Supplementary Information

Synthetic syntrophy: A platform technology for adenine nucleotide cross-feeding between metabolically active vesicles

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1. Selected Abbreviations

AAC	ADP/ATP carrier protein
ABC	ATP-binding cassette
ADP	Adenosine 5'-diphosphate
ArcA	arginine deiminase
ArcB	ornithine transcarbamoylase
ArcC	carbamate kinase
ArcD	arginine/ornithine antiporter
ATP	adenosine-5'-triphosphate
BKA	bongkrekic acid
CATR	carboxyatractyloside
CV	column volume
DDM	n-dodecyl β-D-maltoside
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine, 18:1 (Δ9-Cis) PC
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, 18:1 (Δ9-Cis) PE
DOPG	1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt), 18:1 (Δ9-Cis) PG
EDTA	ethylenediaminetetraacetic acid sodium salt
Egg-PC	L-α-phosphatidylcholine (Egg, Chicken)
KP_i	potassium phosphate buffer (pH 7.0)
LPR	lipid-to-protein ratio (w/w)
MOPS	3-(N-morpholino)propanesulfonic acid
OpuA	osmoregulatory ABC-transporter protein, composed of two OpuABC and two OpuAA subunits
TOCL	1',3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-glycerol (sodium salt), 18:1 Cardiolipin

2. Materials and Methods

Reagents and materials

All lipids were purchased from Avanti Polar Lipids as powder: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, 18:1 (Δ9-Cis) DOPE; 1,2-dioleoyl-sn-glycero-3-phosphocholine, 18:1 (Δ9-Cis) DOPC; 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt), 18:1 (Δ9-Cis) DOPG, 1',3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-glycerol (sodium salt), 18:1 cardiolipin TOCL; L-α-phosphatidylcholine (Egg, Chicken), Egg-PC.

Adenosine-5'-triphosphate sodium salt, ATP (Roche, #44504128); adenosine 5'-diphosphate sodium salt, ADP (Sigma, A2754), betaine ≥99.0% (Sigma, #61962); dipotassium hydrogen phosphate trihydrate (Merck); potassium dihydrogen phosphate (Merck); potassium hydroxide, ca. 85% for analysis, pellets (Acros Organics); sodium hydroxide pellets (Sigma-Aldrich); potassium chloride (Merck); sodium chloride (Sigma-Aldrich); calcium dichloride (Sigma-Aldrich); glycerol, extra pure (Boom, #76050772.1000); ethylenediaminetetraacetic acid sodium salt, EDTA (Acros Organics); imidazole PUFFERAN® ≥99% p.a. (Carl Roth); L-arginine (Sigma); L-ornithine monohydrochloride (Carl Roth); 3-(N-morpholino)propanesulfonic acid, MOPS (Sigma-Aldrich); n-dodecyl β-D-maltoside ≥99%, DDM (GLYCON Biochemicals GmbH, #D97002); Triton™ X-100 (Sigma-Aldrich); magnesium chloride hexahydrate (Sigma); magnesium sulfate (Sigma-Aldrich); Bongkrekic acid solution, BKA (Sigma-Aldrich); carboxyatractyloside potassium salt ≥98%, CATR (Sigma-Aldrich); valinomycin (Sigma-Aldrich); nigericin sodium salt (Sigma); 2-mercaptoethanol, 99% (Sigma-Aldrich); Factor Xa Protease 1mg/mL (New England Biolabs, # P8010L); chloroform, anhydrous, ≥99% (Sigma-Aldrich), diethylether (Sigma-Aldrich).

cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche, #05056489001); PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific™); Coomassie Brilliant Blue R 250 (Serva, #17525.01); Proteus '1-Step-Batch' Midi Spin Columns (Generon, #GEN-1SB08); Bio-Beads SM-2 Adsorbents Media (Bio-Rad Laboratories, #152-3920); ATPlite Luminescence ATP Detection Assay System (Perkin Elmer, #6016947); Pierce™ BCA Protein Assay Kit (Thermo Scientific™ #23225); Ni-Sepharose 6 Fast Flow (GE Healthcare); Whatman™ Nucleopore™ Track-Etch Membrane 0.4µm (cytiva; # 10417104); Amersham™ Protran™ 0.45µm NC Nitrocellulose Blotting Membrane (cytiva, #10600002); Ultima Gold™ MV (Perkin Elmer); N,N,N-trimethyl glycine [methyl-^{14}C-] 0.1mCi/mL in ethanol:water (1:1) solution, 52.0 mCi/mmol (Moravek Inc, #MC-2065); adenosine 5'-triphosphate, tetrasodium salt, [2,8-^{3}H-] 0.250 mCi in ethanol:water (1:1) solution, 21.2 Ci/mmol (Perkin Elmer, #NET420250UC).

3. Experimental Protocols

Protein sources, gene expression and protein purification

The proteins used in this study are based on recombinant genes, expressed in various hosts. The proteins were purified according to established procedures. AAC variant (TtAac_Q302K) from *Thermothelomyces thermophila* was produced in *Saccharomyces cerevisiae* strain WB12 and purified from isolated mitochondria.[1–3] The gene for wild type OpuA was expressed in *Lactococcus lactis* Opu401 and purified from crude membrane vesicles.[4,5] The arginine/ornithine antiporter ArcD from *Lactilactobacillus sakei* was expressed in *L. lactisJP9000 ΔarcD1ΔarcD2* and purified from crude membrane vesicles.[6] The water-soluble enzymes ArcA, ArcB and ArcC1 from the arginine breakdown pathway of *L. lactis* IL1403 were expressed in *L. lactis* NZ9000 and purified by Ni-Sepharose and size-exclusion chromatography.[7] The fluorescent ATP/ADP reporter protein PercevalHR (transferred from the Addgene vector #4908147 into pBAD24) was expressed in *E. coli* MC1061 and purified by Ni-Sepharose and size-exclusion chromatography.[7,8]

Purification of AAC

Solubilization of the yeast mitochondria[2] (250 mg of total protein, 20 g/L, 12.5 mL), was carried out at a concentration of 10 g/L with 2% (w/v) DDM under mild nutation for 1 h at 10 °C in 20 mM MOPS pH 7.0, 150 mM NaCl, 20 mM imidazole and two EDTA-free Complete protease inhibitor tablets. The solubilized mitochondrial membranes were separated from the unsoluble fraction by ultracentrifugation (45 min, 4 °C, 234,000 rcf, Beckman Optima LE-80K Ultracentrifuge, 50.2 Ti Rotor) and then mixed with 375 µL (= 1 CV) Ni^{2+}-Sepharose resin and incubated for 1 h at 10 °C on a nutator. The column material was previously washed with 5 mL (13 CV) ultrapure water and 7.5 mL (20 CV) 20 mM MOPS pH 7.0, 150 mM NaCl, 20 mM imidazole. The protein-loaded resin was washed in a polyprep column by stepwise addition of 25 mL (66 CV) of buffer A (20 mM MOPS pH 7.0, 150 mM NaCl, 50 mM imidazole, 0.03% (w/v) DDM plus 0.03 g/L TOCL) and 15 mL (40 CV) buffer B (20 mM MOPS pH 7.0, 150 mM NaCl, 0.03% (w/v) DDM plus 0.03 g/L TOCL). The column material was resuspended in buffer B to give a total volume of 0.8 mL and then supplemented with imidazole (20 mM) and $CaCl_2$ (1mM). The mixture was incubated overnight at 10°C with Factor Xa protease (10 µg) to cleave AAC from the resin. AAC was recovered from the resin by 3x spin filtration (5 min, 4 °C, 600 rcf) with buffer B. The purified AAC was aliquoted (100 µg), snap-frozen and stored at -80 °C.

Purification of OpuA

Crude membrane vesicles[5] (9.1 mg total protein, 6.5 g/L, 1.4 mL) were solubilized at a concentration of 3 g/L by incubation for 30 min on ice with 0.5% (w/v) DDM in 50 mM KPi pH 7.0, 200 mM KCl plus 20% (v/v) glycerol. After 15 min the sample was mixed gently by once pipetting up and down. The solubilized membrane fractions were separated from the unsoluble material by ultracentrifugation (25 min, 4 °C, 337,000 rcf, Beckman Optima MAX-E, MLA-80 rotor). In the meanwhile, 0.5 mL (=1 CV) Ni^{2+}-Sepharose resin was washed with 6 mL (12 CV) ultrapure water, and 2 mL (4 CV) equilibration 50 mM KPi pH 7.0, 200 mM KCl, 20% (v/v) glycerol, 10 mM imidazole plus 0.04% (w/v) DDM in a polyprep column. The supernatant of the solubilized membrane fractions was supplemented with imidazole (10 mM, pH 7.5) and incubated with the Ni^{2+}-Sepharose resin in the column for 30 min on ice with gently mixing after 15 min by pipetting up and down. The protein-bound resin was washed with 10 mL (20 CV) 50 mM KPi pH 7.0, 200 mM KCl, 20% (v/v) glycerol, 50 mM imidazole plus 0.04% (w/v) DDM, and eluted stepwise with 50 mM KPi pH 7.0, 200 mM KCl, 20% (v/v) glycerol, 200 mM imidazole plus 0.04% (w/v) DDM, applying 0.6 CV for the first elution fraction and 0.4 CV for the subsequent elution fractions. The purified OpuA protein was immediately used for reconstitution into liposomes.

