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Synthetic vesicles for metabolic energy conservation

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Synthetic vesicles for metabolic energy conservation

Tjeerd Pols

2020

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university of
 groningen

Synthetic vesicles for metabolic energy conservation

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

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Chapter 1

General introduction on ATP-regenerating systems & the breakdown of arginine

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Cell fueling systems

All known forms of life use two forms of energy currency: ATP and electrochemical ion gradients. The amount of free energy released upon hydrolysis of ATP to ADP plus inorganic phosphate is the same as that of other nucleoside triphosphates such as GTP, CTP, UTP or TTP, but ATP (and to a lesser extent GTP) is predominantly used when chemical energy needs to be coupled to endergonic reactions or processes (i.e. to shift the equilibrium). The energy stored in ATP is given by the phosphorylation potential (ΔG_p or $\Delta G_p / F$):

$$\Delta G_p = \Delta G^{o'} + 2.3RT \log \frac{[\text{ADP}][\text{Pi}]}{[\text{ATP}]} \quad (\text{kJ/mol}) \quad (1)$$

$$\text{or: } \frac{\Delta G_p}{F} = \frac{\Delta G^{o'}}{F} + \frac{2.3RT}{F} \log \frac{[\text{ADP}][\text{Pi}]}{[\text{ATP}]} \quad (\text{mV})$$

Similarly, electrochemical proton (or sodium) ion gradients are most often used to drive membrane-bound processes, even though other types of ion and solute gradients exist. The F_0F_1 -ATP synthase/hydrolase interconverts the free energy of the phosphorylation potential into an electrochemical proton gradient, hereafter referred to as proton motive force (Δp):

$$\Delta p = \Delta \Psi + \frac{2.3RT}{F} \log \frac{[\text{H}^+]_{\text{in}}}{[\text{H}^+]_{\text{out}}} = \Delta \Psi - Z\Delta \text{pH} \quad (\text{mV}) \quad (2)$$

where $2.3RT/F$ equals 58 mV (at $T = 298 \text{ K}$) and is abbreviated as Z ; F is the Faraday constant, R the gas constant and T is the absolute temperature. $\Delta G^{o'} = -30.5 \text{ kJ/mol}$, and typically ΔG_p ranges from -50 to -65 kJ/mol (or $\Delta G_p/F$ varies from -520 to -670 mV).

Respiratory organisms use the F_0F_1 -ATP synthase to form ATP, whereas fermentative bacteria use the enzyme to hydrolyze part of their ATP obtained in catabolic reactions to generate an electrochemical ion gradient. At thermodynamic equilibrium, the phosphorylation potential equals the proton motive force times the number of protons (n) translocated per ATP. In formula:

$$\frac{\Delta G_p}{F} = n\Delta p \quad (3)$$

where $\Delta G_p/F$ is the phosphorylation potential and Δp is the proton motive force. The number of protons is determined by the c-ring stoichiometry of ATP synthase/hydrolase and varies from 2.7 to 5, depending on the specific enzyme¹. Some organisms exploit an F_0F_1 -ATP synthase/hydrolase that translocates sodium ions instead of protons, hence the formation or utilization of a sodium motive force (Δs). In addition, most forms of life exploit so-called sodium-proton antiporters to interconvert Δp and Δs .

The F_1F_0 -ATP synthase complex is one of the engineering masterpieces in the cell. We briefly discuss two important aspects of the complex, first the c-ring stoichiometry and second the regulation. The architecture of the c-ring, that is, specifically the copy-number of the c-subunit differs per organism from 8 copies for bovine mitochondria to 15 copies in *Spirulina platensis*^{2,3}. This leads to different proton-to-ATP ratios (Equation 3). From an engineering point of view the high-speed gear (low copy-number) works well in organisms that are continuously exposed to a high proton motive force, like in the bovine mitochondria. A high copy number leads to a high torque gear, essential when the proton motive force is low, or variable⁴.

Because the magnitude of the Δp and ΔG_p varies and a cell needs both forms of metabolic energy above some threshold value, it is important to have regulation in place to restrict the directionality of operation. An important regulator of the bacterial F_1F_0 -ATP synthase complex is the ϵ subunit. Structural data for this domain exists for two distinct conformations in different organisms^{5,6}. Tsunoda et al. have used cross-links to trap the ϵ subunit in both of these conformations in *E. coli*⁷. They have shown that in one conformation the synthase works in both directions, whereas in the other conformation the synthesis of ATP remains functional but the ATP hydrolysis is inhibited. Meyrat and von Ballmoos have shown that high ATP/ADP ratios inhibit the ATP synthesis, preventing the proton motive force to be drained completely⁸. These two regulatory mechanisms prevent futile cycling of the ATPase in either direction.

In heterotrophs, the oxidation of organic carbon yields CO_2 plus reducing equivalents such as NADH and FADH_2 . The subsequent oxidation of NADH and FADH_2 results in the formation of an electrochemical proton gradient by the respiratory chain. The usage of the Δp by the F_0F_1 -ATP synthase results in the synthesis of ATP, and the overall process is known as oxidative phosphorylation. This route to Δp and ATP formation is complex and requires numerous enzymes and cofactors. Nature offers alternative mechanisms to conserve metabolic energy through simple metabolic conversion (deamination of amino acids, oxidation of carboxylic acids) or the use of light. In the following sections we discuss a number of simple systems, which can be used as alternatives to oxidative phosphorylation for the synthesis and homeostasis of ATP.

Arginine breakdown pathway. Deamination of arginine yields citrulline plus NH_4^+ , which is catalyzed by the enzyme arginine deiminase (Fig. 1A). Subsequent phosphorolysis of citrulline by ornithine transcarbamoylase yields ornithine plus carbamoyl phosphate (carbamoyl-Pi), a reaction that is thermodynamically unfavorable (see below) but proceeds when the reaction products are drained. Carbamate kinase converts carbamoyl-Pi plus ADP into CO_2 , NH_4^+ and ATP and thereby conserves a large fraction of energy dissipated in the breakdown of the amino acid. Since the

Figure 1: Arginine breakdown pathway. Metabolic energy conservation by breakdown of arginine. (A) Schematic of the arginine breakdown pathway. ADI = arginine deiminase; OTC = ornithine transcarbamoylase; CK = carbamate kinase; AOA = arginine/ornithine antiporter. H_2O , H^+ and inorganic phosphate are not shown. (B) Structures of arginine, citrulline and ornithine at pH 7.

Table 1: Overview of decarboxylation systems

Substrate	Product	Transport mechanism	Ref.
Malonate ²⁻	Acetate ¹⁻	Electrogenic Na ⁺ pump	9
Oxaloacetate ²⁻	Pyruvate ¹⁻	Electrogenic Na ⁺ pump	10
Succinate ²⁻	Propionate ¹⁻	Electrogenic Na ⁺ pump	11
Oxalate ²⁻	Formate ¹⁻	Antiport	12
Malate ²⁻ or H-Malate ¹⁻	Lactate ¹⁻ or Lactic acid	Antiport or H-malate ⁻ uniport plus lactic acid diffusion	13-15
Arginine ¹⁺	Agmatine ²⁺	Antiport	16
Glutamate ¹⁻	γ -amino butyric acid ⁰	Antiport	17,18
Histidine ⁰	Histamine ¹⁺	Antiport	19
Lysine ¹⁺	Cadaverine ²⁺	Antiport	20
Ornithine ¹⁺	Putrescine ²⁺	Antiport	20
Tyrosine ⁰	Tyramine ¹⁺	Antiport	21

Antiport refers to the exchange of the indicated substrate and product. The net predominant charge of the molecules at pH 7 is indicated.

substrate arginine and product ornithine are structurally related (Fig. 1B), they can be transported by one and the same protein via a so-called antiport mechanism²². For every molecule of arginine imported, one molecule of ATP is produced, while the product ornithine is exchanged for arginine; NH₃ (formed from NH₄⁺) and CO₂ can passively diffuse out.

This property of coupling substrate and product fluxes is also possible in many other pathways and aids in keeping the reaction networks away from equilibrium. A pathway similar to that of arginine breakdown can for example be constructed by using the enzymes that convert agmatine into putrescine, CO₂ and 2NH₄⁺, which also yields one ATP per substrate metabolized. In the second part of this chapter, we describe properties

of the arginine deiminase pathway, which encodes the enzymes for arginine breakdown in cells.

Decarboxylation pathways. The free energy released in the decarboxylation of dicarboxylic acids and amino acids is around -20 kJ mol^{-1} (Table 1)²³, which is too little to directly make ATP from ADP plus inorganic phosphate (this requires between -50 and -65 kJ mol^{-1}). The free energy change of a decarboxylation reaction can be stored in the form of an electrochemical ion gradient, which subsequently can be used to synthesize ATP (Equation 3). Biochemical studies of decarboxylation reactions have shown two different mechanisms of energy conservation. In the first, decarboxylation energy is directly converted into an electrochemical Na^+ gradient (Fig. 2A), as first shown for oxaloacetate

Figure 2: Decarboxylation pathways. Metabolic energy conservation by decarboxylation of carboxylic acids (and amino acids, see Table 1). **(A)** Schematic of the oxaloacetate decarboxylase Na^+ pump. For every molecule of oxaloacetate converted into pyruvate, 2 Na^+ ions are pumped out, while one H^+ is imported. The system thus generates an electrochemical sodium gradient ($\Delta\Psi$ plus ΔpNa) and in theory a pH gradient inside acid relative to the outside. Since the outside volume is typically very large, the inverse ΔpH will only be formed if the cell density is high and the external buffering capacity is low. B = Biotin. **(B)** Schematic of the malolactic fermentation pathway. The decarboxylation of malate consumes a H^+ , while its product can be either exchanged for malate (top) or diffuse passively across the membrane (bottom). Both malate $^{2-}$ /lactate $^{1-}$ exchange (top) and H-malate $^{1-}$ uniport with lactic acid diffusion (bottom) generate a $\Delta\Psi$ (inside negative relative to the outside) and ΔpH (inside alkaline relative to the outside).

decarboxylation by Peter Dimroth¹⁰. In the second mechanism, the substrate is decarboxylated and the substrate and product are exchanged across the membrane (Fig. 2B)^{12,13}.

Since the substrate and product carry a different net charge (Table 1), the antiport reaction generates a membrane potential. The chemistry of the decarboxylation reaction requires a proton, hence the formation of a pH gradient when the reaction is performed in confinement, i.e. inside a vesicle system. In a variation on this mechanism, it was demonstrated that monoanionic malate is taken up by uniport and the formed lactic acid leaves the vesicles by passive diffusion (Fig. 2B). In general, biological membranes are highly permeable for weak acids and passive fluxes are considerable, even when the ambient pH is 2-3 pH units higher than the pK_A of the relevant conjugate acid-base pair²⁴. The energetics of the antiport and uniport is the same, but kinetically it can be advantageous to use an antiport mechanism as the product gradient contributes to the driving force for the influx of substrate and *vice versa*.

Artificial photosynthetic cells. Numerous groups have co-reconstituted F_0F_1 -ATP synthase with bacteriorhodopsin to control the synthesis of ATP by light. A disadvantage of this system is that the orientation of the proteins in the membrane is difficult to control. Recently, more advanced systems have been built with the aim of maintaining and controlling the electrochemical proton gradient. Shin and colleagues used the ATP synthase with two photoconverters, a photosystem II and proteorhodopsin²⁵. The three proteins were reconstituted in small lipid vesicles (“artificial organelles”) with the F_1 domain of the ATP synthase on the outside (Fig. 3). Upon activation of photosystem II by red light, protons are pumped into the vesicles (the interior becomes positive and acidic) and the Δp drives the synthesis of ATP. Activation of proteorhodopsin by green light dissipates the Δp or even reverses the polarity of the electrochemical proton gradient, which impedes the synthesis of ATP. The artificial organelles were encapsulated in giant vesicles to provide them with ATP and drive endergonic reactions, such as pyruvate carboxylase-mediated carbon fixation and actin polymerization.

In another study, ATP synthase and bacteriorhodopsin were incorporated in small vesicles and used to drive protein synthesis in giant unilamellar

vesicles²⁶. Remarkably, part of the *de novo* synthesized bacteriorhodopsin and ATP synthase were integrated into the artificial photosynthetic organelle and thereby enhanced the energetic capacity of the system. The proteins are synthesized by the components of the PURE system, but the machinery (Sec, YidC) for insertion of proteins into the membrane is missing. It remains to be established how the membrane proteins are (spontaneously) inserted in the artificial organelle membrane.

Molecular rheostat. Bowie and colleagues have described a molecular rheostat that accounts for ATP demand through switching between an ATP-generating and non-ATP-generating pathway, according to the concentration of inorganic phosphate (Fig. 4)²⁷. The system is based on fourteen purified enzymes in a cell-free system and used to produce

Figure 3: Artificial photosynthetic cells. Schematic of artificial photosynthetic cell. Upon illumination, the vesicle synthesizes ATP by the coordinated activation of two complementary photoconverters (photosystem II, PSII and proteorhodopsin, PR) and an ATP synthase. PSII is activated by red light and acidifies the vesicle lumen, which allows the synthesis of ATP from ADP plus inorganic phosphate to take place on the outside. PR is activated by green light, which at low pH generates an electrochemical proton gradient, inside alkaline and negative, and thus impedes the synthesis of ATP. Figure reproduced with permission from ref. 25.

isobutanol from glucose in solution. The breakdown of glucose is branched at the level of glyceraldehyde phosphate dehydrogenase (GapDH) to keep the synthesis of NADPH and ATP stoichiometrically balanced. In brief, in one branch the glyceraldehyde-3-phosphate (G3P) is metabolized via a mutant GapDH and phosphoglycerate kinase (PGK), yielding ATP and NADPH. In the other branch G3P is converted via a non-phosphorylating glyceraldehyde dehydrogenase (GapN), which eliminates the production of ATP and only generates NADPH. The NADPH is used at the end of the pathway for the production of 2-ketoacid isobutanol. The relative flow through the ATP-generating branch is set by the concentration of inorganic phosphate, which is a substrate of GapDH but not of GapN. Hence, the rheostat responds to the depletion of ATP and restores the ATP level by switching between the branches.

Figure 4: Molecular rheostat to control the ATP and NADPH levels. Schematic of the operation of the molecular rheostat. Left panel: at low Pi concentrations and high levels of ATP, the GapN pathway is used which generates no additional ATP. Right panel: at high Pi concentrations (resulting from the hydrolysis of ATP), the mGapDH-PGK pathway is used to restore the ATP level. G3P = glyceraldehyde-3 phosphate; 3PG = 3-phosphoglycerate; 1,3-BPG = 1,3-bisphosphoglycerate. Figure reproduced with permission from ref. 27.

The arginine deiminase pathway

The arginine deiminase (ADI) pathway is responsible for arginine breakdown in cells, where it provides a source of metabolic energy in the form of ATP^{28,29} and protection from acidic environments by consuming protons^{30,31}. This is very clearly seen in the overall reaction equation, as three protons are consumed for every molecule of ATP formed:

As mentioned above, this reaction is performed by three cytosolic enzymes and usually coupled to a membrane-bound arginine/ornithine antiporter (Fig. 1A). In addition to protection from acidic environments, the pathway has also been linked to tolerance to salt- and temperature stress³² and to the presence of ethanol³³. The pathway is widely used in bacteria and archaea³⁴ but is also present in lower eukaryotes³⁵ and parts of it are even found in mammalian cells (based on our own genome searches, see Table S1, S2, S3 and S4, p. 36-43).

In protozoa the pathway has an important role in pathogenesis^{36,37}, in addition to its function in metabolic energy generation^{38,39}. By secreting arginine deiminase and ornithine transcarbamoylase into the external medium instead of keeping them in the cytosol, the concurrent depletion of arginine reduces the production of antiparasitic (and antimicrobial) nitric oxide in infected tissues^{36,37,40}. Additionally, it has been found that reduced arginine availability affects the production of cytokines by dendritic cells⁴¹, the proliferation of T cells⁴² and inhibits growth of intestinal epithelial cells⁴³.

We found copies of all ADI pathway genes in animals like *Drosophila* but did not find functional data of the pathway in these organisms (Table S1, S2, S3 and S4, p. 36-43). Arginine metabolism in mammalian cells is quite complex, as arginine is involved in synthesis of proteins, urea, creatine, polyamines, nitric oxide, proline, glutamate and agmatine⁴⁴. Mammalian cells use arginases instead of arginine deiminases, which catalyze the hydrolysis of arginine into ornithine and urea, instead of citrulline and ammonium ion. In addition, mammalian cells have only anabolic ornithine

transcarbamoylase (see below; ref. 45) and do not use carbamate kinase (our own genome searches). They utilize arginine/ornithine antiporters in their mitochondria, but make use of arginine uniporters in other membranes^{46,47}.

Interestingly, it has been found that some types of tumors become auxotrophic for arginine, as they lower or eliminate the expression of argininosuccinate synthase⁴⁸. Argininosuccinate synthase is responsible for the conversion of citrulline into argininosuccinate (which can then be broken down into arginine and fumarate), and its lowered expression has been linked to a faster growth rate in tumors⁴⁹. As the arginine auxotrophic tumors rely on the presence of extracellular arginine, treatments with pegylated arginase and pegylated arginine deiminase have been developed^{50,51} and studied in clinical trials⁴⁸.

The ADI pathway has also been studied in the context of the fermentation of e.g. wine, soy sauce and cheese. In wine and soy sauce, a reaction can occur at low pH between ethanol and *N*-carbamyl compounds (like urea, citrulline and carbamoyl-Pi) to form ethyl carbamate⁵². Since both wine and soy sauce contain arginine, the levels of citrulline and carbamoyl-Pi can be enhanced when bacterial species with an active ADI pathway are present⁵³⁻⁵⁵. It is therefore desirable to keep these species out of the fermentation process. In cheeses on the other hand, the formation of carbon dioxide and the release of ammonia from arginine breakdown is important. Carbon dioxide gas can lead to increased eye formation in the cheese, while the ammonia increases the pH and thus proteolysis⁵⁶. These combined effects can lead to more crack formation in the cheese, which can be undesirable in certain types of cheese.

Finally, regulation of the ADI pathway has been well studied in *Lactococcus lactis*. The pathway proteins are encoded by *arcA* (arginine deiminase), *arcB* (ornithine transcarbamoylase), *arcC* (carbamate kinase) and *arcD* (arginine/ornithine antiporter) and are found in the same operon. This *arc* operon in *L. lactis* contains two copies of *arcC* and *arcD* (due to gene duplication), the putative amino acid transaminase *arcT*⁵⁷ and an arginine tRNA ligase *argS*^{58,59}. The operon is regulated by the transcription factors CcpA and AhrC, which respond to the concentrations of a preferred sugar (usually glucose) and arginine, respectively^{60,61}.

Additionally, the transcription factor *argR* and an sRNA (*argX*) are located upstream of the *arc* operon and regulate the pathway in an arginine-dependent manner^{60,62}.

Arginine deiminase. Arginine deiminase (EC 3.5.3.6) is the first enzyme of the ADI pathway, which catalyzes the hydrolysis of arginine:

This reaction is very favorable, as it has an equilibrium constant of 1.2×10^6 , and the hydrolysis of arginine can be performed without an enzyme at high temperatures in highly acidic or alkaline conditions (although the main product is L-ornithine)⁶³.

