lipid ratio of 1:40,000 shows the strongest protein band intensities for the fractions of low density, F1–F3, representing reconstituted TF_OF_1-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 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.

Nominal protein/lipid	final protein/lipid	Reconstitution efficiency
1:40,000	1:29,000	138 %
1:20,000	1:21,000	95 %
1:8,000	1:19,000	42 %

3. Determination of the size distribution of ATPase-liposomes

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

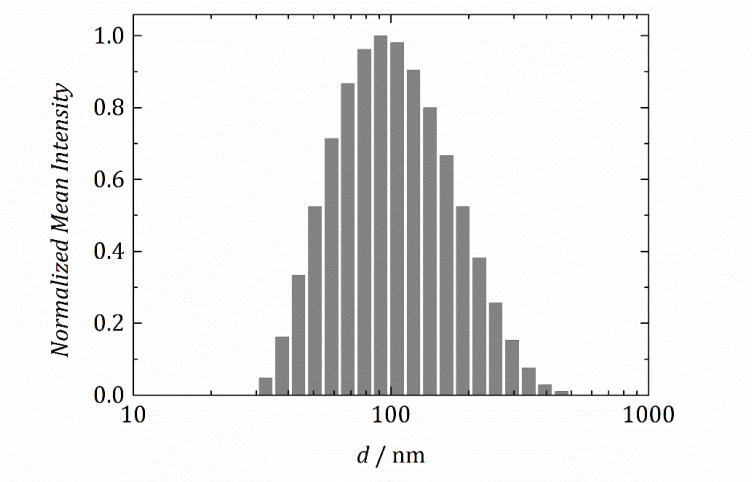

Fig. S4 Intensity dependent size distribution of ATPase-containing liposomes (DOPC/POPE/POPS/cholesterol (50:20:10:20), nominal p/l = 1:20,000, ATPase-buffer: 0.5 mM MOPS, 100 mM KCl, 2 mM $MgCl_2$, pH 7.3) measured by dynamic light scattering (DLS). The proteoliposomes showed a diameter distribution 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 TF_OF_1-ATPase molecules per vesicle N_{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 $h_{bilayer}$ = 4.5 nm,[3] an area of a single ATPase of $a_{protein}$ = 20 nm² [4] were assumed. The average lipid area a_{lipid} = 0.62 nm² of the used lipid mixture was calculated from the values of the CHARMM-GUI Membrane builder (see Table S2).[5]

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.[5]

Lipid	Area/lipid
DOPC (50%)	69.7 Å²
POPE (20%)	58.8 Å²
POPS (10%)	60.4 Å²
Cholesterol (20%)	40.0 Å²
Average	61.6 Å²

$$N_{\mathrm{ATPase}} = N_{\mathrm{lipid}} \cdot \frac{p}{l}. \qquad \text{(S1)}$$

The number of lipids per vesicle (N_{lipid}) is calculated with Eq. (S2), with A_{lipids} being the total area of lipids in a vesicle and a_{lipid} being the average area of a single lipid. For calculation of A_{lipids}, the

total protein area (A_{proteins}) is subtracted from the sum of the outer ($A_{\text{outer surface}}$) and the inner ($A_{\text{inner surface}}$) vesicle surface area:

$$N_{\text{lipid}} = \frac{A_{\text{lipids}}}{a_{\text{lipid}}} = \frac{A_{\text{outer surface}} + A_{\text{inner surface}} - A_{\text{proteins}}}{a_{\text{lipid}}}. \quad \text{(S2)}$$

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

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

$$\chi = \frac{a_{\text{protein}}}{a_{\text{protein}} + 20{,}000 \cdot a_{\text{lipid}}}, \quad \text{(S3)}$$

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

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
χ	0.16 %
A_{proteins} / nm^2	83.1
A_{lipids} / nm^2	51,510
N_{lipid}	83,070
N_{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 µM) 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-chlorphenylhydrazone (CCCP) (0.4 µM, 1.6 µL of a 0.2 mM stock solution in DMSO) was added. ACMA was excited at λ_{ex} = 410 nm and emission was recorded at λ_{em} = 490 nm. The fluorescence intensity was normalized to the intensity obtained immediately after ATP addition (t = 0 s). A characteristic result of an ACMA assay clearly demonstrating the activity of reconstituted ATPase is shown in Fig. S5.