Proteoliposome formation

Formation of LUVs from synthetic lipids:
Liposomes were prepared from mixtures of synthetic lipids with various lipid compositions, following established procedures of thin film rehydration, sonication and extrusion with minor changes.[9] Lipid stocks were obtained by dissolving lyophilized lipid powder in chloroform at a concentration of 20 g/L or 25 g/L. Lipid mixtures were dried in a rotary evaporator at 40 °C, redissolved in diethyl ether and vacuum-dried again. The lipid film was rehydrated in 50 mM KPi, pH 7.0 (vesicles X) or 50 mM KPi, 25 mM KCl, pH 7.0 (vesicles A, vesicles B, and all other vesicles). After 16 sonication cycles (15 s on, 45 s off, 70% amplitude) with a sonication microtip probe 422-A (Cole-Parmer) in an ice bath (ice:water:ethanol), the liposomes were aliquoted (20 mg) and, exposed to three freeze-thaw cycles between liquid nitrogen and ice-cold water (10 °C). After the third freezing the aliquots of the lipid mixtures were stored in liquid nitrogen and used within three months.

Lipid compositions:
A. DOPE:DOPC:DOPG:TOCL = 25:47:25:3 (mol/mol)
B. DOPE:DOPC:DOPG:TOCL = 50:12:35:3 (mol/mol)
C. DOPE:DOPC:DOPG = 25:50:25 (mol/mol)
D. DOPE:DOPC:DOPG:TOCL = 25:40:25:10 (mol/mol)
E. EggPC:TOCL = 97:3 (mol/mol)

Reconstitution of membrane proteins
The membrane proteins used in this study were reconstituted into lipid vesicles by a detergent-destabilization method of preformed liposomes with subsequent detergent removal using polystyrene beads as adsorbents. Specific adaptations to the protocol according to the specific requirements of proteins or for the co-reconstitution of multiple membrane proteins are indicated in ***Supplementary Table 1***.

Supplementary Table 1: Denotation and lipid composition of the proteoliposomes prepared in this study. *Vesicles H went through the whole reconstitution process with detergent-destabilization and detergent removal, but no membrane protein was added. **Vesicles I did not see any detergent nor membrane protein and were extruded only. LPR = lipid-to-protein ratio in w/w.

Proteoliposome	Lipid composition	Membrane protein 1 (LPR, w/w)	Membrane protein 2 (LPR, w/w)
Vesicles X (AAC platform vesicles)	A	AAC (200)	---
Vesicles A (producer)	A	AAC (200)	ArcD (200)
Vesicles B (consumer)	B	AAC (200)	OpuA (200)
Vesicles C	C	AAC (200)	---
Vesicles D	D	AAC (200)	---
Vesicles E	E	AAC (200)	---
Vesicles F	B	---	OpuA (200)
Vesicles G	B	AAC (200)	---
Vesicles H*	B	---	---
Vesicles I**	B	---	---
Vesicles J	B	ArcD (200)	OpuA (200)

Reconstitution procedure for proteoliposomes containing AAC:
The preformed liposomes (20 mg, 1mL; varied lipid composition see ***Supplementary Table 1***) were thawed and 13x extruded through a polycarbonate filter with 400 nm pore size in a mini-extruder (Avanti Polar Lipids) immediately before use in the reconstitution experiment. The extruded liposomes were diluted to 4 g/L in 50 mM KPi, 25 mM KCl, pH 7.0 and subsequently destabilized by titration with Triton X-100 (10% (w/w)) up to a decrease of light scattering to 60 % beyond the saturation point R_{sat} of the lipid membrane. Destabilization of the liposomes was monitored by light scattering measurements at a wavelength of 540 nm (Cary 100 UV-Vis spectrometer).[10] At A_{540nm} of ca. 60% of the initial absorbance value the membrane protein (AAC) was added to the suspension in a lipid-to-protein ratio (LPR) of 200 (w/w). The mixture was incubated for 1 h at 10 °C under mild nutation, before detergents were removed by the stepwise addition of bio-beads SM-2 adsorbents (ca. 220 mg wet weighed beads per 20mg of lipids per addition); additional aliquots of beads were added after 15, 30 and 45 min and the next day (after ca. 16 h). 1 h after the last addition, the beads were removed by filtration through a polyprep column and washed with 1 mL of 50 mM KPi, 25 mM KCl, pH 7.0. The flow-through was collected in a centrifuge tube and immediately spun down in an ultracentrifuge (30 min, 4 °C, 337,000 rcf, Beckman Optima MAX-E, MLA-80 rotor). The obtained pellet was resuspended with 50 mM KPi, 25 mM KCl, pH 7.0 to give a final concentration of 100 g/L. Proteoliposomes were stored in aliquots (6.6 mg lipids per tube) in liquid nitrogen.

Preparation of vesicles **X**, **C**, **D** and **E** was carried out in the same manner, using the lipid and membrane protein composition stated in ***Supplementary Table 1***. **Vesicles A,** that contain additionally the arginine/ornithine antiporter protein ArcD, were reconstituted similarly, that is, in the presence of 2 mM DTT, and they resuspended in 50mM KPi, 58mM NaCl, 2mM DTT, pH 7.0.

Reconstitution procedure for proteoliposomes containing OpuA:
The preformed liposomes (20 mg, 1mL; lipid composition B see ***Supplementary Table 1***) were thawed and 13x extruded through a polycarbonate filter with 400 nm pore size in a mini-extruder (Avanti Polar Lipids) immediately before use in the reconstitution experiment. The extruded liposomes were diluted to 4 g/L in 50 mM KPi, 25% (v/v) glycerol, pH 7.0 and subsequently destabilized by titration with Triton X-100 (10% (w/w)) up to a decrease of light scattering to 60 % beyond the saturation point R_{sat} of the lipid membrane. Destabilization of the liposomes was monitored by light scattering measurements at a wavelength of 540 nm (Cary 100 UV-Vis spectrometer).[10] At A_{540nm} of ca. 60% of the initial absorbance value the membrane protein (OpuA) was added to the suspension in a lipid-to-protein ratio (LPR) of 200 (w/w). The mixture was incubated for 30 min at 10 °C under mild nutation, before detergents were removed by the stepwise addition of bio-beads SM-2 adsorbents (ca. 220 mg wet weighed beads per 20mg of lipids per addition) additional aliquots of beads were added after 30, 60 and 90 min and the next day (after ca. 16 h). After another 2h of incubation the bio-beads were removed by filtration through a polyprep column and washed with 1 mL of 50 mM KPi, pH 7.0. The flow-through was collected in a centrifuge tube filled with 50 mL 50 mM KPi, pH 7.0 to dilute the glycerol. The suspension was immediately spun down in an ultracentrifuge (4 h, 4°C, 125,000 rcf, Beckman Optima LE-80K Ultracentrifuge, 45-Ti rotor). The obtained pellet was resuspended with 50 mM KPi, pH 7.0 to give a final lipid concentration of 100 g/L. Proteoliposomes were stored as aliquots (6.6 mg lipids per tube) in liquid nitrogen.

Preparation of **vesicle B**, **vesicle F**, **vesicle G**, **vesicle H** was carried out in the same manner, using the lipid and membrane protein composition stated in ***Supplementary Table 1***. For the co-reconstitution experiments with two membrane proteins, the two membrane proteins were added subsequently after destabilization of the liposomes and incubated together, and then

the removal of the detergents was started by stepwise addition of the biobeads as described above. **Vesicles J** that contain additionally the membrane protein ArcD were reconstituted and resuspended in the presence of 2 mM DTT.

Loading of the proteoliposomes with luminal assay components

The composition of the encapsulation mixtures, which refers to the luminal loading of the vesicles is specified in the Figure legends and **Supplementary Table *2***. The encapsulation follows a general procedure: to a 1.5 mL microcentrifuge tube filled with the respective vesicles (66 mg of lipid, 100 g of lipid/L), the luminal components were added from stock sock solutions to give a final volume of 200 µL. The stock solutions were prepared in 50 mM KPi pH 7.0. The samples were gently vortexed and then flash-frozen in liquid nitrogen and slowly thawed in a water bath of about 10 °C. After five freeze-thaw cycles the proteoliposomes were extruded 13x through a polycarbonate membrane with 400 nm pore diameter (Whatman, GE Healthcare) in a mini-extruder preequilibrated with a buffer solution according to the composition of the luminal components and washed with 3x 6 mL with iso-osmotic 50 mM KPi pH 7.0, 25 mM KCl; or a different KCl concentration to match with the additions from the different luminal components, using ultracentrifugation (25 min, 4 °C, 337,000 rcf, Beckman Optima MAX-E, MLA-80 rotor) and subsequent resuspension. In the last step, the loaded proteoliposomes were resuspended to a final concentration of 200 mg of lipid/mL. The proteoliposomes were immediately used in the functional assays. The encapsulation procedure of the arginine breakdown pathway is described in detail elsewhere[7] but follows a very similar procedure.