The arginine deiminase from multiple organisms has been studied at various pH values, temperatures and buffer compositions (Table 2). Most proteins were studied between pH 6.0 and 7.4 at a temperature of either 25 or 37 °C in K⁺-MES, K⁺-HEPES or potassium phosphate (KPi). Kinetic parameters have been determined based on the detection of citrulline (through the color forming reaction with diacetyl monoxime; see ref. 64) or of ammonium (through the coupled enzyme assay with glutamate dehydrogenase, that converts ammonium, α-ketoglutarate and NAD(P)H into L-glutamate and NAD(P)⁺; see ref. 65). Most K_M values for arginine lie around 0.2 mM and the k_{cat} values are around 5 s⁻¹. Notable exceptions are the ones from *Lactococcus lactis* ATCC 7962 and *Mycoplasma arthritidis*. The enzyme from *L. lactis* has a K_M of 8.67 mM and a k_{cat} of 790 s⁻¹, which are both much higher than the values from the other organisms, but this could be due to the high temperature (60 °C) at which the enzyme was assayed⁶⁶. The *M. arthritidis* enzyme on the other hand has a much lower K_M (between 4 and 28 μM), but a higher k_{cat} (between 30 and 62 s⁻¹), indicating that it is more specific for arginine and a better catalyst⁶⁷.

Several structures of arginine deiminase have been published, from *Homo sapiens*⁶⁸, *Mycoplasma arginini*⁶⁹, *Mycoplasma penetrans*⁷⁰, *Pseudomonas aeruginosa*⁷¹ and *Streptococcus pyogenes*⁷² (PDB ID: 4N20, 1LXY, 4E4J, 2A9G and 4BOF). All these structures show a dimeric state, except for the one from *P. aeruginosa*, which shows the protein as a tetramer (Fig. 5A).

The structure from *P. aeruginosa* shows bound arginine, and Galkin et al. have described a reaction mechanism on the basis of their structure⁷¹.

According to Galkin et al. the hydrolysis reaction is initiated by the Cys⁴⁰⁶ residue making a nucleophilic attack on the carbon atom of the guanidinium group of arginine (Fig. 5B)⁷¹. This is followed by a proton being transferred from His²⁷⁸ to an amino group and cleavage of that amino group from the carbon atom (and thus formation of ammonia).

Figure 5: Structure of arginine deiminase. (A) Overview picture of arginine deiminase from *Pseudomonas aeruginosa* (PDB ID: 2A9G), showing a homo tetrameric state with the four subunits in different colors. Arginine is shown as a sphere model. (B) Zoom in of the box shown in panel A, indicating the amino acid residues important in the reaction mechanism in cyan (note that this structure has Cys⁴⁰⁶ mutated to Ala⁴⁰⁶). The right panel has been turned by 90 degrees compared to the left panel, as indicated by the arrow. Residues 24 to 40 and 157 to 163 are hidden; arginine is shown as a ball and stick model.

Table 2: Kinetic parameters for arginine deiminases

Organism	AA identity (%)	K_M (mM)	k_{cat} (1/s)
<i>Bacillus cereus</i>	57.1 ^a	0.09	4.4
<i>Burkholderia mallei</i>	35.0 ^a	0.09	1.3
<i>Escherichia coli</i>	38.0 ^a	0.32	3.2
<i>Giardia lamblia</i>		0.16	2.6
<i>Lactococcus lactis</i> ATCC 7962	100 ^a	8.67	790 ^b
<i>Mycoplasma arginini</i>	35.5 ^a	0.2	75 ^b
<i>Mycoplasma arthritidis</i>	37.5 ^a	0.004 0.028	30 ^b 62 ^b
<i>Mycoplasma hominis</i>	37.6 ^a	0.24	0.151
<i>Porphyromonas gingivalis</i>		1.2	0.22
<i>Pseudomonas aeruginosa</i>	34.8 ^a	0.14	6.3
<i>Pseudomonas plecoglossicida</i>	33.9 ^a	0.7 2.88	5.6 7.3 ^b
<i>Pseudomonas putida</i>	35.3 ^a	0.2	120 ^b
<i>Streptococcus pyogenes</i>	46.5 ^a	1.33	1.2 ^b

^a Percentage of amino acid identity with the ArcA protein from *Lactococcus lactis* IL1403. ^b Calculated from reported V_{MAX} and M_W .

Ammonia is then replaced by water, which is deprotonated by His²⁷⁸. The formed hydroxide ion attacks the carbon atom, after which it transfers its proton to Asp²⁸⁰ and cleaves the bond between the carbon atom and Cys⁴⁰⁶. Citrulline is then released from the protein, as well as the proton on Asp²⁸⁰, which according to Galkin et al. is released to the solvent⁷¹. The residues Asp¹⁶⁶, Arg¹⁸⁵, Gly²²⁴, Arg²⁴³ and His⁴⁰⁵ are all involved in stabilization of arginine, citrulline and/or the reaction intermediates.

Conditions	Expression host	Buffer	Assay	Ref.
pH 7.0, 25°C	<i>E. coli</i>	50 mM K-HEPES	Ammonium	73
pH 5.6, 25°C	<i>E. coli</i>	50 mM K-MES	Citrulline	73
pH 6.0, 25°C	Native	50 mM Bis-Tris-HCl	Citrulline	73
pH 7.5, 25°C	<i>E. coli</i>	50 mM K-HEPES	Citrulline	74
pH 7.2, 60°C	<i>E. coli</i>	100 mM KPi	Citrulline	66
pH 7.0, 37°C	Native	100 mM KPi	Citrulline	75
pH 7.2, 25°C pH 7.2, 37°C	Native	50 mM TES	Ammonium	67
pH 7.4, 37°C	<i>E. coli</i>	Not described	Ammonium	76
pH 9.5, 37°C	<i>E. coli</i>	50 mM CHES/HCl + 10 mM dithiothreitol	Citrulline	77
pH 5.6, 25°C	<i>E. coli</i>	50 mM K-MES	Citrulline	73
pH 7.4, 37°C pH 6.0, 37°C	<i>E. coli</i>	500 mM KPi	Citrulline	78
pH 6.0, 37°C	Native	1 M Na-acetate	Citrulline	79
pH 6.5, 37°C	<i>E. coli</i>	100 mM K-MES	Ammonium	80

Ornithine transcarbamoylase. The second enzyme of the ADI pathway is ornithine transcarbamoylase (EC 2.1.3.3), which catalyzes the cleavage of citrulline:

The equilibrium constant of this reaction is very unfavorable ($K_{eq} = 8.5 \times 10^{-6}$) and thus the reaction products need to be removed for substantial flux in the direction of carbamoyl-Pi plus ornithine. This is facilitated by carbamate kinase, which breaks down carbamoyl-Pi, and the arginine/ornithine antiporter, which exports L-ornithine (see below). A consequence of the low value of the equilibrium constant is that cells that utilize the arginine deiminase pathway build up high concentrations of citrulline ($> 20 \text{ mM}$)⁸¹.

Many bacteria have two copies of ornithine transcarbamoylase: a catabolic one catalyzing the reaction in the direction of carbamoyl-Pi plus ornithine, and an anabolic one which catalyzes the opposite reaction to form citrulline for arginine biosynthesis²⁹. In most cases, the anabolic enzymes also catalyze the catabolic reaction *in vitro*, except for the enzyme from *Pseudomonas aeruginosa*. This enzyme appears to be irreversible as it binds citrulline in a dead-end complex, strongly reducing the apparent maximum velocity^{82,83}. The catabolic ornithine transcarbamoylase from *P. aeruginosa* could be turned into an anabolic enzyme with only one mutation⁸⁴: the mutant ornithine transcarbamoylase has a strongly reduced cooperativity (from 4.2 to 1.4) and lower apparent K_M for carbamoyl-Pi, as well as an improved specific activity. This means that less carbamoyl-Pi is needed to saturate the enzyme, but it is more difficult for ornithine to bind to the enzyme when carbamoyl-Pi is already bound. When carbamoyl-Pi and ornithine are both bound, however, they are quickly converted into citrulline. These characteristics have also been observed for the anabolic ornithine transcarbamoylase from *E. coli*⁸⁴.

Most of the studied ornithine transcarbamoylases are anabolic enzymes (Table 3) and therefore K_M values for carbamoyl-Pi and ornithine and k_{cat} in the direction of citrulline synthesis are reported. Notable exceptions are the enzymes from *P. aeruginosa* and *Giardia lamblia*, for which the reactions in both directions have been studied. Here, the catabolic direction was made possible by the usage of arsenate instead of inorganic phosphate⁸⁵ or the addition of carbamate kinase to the reaction mixture⁸⁶. Carbamoyl-arsenate is a very unstable compound, which spontaneously falls apart in carbon dioxide, ammonium ion plus arsenate, thereby stimulating the reaction in the catabolic direction.

Many of the ornithine transcarbamoylases have been studied between pH 8.0 and 9.0, at a temperature of 30 or 37 °C in Tris-HCl buffer. Kinetic parameters have been determined by measuring citrulline formation, through the color-forming reaction with diacetyl monoxime⁶⁴. The K_M values for ornithine are around 0.2 mM, while the values for carbamoyl-Pi lie around 1 mM, that is, under conditions that the other substrate is present at saturating concentrations. The enzymes from *Enterococcus*

Figure 6: Structure of ornithine transcarbamoylase. (A) Overview picture of ornithine transcarbamoylase from *Lactobacillus hilgardii* (PDB ID: 2W37), showing a homo-hexameric state with the six subunits in different colors. C and N indicate the C- and N-terminal tails. The right panel has been turned by 90 degrees compared to the left panel, as indicated by the arrow. (B) Zoom in of the box shown in panel A, with the structure of *L. hilgardii* in green and of *Vibrio vulnificus* (PDB ID: 4JQO) in orange. The structure from *V. vulnificus* contains citrulline and inorganic phosphate, shown as sphere models.

Table 3: Kinetic parameters for ornithine transcarbamoylases

Organism	AA identity (%)	K _M (mM)		k _{cat} (1/s)	
		CP	Orn	CP	Orn
<i>Enterococcus faecalis</i>	35.1 ^a	0.058	36.4	-	48 ^b
<i>Escherichia coli</i>	50.6 ^a	0.2 0.24	2.4 0.86	1300 3450	1300 3450
<i>Giardia lamblia</i>	32.7 ^a	0.13	0.11	82	94.2
<i>Homo sapiens</i>	36.5 ^a	0.13	0.36	55 ^b	55 ^b
<i>Moritella abyssi</i>	34.6 ^a	1.1 - - 1 - 0.9	1.78 3.34 5.67 8 22.5 45	- - - - - -	235 382 546 694 750 690
<i>Mycobacterium bovis</i>	36.3 ^a	0.625	0.85	200 ^b	200 ^b
<i>Ovis aries</i>	36.5 ^a	0.087 0.033 0.25	0.33 0.25 0.45	860 ^b - -	860 ^b - -
<i>Pseudomonas aeruginosa</i>	50.8 ^a	-	0.8	-	2500 ^b
<i>Pseudomonas savastanoi</i>	31.8 ^a	0.09	1.1	-	49
<i>Pyrococcus furiosus</i>	41.6 ^a	0.1 0.1	0.1 0.1	370 500	370 500
<i>Saccharomyces cerevisiae</i>		-	1.6	-	52 ^b

^a Percentage of amino acid identity with the ArcB protein from *Lactococcus lactis* IL1403. ^b Calculated from reported V_{MAX} and M_W. CP = carbamoyl-Pi, Orn = ornithine, PTC = putrescine transcarbamoylase, aOTC = anabolic ornithine transcarbamoylase, cOTC = catabolic ornithine transcarbamoylase.

Conditions	Type	Expression host	Buffer	Assay	Ref.
pH 8.5, 37°C	PTC	<i>E. coli</i>	50 mM Tris-HCl	Citrulline	87
pH 8.0, 37°C pH 8.5, 37°C	aOTC	Native	150 mM Tris-HCl 50 mM TAPS	Citrulline	85 88
pH 8.0, 37°C	cOTC	<i>E. coli</i>	50 mM Tris-HCl	Citrulline	86
pH 7.7, 37°C	aOTC	<i>E. coli</i>	270 mM TEOA	Citrulline	89
pH 9.0, 5°C pH 9.0, 10°C pH 9.0, 15°C pH 9.0, 20°C pH 9.0, 25°C pH 9.0, 30°C	aOTC	<i>E. coli</i>	30 mM Tris-HCl	Citrulline	90
pH 8.5	aOTC	Native	50 mM EDTA-NaOH	Citrulline	91
pH 8.2, 37°C pH 8.6, 37°C pH 9.0, 37°C	aOTC	Native	51 mM DEOA + 100 mM MES + 51 mM N-ethyl-morpholine	Citrulline	92
pH 7.3	cOTC	Native	150 mM imidazole-HCl	Citrulline	84
pH 8.5, 37°C	aOTC	<i>E. coli</i>	50 mM TAPS	Citrulline	88
30°C 55°C	aOTC	<i>S. cerevisiae</i>	PIPES	Not described	93
pH 8.0, 30°C	aOTC	Native	50 mM Tris-HCl	Citrulline	94

faecalis and *Moritella abyssi* have a much lower affinity for carbamoyl-Pi, which seems to be temperature dependent in the case of *M. abyssi*. On the other hand, the K_M for ornithine hardly changes with increasing temperature^{87,90}. The k_{cat} values are between 55 and 370 s⁻¹, except for the enzymes from *Ovis aries* (860 s⁻¹)⁹² and *Escherichia coli* (1300 to 3450 s⁻¹)^{85,88}. The *E. coli* enzyme is clearly the fastest enzyme, without sacrificing much in terms of specificity.

Most of the published structures of ornithine transcarbamoylase are of the anabolic type, which all show a trimeric oligomeric state^{92,95-97} (PDB ID: 2OTC, 1OTH, 1FB5 and 4JQO). There are three structures of the catabolic type, which show a trimeric, hexameric and dodecameric oligomeric state^{86,98,99} (PDB ID: 3GRF, 2W37 and 1ORT). None of the catabolic structures have a bound ligand. Below we focus on the hexameric enzyme (Fig. 6A). The structure reveals an important role for the C-terminus in stabilizing the hexameric state, as deletion of the last six amino acids leads to a mixture of monomeric and tetrameric forms⁹⁸. Additionally, the structure revealed the presence of a metal-binding site that holds three subunits together, which involves a histidine that is conserved in all vertebrates. Since the structure has no bound ligand, we aligned one of the monomers with the anabolic enzyme from *Vibrio vulnificus*, which has citrulline and phosphate bound (Fig. 6B)⁹⁷. The structures align well, indicating that all monomers can bind the substrates. Other studies have shown that the anabolic ornithine transcarbamoylases bind carbamoyl-Pi before binding of ornithine^{85,88,100}; a binding mechanism has not yet been proposed for the catabolic enzymes.

Carbamate kinase. The last cytosolic enzyme of the ADI pathway is carbamate kinase (EC 2.7.2.2), which catalyzes the transfer of the phosphate group from carbamoyl-Pi onto Mg-ADP and the concomitant degradation of carbamoyl-Pi:

This reaction is favored in the direction of product formation with an equilibrium constant of 4.2×10^4 . The reaction without enzyme is unlikely. However, carbamoyl-Pi is a relatively unstable compound, which

decomposes readily in aqueous solutions; the mechanism of spontaneous breakdown depends on the pH of the solution¹⁰¹. At low pH, acid-catalyzed hydrolysis causes the formation of carbon dioxide, ammonium ion plus inorganic phosphate, while at alkaline pH it decomposes into cyanate plus inorganic phosphate¹⁰¹. The half-life of carbamoyl-Pi at a pH between 7.5 and 9.0 is around 50 min at 30 °C, but it drops to 5 min at 50 °C¹⁰².

The instability of carbamoyl-Pi has led to the idea that enzymes involved in carbamoyl-Pi synthesis and breakdown can perform metabolic channeling, so that intermediary metabolites (like carbamoyl-Pi) are always bound to a protein, instead of freely diffusing in solution¹⁰³. Binding of carbamoyl-Pi to either ornithine or aspartate transcarbamoylase has been shown to strongly reduce its rate of thermal decomposition¹⁰⁴. Metabolic channeling was shown for the carbamoyl-Pi synthetase (which uses 2 molecules of ATP, bicarbonate plus ammonia or glutamine to form carbamoyl-Pi) and the ornithine transcarbamoylase of *Pyrococcus furiosus*¹⁰² or the aspartate transcarbamoylase of *Aquifex aeolicus*¹⁰⁵, *Saccharomyces cerevisiae*¹⁰⁶ and *Neurospora crassa*¹⁰⁷. Channeling has also been observed in the mammalian CAD multienzyme complex, which catalyzes the same reactions¹⁰⁸.

In comparison to arginine deiminase and ornithine transcarbamoylase, carbamate kinase has not been studied as extensively (Table 4). Most proteins were studied between pH 6.0 and 8.0 at a temperature of 37 °C in Tris-HCl buffer. The kinetic parameters were determined by measuring the formation of ATP, citrulline or ammonium, by either using radiolabeled compounds or coupled enzyme assays. In the first case, a radiolabeled substrate was enzymatically converted, after which it was separated from the (radiolabeled) product. The difference in concentrations before and after conversion was used to determine the enzymatic activity. In the second case, assays with either hexokinase/glucose-6-phosphate dehydrogenase (converting ATP, D-glucose plus NAD⁺ into ADP, gluconate-6-phosphate and NADH; see ref. 109) or with luciferase (converting luciferin and ATP into light pulses; see ref. 110) were used to determine ATP formation. For ammonium, a coupled assay with glutamate dehydrogenase has been used, which converts ammonium, α -ketoglutarate plus NAD(P)H into L-glutamate and

Table 4: Kinetic parameters for carbamate kinases

Organism	AA identity (%)	K _M (mM)			k _{cat} (1/s)	
		CP	ADP	ATP	CP	ADP
<i>Enterococcus faecalis</i>	54.8 ^a	1.4	0.04	0.62	-	-
<i>Giardia lamblia</i>	41.9 ^a	0.085	0.07	0.035	319	319
<i>Neurospora crassa</i>		-	-	1 - 2	-	-
<i>Pyrococcus furiosus</i>	44.8 ^a	-	0.001	0.017	-	-
<i>Streptococcus pyogenes</i>	53.5 ^a	0.65	0.72	-	1.3 ^b	1.3 ^b
<i>Trichomonas vaginalis</i>	45.7 ^a	0.13	-	-	1.3 ^b	-

^a Percentage of amino acid identity with the ArcC1 protein from *Lactococcus lactis* IL1403. ^b Calculated from reported V_{MAX} and M_w. CP = carbamoyl-Pi.

Table 5: Kinetic parameters for the arginine/ornithine antiporters

Organism	Protein	AA identity (%)	K _M (μM)		
			Arg	Orn	Cit
<i>Lactobacillus brevis</i>	ArcD	46.1 ^a	5.8	15	-
	ArcE1	-	-	27	8.5
	ArcE2	-	0.4	15	-
<i>Lactococcus lactis</i> MG1363	ArcD1	68.3 ^a	5	1	-
	ArcD2	92.0 ^a	4	29	-
<i>Streptococcus pneumoniae</i>	ArcE	-	0.6	1	-

All proteins were expressed in *L. lactis* and assayed with ¹⁴C-L-arginine, ¹⁴C-L-ornithine or ¹⁴C-L-citrulline in 100 mM KPi, pH 6.0 at 30 °C. ^a Percentage of amino acid identity with the ArcD2 protein from *Lactococcus lactis* IL1403. Arg = arginine, Orn = ornithine, Cit = citrulline.