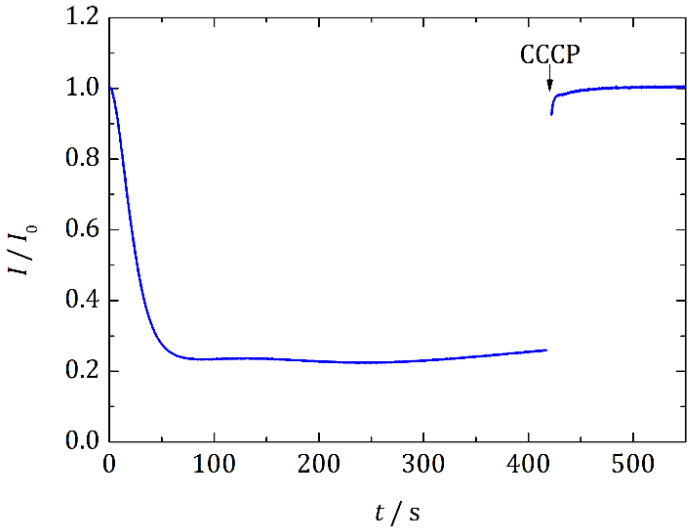

Fig. S5 ACMA-based activity measurement of ATPase-containing liposomes (DOPC/POPE/POPS/cholesterol (50:20:10:20), nominal p/l = 1:20,000) at 37 °C. ATPase buffer (0.5 mM MOPS, 100 mM KCl, 2 mM $MgCl_2$, pH 7.3) was supplemented with 0.9 µM ACMA and 5 nM valinomycin. ATP addition (1.2 mM, t = 0 s) led 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

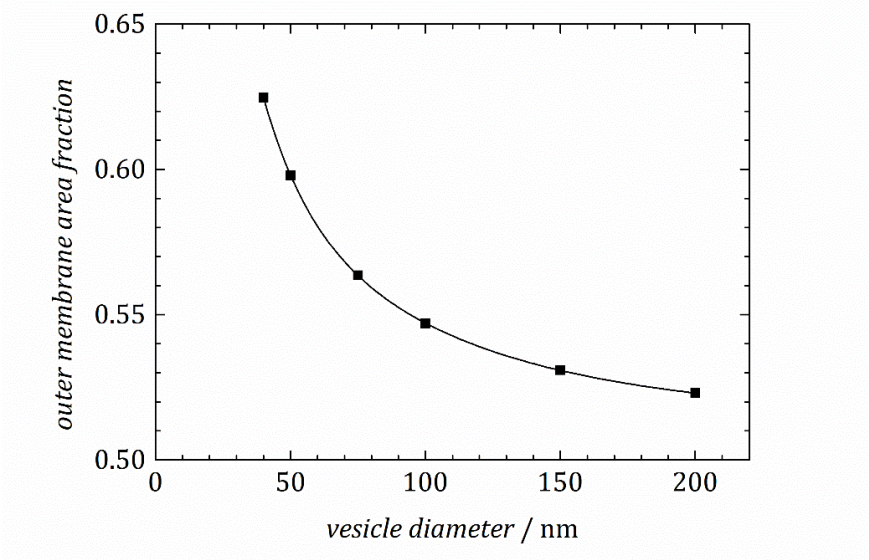

Fig. S6 Calculated outer membrane area fraction as a function of vesicle diameter, assuming a bilayer thickness of 4.5 nm.[3,6] The relative amount of fluorescence intensity emitted from the OG488-DHPE of the outer vesicles membrane leaflet, correlates with the area fraction of the outer vesicle surface. 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

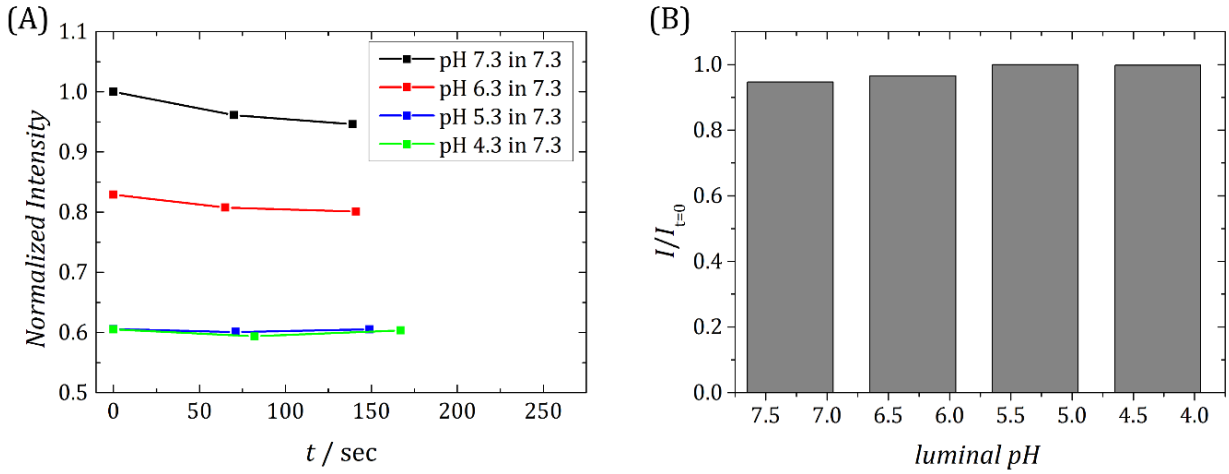

Fig. S1. (**A**) To assess proton permeability, the fluorescence intensities of OG488 DHPE-doped vesicles with luminal pH-values of 7.3 (black), 6.3 (red), 5.3 (blue) and 4.3 (green) were observed in an external buffer of pH 7.3. All intensities were normalized to the fluorescence intensity at t = 0 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) normalized to the initial intensity at t = 0 s ($I_{t=0}$). No significant intensity changes are observed within this time window of observation supporting that proton leakage can be excluded.

References

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