Supplementary Table 2: Overview of the different vesicle loading schemes. Luminal components encapsulated inside the proteoliposomes, and composition of the external reaction buffer used in the functional assays. All internal and external solutions were adjusted to pH 7.0.

Proteoliposomes	Luminal components	Radioactive uptake assay - External reaction buffer	Fluorescence assay – External reaction buffer
Vesicle X (AAC platform vesicles)	5 mM Na-ATP, 50 mM KPi, 25 mM KCl	50 mM KPi, 33 mM KCl, 50 µM Na-ATP (ca. 50 nM ^{3}H-labeled)	
	Vesicle X + 0.0/ 0.05/ 0.1/ 0.25/ 0.5/ 1.0/ 2.5/ 5.0/ 10.0 mM $MgSO_4$	Vesicle X + 0.0/ 0.05/ 0.1/ 0.25/ 0.5/ 1.0/ 2.5/ 5.0/ 10.0 mM $MgSO_4$	
	Vesicle X	Vesicle X +10 µM CATR/ 10 µM BKA/ 10 µM CATR, 10 µM BKA	
Vesicle A (producer)	10 mM Mg-ADP, 50 mM KPi, 2 mM DTT, 1 µM ArcA, 2 µM ArcB, 5 µM ArcC, 0.5 mM L-ornithine		50 mM KPi, 300 mM KCl, 2 mM DTT, 1 µM nigericin, 1 µM valinomycin, 0.5 µM PercevalHR, 5 mM $MgSO_4$, 25/ 50/ 100 µM Na-ADP,10 mM L-Arginine
	Vesicle A + 5 µM PercevalHR		50 mM KPi, 300 mM KCl, 2 mM DTT, 1 µM nigericin, 1 µM valinomycin, 5 mM

			$MgSO_4$, 25/ 50/ 100 µM Na-ADP,10 mM L-Arginine
Vesicle B (consumer)	0.1/ 1.0/ 10 mM Mg-ADP, 50 mM KPi	0.1/ 1.0/ 10 mM Na-ATP, 2 mM EDTA, 50 mM KPi, 300 mM KCl, 200 µM betaine (ca. 20 µM ^{14}C-labeled)	
	10 mM Mg-ATP, 50 mM KPi	0.0/ 10 mM Na-ATP, 2 mM EDTA, 50 mM KPi, 300 mM KCl	
	1 mM Mg-ADP, 50 mM KPi	Vesicle B +10 µM CATR/ 10 µM BKA/ 10 µM CATR, 10 µM BKA	
	20 mM calcein, 50 mM KPi	50 mM KPi, 300 mM KCl/ KPi/ NaCl	
Vesicle C	as vesicle X	as vesicle X	
Vesicle D	as vesicle X	as vesicle X	
Vesicle E	as vesicle X	as vesicle X	
Vesicle F	10 mM Mg-ATP, 50 mM KPi	2 mM EDTA, 50 mM KPi, 300 mM KCl, 200 µM betaine (ca. 20 µM ^{14}C-labeled)	
	20 mM calcein, 50 mM KPi	50 mM KPi, 300 mM KCl/ KPi/ NaCl	
Vesicle G	20 mM calcein, 50 mM KPi	50 mM KPi, 300 mM KCl/ KPi/ NaCl	
Vesicle H*	20 mM calcein, 50 mM KPi	50 mM KPi, 300 mM KCl/ KPi/ NaCl	
Vesicle I**	20 mM calcein, 50 mM KPi	50 mM KPi, 300 mM KCl/ KPi/ NaCl	
Vesicle J	10 mM Mg-ADP, 50 mM KPi, 2 mM DTT, 1 µM ArcA, 2 µM ArcB, 5 µM ArcC, 0.5 mM L-ornithine	50 mM KPi, 300 mM KCl, 2 mM DTT, 1 µM nigericin, 1 µM valinomycin, 5 mM $MgSO_4$, 200 µM betaine (ca. 20 µM ^{14}C-labeled), 10 mM L-Arginine	

Radioactive uptake assays

Transport activity assay of AAC in synthetic vesicles:
Reaction tubes were filled with the reaction buffer of the assay (50 mM KPi, 33 mM KCl, 50 µM Na-[^{3}H]-ATP (ca. 0.1% ^{3}H-labeled), equipped with a stirring bar and equilibrated in a temperature-controlled water bath set to 30 °C. The transport reaction was started by addition of the proteoliposomes (**Vesicle X**, loaded with 5 mM Na-ATP, 50 mM KPi, 25 mM KCl) to the tube filled with reaction buffer at a lipid concentration of 2 g/L (50x dilution). Samples with a volume of 50 µL were taken at defined time points, immediately diluted into 2 mL ice-cold quenching buffer (50 mM KPi, 33 mM KCl, pH 7.0) and filtered through a 0.45 µm cellulose nitrate filter using a filtration manifold. The filter retains ≥99% of the vesicles and was washed with 2 mL quenching buffer. The filter was then dissolved in 1.8 mL scintillation liquid (Ultima Gold^{TM} MV, Perkin Elmer). Radioactivity was quantified in a Tri-Carb 2800 TR liquid scintillation counter.

Variations:
Protocol 1: The transport assay was carried out at a reaction temperature of 10 °C, 20 °C and 30 °C.
Protocol 2: The transport assay was carried out with proteoliposomes of different lipid composition, using **Vesicles X, C, D** and **F**.
Protocol 3: The transport assay was carried out in the presence of $MgSO_4$, supplemented at a concentration of 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 mM $MgSO_4$ to the luminal solution and the external reaction buffer.
Protocol 4: The transport assay was carried out in the presence of specific inhibitors of AAC: 10 µM CATR; 10 µM BKA; or 10 µM CATR + 10 µM BKA.

Transport activity assay of OpuA in synthetic vesicles fueled by externally supplied ATP taken up via AAC:
The proteoliposomes (**Vesicle B**, loaded with 0.1 mM/ 1 mM/ 10 mM Mg-ADP, 50 mM KPi, pH 7.0) were 100x diluted into the reaction buffer of the assay (50 mM KPi, 300 mM KCl, 2 mM EDTA, 0.1 mM/ 1 mM/ 10 mM Na-ATP, pH 7.0) to a lipid concentration of 2 g/L. The reaction tube was equipped with a stirring bar and equilibrated in a temperature-controlled waterbath set to 30 °C. The transport reaction was started by addition of 200 µM [^{14}C]-betaine (ca. 10% ^{14}C-labeled). Samples with a volume of 50 µL were taken at defined time points, immediately diluted into 2 mL ice-cold quenching buffer (50 mM KPi, 300 mM KCl, 2mM EDTA, pH 7.0) and filtered through a 0.45 µm cellulose nitrate filter. The filter retains ≥99% of the vesicles and was washed with 2 mL quenching buffer. The filter was then dissolved in 1.8 mL scintillation liquid (Ultima Gold^{TM} MV, Perkin Elmer). Radioactivity was quantified in a Tri-Carb 2800 TR liquid scintillation counter.

Variations:
Protocol 5: The transport assay was carried out in the presence of the specific inhibitors of AAC: 10 µM CATR + 10 µM BKA, using **Vesicles B** loaded with 1 mM Mg-ADP, and 1 mM externally supplied Na-ATP.
Protocol 6: The transport assay was carried out with **Vesicles B** loaded with 10 mM Mg-ATP in the presence and absence of 10 mM externally supplied Na-ATP.
Protocol 7: The transport assay was carried out with 10 µM [^{14}C]-betaine, using **Vesicles B** loaded with 10 mM Mg-ADP and externally supplied 10 mM Na-ATP.