Conditions	Expression host	Buffer	Assay	Ref.
pH 7.5, 35°C	Native	50 mM Tris-HCl	ATP	111
pH 7.5, 25°C	<i>E. coli</i>	50 mM Tris-HCl	ATP	112
pH 8.2, 37°C	Native	50 μ M Tris-HCl	Citrulline	113
pH 8.0, 37°C	<i>E. coli</i>	100 mM Tris-HCl	ATP	114
pH 6.5, 37°C	<i>E. coli</i>	100 mM K-MES	Ammonium	80
pH 6.0	<i>E. coli</i>	50 mM NaPi + 150 mM NaCl	Not described	115

V_{MAX} (nmol min ⁻¹ mg ⁻¹)			K_i (mM)		Transmembrane helices	Ref.
Arg	Orn	Cit	Cit	Arg		
63 - 12	152 13 20	- 33 -	>10 - 1.9	- 0.15 -	11 13 13	116
30 22	45 128	- -	0.5 -	- -	14 13	117
9	14	-	0.1	-	12	118

NAD(P)⁺ (see ref. 65). Most of the K_M values for carbamoyl-Pi, ADP and ATP lie in the low μM range. Notable exceptions are the enzyme from *Streptococcus pyogenes*, that has a K_M for carbamoyl-Pi and ADP of 0.65 and 0.72 mM, respectively⁸⁰, and the enzyme from *Enterococcus faecalis*, which has a K_M for carbamoyl-Pi of 1.4 mM¹¹¹. The k_{cat} values are either very low at 1.3 s⁻¹ for *S. pyogenes*⁸⁰ and *Trichomonas vaginalis*¹¹⁵ or quite high at 319 s⁻¹ for *Giardia lamblia*¹¹².

Figure 7: Structure of carbamate kinase. (A) Overview picture of carbamate kinase from *Enterococcus faecalis* (PDB ID: 2WE5), showing a homodimeric state with the two subunits in different colors. Mg-ADP is shown as a sphere model. (B) Zoom in of the box shown in panel A, indicating the amino acid residues important in the reaction mechanism in cyan. The right panel has been turned by 90 degrees compared to the left panel, as indicated by the arrow. Residues 227 to 234 and 289 to 304 are hidden in the left panel; residues 152 to 156 are hidden in the right panel; Mg-ADP is shown as a ball and stick model.

Structures of carbamate kinase have been published from *E. faecalis*^{119,120}, *G. lamblia*^{112,121}, *Mycoplasma penetrans*⁷⁰ and *P. furiosus*¹¹⁴, which all show a dimeric oligomeric state (PDB ID: 1B7B and 2WE5, 3KZF and 4JZ7, 4AXS, and 1E19). The structures from *E. faecalis* and *P. furiosus* have ADP bound as a ligand, while the one from *G. lamblia* has AMP-PNP bound. Here, we focus on the structure from *E. faecalis* (Fig. 7A) that has also been published with one sulfate and two fixed water molecules bound, which mimic the binding of a carbamoyl-Pi molecule¹²⁰ (PDB ID: 2WE4).

Additionally, Ramón-Maiques et al. proposed a simple reaction mechanism, in which Lys¹²⁸ and Lys²⁰⁸ play a key role in carbamoyl-Pi binding and catalysis (Fig. 7B)¹²⁰. The amino groups of Gly¹¹ and Asn¹² can bind the phosphoryl group that needs to be displaced by 3.5 ångström between the ADP bound and the sulfate/water molecule bound structures, so it is likely that they are responsible for the transfer of the phosphoryl group. Finally, the Asp²¹⁰ and Lys²⁷¹ residues seem to be involved in stabilization of the ADP molecule.

Arginine/ornithine antiporter. The final enzyme of the ADI pathway is the arginine/ornithine antiporter. Although it is not directly involved in arginine breakdown, it supplies the cell with arginine from the external medium and exchanges it for internal ornithine in a one-to-one stoichiometry²²:

After the initial studies on the antiporters from *Lactococcus lactis* and *Pseudomonas aeruginosa*^{22,122,123}, little work has been reported on arginine/ornithine antiporters. Most studies focused on the effects of deleting the *arcD* or *arcE* genes (see below) on a bacterium's virulence or survival in acidic environments. In *Staphylococcus aureus*, the deletion of *arcD* did not lead to significant changes in the pH of its biofilm (although the ammonia production decreased), nor did it affect formation of the biofilm or the bacterium's survivability in mice¹²⁴. In *Streptococcus pneumoniae* on the other hand, deletion of *arcE* led to impairment of the bacterial capsule, which is normally used to protect the bacterium from phagocytosis^{125,126}. Gupta et al. ruled out that this impairment was due

to a lack of arginine transport and hypothesized that the arginine/ornithine antiporter might be (indirectly) responsible for anchoring of the capsular polysaccharides to the bacterial surface¹²⁵. In *Streptococcus gordonii*, the export of ornithine enabled formation of a dual-species biofilm with *Fusobacterium nucleatum*¹²⁷. Deletion of *arcE* also led to less export of ammonia and a clear decrease in external pH, in contrast to the finding in *S. aureus*^{124,127}. Finally, in *Streptococcus suis*, arginine is an essential amino acid, thus knockout of the arginine/ornithine antiporter encoded by *arcE* compromises growth and the bacterium is no longer protected against acid stress¹²⁸.

More recently, the arginine/ornithine antiporters of *L. lactis*, *S. pneumoniae* and *Lactobacillus brevis* have been characterized in whole cells (Table 5)¹¹⁶⁻¹¹⁸. The K_M and maximal rates for arginine and ornithine of the different proteins were fairly similar, and the ArcE protein from *S. pneumoniae* could perform citrulline/ornithine antiport as efficiently as arginine/ornithine antiport¹¹⁸. The ArcD1 protein from *L. lactis* and the ArcD and ArcE2 proteins from *L. brevis*, however, could not perform efficient citrulline/ornithine antiport^{116,118}. In contrast, the ArcE1 protein

Figure 8: Models for the arginine breakdown pathway with and without citrulline/ornithine antiport. (A) Simplified schematic of the arginine breakdown pathway, see Fig. 1A for more details. (B) Simplified schematic of the arginine breakdown pathway, including the citrulline/ornithine antiport reaction (here represented by ArcE). Ornithine transcarbamoylase and the arginine/ornithine antiporter compete for internal citrulline. ADI = arginine deiminase; OTC = ornithine transcarbamoylase; CK = carbamate kinase; AOA = arginine/ornithine antiporter. NH_4^+ , CO_2 , H_2O , H^+ and inorganic phosphate are not shown. Adapted from ref. 116.

from *L. brevis* could not perform efficient arginine/ornithine antiport, but showed effective citrulline/ornithine antiport¹¹⁶.

The presence of efficient citrulline/ornithine antiport can lead to two additional physiological functions for the ADI pathway. If internal citrulline is exported, which has been observed in some lactic acid bacteria (e.g. ref. 129) the citrulline may not build up to very high levels (Fig. 8)¹¹⁶. This overflow mechanism balances the favorable equilibrium constant of arginine deiminase with the unfavorable one of ornithine transcarbamoylase and serves as an energy uncoupling mechanism, which may offer protection in acidic environments because arginine to citrulline conversion still produces an ammonium ion^{130,131}.

Figure 9: Structure of arginine/agmatine antiporter AdiC. Overview picture of arginine/agmatine antiporter AdiC from *Escherichia coli* (PDB ID: 5J4N), showing a homodimeric state with the two subunits in different colors. Agmatine is shown as a sphere model. The top panel shows the membrane plane view, while the bottom panel shows the periplasmic view (it has been turned 90 degrees compared to the top panel, as indicated by the arrow).

The ArcD and ArcE proteins share no sequence identity and belong to different protein families. While ArcD proteins belong to the basic amino acid/polyamine antiporter (APA) family (TC 2.A.3.2) in the amino acid/polyamine/organocation (APC) superfamily, ArcE proteins belong to the basic amino acid antiporter (ArcD) family (TC 2.A.118) in the ion transporter (IT) superfamily¹¹⁶. Most proteins in the APC superfamily contain 12 transmembrane helices and published protein structures all show the ‘LeuT fold’^{132,133}. No structures of arginine/ornithine antiporters have been resolved yet, and therefore we show the structure of the arginine/agmatine transporter AdiC, which has 27.6% amino acid identity with ArcD2 from *L. lactis* IL1403 (Fig. 9)¹⁶. ArcD2 and ArcD1 from *L. lactis*, however, have one and two additional transmembrane segment(s), respectively, when compared to AdiC (Table 5). It is possible that the ArcD proteins primarily function as arginine/ornithine antiporters, while the ArcE proteins are citrulline/ornithine antiporters; there are also proteins that can perform both functions, probably because arginine and citrulline are structurally very similar, see Fig. 1B). The ArcD and ArcE transporters are examples of convergent evolution, as the proteins evolved independently of each other, but still arrived at very similar functions¹¹⁶.

Outline of this thesis

In this thesis, I describe the purification and characterization of the enzymes of the arginine deiminase pathway from *Lactococcus lactis* IL1403, and the use of this pathway as an ATP-regenerating system in synthetic vesicles. In Chapter 2, I describe the kinetic characterization of the individual enzymes and determine the effect of conditions that are relevant to the construction of the vesicles and ultimately synthetic cells. In Chapter 3, I present the reconstitution of the ATP-regenerating system together with the ATP-driven glycine betaine transporter OpuA. We show that this system can maintain a far-from-equilibrium metabolic state for many hours, as well as a basic level of physicochemical homeostasis. Finally, in Chapter 4, I discuss modules that are important for building synthetic cells, as well as possible improvements to our system for metabolic energy conservation and the challenges we faced in constructing it.

Methods

Equilibrium constants. Equilibrium constants were calculated for pH 7.0 and an ionic strength of 0.1 M using eQuilibrator 2.2 (equilibrator.weizmann.ac.il).

Protein structures. Structure files were downloaded from the RCSB protein data bank (<https://www.rcsb.org>) and structure figures were made with UCSF Chimera v1.14 (www.rbvi.ucsf.edu/chimera).

Protein parameter tables. Table 2, 3 and 4 were made from a search in the BRENDA database (<https://www.brenda-enzymes.org>), after which individual entries were checked in the cited literature. Some entries were discarded since the cited literature did not have values for either K_M or V_{MAX} . Table 5 was made by combining the data from the three cited references. Amino acid identity was calculated from searches with pBLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), using the non-redundant protein sequences database. Identity percentages are only shown for proteins that had >90% query coverage with the corresponding *Lactococcus lactis* IL1403 protein. The number of transmembrane helices of the proteins were determined with TMHMM v2.0 (<https://services.healthtech.dtu.dk/>).

Alignment tables. Table S1, S2, S3 and S4 were created from searches with pBLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), using the non-redundant protein sequences database, from which all entries from bacteria (taxid: 2) and archaea (taxid: 2157) were excluded. Duplicate entries, as well as entries from uncultured organisms and synthetic constructs were discarded and the top 20 hits are shown.

Table S1: Alignment table for *L. lactis* IL1403 ArcA

Hit	Description	Organism	Query cover (%)	AA identity (%)	Accession
1	Predicted: Arginine deiminase	<i>Drosophila kikkawai</i>	91	60.4	XP_017032402.1
2	Predicted: Arginine deiminase	<i>Drosophila bipectinata</i>	75	62.5	XP_017086811.1
3	Predicted: Arginine deiminase	<i>Drosophila eugracilis</i>	76	56.4	XP_017070092.1
4	Unnamed protein product	<i>Callosobruchus maculatus</i>	98	37.1	VEN63481.1
5	Arginine deiminase	<i>Beauveria bassiana</i> D1-5	98	36.7	KGQ13521.1
6	Arginine deiminase	<i>Gregarina niphandrodes</i>	99	27.7	XP_011128800.1
7	Arginine deiminase 2	<i>Nosema bombycis</i> CQ1	86	29.4	EOB12249.1
8	Arginine deiminase	<i>Lasius niger</i>	30	32.3	KMQ98347.1
9	Hypothetical protein B4U79_16161	<i>Dinothrombium tinctorium</i>	62	27.7	RWS07053.1
10	Amidinotransferase family protein	<i>Tritrichomonas foetus</i>	66	26.6	OHT06876.1

Bacterial and archaeal genes were excluded from analysis.

Hit	Description	Organism	Query cover (%)	AA identity (%)	Accession
11	Amidinotransferase family protein	<i>Trichomonas vaginalis</i> G3	67	25.4	XP_001325990.1
12	Uncharacterized protein LOC111078062	<i>Drosophila obscura</i>	66	22.7	XP_022228292.1
13	Hypothetical protein PBRA_001091	<i>Plasmodiophora brassicae</i>	58	26.1	CE099185.1
14	Hypothetical protein Angca_004807	<i>Angiostrongylus cantonensis</i>	60	22.9	KAE9421491.1
15	Hypothetical protein ES319_D11G046900v1	<i>Gossypium barbadense</i>	23	26.7	KAB2002164.1
16	Hypothetical protein E1A91_D11G048000v1	<i>Gossypium mustelinum</i>	23	26.7	TYI54042.1
17	Hypothetical protein ES332_D11G049200v1	<i>Gossypium tomentosum</i>	23	25.7	TYH42226.1
18	Predicted: Thaumatin protein 1	<i>Gossypium raimondii</i>	24	28.2	XP_012488182.1
19	Thaumatin protein 1 isoform B	<i>Glycine soja</i>	20	26.7	RZB78836.1
20	Thaumatin protein 1	<i>Glycine max</i>	20	26.7	XP_003538880.1

Table S2: Alignment table for *L. lactis* IL1403 ArcB

Hit	Description	Organism	Query cover (%)	AA identity (%)	Accession
1	Predicted: Ornithine carbamoyltransferase, catabolic	<i>Drosophila kikkawai</i>	92	62.5	XP_017032401.1
2	Elongation factor 4	<i>Platysternon megacephalum</i>	91	51.2	TFJ95347.1
3	Ornithine carbamoyltransferase chain F	<i>Beauveria bassiana</i> D1-5	93	48.8	KGQ13520.1
4	Ornithine carbamoyltransferase	<i>Eumeta japonica</i>	92	49.2	GBP02644.1
5	OTCase N and OTCase domain containing protein	<i>Trichuris trichiura</i>	92	48.4	CDW60281.1
6	Unnamed protein product	<i>Callosobruchus maculatus</i>	89	49.4	VEN63474.1
7	Uncharacterized protein LOC111676694	<i>Lucilia cuprina</i>	84	48.2	XP_023293434.1
8	Hypothetical protein FOCC_FOCC015949	<i>Frankliniella occidentalis</i>	65	47.5	KAE8738541.1
9	Predicted: Ornithine carbamoyltransferase, mitochondrial, partial	<i>Gavialis gangeticus</i>	94	37.3	XP_019379413.1
10	Ornithine carbamoyltransferase, mitochondrial	<i>Alligator mississippiensis</i>	93	37.9	KYO40176.1

Bacterial and archaeal genes were excluded from analysis.

Hit	Description	Organism	Query cover (%)	AA identity (%)	Accession
11	Ornithine carbamoyltransferase, mitochondrial	<i>Esox lucius</i>	97	38.3	XP_010867194.3
12	Predicted: Ornithine carbamoyltransferase, mitochondrial	<i>Crocodylus porosus</i>	94	37.9	XP_019384650.1
13	Uncharacterized protein FWK35_00033338	<i>Aphis craccivora</i>	74	43.8	KAF0716762.1
14	Ornithine carbamoyltransferase, mitochondrial	<i>Alligator sinensis</i>	93	37.6	XP_006015714.1
15	Ornithine carbamoyltransferase, mitochondrial	<i>Rana catesbeiana</i>	94	37.5	P31326.1
16	Ornithine carbamoyltransferase, mitochondrial	<i>Pelodiscus sinensis</i>	94	38.1	XP_006133999.1
17	Ornithine carbamoyltransferase, mitochondrial	<i>Rhinatrema bivittatum</i>	95	38.1	XP_029459052.1
18	Predicted: Ornithine carbamoyltransferase, mitochondrial	<i>Nanorana parkeri</i>	94	37.2	XP_018409018.1
19	Ornithine carbamoyltransferase, mitochondrial	<i>Xenopus tropicalis</i>	96	38.6	NP_001005795.1
20	Ornithine transcarbamylase	<i>Xenopus laevis</i>	96	37.7	AAF61406.1

Table S3: Alignment table for *L. lactis* IL1403 ArcC1

Hit	Description	Organism	Query cover (%)	AA identity (%)	Accession
1	Predicted: carbamate kinase	<i>Drosophila kikkawai</i>	99	50.6	XP_017032400.1
2	Uncharacterized protein LOC111361385	<i>Spodoptera litura</i>	98	52.4	XP_022833529.1
3	Carbamate kinase	<i>Trichomonas vaginalis</i> G3	99	45.7	XP_001328170.1
4	Carbamate kinase	<i>Lacustera cypriaca</i>	99	45.1	AMQ24253.1
5	Carbamate kinase	<i>Tritrichomonas foetus</i>	99	43.1	OHT17023.1
6	Carbamate kinase	<i>Trichomonadida</i> sp. LN-2016a	85	48.5	AMQ24262.1
7	Carbamate kinase	<i>Chilomastix cuspidata</i>	98	45.2	AMQ24248.1
8	Carbamate kinase	<i>Giardia lamblia</i> P15	99	42.2	EFO64971.1
9	Carbamate kinase	<i>Ergobibamus cyprinoides</i>	99	44.6	AMQ24271.1
10	Carbamate kinase	<i>Giardia intestinalis</i> ATCC 50581	99	41.9	EET00411.1

Bacterial and archaeal genes were excluded from analysis.

Hit	Description	Organism	Query cover (%)	AA identity (%)	Accession
11	Carbamate kinase	<i>Hexamita</i> sp.	98	42.7	AAF08984.1
12	Carbamate kinase	<i>Pyrsonympha</i> sp. LN-2016a	99	43.1	AMQ24257.1
13	Carbamate kinase	<i>Monocercomonoides</i> sp. PA203	99	45.0	AMQ24265.1
14	Carbamate kinase	<i>Kipferlia bialata</i>	99	43.8	GCA62000.1
15	Carbamate kinase	<i>Trimastix marina</i>	99	42.6	AMQ24259.1
16	Hypothetical protein LaI_00012736	<i>Lupinus albus</i>	98	42.2	KAF1854737.1
17	Carbamate kinase	<i>Giardia muris</i>	99	42.1	TNJ29176.1
18	Carbamate kinase	<i>Gracilariopsis chorda</i>	100	39.5	PXF40110.1
19	Uncharacterized protein LOC111361243	<i>Spodoptera litura</i>	91	44.8	XP_022833380.1
20	Carbamate kinase	<i>Spironucleus salmonicida</i>	98	39.9	EST43735.1

Table S4: Alignment table for *L. lactis* IL1403 ArcD2

Hit	Description	Organism	Query cover (%)	AA identity (%)	Accession
1	Ornithine carbamoyltransferase, catabolic	<i>Beauveria bassiana</i> D1-5	90	37.7	KGQ13465.1
2	Hypothetical protein EVAR_35081_1	<i>Eumeta japonica</i>	91	31.1	GBP45543.1
3	Uncharacterized protein LOC115259013	<i>Aedes albopictus</i>	78	31.6	XP_029715379.1
4	Arginine/ornithine antiporter domain protein	<i>Necator americanus</i>	52	38.2	XP_013293730.1
5	Uncharacterized protein LOC107885343	<i>Acyrtosiphon pisum</i>	46	32.5	XP_016664454.1
6	Uncharacterized protein LOC105827954	<i>Monomorium pharaonis</i>	75	25.1	XP_012521610.1
7	B(0,+)-type amino acid transporter 1	<i>Trichuris trichiura</i>	70	27.6	CDW56996.1
8	Predicted: histidine--tRNA ligase	<i>Drosophila bipectinata</i>	34	29.7	XP_017093671.1
9	arcD	<i>Trichonephila clavipes</i>	30	31.1	PRD19625.1
10	Arginine/ornithine antiporter	<i>Nosema bombycis</i> CQ1	18	39.4	EOB12250.1

Bacterial and archaeal genes were excluded from analysis.