Adenosine nucleotide cross-feeding between Vesicles A and Vesicles B with concomitant uptake of betaine by Vesicles B:
Vesicle A (8 g of lipid/L, loaded with 10 mM Mg-ADP, 50 mM KPi, 2 mM DTT, 1 µM ArcA, 2 µM ArcB, 5 µM ArcC plus 0.5 mM L-ornithine) and **Vesicle B** (2 g of lipid/L, loaded with1 mM Mg-ADP, 50 mM KPi, pH 7.0) were mixed in a 4:1 vesicle ratio in the reaction buffer of the assay (50 mM KPi, 300 mM KCl, 2 mM EDTA, 2 mM DTT, 1 µM nigericin, 1 µM valinomycin plus 100 µM Na-ADP, pH 7.0. The reaction tube was equipped with a stirring bar and equilibrated in a temperature-controlled water bath set to 30 °C. The transport reaction was started by addition of 34 µM [^{14}C]-betaine plus 10 mM L-arginine. After 3 h, 6 h, 18 h and 20 h, additional 10 mM L-arginine were added. Samples with a volume of 50 µL were taken at defined time points, immediately diluted into 2 mL ice-cold quenching buffer (50 mM KPi, 300 mM KCl, 2mM EDTA, pH 7.0) and filtered through a 0.45 µm cellulose nitrate filter. The filter retains ≥99% of the vesicles and was washed with 2 mL quenching buffer. The filter was then dissolved in 1.8 mL scintillation liquid (Ultima Gold^{TM} MV, Perkin Elmer). Radioactivity was quantified in a Tri-Carb 2800 TR liquid scintillation counter.

Variations:
Protocol 8: The transport assay was carried out in the presence of the specific inhibitors of AAC, *i.e.* with 10 µM CATR + 10 µM BKA in the external reaction buffer.
Protocol 9: The transport assay was carried out in the absence of the fuel L-arginine.
Protocol 10: The transport assay was carried out with a varied vesicle ratio A:B = 4:1, 1:1, 0.5:1, which corresponds to vesicle concentrations A:B of 8g/L:2g/L, 2g/L:2g/L and 1g/L:2g/L in the reaction buffer, respectively.
Protocol 11: The transport assay was carried out with overall diluted vesicles concentrations at constant vesicle ratios A:B = 4:1. Thus, yielding vesicle concentrations A:B of 8g/L:2g/L, 4g/L:1g/L and 0.8g/L:0.2g/L in the reaction buffer.

Manipulation of OpuA transport activity:
The following procedure describes the conditions for ionic strength gated betaine transport, and the inhibition of both orientations of OpuA by omitting Mg^{2+} ions (protocol 12), chelating Mg^{2+} ions by EDTA (protocol 13) or treatment of the protein with ortho-vanadate (protocol 14). To only inhibit inside-out oriented OpuA Mg^{2+} ions are omitted from the external medium either with or without EDTA in the assay buffer (protocol 15).

The proteoliposomes (**Vesicle F**, loaded with 10 mM Mg-ATP, 50 mM KPi, pH 7.0) were 100x diluted into the reaction buffer of the assay (50 mM KPi, 300 mM KCl, pH 7.0) to a lipid concentration of 2 g/L. The dilution into hypertonic medium causes shrinkage of the vesicles, which increases the internal ionic strength and activates OpuA.[11] The ionic strength gating of transport is also used in the assays described in the next sections. The reaction tube was equipped with a stirring bar and equilibrated in a temperature-controlled water bath set to 30 °C. The transport reaction was started by addition of 200 µM [^{14}C]-betaine (ca. 10% ^{14}C-labeled). Samples with a volume of 50 µL were taken at defined time points, immediately diluted into 2 mL ice-cold quenching buffer (50 mM KPi, 300 mM KCl, pH 7.0) and filtered through a 0.45 µm cellulose nitrate filter. The filter retains ≥99% of the vesicles and was washed with 2 mL quenching buffer. The filter was then dissolved in 1.8 mL scintillation liquid (Ultima Gold^{TM} MV, Perkin Elmer). Radioactivity was quantified in a Tri-Carb 2800 TR liquid scintillation counter.

Variations:

Protocol 12: The transport assay was carried out under Mg^{2+}-free conditions: **Vesicles F** were loaded with 10 mM Na-ATP (instead of Mg-ATP) and 20 mM EDTA was added both to the luminal and the external reaction solution of the vesicles.

Protocol 13: The transport assay was carried out with **Vesicles F** loaded with 10 mM Mg-ATP. 20 mM EDTA was added both to the luminal and the external reaction solution of the vesicles.

Protocol 14: The transport assay was carried out with **Vesicles F** loaded with 10 mM Mg-ATP. 5 mM sodium ortho-vanadate was added both to the luminal and the external reaction solution of the vesicles.

Protocol 15: The transport assay was carried out with **Vesicles F** loaded with 10 mM Mg-ATP. 2 mM EDTA was added to the external reaction solution of the vesicles.

Monitoring of ATP excretion from ATP-producing vesicles by fluorescent reporter protein PercevalHR

Vesicles A (loaded with 10 mM Mg-ADP, 50 mM KPi, 2 mM DTT, 1 µM ArcA, 2 µM ArcB, 5 µM ArcC, 0.5 mM L-ornithine) were 50x diluted into the reaction buffer of the assay (50 mM KPi, 300 mM KCl, 2 mM EDTA, 2 mM DTT, 1 µM nigericin, 1 µM valinomycin, 25 µM /50 µM/100 µM Na-ADP, pH 7.0, supplemented with 0.5 µM PercevalHR) to yield a lipid concentration of 2 g/L. They were mixed gently by pipetting, and then transferred to a fluorescence quartz glass ultra-microcuvette (Hellma Analytics, #105.252QS). The cuvette was placed in a temperature-controlled sample holder and equilibrated to 30 °C.
The *in-situ* production of ATP inside the vesicles with subsequent export was initiated by addition of 10 mM L-arginine (from a 250 mM L-arginine stock solution in 50 mM KPi pH 7.0). ATP excretion from the vesicles was continuously monitored by time-dependent fluorescence excitation spectra (400−520 nm, bandwidth = 5 nm) of the fluorescent reporter protein PercevalHR at an emission wavelength of 550 nm (bandwidth = 5 nm) in a Jasco FP-8300 spectrofluorometer.

Variations:

Protocol 16: *In-situ* ATP production was monitored on the inside of **Vesicles A** by encapsulating additionally 5 µM PercevalHR and leaving out PercevalHR from the external reaction buffer.

Protocol 17: The fluorescence assay was carried out with **Vesicles A** in an external reaction buffer supplemented with 10 µM Na-ADP in the presence and absence of AAC-inhibitors CATR (10 µM) and BKA (10 µM).

Quantification of excreted ATP from ATP-producing organelles by the luciferin/luciferase reaction (bioluminescence)

To quantify the amount of ATP exported to the external reaction solution, time-dependent samples (50 µL) of the reaction mixture were taken: one before addition of L-arginine (t=0) and then 1, 3 and 24 h after addition of L-arginine. The samples were immediately diluted into 200 µL ice-cold quenching buffer (50 mM KPi, pH 7.0, 10 µM BKA, 10 µM CATR) in a mini-centrifuge tube. The proteoliposomes were pelleted by ultracentrifugation (25 min, 4 °C, 229,000 rcf, Beckman Optima MAX-E, TLA-120.1 rotor, Beckman Coulter). 200 µL of the supernatant was taken and stored at -20°C in an microreaction tube and later analyzed by the commercially available ATPlite kit (Perkin Elmer), using appropriate calibration curves.

Membrane permeability measurements with a stopped-flow fluorescence spectrometer

To quantify the membrane permeability of the proteoliposomes, the volume changes of the vesicles after hyperosmotic upshift were analyzed by stopped-flow fluorescence measurements. The method is described in detail elsewhere.[9,12] The vesicles were loaded with a self-quenching concentration (20 mM) of calcein in 50 mM KPi, pH 7.0. The vesicles were extruded 13x through a polycarbonate filter with a pore size of 400 nm and purified via 22 cm long gel filtration column packed with Sephadex G-75 (Sigma) column equilibrated in 90 mM KPi pH 7.0. Vesicles were collected and diluted to a total volume of 12 mL (ca. 0.5 g/L.

Vesicles with lipid composition **B.** DOPE:DOPC:DOPG:TOCL = 50:12:35:3 (mol/mol) were systematically analyzed in terms of permeability of the membrane for betaine, KCl, KPi and NaCl: **Vesicles B** (AAC+OpuA), **Vesicles G** (AAC), **Vesicles F** (OpuA), **Vesicles H** (mock reconstitution without membrane proteins, with detergent destabilization), **Vesicle I** (no reconstitution procedure, no detergent destabilization). Stocks of the respective solutes of

interest, whose membrane permeability was tested, were prepared as 2x concentrated solutions in 90 mM KPi pH 7.0. Thus, solution with 400 mM betaine, 600 mM KCl, 600 mM KPi and 600 mM NaCl were prepared in 90 mM KPi pH 7.0.

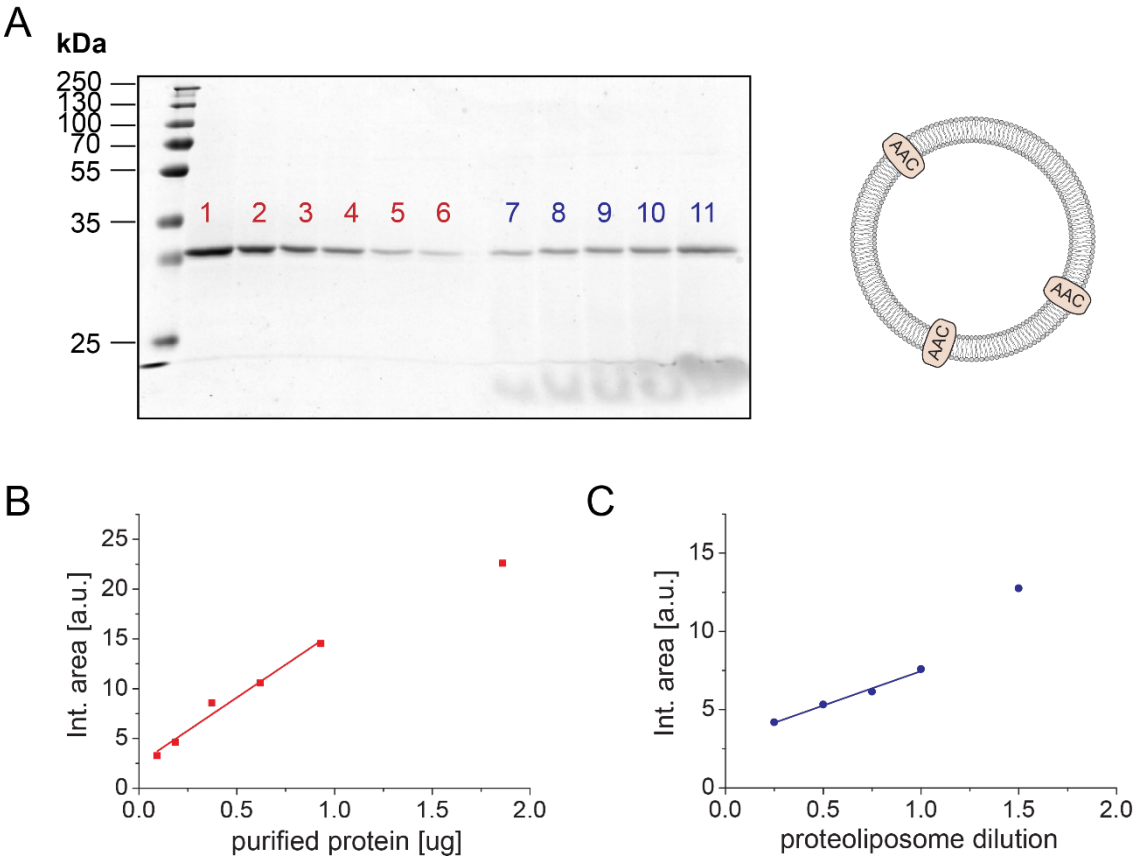
For a stopped-flow mixing experiment, the two syringes of the stopped-flow apparatus (SX20, Applied Photophysics Lim., Leatherhead) are filled: one with the concentrated osmolyte solution and the other with the calcein-loaded vesicles. The system was equilibrated to 30 °C. Upon mixing the solutions from the two syringes are forced through the mixer (1:1 mixing ratio, 2 ms dead time) into the optical cell (20 µL, 2 mm optical pathlength) of the device. Calcein fluorescence was excited at 495 nm, and the emitted light collected by a photomultiplier tube (Hamamatsu R6095) at 90° filtered by a Schott long-pass filter (cut-off at 515 nm). The fluorescence intensity after the osmotic upshift was recorded with logarithmically spaced time points to better resolve the fast kinetics of the fluorescence quenching upon the mixing process. For each experimental condition, multiple acquisitions were performed, and $n \geq 3$ measurements were averaged.

Determination of the reconstitution efficiency of membrane proteins into vesicles by SDS-PAGE

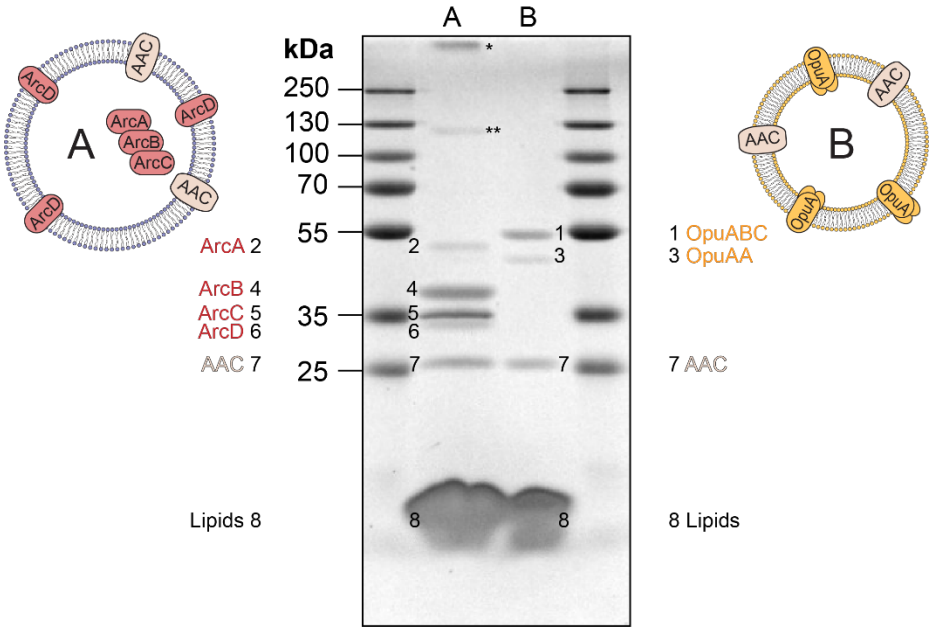
To quantify the concentration of the reconstituted membrane protein(s) in the proteoliposomes, a dilution series of the purified, detergent-solubilized membrane protein (AAC, ArcD and OpuA) together with a dilution series of the proteoliposomes (lipid mass) were run in parallel on the same SDS-polyacrylamide gel (12%). The Coomassie-Brilliant Blue stained bands were analyzed by gray scale analysis in Image J. The gray scale values of the dilution series of the detergent-solubilized membrane protein(s) served as calibration series and was fitted with a linear regression line (Origin). The obtained calibration line was used to calculate the protein concentration from the gray scale analysis of the bands of the diluted proteoliposomes.The reconstitution efficiency was determined from the averaged protein amount quantified in the proteoliposomes by SDS-PAGE in relation to the initially fed protein amount during the reconstitution procedure.

4. Supplementary Data

Membrane reconstitution efficiency estimated by SDS-PAGE

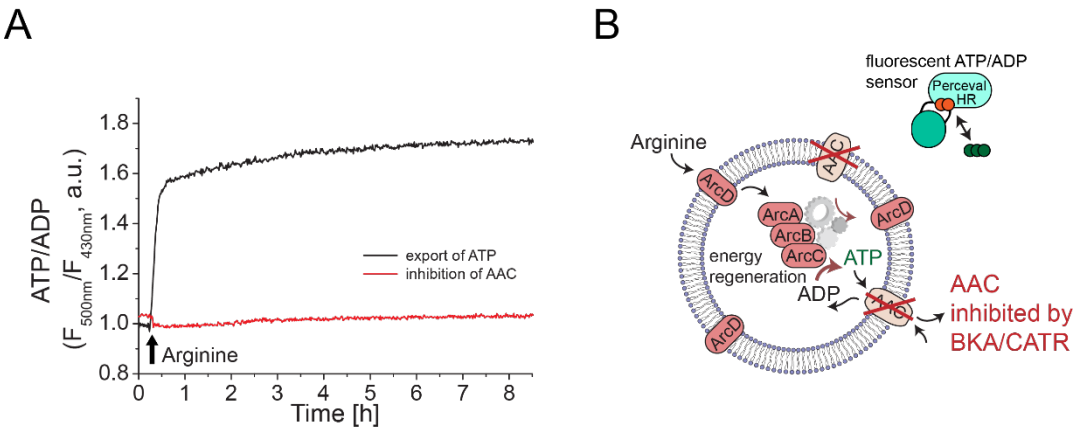


Supplementary Figure 1: Protein quantification in proteoliposomes. Determination of the reconstitution efficiency of membrane proteins by SDS-PAGE. Here, AAC in liposomes composed of DOPE:DOPC:DOPG:TOCL= 25:47:25:3 (mol%) and a lipid:protein-ratio of 200 (mol/mol). (A) Coomassie Brilliant Blue-stained SDS-PAGE (12 wt% of polyacrylamide) of dilution series of solubilized AAC and corresponding proteoliposomes. A dilution series of the detergent-stabilized AAC (lane 1-6: 1x/2x/3x/5x/10x/20x dilution factor with c_{1x}=1.86g of protein/L) was used for calibration of the integrated gray scale values (Gel Analyzer, ImageJ) and subsequently used to quantify the protein bands in the proteoliposomes (lane 7-11: 6x/4x/3x/2x/1x dilution factor). The dilution series of the proteoliposomes shows a stained blob at the bottom of the gel, which is characteristic of the solubilized lipids. (B) Calibration line of the integrated band areas (=intensities) as a function of applied mass of purified protein. (C) Integrated band intensities from the gray scale plots of the dilution series of the proteoliposomes. The protein content was calculated for each dilution of the proteoliposomes and then averaged to determine the reconstitution efficiency. The intensities of the highest applied concentrations were neglected because they deviate from the otherwise linear relationship. AAC reconstitutes on average with an efficiency of 43±10% relative to the initial feed. All specific uptake activities presented in this article are calculated with the experimentally determined average protein concentration in the proteoliposomes according to this method.