Hit	Description	Organism	Query cover (%)	AA identity (%)	Accession
11	Lysine-specific permease	<i>Choanephora cucurbitarum</i>	71	22.5	OBZ91216.1
12	Hypothetical protein HMPREF1544_09648	<i>Mucor circinelloides</i>	70	21.8	EPB83605.1
13	Hypothetical protein CXQ85_001676	<i>Candida haemulonis</i>	69	23.8	XP_025340839.1
14	Hypothetical protein RMATCC62417_13486	<i>Rhizopus microsporus</i>	70	21.3	CEG78958.1
15	Gamma-aminobutyrate permease	<i>Mucor ambiguus</i>	70	21.8	GAN11652.1
16	Hypothetical protein	<i>Parasitella parasitica</i>	69	21.7	CEP18212.1
17	Hypothetical protein CU097_011584	<i>Rhizopus azygosporus</i>	70	21.3	RCH92702.1
18	Hypothetical protein CJJ07_000032	<i>Candida auris</i>	69	23.6	PSK79971.1
19	Cationic amino acid transporter 2	<i>Melanaphis sacchari</i>	59	24.0	XP_025199528.1
20	Hypothetical protein PHYBLDRAFT_119836	<i>Phycomyces blakesleeanus</i>	71	22.4	XP_018283762.1

Chapter 2

Enzymology of the pathway for ATP production by arginine breakdown

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Abstract

2 In cells the breakdown of arginine to ornithine, ammonium ion plus carbon dioxide is coupled to the generation of metabolic energy in the form of ATP. The arginine breakdown pathway is minimally composed of arginine deiminase, ornithine transcarbamoylase, carbamate kinase and an arginine/ornithine antiporter; ammonia and carbon dioxide most likely diffuse passively across the membrane. The genes for the enzymes and transporter have been cloned and expressed and the proteins have been purified from *Lactococcus lactis* IL1403 and incorporated into lipid vesicles for sustained production of ATP. Here, we study the kinetic parameters and biochemical properties of the individual enzymes and the antiporter, and we determine how the physicochemical conditions, effector composition and effector concentration affect the enzymes. We report the K_M and V_{MAX} values for catalysis and the native oligomeric state of all proteins, and we measured the effect of pathway intermediates, pH, temperature, freeze-thaw cycles and salts on the activity of the cytosolic enzymes. We also present data on the protein-to-lipid ratio and lipid composition dependence of the antiporter.

Introduction

The arginine deiminase pathway is one of the simplest routes for the generation of ATP and alkalinization of the internal pH. With only three cytosolic enzymes, arginine is converted into ornithine, ammonium ion plus carbon dioxide, while ATP is created from ADP and phosphate (Fig. 1); the reaction equation is:

The enzymes of the pathway are arginine deiminase (ADI), which hydrolyzes arginine into citrulline plus ammonium ion; ornithine transcarbamoylase (OTC), which converts citrulline plus phosphate into carbamoyl-phosphate (carbamoyl-Pi) plus ornithine; and carbamate kinase (CK), which hydrolyzes carbamoyl-Pi to form carbon dioxide plus ammonium ion under concomitant formation of ATP from ADP and the

phosphate moiety of carbamoyl-Pi. The ADI pathway also employs a membrane-bound arginine/ornithine antiporter (AOA) to couple the import of the substrate arginine to export of the product ornithine.

The ADI pathway is widely used in bacteria to generate metabolic energy^{28,29} and to protect cells in acidic environments^{30,31}; per molecule of arginine metabolized three protons are used (see reaction equation). The enzymes of the pathway are also found in archaea³⁴, lower eukaryotes³⁵ and some are also present in mammalian cells (based on our own genome searches, see Chapter 1). In some protozoa, the pathway is used for energy generation^{38,39} but is also important for pathogenesis^{36,37}. By secreting ADI and OTC into the external medium, the concurrent depletion of arginine reduces the production of antiparasitic (and antimicrobial) nitric oxide in infected tissues^{36,37}. Some bacteria have an anabolic OTC in addition to the catabolic one, which is used for arginine biosynthesis instead of arginine breakdown⁸⁴. Interestingly, a catabolic OTC can be changed into an anabolic OTC with only one mutation⁸⁴. The anabolic OTCs have a strongly reduced cooperativity and lower apparent K_M for carbamoyl-Pi. In mammalian cells arginine metabolism is rather complex, as arginine is involved in synthesis of proteins, urea, creatine, polyamines, nitric oxide, proline, glutamate and agmatine⁴⁴. Mammalian cells use arginases that catalyze the reaction of arginine plus water into ornithine plus urea, and they have anabolic but not catabolic OTCs⁴⁵. Furthermore, mammalian cells do not use CKs but have AOAs in their mitochondria and make use of arginine uniporters^{46,47}.

Figure 1: Schematic of the arginine breakdown pathway. AOA = arginine/ornithine antiporter; ADI = arginine deiminase; OTC = ornithine transcarbamoylase; CK = carbamate kinase.

The proteins for breakdown of arginine are encoded by *arcA* (ADI), *arcB* (OTC), *arcC* (CK) and *arcD* (AOA), and in bacteria they are usually present in a gene cluster¹³⁴, although the arrangement of the genes varies between species. In archaea the proteins have been found in a gene cluster but also dispersed over the chromosome¹³⁴. The *arc* operon in *Lactococcus lactis* contains two copies of *arcC* and *arcD* (due to gene duplication) as well as *arcT*, a putative amino acid transaminase⁵⁷ and *argS*, an arginine-tRNA ligase^{58,59}. Additionally, the transcription factor *argR* and an sRNA (*argX*) are located upstream of the *arc* operon, which are arginine-dependent regulators of the pathway^{60,62}. The *arc* operon is further regulated by the transcription factors CcpA and AhrC, which respond to the concentrations of a preferred sugar (usually glucose) and arginine, respectively^{60,61}.

Individual enzymes for arginine breakdown have been studied, but to our knowledge a comprehensive and systematic characterization of all enzymes from a single organism has not been carried out. One study characterizes ADI and CK from *Streptococcus pyogenes*⁸⁰, while three separate studies report on ADI, OTC and CK from *Giardia lamblia*^{74,86,112}. In addition, ADI and OTC from *Pseudomonas aeruginosa* have been studied^{84,135}, as well as OTC and CK from *Enterococcus faecalis*^{87,111,136} and from *Pyrococcus furiosus*^{93,114}. Other studies focus on individual enzymes (e.g. ref. 66). Arginine/ornithine antiport activity was first studied in *L. lactis*, both in membrane vesicles²² and in whole cells⁸¹. Later it was shown in whole cells that *L. lactis* has two AOAs, namely ArcD1 and ArcD2, which have a similar affinity for arginine but very different affinities for ornithine, lysine, histidine and alanine¹¹⁷. The archaeal ArcD antiporter from *Halobacterium salinarum* has been studied in membrane vesicles, which showed a similar K_M but a lower specific activity for arginine than the protein from *L. lactis*¹³⁷.

Recently, we used ArcA, ArcB, ArcC1 and ArcD2 from *L. lactis* to construct an ATP-regenerating system in synthetic vesicles (see Chapter 3). The system is fed with external arginine and can maintain constant levels of ATP for up to at least 6 hours, even when ATP is consumed. The produced ATP has been used to fuel the gated transport of an osmolyte, which allows the vesicles to respond to osmotic stress. We now characterize ArcA, ArcB, ArcC1 and ArcD2 from *L. lactis* IL1403; we determined the

Table 1: Properties of the proteins

Protein	Monomeric molar extinction coefficient ($M^{-1} \text{ cm}^{-1}$)	<i>pI</i>	Monomeric M_w (kDa)	Native M_w (kDa)	Oligomeric state
ArcA	34,505	5.45	47.7	194	Tetramer
ArcB	36,900	5.78	40.9	245	Hexamer
ArcC1	21,430	5.08	36.2	73	Dimer
ArcD2	109,445	9.38	56.7	53	Monomer

Values for molar extinction coefficient, isoelectric point (*pI*) and monomeric molecular weight (M_w) were calculated with ExPASy ProtParam. Native M_w as observed with SEC-MALS (see below, Fig. 2B).

oligomeric state and kinetic parameters of enzymatic conversion or transport of the purified proteins, determined the pH and temperature dependence of the enzymes, and the role of small molecule effectors on their catalytic performance.

Results & Discussion

Choice of the proteins for arginine breakdown. We have studied ArcA, ArcB, ArcC1 and ArcD2 from *Lactococcus lactis* IL1403. We chose to work with ArcC1, as initial expression tests showed better production of ArcC1 than ArcC2. We had to work with ArcD2 as ArcD1 from *L. lactis* IL1403 misses the 14th transmembrane helix when aligned to ArcD1 from *L. lactis* MG1363¹³⁸, rendering the transporter inactive (data not shown). The properties of the proteins, including molar extinction coefficient, isoelectric point (*pI*), molecular weights (M_w) and oligomeric state, are summarized in Table 1.

Expression and purification of proteins. ArcA, ArcB, ArcC1 and ArcD2 from *L. lactis* IL1403 were expressed in *L. lactis* NZ9000 (cytosolic proteins) or JP9000 (ArcD2), as described in materials & methods. The proteins were purified with metal-affinity chromatography, followed by

Figure 2: SDS-PAGE and static light scattering analysis of proteins. (A) SDS-PAGE analysis of purified ArcA, ArcB, ArcC1 and ArcD2, of which the theoretical molecular weights of the monomers are 47.7, 40.9, 36.2 and 56.7 kDa, respectively. Full SDS-PAA gels are shown in Fig. 7. (B) SEC-MALS chromatograms of ArcA (top left), ArcB (top right), ArcC1 (bottom left) and ArcD2 (bottom right), showing refractive index (blue traces), light scattering (green traces) and absorption at 280 nm (pink traces). The peaks (yellow traces) indicate molecular weights of 194, 245, 73 and 53 kDa for native ArcA, ArcB, ArcC1 and ArcD2, respectively, which indicates that ArcA is tetrameric, ArcB is hexameric, ArcC1 is dimeric and ArcD2 is monomeric under native conditions. Additionally, the molecular weight of the DDM mass associated with ArcD2 is indicated and corresponds to 156 kDa (light blue trace). The chromatograms for BSA and aldolase standards are shown in Fig. 8.

size-exclusion chromatography, which resulted in single bands on a SDS-PAA gel (Fig. 2A). The molecular weights of the SDS-denatured proteins matched well with the theoretical monomeric molecular weights (ArcA: 47.7 kDa, ArcB: 40.9 kDa, ArcC1: 36.2 kDa and ArcD2: 56.7 kDa), except for ArcD2, which migrates at a lower molecular weight than expected. This has been observed for many other membrane proteins and is probably due to incomplete denaturation by SDS¹³⁹.

Oligomeric state analysis. We have determined the oligomeric state of the proteins by coupling size-exclusion chromatography to multi-angular light scattering (SEC-MALS), of which the chromatograms are shown in Fig. 2B. SEC-MALS determines the absolute molar mass and average size of a protein by detecting how it scatters light. For ArcA, we found a native molecular weight of 194 kDa, indicating a tetrameric oligomeric state. The native molecular weight of ArcA from *L. lactis* ATCC 7962 (which has 100% identity with the ArcA studied here) was measured before by ref. 66. They found by size-exclusion chromatography a native molecular weight of 140 kDa and concluded that the protein is a trimer. We obtained the same molecular weight in our size-exclusion chromatography analysis, but find a higher native molecular weight when the multi-angular light scattering is included in the analysis. In general, SEC-MALS is the method of choice to analyze the oligomeric states of proteins, as it allows correcting for differences in shape and e.g. the presence of bound detergent in case of membrane proteins¹⁴⁰. A structural study on arginine deiminase from *Streptococcus pyogenes* (44.5% amino acid identity with *L. lactis* ArcA) showed a mix of monomeric and dimeric states⁷², while the protein from *Pseudomonas aeruginosa* (32.1% identity) showed a tetrameric oligomeric state⁷¹.

The native molecular weight of ArcB was 245 kDa, indicating a hexameric state. Ornithine transcarbamoylase from *Lactobacillus hilgardii* (69.8% identity with *L. lactis* ArcB) and *Gloeobacter violaceus* (35.9% identity) were also found to be hexamers^{98,141}. For ArcC1 we found a native molecular weight of 73 kDa, which corresponds to a dimer. Carbamate kinase from *Enterococcus faecalis* (48.4% identity with *L. lactis* ArcC1) and *Pyrococcus furiosus* (42.6% identity) both showed a dimeric state^{114,120}. Finally, we found a native molecular weight of 53 kDa for ArcD2, which indicates that the arginine/ornithine antiporter is

monomeric, that is, at least in the DDM-solubilized state. The arginine/agmatine antiporter AdiC from *Escherichia coli* (22.5% amino acid identity with *L. lactis* ArcD2), which belongs to the same amino acid/polyamine/organocation (APC) superfamily of transporters as ArcD2, was found to be a dimer¹⁴².

When analyzing the protein database (PDB) structures of Arc homologs with bound substrates (e.g. ADI from *Pseudomonas aeruginosa*, 32% identity with *L. lactis* ArcA, PDB ID: 2A9G; OTC from *Vibrio vulnificus*, 48% identity with *L. lactis* ArcB, PDB ID: 4JQO; CK from *E. faecalis*, 52% identity with *L. lactis* ArcC1, PDB ID: 2WE5), we find that the proteins have one active site per monomer^{69,97,120}. Therefore the k_{cat} values were calculated with the assumption that every monomer is catalytically active.

Characterization of arginine deiminase. We first determined the kinetic parameters of *L. lactis* IL1403 ArcA, using arginine as the substrate (Fig. 3A). The Michaelis-Menten fit of the datapoints is shown in Fig. 3B, giving a K_M value of $34.5 \pm 6.6 \mu\text{M}$ and a V_{MAX} of $4.2 \pm 0.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$ at 30 °C in 50 mM KPi, pH 7.0. The k_{cat} was calculated at $3.3 \pm 0.2 \text{ s}^{-1}$, with the assumption of one active site per monomer, hence four sites per tetramer. When the concentration of arginine is increased beyond 0.5 mM, substrate inhibition is observed with a K_I of 3.2 mM (Fig. 3C). An earlier study by Kim et al. on ArcA from *L. lactis* ATCC 7962 (100% amino acid identity with *L. lactis* IL1403 ArcA) reported a K_M of $8.7 \pm 0.05 \text{ mM}$ and a V_{MAX} of $345 \pm 2 \mu\text{mol min}^{-1} \text{mg}^{-1}$ at 60 °C in 100 mM KPi, pH 7.2⁶⁶. These V_{MAX} and K_M values are approximately two orders of magnitude higher than the ones we found, but also much higher when compared to ArcA from other organisms in the BRENDA database (www.brenda-enzymes.org; see Chapter 1). We can partly explain the higher V_{MAX} and K_M by the higher temperature at which the ATCC 7962 enzyme was assayed (60 °C versus 30 °C), but intriguingly the ATCC 7962 and IL1403 ArcA proteins also differ in temperature dependence (see below).

Kim et al. measured citrulline production like we did via the reaction with 2,3-butanedione monoxime (DAMO), but they did not use a reducing agent (thiosemicarbazide in our assay) or an iron catalyst (we use ammonium iron(III) sulfate)^{66,143}. The ratio between reaction and acid mixture is also

Figure 3: Arginine deiminase (ADI, Arca) activity. Standard conditions are: 42 nM Arca, 480 μM arginine in 50 mM KPi, pH 7.0 at 30 °C. Error bars represent the standard deviation between replicates, grey lines mark the activity of Arca under standard conditions, which is normalized to 100%. **(A)** Schematic of the Arca reaction. K_{eq} value was calculated with an ionic strength of 0.1 M at pH 7.0 using eQuilibrator 2.2. **(B)** Michaelis-Menten plot of Arca activity versus arginine concentration, giving a K_{M} of 34.5 ± 6.6 μM, a V_{MAX} of 4.2 ± 0.2 μmol min⁻¹ mg⁻¹ and a k_{cat} of 3.3 ± 0.2 s⁻¹. Data from biological triplicates. **(C)** Substrate inhibition observed for Arca at higher arginine concentrations, using 3.2 nM Arca. Fitting with uncompetitive inhibition gives a K_{M} of 5.8 ± 0.9 μM, a V_{MAX} of 9.0 ± 0.4 μmol min⁻¹ mg⁻¹ and a K_{I} of 3.2 ± 0.4 mM. Data from a single experiment. **(D)** Influence on Arca activity of increasing amounts of ornithine (blue bars) and carbamoyl-Pi (green bars); data from biological duplicates, 100% activity equals 4.5 μmol min⁻¹ mg⁻¹. **(E)** Influence on Arca activity of pH (left), temperature (middle) and freeze-thaw (FT) cycles (right); data from biological duplicates, 100% activity equals 4.2 μmol min⁻¹ mg⁻¹. **(F)** Influence on Arca activity of different (concentrations of) salts, namely potassium phosphate (KPi; blue bars), sodium phosphate (NaPi; green bars), potassium chloride (KCl; pink bars), sodium chloride (NaCl; yellow bars) and Tris-HCl (Tris; light blue bars). 50 mM KPi was added to all the KCl and NaCl measurements; data from biological duplicates, 100% activity equals 5.0 μmol min⁻¹ mg⁻¹.

different, where we add 150 μL of a 3:2:5 mixture (v/v) of 95% H_2SO_4 , 85% H_3PO_4 and H_2O to 50 μL of reaction mix, Kim et al. adds 250 μL of a 1:3 mixture (v/v) of 95% H_2SO_4 and 85% H_3PO_4 to 500 μL of reaction mix⁶⁶. Finally, the concentration of DAMO is very different, as we use about 0.16% (w/v), while the previous study uses 0.75%. We therefore think that their assay is less sensitive to citrulline production than the assay that we used. This could explain why they used 10 mM arginine as the lowest concentration, even though it is higher than the K_M of 8.7 mM that they report for arginine.

Next we investigated the effect of ADI pathway intermediates on the activity of ArcA. The addition of up to 10 mM carbamoyl-Pi did not influence the activity of ArcA (Fig. 3D, green bars), while 10 mM ornithine (20 times in excess of arginine) decreased the activity by 30% (Fig. 3D, blue bars). The inhibition by ornithine is most likely competitive in nature as the amino acid may fit in the same binding pocket as arginine. Carbamoyl-Pi, on the other hand, is structurally very different and will most likely not compete with arginine for binding to ArcA. Mg-ATP, Mg-ADP, Mg-AMP or ammonia at a concentration of 2 mM did not affect the activity of ArcA, but 20 mM of ammonia decreased activity by 30% (data not shown).

The optimum pH of ArcA is pH 7.2 at 30 °C (Fig. 3E, left), while the optimum temperature is 41 °C at pH 7.0 (Fig. 3E, middle). Between pH 6.8 and 7.2, the change in activity is small, but lowering the pH to 5.8 decreases activity by 14%, while increasing the pH to 8 drops activity by 30%. When the temperature of the reaction mix was increased from 26 °C to 41 °C, the activity of ArcA increased. Any further increase in temperature led to a decrease in activity and full inactivation was seen at 62 °C. The same optimal pH of 7.2 was found at 60 °C with ArcA from *L. lactis* ATCC 7962, but in the study of Kim et al. the enzyme was completely inactive at pH 6.4 or 7.8⁶⁶. Additionally, Kim et al. found an optimal temperature of 60 °C and a decrease of 85% in activity at 40 °C, which is almost the opposite effect of what we observe here⁶⁶. They observed inactivation of the enzyme at 60 °C in about 40 min, while we see that ArcA is completely inactivated within 3 minutes (Fig. 3E, middle). We have no explanation for the discrepancy between the studies of Kim et al. and ours⁶⁶.

Freezing the enzyme in liquid nitrogen and subsequently thawing it has little or no effect on ArcA activity (Fig. 3E, right). This finding is important for studies where the enzyme has to be stored or encapsulated into synthetic vesicles, as described in Chapter 3. Finally, increasing the concentration of potassium phosphate (KPi) does not have an effect on ArcA activity (Fig. 3F, blue bars). Changing to sodium phosphate (NaPi) and increasing its concentration to 100 mM or higher seems to increase ArcA activity, although the error of the measurements was relatively large (Fig. 3F, green bars). Adding 50 mM of potassium chloride (KCl) or sodium chloride (NaCl) to 50 mM KPi had some effect (Fig. 3F, pink and yellow bars), but overall ArcA is not very sensitive to changes in ionic strength. Changing from a phosphate buffer to Tris-HCl reduced activity by 90% (Fig. 3F, light blue bars).

Characterization of ornithine transcarbamoylase. The kinetic parameters of *L. lactis* IL1403 ArcB were characterized with ornithine and carbamoyl-Pi as substrates (Fig. 4A). In the breakdown of arginine, this reaction runs in the opposite direction, but the equilibrium constant (K_{eq}) favors formation of citrulline plus inorganic phosphate by a factor of $\sim 10^5$. The forward reaction has been studied for ArcB from *E. coli*, using arsenate instead of phosphate, yielding the formation of ornithine plus carbamoyl-arsenate⁸⁵; the latter decomposes spontaneously into carbon dioxide, ammonium ion and arsenate. The Michaelis-Menten fits of the datapoints of Fig. 4B yield a K_M value of 0.7 ± 0.2 mM and a V_{MAX} of 600 ± 40 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ for ornithine (left panel), when the concentration of carbamoyl-Pi is kept at 5 mM. When the concentration of carbamoyl-Pi is varied, we obtain a K_M of 2.7 ± 0.4 mM and a V_{MAX} of 830 ± 65 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ (right panel), with the concentration of ornithine fixed at 5 mM. The k_{cat} values are 410 ± 30 s^{-1} and 565 ± 45 s^{-1} for ornithine and carbamoyl-Pi, respectively, assuming one active site per monomer. When we compare our values with those of other ArcB-like enzymes in the BRENDA database (Table 3 in Chapter 1), we find that the K_M for carbamoyl-Pi is the highest, while the K_M for ornithine is one of the lowest; our k_{cat} values are among the highest reported up to now.

With 10 mM of arginine (2 times more than the concentrations of ornithine and carbamoyl-Pi) the activity of ArcB decreased by 25%, (Fig. 4C). Like the inhibition of ArcA by ornithine, ArcB may be inhibited by arginine

Figure 4: Ornithine transcarbamoylase (OTC, ArcB) activity. Standard conditions are: 1 nM ArcB, 5 mM ornithine and 5 mM carbamoyl-Pi in 50 mM KPi, pH 7.0 at 30 $^{\circ}\text{C}$. Error bars represent the standard deviation between replicates, grey lines mark the activity of ArcB under standard conditions, which is normalized to 100%. **(A)** Schematic of the ornithine transcarbamoylase reaction in the direction of citrulline formation, which is opposite of how the reaction runs in the arginine breakdown pathway. K_{eq} value was calculated with an ionic strength of 0.1 M at pH 7.0 using eQuilibrator 2.2. **(B)** Michaelis-Menten plot of ArcB activity versus ornithine (left) and carbamoyl-Pi (right) concentration. Fitting gives a K_{M} of 0.7 ± 0.2 mM, a V_{MAX} of 600 ± 40 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ and a k_{cat} of 410 ± 30 s^{-1} when ornithine is varied and carbamoyl-Pi is kept at 5 mM. Additionally, a K_{M} of 2.7 ± 0.4 mM, a V_{MAX} of 830 ± 65 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ and a k_{cat} of 565 ± 45 s^{-1} are obtained when carbamoyl-Pi is varied and ornithine is kept at 5 mM; data from biological duplicates. **(C)** Influence on ArcB activity of increasing amounts of arginine; data from biological duplicates, 100% activity equals 340 $\mu\text{mol min}^{-1} \text{mg}^{-1}$. **(D)** Influence on ArcB activity of pH (left), temperature (middle) and freeze-thaw (FT) cycles (right). Effect of FT cycles was studied for ArcB in solution (blue squares) and encapsulated in proteoliposomes (green circles); data from biological duplicates, 100% activity equals 480 $\mu\text{mol min}^{-1} \text{mg}^{-1}$. **(E)** Influence on ArcB activity of different (concentrations of) salts. 10 mM KPi was added to all the KCl measurements; data from biological duplicates, 100% activity equals 310 $\mu\text{mol min}^{-1} \text{mg}^{-1}$.

because it is structurally similar to ornithine. The addition of 2 mM Mg-ATP, Mg-ADP, Mg-AMP or 20 mM ammonia had no effect on ArcB (data not shown). The optimum pH of ArcB was pH 7.5 at 30 °C (Fig. 4D, left) and the optimum temperature was 35 °C at pH 7.0 (Fig. 4D, middle). Lowering of the pH from 7.0 to 5.8 inactivated the enzyme for more than 90%, while increasing the pH to 8.0 reduced the activity by 25%. Lowering the temperature from 35 to 25 °C lowered activity by 75%, while increasing the temperature to 62 °C inactivated ArcB, similar to what we observed for ArcA.

We find that ArcB in solution is sensitive to cycles of freezing and thawing (FT) (Fig. 4D, right). After just one FT cycle, we observed an 80% loss in activity. To check if this inactivation also happens when ArcB is encapsulated in vesicles (as done in Chapter 3), we mimicked those conditions by adding ArcD2 proteoliposomes, Mg-ADP and ornithine to the FT mix; we omitted ArcA and ArcC1 to be able to measure ArcB activity. After a varying number of cycles of freezing and thawing, the vesicles were dissolved with 0.1% (v/v) Triton X-100 and ArcB activity was determined. Importantly, ArcB in the vesicles was much less affected by freezing and thawing, as a loss of activity of only 25% was observed after five FT cycles. Most likely the encapsulation of ArcB in the vesicles and the presence of the substrate (ornithine) stabilizes the enzyme.

Increasing the concentration of KPi or NaPi decreased the activity of ArcB, probably because the reactant inorganic phosphate becomes inhibitory (Fig. 4E, blue and green bars). This notion is supported by the finding that increasing the salt concentration (addition of KCl) did not inhibit the enzyme. In fact, concentrations of KCl above 50 mM increased the activity of ArcB (Fig. 4E, pink bars). ArcB was inhibited by Tris-HCl at concentrations higher than 50 mM (Fig. 4E, yellow bars).

Characterization of carbamate kinase. We used carbamoyl-Pi and Mg-ADP as substrates to determine the kinetic parameters of the reaction catalyzed by ArcC1 from *L. lactis* IL1403 (Fig. 5A). The Michaelis-Menten fits of the datapoints of Fig. 5B give a K_M value of 0.6 ± 0.2 mM and a V_{MAX} of 330 ± 30 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ for carbamoyl-Pi (left panel), when the concentration of Mg-ADP is fixed at 5 mM. For Mg-ADP, a K_M of 1.6 ± 0.7 mM and a V_{MAX} of 310 ± 40 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ were determined (right panel),

Figure 5: Carbamate kinase (CK, ArcC1) activity. Standard conditions are: 46 nM ArcC1, 5 mM carbamoyl-Pi and 5 mM Mg-ADP in 50 mM KPi, pH 7.0 at 30 °C. Error bars represent the standard deviation between replicates, grey lines mark the activity of ArcC1 under standard conditions, which is normalized to 100%. (A) Schematic of the carbamate kinase reaction. K_{eq} value was calculated with an ionic strength of 0.1 M at pH 7.0 using eQuilibrator 2.2. (B) Michaelis-Menten plot of ArcC1 activity versus carbamoyl-Pi (left) and ADP (right) concentration. Fitting gives a K_M of 0.6 ± 0.2 mM, a V_{MAX} of 330 ± 30 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ and a k_{cat} of 200 ± 20 s^{-1} when carbamoyl-Pi is varied and Mg-ADP is kept at 5 mM. A K_M of 1.6 ± 0.7 mM, a V_{MAX} of 310 ± 40 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ and a k_{cat} of 190 ± 15 s^{-1} are obtained when Mg-ADP is varied and carbamoyl-Pi is kept at 5 mM; data from biological duplicates. (C) Influence on ArcC1 activity of increasing amounts of arginine (blue bars), citrulline (green bars) and ornithine (pink bars); data from biological duplicates; 100% activity equals 390 $\mu\text{mol min}^{-1} \text{mg}^{-1}$. (D) Influence on ArcC1 activity of pH (left), temperature (middle) and freeze-thaw (FT) cycles (right); data from biological duplicates, 100% activity equals 380 $\mu\text{mol min}^{-1} \text{mg}^{-1}$. (E) Influence on ArcC1 activity of different (concentrations of) salts and buffers. 50 mM KPi was added to all the KCl and NaCl measurements; data from biological duplicates, 100% activity equals 400 $\mu\text{mol min}^{-1} \text{mg}^{-1}$.

when carbamoyl-Pi is kept constant at 5 mM. The k_{cat} values of ArcC1 are $200 \pm 20 \text{ s}^{-1}$ and $190 \pm 15 \text{ s}^{-1}$ when carbamoyl-Pi and Mg-ADP are varied, respectively, assuming one active site per monomer. The error bars in Fig. 5B are relatively large, as the data was obtained from the combination of two independent ratiometric fluorescent measurements, using PercevalHR for determination of the ATP/ADP ratio and pyranine for pH. Since PercevalHR readout is sensitive to pH (Fig. 3 in Chapter 3; ref. 144), we used the pyranine data to correct the Perceval HR fluorescence for the increase in pH. According to the BRENDA database very few ArcC-type enzymes have been studied (Table 4 in Chapter 1). The k_{cat} values reported here for ArcC1 are the highest reported so far.

Adding up to 10 mM of arginine did not negatively affect the activity of ArcC1 (Fig. 5C, blue bars). Citrulline at 10 mM decreased the activity by 40 to 50% (Fig. 5C, green bars), while ornithine was slightly inhibitory (Fig. 5C, pink bars). The optimum pH for ArcC1 activity is 7.0 at 30 °C (Fig. 5D, left), while the optimum temperature is 25 °C at pH 7.0 (Fig. 5D, middle). The enzyme has a narrow pH window for its activity, as ArcC1 is fully inactivated below pH 5.8 or above pH 8.0. ArcC1 is also much more sensitive to temperature than ArcA and ArcB. Freeze-thaw cycles, on the other hand, had little influence on the activity of ArcC1 (Fig. 5D, right).

KPi and NaPi decreased the activity of ArcC1 when their concentrations were above 50 mM (Fig. 5E, blue and green bars). The inhibition by increasing NaPi or KPi concentration may reflect competitive inhibition as inorganic phosphate may compete with carbamoyl-Pi for binding to ArcC1. Interestingly, NaPi inhibits the activity of ArcC1 more than KPi does, an effect that is also seen when adding NaCl (70% inhibition at 300 mM) instead of KCl (35% inhibition at 300 mM; Fig. 5E, pink and yellow bars). As observed for ArcA and ArcB, Tris-HCl inhibited ArcC1 at concentrations above 50 mM (Fig. 5E, light blue bars).

Characterization of the arginine/ornithine antiporter. The kinetic parameters of ArcD2 from *L. lactis* IL1403 were determined in proteoliposomes with external arginine as substrate and internal ornithine or citrulline as counter-substrate for the antiport reaction (Fig. 6A). The standard proteoliposomes consisted of 50 mole% 1,2-dioleoyl-

Figure 6: Arginine/ornithine antiporter (AOA, ArcD2) activity. Standard conditions are: 1:200 (w/w) ArcD2:lipid ratio, using 50:38:12 (w/w) DOPE:DOPG:DOPC lipids extruded through 200 nm polycarbonate filters. In transport assays: 176 nM ArcD2, 25 μM arginine externally and 10 mM ornithine or citrulline internally in 50 mM KPi, pH 7.0 at 30 °C. Error bars represent the standard deviation between replicates; grey lines mark the activity of ArcD2 under standard conditions, which is normalized to 100%. **(A)** Schematic of the arginine/ornithine antiport reaction. **(B)** Michaelis-Menten plot of ArcD2 activity versus external arginine (left) and internal ornithine (right) concentration. Fitting gives a K_M of 6.0 ± 0.7 μM, a V_{MAX} of 120 ± 5 nmol min⁻¹ mg⁻¹ and a k_{cat} of 0.11 ± 0.005 s⁻¹ when external arginine is varied and internal ornithine is kept at 10 mM. Additionally, a K_M of 2.5 ± 0.7 mM, a V_{MAX} of 95 ± 10 nmol min⁻¹ mg⁻¹ and

sn-glycero-3-phosphoethanolamine (DOPE), 38 mole% 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (DOPG) and 12 mole% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). The Michaelis-Menten fits of arginine/ornithine antiport are shown in Fig. 6B, giving a K_M value of $6.0 \pm 0.7 \mu\text{M}$ and a V_{MAX} of $120 \pm 5 \text{ nmol min}^{-1} \text{ mg}^{-1}$ for external arginine (left panel) when 10 mM of ornithine is present on the inside. Additionally, a K_M of $2.5 \pm 0.7 \text{ mM}$ and a V_{MAX} of $95 \pm 10 \text{ nmol min}^{-1} \text{ mg}^{-1}$ were obtained for internal ornithine and 25 μM of arginine on the outside (right panel). The k_{cat} values are $0.11 \pm 0.005 \text{ s}^{-1}$ and $0.09 \pm 0.009 \text{ s}^{-1}$ when arginine and ornithine, respectively, were varied in the kinetic analysis. Previously, ArcD2 was studied in whole cells¹¹⁷, who found a K_M of $4 \pm 2 \mu\text{M}$ and a V_{MAX} of $22 \pm 17 \text{ nmol min}^{-1} \text{ mg}^{-1}$ for external arginine. Surprisingly, Noens et al. found a 5 to 6-fold higher V_{MAX} for ornithine/ornithine than for arginine/ornithine antiport¹¹⁷. It is possible that the different lipid composition, varying internal substrate concentration(s) or the presence of additional arginine and/or ornithine transporters have affected the whole cell measurements. We therefore do not further compare our measurements with those in intact cells.

a k_{cat} of $0.09 \pm 0.009 \text{ s}^{-1}$ are obtained when ornithine is varied and arginine is kept at 25 μM . Arginine data from biological triplicates, ornithine data from technical duplicates. (C) Michaelis-Menten plot of ArcD2 activity versus internal citrulline concentration, with and without a membrane potential ($\Delta\Psi$) of -120 mV, giving a K_M of $6.0 \pm 2.1 \text{ mM}$ and a V_{MAX} of $20 \pm 3 \text{ nmol min}^{-1} \text{ mg}^{-1}$ for measurements in the presence of $\Delta\Psi$; data from technical duplicates. (D) Influence on ArcD2 activity of protein-to-lipid ratio (left), lipid headgroups (middle) and lipid tail types (right). Protein-to-lipid ratios range from 1:800 (w/w), to 1:400, 1:200 and 1:100. Lipid headgroups were varied from 50:50:0 (w/w) (DOPE:DOPG:DOPC) to 50:38:12, 50:25:25, 50:13:37 and 50:0:50. The 50:38:12 lipid composition was studied with DO and PO acyl tails. Data from technical duplicates, with 10 mM ornithine internally; protein-to-lipid ratios data with 25 μM arginine externally; lipid headgroups and tail types data with 10 μM arginine externally; 100% activity equals $120 \text{ nmol min}^{-1} \text{ mg}^{-1}$. (E) Influx of arginine into empty proteoliposomes, with and without a membrane potential ($\Delta\Psi$) of -120 mV. Inset: influx of arginine into proteoliposomes filled with 10 mM ornithine. Empty proteoliposomes data from a single experiment; ornithine data from biological triplicates; with 1 μM arginine outside. (F) Initiation of the antiport reaction after different periods of imposing a membrane potential ($\Delta\Psi$) of -120 mV, in the presence (blue squares) and absence (green circles) of an osmotic shock. Data from a single experiment, with 10 mM citrulline internally.

Next, we characterized ArcD2-mediated arginine/citrulline antiport. Without a membrane potential ($\Delta\Psi$), no arginine/citrulline antiport was observed, even with 20 mM internal citrulline (Fig. 6C, green circles). When a $\Delta\Psi$ of -120 mV was applied (by diluting the KPi-filled proteoliposomes 100-fold into NaPi plus valinomycin; see materials & methods), we observed transport. We determined a K_M of 6.0 ± 2.1 mM and a V_{MAX} of 20 ± 3 nmol min⁻¹ mg⁻¹ when internal citrulline was varied with 25 μ M arginine on the outside (Fig. 6C, blue squares). For arginine/ornithine antiport we do not observe an effect of imposing a $\Delta\Psi$ (Fig. 2 in Chapter 3). The K_M values for internal ornithine and citrulline are similar, while in the presence of a $\Delta\Psi$ the V_{MAX} for arginine/citrulline antiport is about 5 times lower than the one found for arginine/ornithine antiport; in the absence of a $\Delta\Psi$ the V_{MAX} the arginine/citrulline antiport is below our detection limit. The stimulation of arginine/citrulline antiport by a membrane potential (inside negative relative to the outside) is in accordance with the difference in charge of arginine (cationic) and citrulline (neutral); hence this translocation cycle is electrogenic.

We tested the activity of ArcD2 at protein-to-lipid ratios (w/w) of 1:800, 1:400, 1:200 and 1:100 (Fig. 6D, left). The highest uptake rate was observed at a ratio of 1:200 but the highest specific activity was found at 1:800, that is, when we assume that equal fractions of ArcD2 are reconstituted at each protein-to-lipid ratio. Next, we varied the amount of DOPG (and inversely DOPC) while keeping DOPE constant (Fig. 6D, middle). The extremes of 50 mole% DOPG and 0 mole% DOPG (and thus 0 mole% DOPC and 50 mole% DOPC, respectively) lowered the activity by 80 to 90%. Lowering the concentration of DOPG from 38 mole% to 25 or 13 mole% however increased the activity by 30 and 60%. Finally, we replaced one of the dioleoyl tails by a palmitoyl tail in the lipid mixture with 38 mole% of PG, 12 mole% PC plus 50 mole% PE. Surprisingly, we find that the activity of ArcD2 in POPX lipids is reduced by 60% when compared to DOPX (Fig. 6D, right). Thus, the increased packing and presumably lower fluidity of membranes composed of POPX lipids reduce the activity of the ArcD2 transporter^{24,145}. We recently made the opposite observation for an eukaryotic amino acid transporter¹⁴⁶.