Supplementary Figure 2: SDS-PAGE (12%) of proteoliposomes with fully reconstituted metabolic pathways as applied in the energy-producing, -consuming or -cross-feeding vesicles. (A) Electrophoretic separation of the solubilized energy-producing **Vesicles A** with the characteristic bands of **2** ArcA, **4** ArcB, **5** ArcC, **6** ArcD, **7** AAC, **8** Lipids, *aggregated protein, **contamination co-purified with ArcD. (B) Electrophoretic separation of the solubilized energy-consuming **Vesicles B** with the characteristic bands of **1** OpuABC, **3** OpuAA, **7** AAC, **8** Lipids.

Proof-of-concept: ATP-excreting vesicles



Supplementary Figure 3: Inhibition of ADP/ATP-carrier in ATP-producing vesicles blocks export of ATP. (A) Control experiment with AAC inhibited by 10 µM BKA plus 10 µM CATR (red curve) shows that PercevalHR reports the ATP that is produced in the vesicles and exported into the external medium via AAC (black curve). Neither PercevalHR nor ATP permeate the vesicle membrane. (B) Schematic illustration of ATP-producing vesicle with AAC inhibited by BKA/CATR and external ATP-sensor PercevalHR. Fluorescent assay of ATP-excretion was carried out in 50 mM KP_i pH7.0, 300 mM KCl, 2 mM DTT, 1 µM nigericin, 1 µM valinomycin, 0.5 µM PercevalHR, 5 mM $MgSO_4$ and 5 µM Na^+-ADP with and without the inhibitors BKA and CATR at T = 30 °C. Proteoliposomes were loaded with 10 mM Mg^{2+}-ADP, 50 mM KP_i pH7.0, 2 mM DTT, 1 µM ArcA, 2 µM ArcB, 5 µM ArcC, 0.5 mM L-ornithine and added at a lipid concentration of 2 gL^{-1}. Assay was started by addition of 10 mM L-arginine.

Kinetic parameters of membrane proteins and thermodynamic considerations of the transmembrane equilibration of the nucleotides

Supplementary Table 3: Kinetic parameters of the membrane proteins. The Michaelis-Menten constant (K_M), initial uptake rate ($k_{initial}$) under saturating conditions (=V_{max}), and inhibition constant (K_I) are given as determined in transport assays in proteoliposomes. The molecular weight (MW) of the functional protein complexes is given. (a) Biemans-Oldehinkel *et al.*[13] in proteoliposomes, T= 30°C. (b) Patzlaff *et al.*[14] in proteoliposomes, T= 30°C. (c) King *et al.*[1] in fused membrane vesicles of *L. Lactis*, T= not reported, (d) This study, in proteoliposomes fueled via AAC (ATP_{in}=10 mM, ATP_{ext}= 10min), T= 30°C. (e) This study, in proteoliposomes encapsulated ATP (ATP_{in}=10 mM), T= 30°C. (f) This study, in proteoliposomes (ATP_{in}=5 mM, ATP_{ext}= 50 µM), T= 30°C.

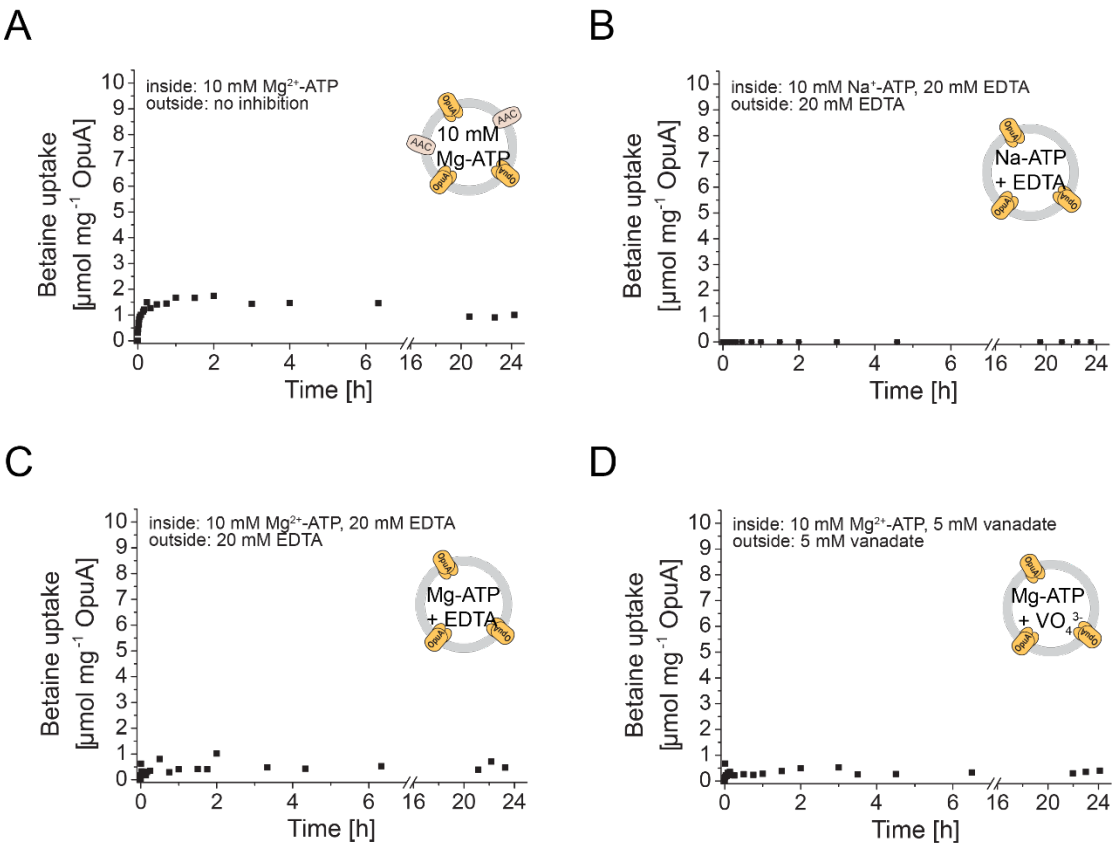
	K_M	$k_{initial}$	K_I
OpuA, WT (218 kDa)	1.9 µM (Betaine)[(a)]	1.3 µmol Betaine mg^{-1} OpuA min^{-1}[(d)]; 0.8 µmol Betaine mg^{-1} OpuA min^{-1}[(e)]	12 mM (ADP)[(b)]
	3 mM (ATP)[(b)]		
AAC, Q302K (33 kDa)	2.2 µM (ADP) [(c)]	3.5 µmol ATP mg^{-1} AAC min^{-1}[(f)]	

Supplementary Table 4: Maximum theoretical ATP concentration in the lumen of vesicles that can be reached by fueling of ATP via the ADP/ATP-carrier. Start denotes the initial concentration of ATP on the outside and ADP on the inside of the vesicles. With time AAC equilibrates both nucleotides across the membrane. This estimation considers only the chemical gradients of the nucleotides and no effects of a membrane potential. Further it assumes an internal volume of the proteoliposomes of 0.2 vol% and no ATP consumption. The initially set ratio of ATP_{ext}/ADP_{in} can be used to control the maximum internal concentration of ATP and ADP, which determines the driving force of the ATP-consuming reaction in the lumen of the vesicles.

	External Solution		**Inside (Lumen)**		**Max. internal molar ratio ATP/ADP**	**Max. internal c(ATP)**
	c(ATP)	c(ADP)	c(ATP)	c(ADP)		
Start	100 µM	---	---	1 mM		
Equilibration	100 µM	2 µM	100 µM	2 µM	50	0.98 mM
Start	100 µM	---	---	10 mM		
Equilibration	100 µM	20 µM	100 µM	20 µM	5	8.33 mM
Start	1 mM	---	---	1 mM		
Equilibration	1 mM	2 µM	1 mM	2 µM	500	0.998 mM
Start	1 mM	---	---	10 mM		
Equilibration	1 mM	20 µM	1 mM	20 µM	50	9.8 mM
Start	10 mM	---	---	1 mM		
Equilibration	10 mM	2 µM	10 mM	2 µM	5000	0.9998 mM
Start	10 mM	---	---	10 mM		
Equilibration	10 mM	20 µM	10 mM	20 µM	500	9.98 mM

Inhibition of inside-out oriented OpuA

To inhibit inside-out oriented OpuA and rule out active export of betaine from the vesicles when ATP is present externally, different inhibitors were tested.