It has been reported that (small) peptides rich in arginine permeate lipid membranes without the need for a membrane protein^{147,148}. This

prompted us to study the passive influx of arginine into vesicles with and without ArcD2 (Fig. 6E) and thus discriminate passive influx from carrier-mediated uniport. Empty ArcD2 proteoliposomes were incubated with 1 μM of radiolabeled arginine for one hour, which shows very slow uptake at a rate of about $0.09 \text{ nmol min}^{-1} \text{ mg}^{-1}$. The same experiment was also performed with liposomes without ArcD2, which showed no arginine uptake at all (data not shown). It thus seems that ArcD2 can uniport arginine slowly, albeit at a rate of no more than 0.4% the speed of arginine/ornithine antiport.

Finally, we determined how long an artificially imposed membrane potential ($\Delta\Psi$) lasts in vesicles composed of 38 mole% DOPG, 12 mole% DOPC plus 50 mole% DOPE (Fig. 6F). We diluted ArcD2 proteoliposomes, filled with 10 mM citrulline and 50 mM KPi, pH 7.0 into 50 mM NaPi, pH 7.0 with valinomycin (see materials & methods), and after 0, 10, 20, 30, 45 and 60 minutes we added radiolabeled arginine. Fig. 6C shows arginine/citrulline antiport only when a $\Delta\Psi$ is present. We find the highest rate of transport when uptake of arginine is assayed from the moment the membrane potential is imposed. As expected the rate decreases at later times, which reflects the transient nature of the $\Delta\Psi$. The membrane potential is maintained for at least 60 minutes (albeit at a lower value than initially) when the proteoliposomes are osmotically shocked by addition of 250 mM NaCl (Fig. 6F, blue squares). In the unshocked condition, the $\Delta\Psi$ drops to zero within 60 minutes (Fig. 6F, green circles).

Conclusions

We report the characterization of the enzymes and transporter responsible for breakdown of arginine and production of ATP from a single organism. As presented in the introduction, this pathway has recently been incorporated into lipid vesicles to form an ATP-regenerating system that can perform long-term energy homeostasis (Chapter 3). We find that the import of arginine into cells or vesicles by ArcD2 and the concomitant export of the product of arginine breakdown, that is ornithine, is relatively slow and presumably rate-determining for the production of ATP. The k_{cat} of ArcD2 is more than an order of magnitude lower than that of the slowest enzyme, and the maximal rate of arginine/ornithine

antiport is reached at a concentration of about 60 μM ($K_M = 6.0 \mu\text{M}$). ArcA or ArcB may catalyze the second slowest step. ArcA has a lower k_{cat} than ArcB, but the latter enzyme was assayed in the backward reaction; the forward reaction requires a high concentration of citrulline plus inorganic phosphate and a low concentration of ornithine and carbamoyl-Pi. The high k_{cat} of ArcC1 ensures that carbamoyl-Pi is rapidly converted into ammonia plus carbon dioxide (with concomitant production of ATP from ADP).

One of the first improvements for the ATP-regenerating system in synthetic vesicles would thus be to incorporate a faster arginine/ornithine antiporter, one with a higher K_M for arginine and corresponding increase in k_{cat} . ArcD2 is a well-coupled antiporter, as arginine uniport occurs at less than 1% of the arginine/ornithine antiport activity. The protein also mediates arginine/citrulline antiport, but this activity is very low in the absence of a membrane potential. These two qualities are important to keep in mind and to test for when looking for alternative arginine/ornithine antiporters.

The enzymes of the pathway are not very sensitive to pathway intermediates, albeit that some inhibition by inorganic phosphate of the backward reaction of ArcB and the forward reaction of ArcC1 is observed. The inhibition of ArcB will however not pose a problem for the breakdown of arginine, because the inorganic phosphate is used as a substrate in the forward reaction of ArcB. The pH and temperature ranges in which the system work optimally are quite narrow, as the activity of the enzymes (in particular of ArcC1) is strongly decreased when deviating from pH 7.0 and 30 °C. A second improvement to the ATP-regenerating system would be to use a more robust carbamate kinase, that is, an enzyme that is influenced less by pH and temperature.

The freeze-thaw cycles needed for inclusion of the enzymes into vesicles and future synthetic cells do not have significant adverse effects on the reconstitution of the arginine breakdown pathway. This is an important finding, as the freeze-thaw cycles are an essential step in the creation of synthetic cell-like systems. Furthermore, the enzymes work well in phosphate buffers with potassium or sodium as the counter ion, which are physiological conditions.

The enzymes characterized in this chapter have been coupled to ATP consumption by reconstituting the metabolic network with the ATP-driven glycine betaine transporter OpuA (Chapter 3). We have observed that without ATP consumption, the ATP/ADP ratio increases until all ADP is consumed. The full pathway then stops but surprisingly arginine uptake and deamination continues, leading to a futile cycle with acidification of the interior of the cell. With OpuA activity present, the full pathway continues with limited futile hydrolysis of arginine and the ATP/ADP ratio stays constant for several hours. Thus, adding an ATP consuming reaction leads to greater stability (and basic homeostasis) of the pathway, which is what one expects for an out-of-equilibrium metabolic network.

Materials & Methods

Materials. Common chemicals were of analytical grade and ordered from Sigma-Aldrich Corporation, Carl Roth GmbH & Co. KG or Merck KGaA. The lipids were obtained from Avanti Polar Lipids, Inc. (>99% pure, in chloroform): 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) [850725C], 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) [850375C], 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DOPG) [840475C], 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) [850757C], 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) [850457C] and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG) [840457C]. *n*-dodecyl- β -D-maltoside (DDM) [D97002] was purchased from Glycon Biochemicals GmbH and Triton X-100 [T9284] from Sigma-Aldrich Corporation. ^{14}C -L-arginine was purchased from Moravsek, Inc. [MC-137, 338 mCi mmol $^{-1}$].

Construction of expression strains. The *arcX* genes were PCR-amplified from the genome of *Lactococcus lactis* IL1403 with primers *arcX*-Fw and *arcX*-rev (Table 2), using Phusion HF DNA polymerase (Thermo Fisher Scientific, Inc.). The *arcA* and *arcB* PCR inserts, and the pNZcLICoppA vector¹⁴⁹, were digested with *Nco*I and *Bam*HI and subsequently ligated to yield pNZ*arcA* and pNZ*arcB*. These vectors contain the corresponding genes under the control of the nisin-inducible P_{NIS} promoter¹⁵⁰ and the genes have a cleavable 6His-tag at the N-terminus.

The *arcC1* and *arcD2* PCR inserts were used for ligation-independent cloning as described in ref. 151. This yielded pNZ*arcC1* and pNZ*arcD2* with the genes under the control of the P_{NIS} promoter and with a cleavable 10His-tag at the N- and C-terminus, respectively. To construct the cysteine-less variant of *arcD2* (*arcD2* Δ C), two mutations were made, namely C395T and C487T. The *arcD2* gene was PCR-amplified from pNZ*arcD2* with uracil-containing primers (*arcD2* Δ C-X), using PfuX7 DNA polymerase¹⁵². The two amplified fragments were ligated with USER enzyme (New England Biolabs, Inc.) to create the pNZ*arcD2* Δ C vector.

The pNZ*arcA*, pNZ*arcB* and pNZ*arcC1* vectors were transformed into *L. lactis* NZ9000¹⁵⁰, while pNZ*arcD2* and pNZ*arcD2* Δ C were transformed into

Table 2: Primers used for cloning

Primer	Sequence (5' -> 3')
<i>arcA</i> -Fw	AAACCATGGGTATGAACAATGGAATTAAATGTTAACTCAGAAATTGGG
<i>arcA</i> -Rv	ATAAGGATCCCAAATCTTCACGCCAAAGTGGTTGTGAC
<i>arcB</i> -Fw	AAACCATGGGTATGACATCACCACCTTATTACAAAAGCAGAAGTAAAC
<i>arcB</i> -Rv	ATAAGGATCCTTTAAAATCTTCAGGAAGCTGCTGGGATAAATAAATTAC
<i>arcC1</i> -Fw	ATGGTGAGAATTTATATTTTCAAGGTGTAAAACGTATTGTTGTAGCCC
<i>arcC1</i> -Rv	TGGGAGGGTGGGATTTTCATTAAGGAACAATTCTTGTTCCTACTAC
<i>arcD2</i> -Fw	ATGGGTGGTGGATTTGCTATGGAAAACAAGAAAACAAAAGGG
<i>arcD2</i> -Rv	TTGGAAGTATAAATTTTCGTAGCCTAGTAATCCCCG
<i>arcD2ΔC</i> -1	AGCTGTAATUATGATTACTTATGCTTTGGTTGGAGC
<i>arcD2ΔC</i> -2	AGACCAGAAUTGCTAAAACTCCTAGTAAGG
<i>arcD2ΔC</i> -3	ATTCTGGTCUTGATTACTGGCGCAAATCAGGAAC
<i>arcD2ΔC</i> -4	AATTACAGCUGTTGCCATATAAATAAAGAC

L. lactis JP9000 $\Delta arcD1D2^{117}$ (Table 3). The pRsetB-PercevalHR plasmid was a gift from professor Gary Yellen (Addgene plasmid 49081; ref. 144) and transformed into *E. coli* BL21-DE3 (Table 3).

Expression of genes. *L. lactis* cells were grown in rich medium: 2% (w/v) Gistex from Brenntag AG, 65 mM NaPi, pH 7.0, 1% (w/v) glucose plus 5 $\mu\text{g mL}^{-1}$ chloramphenicol at 30 °C with stirring (200 rpm). The strains for ArcA, ArcB and ArcC1 production were grown as 3 L cultures in 5 L flasks and induced at an optical density at 600 nm (OD_{600}) of 0.5 with 0.05% (v/v) of culture supernatant from a nisin A-producing strain¹⁵⁰. In contrast, the strain for ArcD2 was grown as a 2 L culture in a 3 L bioreactor stirred at 200 rpm with pH control (kept above pH 6.5 with 4 M KOH) and induced at an OD_{600} of 2.0 with 0.05% (v/v) of culture supernatant from a nisin A-producing strain. After induction, all strains

Table 3: Strains used in this chapter

Strain	Genotype	Vector	References
<i>L. lactis</i> NZ9000	MG1363 with <i>nisRK</i> in <i>pepN</i> locus		150
<i>L. lactis</i> NZ9000-A		pNZarcA	This chapter, 153
<i>L. lactis</i> NZ9000-B		pNZarcB	This chapter, 153
<i>L. lactis</i> NZ9000-C1		pNZarcC1	This chapter, 153
<i>L. lactis</i> JP9000	MG1363 with <i>nisRK</i> in <i>pseudo_10</i> locus		117
<i>L. lactis</i> JP9000-D2		pNZarcD2	This chapter, 153
<i>L. lactis</i> JP9000-D2ΔC		pNZarcD2ΔC	This chapter, 153
<i>E. coli</i> BL21(DE3)	F ⁻ <i>ompT gal dcm</i> <i>lon hsdS_B(r_B⁻ m_B⁻)</i> λ(DE3 [<i>lacI lacUV5-T7 ind1 sam7 nin5</i>])	pRsetB- PercevalHR	This chapter, 153

were grown for an additional 2 hours before harvesting. Harvesting and washing was done by centrifugation (15 min, 6.000 x g, 4 °C) and resuspension of the cells in ice-cold 100 mM KPi, pH 7.0 (buffer A, Table 4). Finally, cells were centrifuged again and resuspended to an OD₆₀₀ of 100 in ice-cold 50 mM KPi, pH 7.0 (buffer B), flash-frozen in liquid nitrogen in aliquots of 50 mL and stored at -80 °C.

Preparation of cell lysates and membrane vesicles. The preparation of cell lysate and membrane vesicles was done as follows. After cells were thawed on ice, 100 μg mL⁻¹ DNase and 2 mM MgSO₄ were added. Cells were lysed by high-pressure disruption (Constant Systems, Ltd.) with two passages at 39 kpsi and 4 °C. After lysis, 5 mM Na₂-EDTA (pH 8.0) and 1 mM PMSF (100 mM stock in isopropanol) were added and cell debris was removed by centrifugation (15 min, 22.000 x g, 4 °C). Next, the supernatant was centrifuged for 90 min at 125.000 x g at 4 °C. For ArcA, ArcB and ArcC1, the supernatant (containing cell lysate) was flash-frozen in liquid nitrogen in 10 mL aliquots and stored at -80 °C. For ArcD2, the pellet (containing membrane vesicles) was resuspended in ice-cold buffer B to a 10 mg mL⁻¹ protein concentration, before freezing in liquid nitrogen (2 mL aliquots) and storage.

Table 4: Buffers used in this chapter

Buffer	Composition
A	100 mM KPi, pH 7.0
B	50 mM KPi, pH 7.0
C	50 mM KPi, pH 7.0 with 200 mM NaCl
D	50 mM KPi, pH 7.0 with 100 mM NaCl
E	50 mM KPi, pH 7.0 with 200 mM KCl
F	25 mM KPi, pH 8.0 with 500 mM NaCl plus 5% (v/v) glycerol
G	50 mM NaPi, pH 7.0

E. coli BL21-DE3 pRsetB-PerCHR cells were grown in lysogeny broth (LB) with 100 $\mu\text{g mL}^{-1}$ ampicillin. 0.5 L cultures were grown in 5 L flasks at 30 °C with shaking (200 rpm) to an OD₆₀₀ of 0.7, after which they were cooled to 22 °C, induced with 100 μM isopropyl β -D-1-thiogalactopyranoside (IPTG) and grown for an additional 72 hours before harvesting as described in ref. 144. Harvesting and washing was done by centrifugation (15 min, 6.000 x g, 4 °C) and resuspension in ice-cold 50 mM NaPi, pH 7.0 with 100 mM NaCl. Finally, cells were centrifuged again and resuspended to an OD₆₀₀ of 100 in ice-cold 20 mM NaPi, pH 7.0 with 100 mM NaCl, flash-frozen in liquid nitrogen in 50 mL aliquots and stored at -80 °C. Cell lysate was prepared by adding 250 μg of DNase to thawed cells, after which they were lysed by high-pressure disruption in a single passage at 25 kpsi and 4 °C. After lysis, 0.1 mM PMSF was added and cell debris was removed by centrifugation for 60 min at 145.000 x g at 4 °C. The supernatant (containing cell lysate) was flash-frozen in liquid nitrogen in 15 mL aliquots and stored at -80 °C.

Purification of ArcA, ArcB and ArcC1. All protein purification and handling steps were performed on ice or at 4 °C, except when specified otherwise. Ni²⁺-Sepharose resin was pre-equilibrated in 50 mM KPi, pH 7.0, with 200 mM NaCl (buffer C) with either 10 mM imidazole for ArcA and ArcB or 5 mM imidazole and 10% (v/v) glycerol for ArcC1. Cell lysate was thawed on ice, added to the Ni²⁺-Sepharose resin (0.5 mL bed volume per 10 mg total protein) and nutated for 1 hour. The mixture was poured over a polyprep column (Bio-Rad Laboratories, Inc.), after which the resin was washed with 20 column volumes of buffer C with 50 mM imidazole [plus 10% (v/v) glycerol for ArcC1]. Proteins were then eluted with 3 column volumes of buffer C with 500 mM imidazole [plus 10% (v/v)

glycerol for ArcC1]. The most concentrated fractions were run on a Superdex 200 Increase 10/300 GL size-exclusion column (GE Healthcare) in 50 mM KPi, pH 7.0 with 100 mM NaCl (buffer D) [plus 10% (v/v) glycerol for ArcC1]. Protein containing fractions were pooled and concentrated to 4-8 mg mL⁻¹ in a Vivaspin 500 (30.000 kDa) centrifugal concentrator (Sartorius AG), after which they were aliquoted, flash-frozen in 100 µL aliquots and stored at -80 °C.

Purification of ArcD2. ArcD2 membrane vesicles were quickly thawed and diluted to a total protein concentration of 7 mg mL⁻¹ in 50 mM KPi, pH 7.0 plus 200 mM KCl (buffer E). 0.5% (w/v) *n*-dodecyl-β-D-maltoside (DDM) was added to the vesicles for solubilization and the mixture was nutated for 30 min. Unsolubilized material was removed by centrifugation (20 min, 270.000 x g, 4 °C). Ni²⁺-Sephacrose resin (0.5 mL of Ni²⁺-Sephacrose resin per 20 mg total protein) was pre-equilibrated in buffer E with 10 mM imidazole plus 0.03% (w/v) DDM. The supernatant was diluted 1.6-fold to reduce the DDM concentration and then added to the Ni²⁺-Sephacrose column material and nutated for 1 hour at 4 °C. The mixture was poured into a poly-prep column, after which the resin was washed with 20 column volumes of buffer E containing 50 mM imidazole plus 0.02% (w/v) DDM. Proteins were eluted in 3 column volumes of buffer E with 500 mM imidazole plus 0.02% (w/v) DDM.

Reconstitution and storage of ArcD2. Synthetic lipids were mixed from chloroform stocks in the ratios indicated in Table 5. Lipids were dried in a rotary vacuum setup (Büchi Labortechnik AG), dissolved in diethyl ether, dried again and rehydrated in buffer B to a final concentration of 20 mg of lipids x mL⁻¹. Dissolved lipids, cooled with ice water, were sonicated with a tip sonicator (Sonics and Materials, Inc.) (15 sec on, 45 sec off, 70% amplitude, 16 cycles), subjected to three freeze-thaw cycles, alternating between liquid nitrogen and a water bath at room temperature, and extruded 13 times through a 400 nm pore size polycarbonate filter (Avestin Europe GmbH) to obtain liposomes.

Using an established protocol¹⁵⁴, ArcD2 was reconstituted in preformed liposomes at a protein-to-lipid ratio of 1:800 (w/w), 1:400, 1:200 and 1:100. The liposomes were first diluted 5 times to a final concentration of 4 mg of lipids mL⁻¹ in buffer B and then destabilized by a stepwise

Table 5: Lipid compositions used in this chapter

Name	DOPE	DOPG	DOPC
50% DOPG	50	50	0
38% DOPG	50	38	12
25% DOPG	50	25	25
13% DOPG	50	13	37
0% DOPG	50	0	50
	POPE	POPG	POPC
38% POPG	50	38	12

All values are given in mole%.

titration with 10% (v/v) Triton X-100, until the membrane was saturated with detergent (R_{sat} ; ref. 155), after which the purified ArcD2 was added. The protein and destabilized liposomes were mixed for 15 min at 4 °C, after which detergent was removed by adding SM2 biobeads (600 mg per 20 mg of lipids) in three equal aliquots with 15 min incubation in between each addition. After the third addition, the mixture was incubated overnight, which was followed by one additional addition (200 mg per 20 mg of lipids) of SM2 biobeads and incubation for 1 hour. Finally, the proteoliposomes were collected by centrifugation (30 min, 325.000 x g, 4 °C) and resuspended in 200 μL buffer B per 20 mg of lipids, yielding a final concentration of 100 mg of lipids x mL^{-1} . Proteoliposomes were divided into three aliquots and stored in liquid nitrogen.