Supplementary Figure 4: Manipulation of OpuA activity. (A) Positive control experiment in which OpuA is fueled by internal Mg^{2+}-ATP (vesicles B) without inhibition. OpuA was reconstituted in proteoliposomes as the only membrane protein and internally fueled by encapsulated 10 mM ATP (vesicles F). (B) Na^{+}-ATP was loaded into the proteoliposomes and excess EDTA added on both sides of the membrane to ensure completely Mg^{2+}-free conditions. OpuA-driven transport of betaine depends on the presence of Mg^{2+}-ions. (C) Excess of EDTA is also sufficient to inhibit the uptake of betaine when the proteoliposomes are loaded with Mg^{2+}-ATP. Mg^{2+} is fully chelated and no uptake of betaine was observed. (D) Ortho-vanadate, structurally similar to the phosphate ion, also functions as an effective inhibitor of OpuA. Ortho-vanadate has been shown to inhibit several ABC-transporter proteins by trapping ADP in the nucleotide-binding domains after hydrolysis of ATP.[15,16] [^{14}C]- betaine uptake assays were carried out in 50 mM KP_i pH7.0, 300 mM KCl, 2 mM EDTA, 200 µM [^{14}C]-betaine (ca.10%-labeled) at T = 30 °C. Proteoliposomes were loaded with 10 mM ATP, 50 mM KP_i and assayed at a lipid concentration of 2 gL^{-1}. Mg^{2+}-ion and inhibitor concentrations are indicated in the graphs.

Membrane permeability of proteoliposomes

Principle of the method

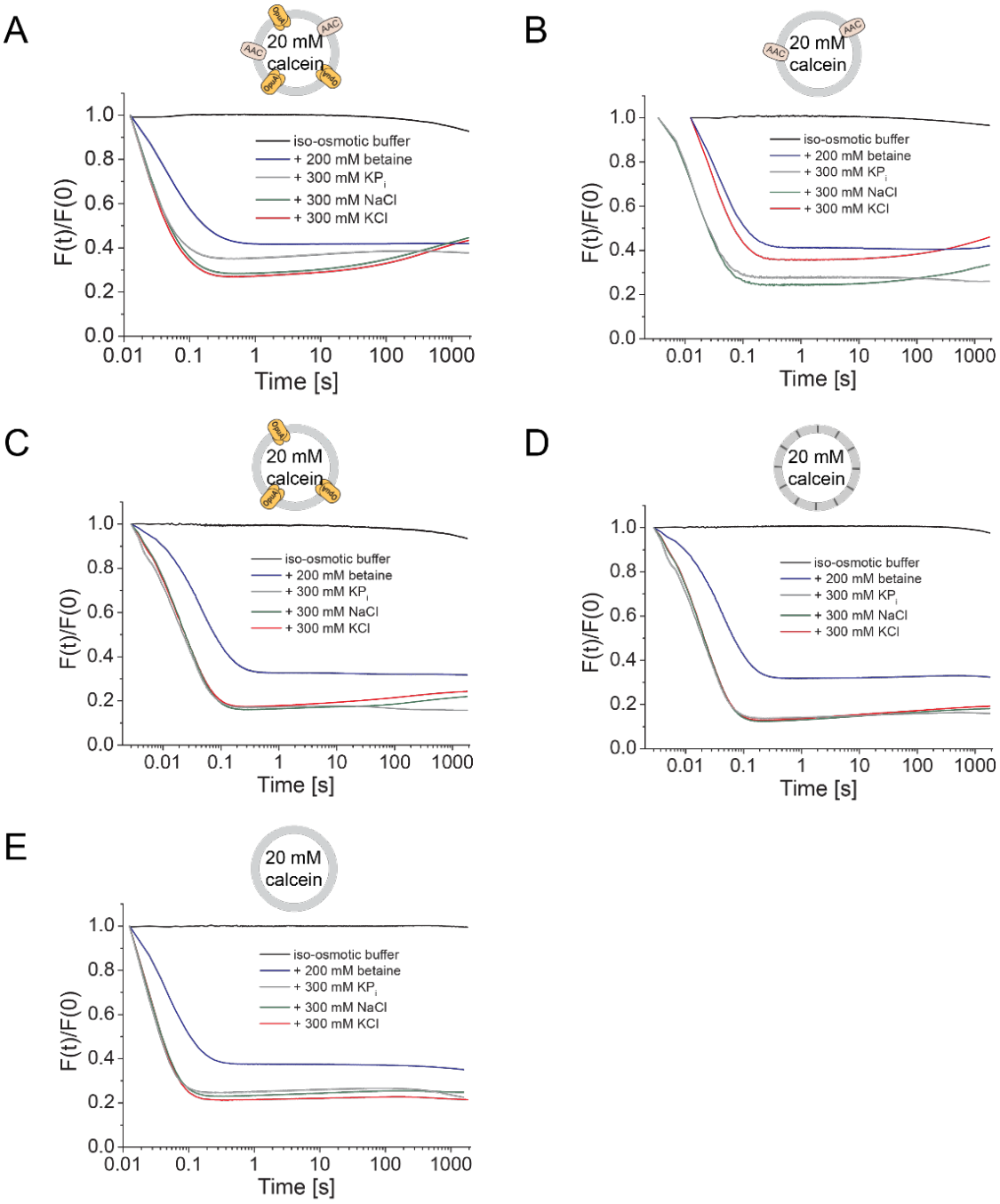
Membrane permeability of (proteo-)liposomes was assessed by imposition of an osmotic upshift and concurrent monitoring the quenching of the fluorescence of calcein inside the vesicles. To this end, the vesicles are filled with calcein (Na-salt) at a self-quenching concentration of 20 mM.

The stopped flow apparatus measures the out-of-equilibrium relaxation kinetics of the vesicles after the osmotic upshift, i.e., the addition of a solute solution with higher osmolality to the vesicle solution. After the osmotic upshift, the thermodynamic equilibrium is re-established by (i) water efflux (to reequilibrate the chemical potential of water) and/or (ii) solute influx (to dissipate the solute concentration gradient). The contribution of the two fluxes to the recovery kinetics depends on the relative permeability of water and the solutes, which is be assessed by the quenching and recovery of the fluorescence signal of calcein.

Experiment:

The solute solution (200 mM betaine; 300 mM KPi, KCl, NaCl *after* mixing plus 90 mM KPi) and the vesicle solution (pre-equilibrated with 90 mM KPi) were loaded into syringes of a stopped-flow apparatus each, forced through a mixer (1:1 mixing ratio and 2 ms dead time) into the optical cell of the spectrometer. Calcein was excited at 495 nm and the emitted light, collected at 90°, was filtered by a Schott long-pass filter (cut-off wavelength at 515 nm) and detected by a photomultiplier tube (Hamamatsu R6095) with 10 µs time resolution. The voltage of the photomultiplier was automatically selected and kept constant during each set of experiments. The fluorescence intensity kinetics after the osmotic shock was recorded with logarithmically spaced time points to better resolve faster processes. For noise reduction, multiple acquisitions ($n \geq 3$) were performed for each experimental condition.