Purification of PercevalHR. PercevalHR was purified in a manner similar to ArcA, ArcB and ArcC1, except that different buffers were used. Ni^{2+} -Sephacrose resin was pre-equilibrated in 25 mM KPi, pH 8.0 with 500 mM NaCl with 5% (v/v) glycerol (buffer F) plus 10 mM imidazole. The resin was washed with buffer F plus 25 mM imidazole and protein was eluted with buffer F plus 250 mM imidazole. The most concentrated fractions were run on a Superdex 200 Increase 10/300 GL size-exclusion column (GE Healthcare) in 10 mM NaPi, pH 7.4 with 150 mM NaCl and 5% (v/v) glycerol. Protein containing fractions (1-2 mg mL^{-1}) were aliquoted in volumes of 50 μL , flash-frozen and stored at -80 °C.

Protein purity and concentration determination. Protein purity was determined with SDS-PAGE, using a 4% stacking gel and 12.5% separating gel, as described in ref. 156. Cell lysate at 10 mg/ml and Ni^{2+} -Sephacrose resin flow through fractions were diluted 15-fold, Ni^{2+} -Sephacrose resin

Figure 7: Full SDS-PAGE gels showing the purification of ArcA, ArcB, ArcC1 and ArcD2. Molecular weight (M_w) markers at 97, 66, 45, 30, 20 and 14 kDa. Cell lysate fractions equals raw cell lysate before purification, column flow through, and column wash fractions of the Ni^{2+} -Sephacrose resin purifications. Metal-affinity and size-exclusion chromatography (SEC) purified fractions are also shown. (A) SDS-PAGE gel for ArcA and ArcB. (B) SDS-PAGE gel for ArcC1. (C) SDS-PAGE gel for ArcD2.

wash fractions were diluted 1.25-fold and protein fractions were diluted to 0.2 mg mL⁻¹ with loading buffer (final concentrations: 2% (v/v) SDS, 4% glycerol, 0.01% bromophenol blue plus 5% β-mercaptoethanol in 50 mM Tris-HCl, pH 6.8). Protein gels were stained with Lonza Prosieve EX Safe Stain (Fig. 7). Protein concentration was calculated from the extinction coefficient and the absorbance at 280 nm, measured with a microvolume UV-Vis spectrophotometer (NanoDrop Technologies, Inc.).

Oligomeric state determination of proteins. Ni²⁺-Sepharose/size-exclusion chromatography-purified fractions of ArcA, ArcB, ArcC1 and ArcD2 were analyzed on a second Superdex 200 Increase 10/300 GL size-exclusion column (GE Healthcare) in buffer D [with 0.02% (w/v) DDM for ArcD2], which was coupled to a multi-angle light scattering system with detectors for absorbance at 280 nm (Agilent Technologies, Inc.), static light scattering (Wyatt Technology Corporation) and differential refractive index (Wyatt Technology Corporation). Data analysis was performed with the ASTRA software package (Wyatt Technology Corporation), using a value for the refractive index increment (dn/dc)_{protein} of 0.180 mL mg⁻¹ and (dn/dc)_{detergent} of 0.143 mL mg⁻¹ as described in ref. 140. We calibrated the detectors with BSA for ArcA, ArcB and ArcC1 and with aldolase for ArcD2 (Fig. 8).

Figure 8: Static light scattering chromatograms of BSA and aldolase. Static light scattering (SLS) chromatograms for bovine serum albumin (BSA; left) and aldolase (right), with refractive index (blue traces), light scattering (green traces) and absorption at 280 nm (pink traces). The molecular weight at the peaks (yellow traces) indicate 66 and 157 kDa for BSA and aldolase, respectively. BSA was used as a standard protein for ArcA, ArcB and ArcC1; aldolase was used for ArcD2.

Enzymatic assays for ArcA and ArcB. Activity of ArcA and ArcB was measured with the COLDER assay, originally described in ref. 143. Either 2 $\mu\text{g mL}^{-1}$ ArcA or 0.25 $\mu\text{g mL}^{-1}$ ArcB was incubated in buffer B for 3 min at 30 °C in a total volume of 275 μL . To start the reaction, varying concentrations of either 0 to 480 μM L-arginine for ArcA, or 0 to 10 mM L-ornithine plus 0 to 5 mM carbamoyl-Pi for ArcB were added and citrulline formation was analyzed every 30 sec over a 2.5 min period. 200 μL of COLDER solution (20 mM 2,3-butanedione monoxime (DAMO), 0.5 mM thiosemicarbazide, 2.25 M phosphoric acid, 4.5 M sulfuric acid plus 1.5 mM ammonium iron(III) sulfate) was pipetted into each well of a 96-well flat-bottom transparent polystyrene plate (Greiner Bio-One International GmbH), to which 50 μL of reaction mixture was added to stop the enzymatic conversion. Additionally, a set of calibration samples with L-citrulline concentrations ranging from 0 to 250 μM was added to the COLDER solution. To allow color development, the plate was sealed with thermo resistant tape (Nalge Nunc International) and incubated for 20 min at 80 °C in a block heater (Cole-Parmer Instrument Co Ltd). Afterwards, the plate was cooled down to room temperature for 30 min, the condensate was centrifuged (1 min, 1000 x g, 20 °C) and the absorbance was measured at 540 nm in a plate reader (BioTek Instruments, Inc.). Enzyme activity (in nmol L-citrulline x min⁻¹ x mg protein⁻¹) was determined by the formula:

$$\text{Act}_{\text{enz}} = \frac{\Delta_{\text{enz}}}{\Delta_{\text{cal}}} * \frac{1}{c_{\text{enz}} * \text{vol}_{\text{rm}}} \quad (1)$$

where Δ_{enz} (AU min⁻¹) and Δ_{cal} (AU x nmol L-citrulline⁻¹) are the slopes of the enzyme and calibration curves, respectively; c_{enz} is the final concentration of enzyme in mg mL⁻¹ and vol_{rm} is the volume of the reaction mixture in mL. After plotting the enzyme activities (reaction rates) at various concentrations of substrate, the datapoints were fitted with the Michaelis-Menten equation:

$$v = \frac{V_{\text{MAX}} * [\text{S}]}{K_{\text{M}} + [\text{S}]} \quad (2)$$

where v is the reaction rate, V_{MAX} is the maximum reaction rate, K_{M} is the Michaelis constant and $[\text{S}]$ is the substrate concentration. In addition, we

used the Michaelis-Menten equation with uncompetitive inhibition to fit the substrate inhibition of ArcA (Fig. 3C), for which we modified equation 2 as follows:

$$v = \frac{V_{MAX} * [S]}{K_M + \alpha * [S]} \quad (3)$$

where α is defined as:

$$\alpha = 1 + \frac{[I]}{K_I} \quad (4)$$

in which $[I]$ is the apparent inhibitor concentration and K_I is the inhibition constant:

$$K_I = \frac{[ES] * [I]}{[ESI]} \quad (5)$$

where ES is the enzyme-substrate complex and ESI is the enzyme-inhibitor-substrate complex.

To determine the pH and temperature dependence, as well as the role of small molecule effectors, experiments were done following the same protocol, but with either 480 μ M L-arginine for ArcA or 5 mM L-ornithine plus 5 mM carbamoyl-Pi for ArcB as the substrates and with the following adjustments. For the experiments with ADI pathway intermediates, 0 to 10 mM of L-ornithine, carbamoyl-Pi or L-arginine were added to the reaction mixtures. To determine the pH dependence of the enzymes, the 50 mM KPi in the reaction mixture was adjusted to a pH between 5.8 and 8.0 by mixing the appropriate amounts of KH_2PO_4 and K_2HPO_4 . To determine the temperature dependence of the enzymes, the reaction mixture was incubated between 26 °C and 62 °C. To determine the effect of freeze-thaw cycles on the enzymes, the protein stocks (4.2 to 7.2 mg of protein mL^{-1}) were either not frozen (control sample) or frozen in liquid nitrogen and thawed in an ice-water bath at 10 °C; the freeze-thaw cycles were repeated up to 8 times prior to determining the enzymatic activity. Alternatively, 2 μ M ArcB was encapsulated into ArcD2 proteoliposomes (66 μ L, 6.6 mg of lipid) with 5 mM Mg-ADP ($MgSO_4$ plus ADP) and 0.5 mM L-ornithine in buffer B, the samples were frozen and

thawed 0 to 5 times. Next, the proteoliposomes were dissolved with 0.1% (v/v) Triton X-100 and the enzymatic activities of ArcA and ArcB were determined; control experiments showed that Triton X-100 had no effect on the enzymes or the COLDER assay. To determine the salt dependence of the enzymes, buffer B was supplemented with 50 to 300 mM of KCl or NaCl in the reaction mixture. Alternatively, the reaction mixture was composed of 50 to 300 mM of KPi, NaPi or Tris-HCl at pH 7.0.

Enzymatic assays for ArcC1. The activity of ArcC1 was measured from changes in ATP/ADP ratio with the ratiometric fluorescent sensor PercevalHR¹⁴⁴ (for the K_M and V_{MAX} data), or by direct measurements of ATP with the ATPliteTM Luminescence Assay System (PerkinElmer, Inc.).

PercevalHR measurements: For the measurements with PercevalHR, 3.3 $\mu\text{g mL}^{-1}$ ArcC1 was incubated in buffer B supplemented with 5 mM of MgSO_4 and 10 $\mu\text{g mL}^{-1}$ of purified PercevalHR in 105.250-QS cuvettes (Hellma Analytics) in a FP-8300 spectrofluorometer (Jasco, Inc.). ADP was added in varying concentrations (0.1 to 10 mM) and the mixture was incubated for 5 min at 30 °C. To start the reaction, carbamoyl-phosphate was added in varying concentrations (0.2 to 10 mM). The fluorescence spectrum of PercevalHR was measured by excitation from 400 ± 5 nm to 510 ± 5 nm, while the emission was recorded at 550 ± 5 nm. As the pH of the reaction mixture changes in time and PercevalHR is sensitive to pH (Fig. 3 in Chapter 3; ref. 144), it was necessary to measure the pH changes and correct the Perceval HR readout accordingly. In the pH experiments PercevalHR was substituted with pyranine and the fluorescence spectrum of pyranine was measured by excitation from 380 ± 5 nm to 480 ± 5 nm, while the emission was recorded at 512 ± 5 nm.

Pyranine calibration: The pyranine signal was calibrated at a sensor concentration of 0.1 μM in 50 mM KPi with varying pH values (from 6.0 to 9.0). The pH in solution was plotted against the ratio of the peaks at 450 nm and 405 nm and fitted using the following logistic function:

$$y = \frac{L}{1 + e^{-k * (x - x_0)}} \quad (6)$$

where L is the curve's maximum value; k is the logistic growth rate and x_0 is the x-value of the sigmoid's midpoint. Equation 6 was re-written

with the parameters: $x = \text{pH}$ and $y = F_{450}/F_{405}$ to obtain the following equation:

$$\text{pH} = \frac{\ln(L * \frac{F_{405}}{F_{450}} - 1)}{-k} + x_0 \quad (7)$$

By fitting equation 7 to the data points in 50 mM KPi (Fig. S4B in Chapter 3), the following values were obtained: $L = 3.60$, $k = 2.38$ and $x_0 = 7.84$.

PercevalHR calibration: The PercevalHR signal was calibrated at a sensor concentration of $10 \mu\text{g mL}^{-1}$ in 50 mM KPi with varying pH values (from 6.6 to 7.6), containing a mixture of ATP and ADP at a total concentration of 5 mM and a total MgSO_4 concentration of 5 mM. The ATP/ADP ratio was plotted against the ratio of the two excitation peaks at 430 nm and 500 nm and were fitted using the Hill equation:

$$\frac{F_{500}}{F_{430}} = \text{start} + (\text{end} - \text{start}) \frac{(\frac{\text{ATP}}{\text{ADP}})^n}{k^n + (\frac{\text{ATP}}{\text{ADP}})^n} \quad (8)$$

where k is the apparent affinity constant; n is the Hill coefficient; start and end refer to the y -values at the vertical asymptotes. The Hill coefficient was constrained to 1. When the parameters of datasets recorded at varying pH values were compared, it was evident that only start and end were affected by pH, k remained constant. The start and end values were plotted against the pH and were fitted by using the following logistic equations (Fig. S2C in Chapter 3):

$$\text{start} = y_0 + A * e^{R_0 * \text{pH}}, \text{end} = y_0 + A * e^{R_0 * \text{pH}} \quad (9)$$

where y_0 , A and R_0 are the fit parameters. The resulting equations were incorporated into equation 8 to yield a formula in which the ATP/ADP ratio is dependent on the pH of the solution and the ratio of the excitation peaks at 430 nm and 500 nm of PercevalHR:

$$\frac{\text{ATP}}{\text{ADP}} = \frac{4.11 * \left(\frac{F_{500}}{F_{430}} - (-0.021 + 2 * 10^{-6} * e^{1.76 * \text{pH}}) \right)}{(-1.15 + 3.5 * 10^{-3} * e^{0.96 * \text{pH}}) - \frac{F_{500}}{F_{430}}} \quad (10)$$

ATP luminescence measurements: For the ATP luminescence measurements, $0.25 \mu\text{g mL}^{-1}$ ArcC1 was incubated in buffer B for 3 min at 30°C , in a total volume of $275 \mu\text{L}$. To start the reaction, 5 mM Mg-ADP plus 5 mM carbamoyl-Pi were added and ATP formation was analyzed every 30 sec for up to 2.5 min. To stop the reaction, $50 \mu\text{L}$ of the mixture was pipetted into $50 \mu\text{L}$ of ATPlite™ mammalian cell lysis solution plus $50 \mu\text{L}$ of buffer B, which was present in each well of a 96-well flat-bottom white polystyrene plate (Greiner Bio-One International GmbH). Additionally, a set of calibration samples with Mg-ATP concentrations ranging from 0 to 0.5 mM was added to the ATPlite™ mammalian cell lysis solution. The plate was then shaken for 5 min on an orbital shaker at 700 rpm, after which $50 \mu\text{L}$ of ATPlite™ substrate solution was added and the plate was shaken for another 5 min at 700 rpm. Next, the plate was dark adapted for 10 min before luminescence was read in a plate reader (BioTek Instruments, Inc.). Enzyme activity (in $\text{nmol Mg-ATP} \times \text{min}^{-1} \times \text{mg protein}^{-1}$) was determined by equation 1 with the modification that Δcal is the slope of the calibration curve in $\text{AU} \times \text{nmol Mg-ATP}^{-1}$.

For the experiments with ADI pathway intermediates, 0 to 10 mM of L-arginine, L-ornithine or L-citrulline were added to the reaction mixtures. To determine the effects of pH, temperature, freeze-thaw cycles, salt concentration and buffer composition, the same adjustments were made as described in the previous section for ArcA and ArcB.

Transport assays for ArcD2. Proteoliposomes with ArcD2 in the membrane at a protein-to-lipid ratio of 1:200 (w/w) ($66 \mu\text{L}$, 6.6 mg of lipid) were encapsulated with L-ornithine (0, 0.5, 1, 2, 3.5, 5 or 10 mM) or L-citrulline (0, 1, 2, 5, 10 or 20 mM) in buffer B with 5 freeze-thaw cycles in a total volume of $200 \mu\text{L}$. The proteoliposomes were extruded 13 times through a 200 nm pore size polycarbonate filter and diluted to 6 mL in buffer B with or without the same concentration of L-ornithine or L-citrulline as present on the inside. Proteoliposomes were collected by centrifugation (20 min, $225,000 \times g$, 4°C), washed with buffer B (6 mL), centrifuged again and resuspended in $30 \mu\text{L}$ buffer B per 6.6 mg of lipid, yielding a final concentration of $220 \text{ mg of lipid} \times \text{mL}^{-1}$. For the transport assay, proteoliposomes were diluted 100-fold to a final concentration of $2.2 \text{ mg of lipid} \times \text{mL}^{-1}$, in pre-heated (3 min, 30°C) buffer B with $25 \mu\text{M}$ L-arginine [of which 4% (mol/mol) was ^{14}C -radiolabeled] and $100 \mu\text{L}$

samples were taken every 20 sec over an 80 sec period. After given reaction times, the samples were diluted into 2 mL of ice-cold buffer B and filtered over 0.45 μm pore size cellulose nitrate filters to stop the uptake of amino acid. The filter was then washed with another 2 mL of buffer B. Radioactivity on the filter was quantified by liquid scintillation counting using Ultima Gold MV scintillation fluid (PerkinElmer) and a Tri-Carb 2800TR scintillation counter (PerkinElmer). To impose a membrane potential, proteoliposomes were diluted 100-fold into 50 mM NaPi, pH 7.0 (buffer G) instead of buffer B, supplemented with 1 μM of the potassium ionophore valinomycin ($\Delta\Psi = -120 \text{ mV}$).

To determine the K_M and V_{MAX} values of arginine/ornithine and arginine/citrulline transport, the same protocol was used except that the external concentration of arginine or the internal concentration of ornithine or citrulline were varied. To determine the optimal reconstitution conditions, ArcD2-to-lipid ratios of 1:800 (w/w), 1:400 1:200 and 1:100 were used, and the proteoliposomes were filled with 10 mM L-ornithine. To determine the lipid dependence of ArcD2, the protein was reconstituted into the lipid mixtures specified in Table 5; the proteoliposomes were filled with 10 mM L-ornithine and were assayed with 10 μM L-arginine on the outside [of which 10% (mol/mol) was ^{14}C -radiolabeled].

To determine the influx of arginine (without counter-substrate present on the inside), the proteoliposomes were filled with buffer B and frozen-thawed as described heretofore. Proteoliposomes were incubated in buffer B or buffer G plus 1 μM valinomycin and were assayed with 1 μM ^{14}C -L-arginine. Samples were taken every 10 to 15 min; the transport reactions were terminated and the vesicles were collected by ultrafiltration as described above. To determine the duration of the artificially-imposed membrane potential, proteoliposomes were filled with 10 mM L-citrulline and diluted in buffer G plus 1 μM valinomycin with or without 250 mM NaCl. 25 μM ^{14}C -L-arginine was added 0, 10, 20, 30, 45 and 60 min after the imposition of the membrane potential.

Chapter 3

A synthetic metabolic network for physicochemical homeostasis

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Abstract

One of the grand challenges in chemistry is the construction of functional out-of-equilibrium networks, which are typical of living cells. Building such a system from molecular components requires control over the formation and degradation of the interacting chemicals and homeostasis of the internal physical-chemical conditions. The provision and consumption of ATP lies at the heart of this challenge. We report the in vitro construction of a pathway in vesicles for sustained ATP production that is maintained away from equilibrium by control of energy dissipation. We maintain a constant level of ATP with varying load on the system. The pathway enables us to control the transmembrane fluxes of osmolytes and to demonstrate basic physicochemical homeostasis. Our work demonstrates metabolic energy conservation and cell volume regulatory mechanisms in a cell-like system at a level of complexity minimally needed for life.