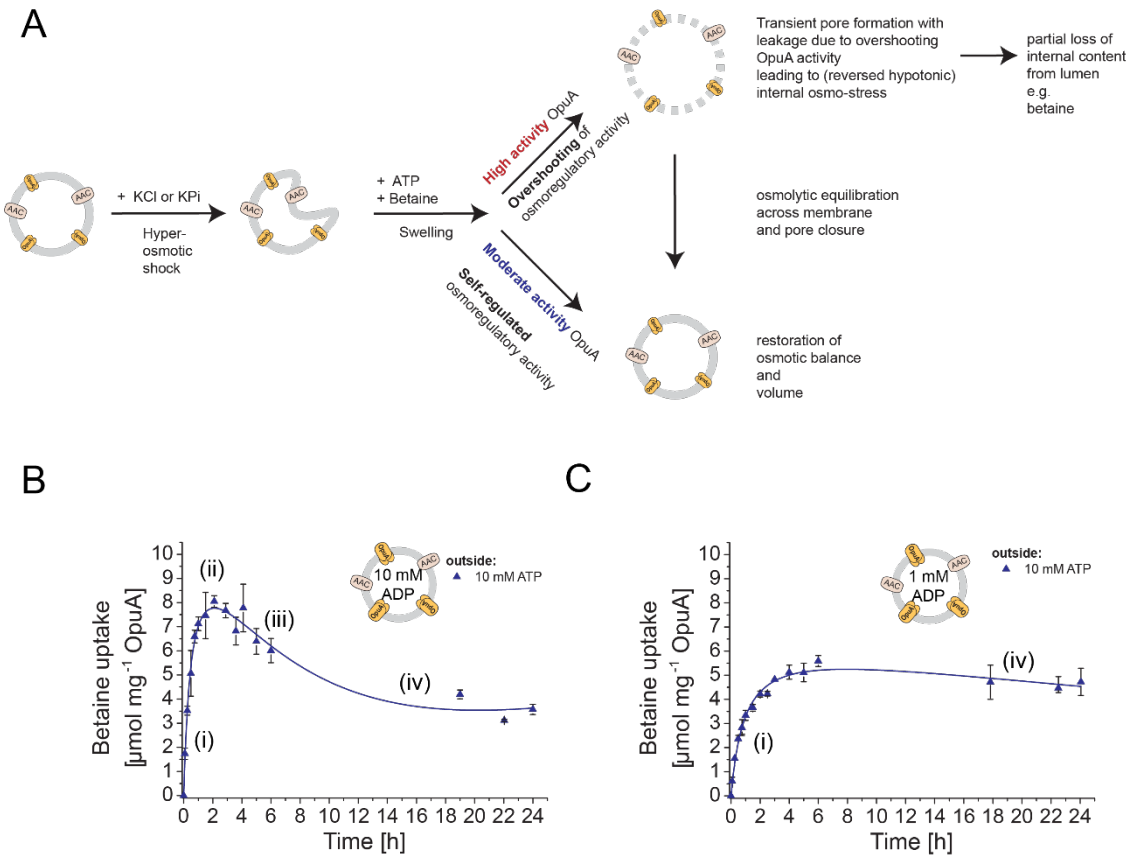
Upon osmotic upshift the vesicles shrink, which is seen as decrease in F(t)/F(0). Permeable solutes will diffuse through the membrane and gradually restore the volume of the vesicles, which is seen as an increase in F(t)/F(0) at later times. We observe a restoration of volume with NaCl and KCl in vesicles in which both OpuA and AAC (**Supplementary Figure *5*A**), AAC (**Supplementary Figure *5*B**) or OpuA (**Supplementary Figure *5*C**) was reconstituted, but not or very little in empty liposomes (**Supplementary Figure *5*D,E**). Betaine or KPi did not elicit any dequenching, showing that these osmolytes are membrane impermeable irrespective of whether AAC or OpuA is present. The difference between KPi and KCl suggests that the vesicles are somewhat permeable for chloride ions when AAC or OpuA is incorporated in the membrane. Fluorescence was monitored at an excitation wavelength of 495 nm.



Supplementary Figure 5: Membrane permeability of (proteo-)liposomes. The membrane permeability of different types of calcein-loaded vesicles was assessed by osmotic upshift: (A) Proteoliposomes with reconstituted AAC and OpuA. (B) Proteoliposomes with reconstituted AAC. (C) Proteoliposomes with reconstituted OpuA. (D) Liposomes that went through a mock reconstitution, *i.e.* vesicles were destabilized by detergent, but no membrane protein was added, followed by removal of detergents, and washing of the vesicles. (E) Empty liposomes without any detergent treatment. Vesicles were loaded with 50 mM KP_i (pH 7.0) plus 20 mM Na-calcein (a self-quenching concentration) and stored in iso-osmotic buffer (90 mM KPi, pH 7.0). During the stopped flow measurement, the vesicle suspension is rapidly mixed with a hyperosmotic solution with the molecule of interest in 90 mM KP_i (pH 7.0). The hyperosmotic solutions were composed of 90 mM KPi, pH 7.0 plus 200 mM betaine (blue) or 300 mM KP_i (gray) or 300mM NaCl (green) or 300 mM KCl. Lipid composition of the vesicles was DOPE:DOPC:DOPG:TOCL=50:38:12:3 (mol%). The lipid:protein ratio was 200 (*w/w*) for each of the reconstituted membrane proteins. The measurements were performed at 30 °C.

Explanation of the transient betaine uptake through unconstrained OpuA activity

Exposure of the **Vesicles B** to hypertonic conditions (e.g. 300 mM KCl) shrinks the vesicles and activates OpuA, because the internal ionic strength reaches threshold values. The uptake of betaine, driven by the ATP supplied by AAC, will increase the internal osmolality and the vesicles will swell again; too much uptake of betaine can cause lysis of the vesicles. The activity of OpuA is dependent on the concentration of ATP and is set by the initial ratio of $[ATP_{ext}]/[ADP_{in}]$, see also **Supplementary Table *4***. At ATP concentrations > $K_{m,ATP}$ of OpuA, the turnover of OpuA is so high, that the self-regulated ionic-strength dependent gating mechanism does not suffice to prevent overaccumulation. As the **Vesicles B** will also accumulate phosphate ions from the hydrolysis of ATP to ADP, the internal osmolality will not only increase by the betaine uptake but also by the phosphate accumulation. When the internal osmotic pressure becomes too high transient pores may form[17] and lower the internal pressure, which is seen as decrease in betaine concentration over time (**Figure 3D** and **3F**; **Supplementary Figure *6*B**). At low nucleotide concentrations, that is below $K_{m,ATP}$ of OpuA, the activity of OpuA is reduced and the increase in internal osmotic pressure is moderate and the vesicles stay intact (**Figure 3E**; **Supplementary Figure *6*C**). We note that in living cells OpuA activity can rupture cells if, in addition to gating by ionic strength, a regulatory mechanism involving the second messenger cyclic-di-AMP is not in place.[4,18]



Supplementary Figure 6: Explanation of the (transient) betaine uptake in hyperosmotically stressed Vesicles B. (A) Schematic representation of the different stages of the vesicle morphology upon osmotic upshift at high and low OpuA activity. Time dependence of betaine accumulation at high (B) and low (C) OpuA activity: (i) initial fast uptake of betaine, (ii) reaching of critical point where the osmotic pressure reverses and transient pores are formed, (iii) release of betaine down the concentration gradient, possibly through pulsating swell-burst cycles, (iv) a new steady state osmotic gradient is reached after several hours. On a general note, the observed uptake curves are the bulk average of a polydisperse suspension of proteoliposomes that is inhomogeneous in terms of size and protein reconstitution. Individual vesicles might behave differently.

Retention of betaine when vesicles are not under built-up of internal osmotic pressure as a result of massive accumulation of betaine

The Vesicles B can retain a small amount of accumulated betaine without significant leakage over time: the uptake of 10 µM [^{14}C]-betaine is complete (all radiolabel is taken up by the vesicles) and retained for at least 6 h, showing that the membranes are well sealed when the vesicles are not osmotically challenged by the overaccumulaton of betaine and phosphate. Thus, a high initial uptake activity of betaine (as facilitated by the constant ADP/ATP exchange of the AAC) is not damaging the vesicles but rather the massive uptake of betaine at higher concentrations (cf. **Figure 3D** and **F**, main text) is causing osmotic effects.

A

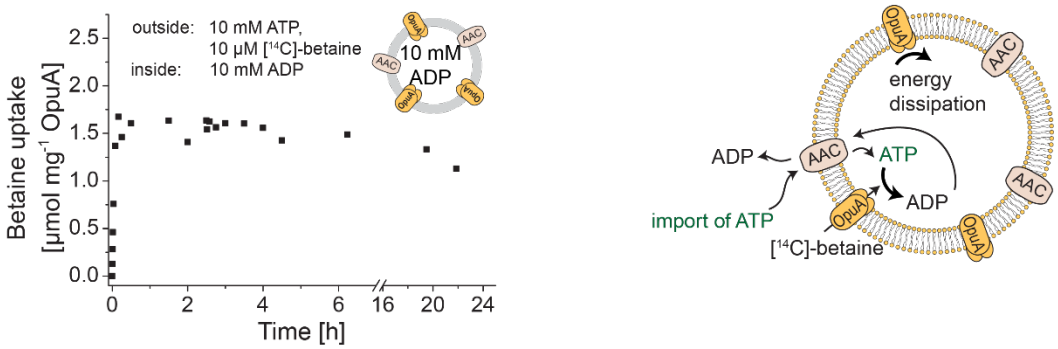


Figure 7: Uptake and retention of betaine by Vesicles B. (A) AAC-mediated uptake of 10 µM *[*^{14}*C]*-betaine is complete and retained for at least 6h. Betaine uptake assays were carried out in 50 mM KP_i pH 7.0, 300 mM KCl, 2 mM EDTA, 10 µM [^{14}C]-betaine plus 10 mM external Na^+-ATP at T = 30 °C. Proteoliposomes were loaded with 10 mM Mg^{2+}-ATP, 50 mM KP_i pH7.0 and used at a lipid concentration of 2 gL^{-1}.

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