Introduction

The generation and consumption of ATP lies at the heart of life. Complex networks of proteins, nucleic acids and small molecules sustain the essential processes of gene expression and cell division that characterize living cells, but without ATP they are non-functional. Herein lies one of the major challenges in the construction of synthetic cell-like systems. Other processes, such as achieving tunable DNA replication, efficient transcription and translation, and vesicle division^{157,158} are essentially secondary to the solution of a controlled energy supply. Metabolic energy conservation is a prerequisite for synthetic systems no matter how complex. Energy is critical not just for (macro)molecular syntheses but also for maintaining the cytoplasm in a state compatible with metabolism through control over pH, ionic strength and solute composition. Here we have addressed that issue and show that we can control ATP production and ionic homeostasis in synthetic vesicles.

The bottom-up construction of synthetic cells from molecular components¹⁵⁹ differs in concept and strategy from the top-down approach to engineer minimal cells, pioneered by the J. Craig Venter

institute¹⁶⁰. Yet, both approaches address what tasks a living cell should minimally perform and how this can be accomplished with a minimal set of components. New biochemical functions and regulatory principles will be discovered as we make progress towards constructing a minimal cell.

In the field of bottom-up synthetic biology (perhaps better called synthetic biochemistry), work is progressing towards establishing new information storage systems¹⁶¹, replication of DNA by self-encoded proteins¹⁶², the engineering of gene and protein networks^{163,164}, formation of skeletal-like networks¹⁶⁵, biosynthesis of lipids¹⁶⁶⁻¹⁶⁸, division of vesicles^{169,170}, development of non-lipid compartment systems^{171,172} and chemical homeostasis through self-replication^{173,174}. Protein synthesis has been realized using recombinant elements¹⁷⁵, which have been incorporated into vesicles^{176,177} or water-in-oil droplets¹⁷². However, long-term sustained synthesis of chemicals is a bottleneck in the development and application of synthetic cell-like systems^{178,179}. At the root of the poor performance of reconstituted systems are challenges that relate to sustained production of nucleotides, import of substrate(s) and export of waste product(s), control of the internal physicochemical conditions (pH, ionic strength, crowding) and stability of the lipid-bounded compartment, all of which require constant energy dissipation.

Inspired by the challenges of the bottom-up construction of a living cell, we focus on the development of new open vesicle systems that sustain nucleotide levels and electrochemical gradients to allow further functionalities to be integrated. ATP is especially crucial, not only as a source of metabolic energy for most biological processes, but also as a hydrotrope, influencing the viscosity and possibly the structure of the cytosol¹⁸⁰. Energy consumption in a growing cell is dominated by polymerization reactions and maintenance processes¹⁸¹, so regeneration of ATP is required to keep the cell running. Recent developments in the field of synthetic biochemistry have started to address the issue of ATP homeostasis. A cell-free molecular rheostat for control of ATP levels has been reported, employing two parallel pathways and regulation by free inorganic phosphate²⁷, but the system has not been implemented in vesicles. Photosynthetic artificial organelles have been constructed that form ATP on the outside of small vesicles, encapsulated in giant vesicles, allowing optical control of ATP dependent reactions^{25,26}.

Here, we present the construction of a molecular system integrated into a cell-like container with control of solute fluxes and tunable supply of energy to fuel ATP-requiring processes. We have equipped the vesicles with sensors for online readout of the internal ATP/ADP ratio and pH, allowing us to conclude that the system enables long-term metabolic energy conservation and physicochemical homeostasis.

Figure 1: Layout and thermodynamics of the system. (A) Simplified schematic of the arginine breakdown pathway. (B) Structures of arginine, citrulline and ornithine at neutral pH. (C) Thermodynamics of arginine conversion for the reactions of ArcA, ArcB and ArcC1. G^0 values were calculated for pH 7.0 and an ionic strength of 0.1 M using eQuilibrator 2.2. (D) Thermodynamics of the arginine/ornithine antiport reaction. G values were calculated at varying concentration gradients of arginine (outside to inside) and ornithine (inside to outside). When the arginine and ornithine gradients are opposite, that is, $[\text{Arg}]_{\text{in}} < [\text{Arg}]_{\text{out}}$ and $[\text{Orn}]_{\text{in}} > [\text{Orn}]_{\text{out}}$, then a negative (and thus favorable) G value is obtained. Four scenarios for the product of the arginine and ornithine gradients are indicated.

Results & Discussion

A system for sustained production of ATP. The conversion of arginine into ornithine, ammonium ion plus carbon dioxide yields ATP in three enzymatic steps (Fig. 1A)³⁵. For sustainable energy conservation in a compartmentalized system, the import of substrates and excretion of products have to be efficient, which can be achieved by coupling the solute fluxes. The antiporter ArcD2 facilitates the stoichiometric exchange of the substrate arginine for the product ornithine (Fig. 1B)²², which is important for maintaining the metabolic network away from equilibrium. The thermodynamics of the arginine conversion under standard conditions are given in Figure 1C. The equilibrium constant of the conversion of citrulline plus phosphate into ornithine plus carbamoyl-phosphate is highly unfavorable, but the overall standard Gibbs free energy difference (ΔG^0) of the breakdown of arginine is negative. Since the actual ΔG is determined by ΔG^0 and the concentration of the reactants, the antiport reaction favors an even more negative ΔG by maintaining an out-to-in gradient of arginine and in-to-out gradient of ornithine (Fig. 1D). We anticipate that NH_3 and CO_2 will passively diffuse out of the cell.

Engineering of the metabolic network for ATP. To construct the system for ATP regeneration, we purified and characterized arginine deiminase (ArcA), ornithine transcarbamoylase (ArcB), carbamate kinase (ArcC1), and the arginine/ornithine antiporter ArcD2. Their kinetic and molecular properties are summarized in Figure 2A. The enzymatic network is enclosed with inorganic phosphate and Mg-ADP in vesicles composed of synthetic lipids, while ArcD2 is reconstituted in the membrane. The concentration and number of reporters, ions and metabolites per vesicle is given in Table 1. The lipid composition of the vesicles is based on general requirements for membrane transport (bilayer and non-bilayer-forming lipids, anionic and zwitterionic lipids), which is tuned to our needs (see below; ref. 182).

Vesicles obtained by extrusion through 400 and 200 nm filters have an average radius of 84 nm (SD = 59 nm; $n = 2090$) and 64 nm (SD = 39 nm; $n = 2092$) respectively (Fig. 2B and S1, p. 113), as estimated from cryo-electron microscopy images. The average internal volumes of the vesicles

Figure 2: Characterization of the components of the system. (A) Molecular and kinetic properties of the enzymes; K_{eq} was calculated as in Figure 1C. The K_M values of a given substrate were determined under conditions of excess of the other substrate. The number of molecules per vesicle was calculated from the internal concentration of the enzymes and the average size of the vesicles, when extruded through a 400 nm (left column) and 200 nm (right column) filter (Appendix 1). Kinetic parameters of ArcB are given for the back reaction. The kinetic parameters of ArcD2 were estimated from measurements in cells¹¹⁷, assuming that ArcD2 constitutes 1% of membrane protein; the data for OpuA are from ref. 183. (B) Distribution of the radius of lipid vesicles extruded through a 400 nm (blue bars) and 200 nm (black bars) filter, as estimated from CryoTEM micrographs (Fig. S1, p. 113). The diameter of 2090 vesicles (400 nm filter) and 2092 vesicles (200 nm filter) were measured with ImageJ. (C) Distribution of the internal volume, based on the distribution of radii, assuming that all vesicles are

Table 1: Average number of molecules per vesicle

Compound	Internal concentration	Molecules per vesicle (84 nm radius)	Molecules per vesicles (226 nm radius)
L-Ornithine	0.5 mM	740	14,500
ADP	5 mM	7,400	144,600
Mg ²⁺	5 mM	7,400	144,600
Pyranine	100 μ M	150	2,900
PercevalHR	2.9 μ M	4	83
Phosphate	50 mM	73,700	1,446,200
K ⁺	50 mM	73,700	1,446,200
Na ⁺	25 / 40 mM	36,800 / 59,000	723,100 / 1,157,000
Cl ⁻	25 / 40 mM	36,800 / 59,000	723,100 / 1,157,000
ATP/ADP ratio = 3 (no GB uptake)	3.75 / 1.25 mM	5,500 / 1,800	108,500 / 36,200
ATP/ADP ratio = 2 (with GB uptake)	3.33 / 1.67 mM	4,900 / 2,500	96,400 / 48,200
Glycine betaine (imported)	50 mM	73,700	1,446,200

center around radii of 226 nm (SD = 113 nm) and 123 nm (SD = 49 nm), respectively (Fig. 2C). Although a fraction of the vesicles is multi-lamellar, it is likely that all layers of the vesicles are active because we reconstitute the membrane proteins in liposomes prior to the inclusion of the enzymes, sensors and metabolites (protocol A1). The encapsulation of the luminal components is done by five freeze-thaw cycles, which induce content exchange between vesicles and homogenize the

spherical. (D) Kinetics of arginine uptake in proteoliposomes with 1 mM (blue circles) and 0.1 mM (black squares) ornithine on the inside (protocol B2); ¹⁴C-arginine concentration of 10 μ M. Inset: influence of a membrane potential ($\Delta\Psi$) on arginine uptake with 1 mM ornithine on the inside. Data from biological duplicates, panel error bars indicate standard deviation, inset error bars indicate standard error. (E) Kinetics of arginine uptake in proteoliposomes with 10 mM (blue circles) and 1 mM (black squares) citrulline on the inside (protocol B2); ¹⁴C-arginine concentration of 10 μ M. Inset: influence of a $\Delta\Psi$ on arginine uptake with 10 mM citrulline on the inside. Data and error bars as in panel D.

membranes. The vesicles obtained by extrusion through 200 nm filters are more homogenous in size (Fig. 2B and 2C) but contain a smaller number of components (Fig. 2A and Table 1), yet the performance of the metabolic network is similar in both types of compartments (see below).

We first characterized ArcD2 in lipid vesicles without the enzymatic network and demonstrated exchange of arginine for ornithine (Fig. 2D). ArcD2 transports arginine in and ornithine out in both membrane orientations, which is a property of this type of secondary transporter. The direction of transport is determined by the concentration gradients of the amino acids, not by the orientation of the protein. The arginine/ornithine antiport reaction is not affected by an imposed membrane potential ($\Delta\Psi$) (Fig. 2D, inset). Surprisingly, we also detect that ArcD2 exchanges arginine for citrulline, albeit at a much lower rate than the arginine/ornithine antiport (Fig. 2E). The arginine/citrulline antiport is electrogenic (Fig. 2E, inset), which agrees with arginine (and ornithine) being cationic and citrulline being neutral at pH 7 (Fig. 1B).

The turnover number (k_{cat}) and equilibrium constant (K_{eq}) of the enzymes were used to guide the initial design of the pathway, and the enzymes were incorporated in the vesicles at a copy number well above the stochastic threshold (Fig. 2A). The ArcD2 protein is reconstituted at a protein-to-lipid ratio of 1:400 (w/w), yielding on average 61 and 18 antiporters per vesicle with radii of 226 and 123 nm, respectively. Since arginine is imported when a counter solute is present on the inside, we include L-ornithine in the vesicles to enable the metabolism of arginine.

For readout of ATP production, we enclosed PercevalHR¹⁴⁴, a protein-based fluorescent reporter of the ATP/ADP ratio (Fig. 3A); the calibration and characterization of the sensor are shown in Figure S2 (p. 114). Upon addition of arginine, the vesicles produce ATP. Thus, after an initial, rapid, increase in the ratio of the excitation maxima at 500 nm and 430 nm (representing an increase in ATP/ADP ratio) the ratio unexpectedly declines after 30 min (Fig. 3B, blue trace). The ATP/ADP ratio increases again and stabilizes (Fig. 3B, black trace) in the presence of the protonophore carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), suggesting that arginine conversion by the metabolic network changes the internal pH of the vesicles (see below). The drop in

fluorescence signal without FCCP is explained by the pH-dependent binding of nucleotides to PercevalHR (Fig. S2B and S2C, p. 114). The decrease in F500/430 signal suggests that the internal pH is decreasing, because the fluorescence of PercevalHR increases with increasing pH (Fig. S2B, p. 114).

Figure 3: Generation of ATP and pH changes. The internal composition of the vesicles at the start of the experiment is given in Figure 2A (enzymes) and Table 1 (ions, metabolites) and the preparation of vesicles is given in protocol A1, unless specified otherwise. Representative traces from biological duplicates are shown. **(A)** Schematic of PercevalHR; adapted from ref. 144. **(B)** Effect of FCCP on the fluorescence readout of PercevalHR (protocol A1); the ratio of the fluorescence peaks at 500 nm and 430 nm is shown. In the absence of FCCP, the fluorescence readout declines 30 min after addition of 10 mM arginine (at t = 0) due to changes in the internal pH (addition of FCCP after 2 hours increases the signal, indicated by the black arrow). The fluorescence signal is constant for several hours in the presence of 10 μM FCCP. **(C)** External pH change (protocol B5) of arginine metabolizing vesicles in outside medium with 10 mM KPi, pH 7.0 plus 355 mM KCl. The ATP production was started by adding 5 mM arginine at t = 0. **(D)** Schematic of the pH effects caused by ammonia and carbon dioxide diffusion. **(E)** Internal pH change (protocol B4) of arginine metabolizing vesicles with either 50 mM (blue trace) or 15 mM KPi plus 40 mM KCl, pH 7.0 on the inside (protocol A2; black trace). 5 mM arginine was added at t = 0.

We found that, in the initial design of the pathway, a substantial amount of citrulline is formed on the outside, which is due to residual binding of ArcA to the outer membrane surface even after repeated washing of the vesicles; control experiments rule out that ArcA is binding to ArcD2 or OpuA or due to a high or low concentration of anionic lipid. We thus inactivated external ArcA by treatment of the vesicles with the membrane-impermeable sulfhydryl reagent *p*-chloromercuribenzenesulfonate (pCMBS). To avoid inhibition of the antiporter ArcD2, we engineered a cysteine-less variant that is fully functional and insensitive to sulfhydryl reagents (Fig. S3, p. 115). This optimized system is used for further characterization and application.

Arginine breakdown and control of futile hydrolysis and pH. The breakdown of arginine, in the lumen of the vesicle, is given by the reaction equation:

The external pH increases upon the addition of arginine (Fig. 3C), which is in accordance with the reaction equation when the products (except for ATP) end up in the outside medium (Fig. 3D). Unexpectedly, the vesicle lumen acidifies over longer timescales, that is, after an initial increase of the internal pH (Fig. 3E, blue line; pyranine calibration shown in Fig. S4, p. 116); the transient in the internal pH is more evident when the internal buffer capacity is decreased (Fig. 3E, black line). The acidification of the vesicle lumen cannot be readily explained if arginine is solely converted into ornithine. Indeed, we found citrulline as an end product in addition to ornithine (Fig. 4A).

What is the basis for the futile hydrolysis of arginine? Since external ArcA was inactivated by pCMBS, we infer that part of the citrulline is not metabolized further but exported and accompanied by diffusion of NH_3 through the membrane (Fig. 4A). This side reaction is possible if steps in the pathway downstream of ArcA are limiting the breakdown of arginine (Fig. 4B, bold arrow) and when citrulline is exchanged for arginine (Fig. 4B, dashed arrow). In the vesicles with the full arginine breakdown pathway, citrulline will compete with ornithine for export, when the

internal citrulline concentration is high. The equilibrium constant of the reaction catalyzed by ArcB ($K_{eq} = 8.5 \times 10^{-6}$, see Fig. 2A) predicts high citrulline-to-ornithine ratios in the vesicles. Indeed, we find that the internal citrulline-to-ornithine ratio increases from 0 to more than 10 when arginine is converted for one hour. Thus, the K_{eq} values of

Figure 4: Control of futile hydrolysis of arginine and pH homeostasis. The internal composition of the vesicles at the start of the experiment is given in Figure 2A (enzymes) and Table 1 (ions, metabolites) and the preparation of vesicles is given in protocol A1, unless specified otherwise. Representative traces from biological duplicates are shown. **(A)** External concentration of metabolites (protocol B6): arginine (blue circles), citrulline (pink triangles), ornithine (yellow diamonds) and NH₃ (black squares), as measured by HPLC in vesicles treated with 25 μ M pCMBS. 5 mM arginine was added at $t = 0$. **(B)** Full schematic of the arginine breakdown pathway; the futile hydrolysis of arginine and arginine/citrulline exchange are depicted by bold and dashed arrows, respectively. **(C)** Stopped-flow fluorescence measurements to determine the permeability of the vesicles for NH₄Cl (blue trace), NH₄-acetate (black trace), potassium phosphate (pink trace) and sodium acetate (yellow trace); pyranine inside the vesicles was used as pH indicator. **(D)** Internal pH change (protocol B4) of arginine metabolizing vesicles with either 5 mM Mg-ADP (protocol A1; blue trace) or 15 mM Mg-ADP on the inside (protocol A3; black trace). 5 mM arginine was added at $t = 0$. **(E)** Homology model of OpuA and structure of the compatible solute glycine betaine. Glycine betaine import via OpuA consumes the ATP as indicated in panel B.

the reactions of citrulline formation and breakdown (Fig. 2A), and the substrate promiscuity of ArcD2 (Fig. 2D and 2E) enable arginine/citrulline in addition to arginine/ornithine antiport.

How do the side reactions of the arginine breakdown pathway lead to acidification of the vesicle lumen? The pK_A of $NH_4^+ \leftrightarrow NH_3 + H^+$ is 9.09 at 30 °C¹⁸⁴, and thus at pH 7.0 the fraction of NH_3 is small, but the base/conjugated acid reaction is fast. If NH_3 diffuses across the membrane, it will leave a proton behind in the vesicle lumen. Since the external volume is large compared to the internal one, there will be a net flux of NH_3 from the vesicle lumen to the medium. Using stopped-flow fluorescence-based flux measurements to probe the permeability of the vesicles for small molecules, we confirmed that NH_3 diffuses out rapidly, but that the membrane is highly impermeable for inorganic phosphate, K^+ , and Cl^- ions (Fig. 4C). CO_2 also diffuses rapidly across the membrane, down its concentration gradient, but only NH_3 leaves behind protons, therefore it is this that causes the pH change in the vesicle lumen.

Finally, the breakdown of arginine to ornithine plus NH_4^+ and CO_2 is a dead-end process, which reaches equilibrium if the produced ATP is not utilized; the system runs out of ADP in about 30 min. The production of NH_4^+ from the conversion of arginine to citrulline then takes over, and the accompanying diffusion of NH_3 out of the vesicles leads to a net acidification of the vesicle lumen (Fig. 4D). Indeed, in Figure 4D we show that the vesicles acidify significantly less when the vesicles are loaded with a higher concentration of ADP and the ATP synthesis is extended.

Load on the metabolic network. Cell growth is impacted by the solute concentration of the environment. Control of osmolyte import and export under conditions of osmotic stress allows cells to maintain their volume and achieve physicochemical homeostasis¹⁸⁵⁻¹⁸⁷. Potassium is the most abundant osmolyte in many (micro)organisms, but excessive salt accumulation increases the ionic strength, which diminishes enzyme function. To control the volume, internal pH, and ionic strength, bacteria modulate the intake of potassium ions. When needed, they replace the electrolyte for so-called compatible solutes, like glycine betaine, proline and/or sugars¹⁸⁸. Compatible solutes like glycine betaine not only act in

volume regulation but also prevent aggregation of macromolecules by affecting protein folding and stability^{189,190}.

The energy produced by the ATP breakdown pathway has been used to modulate the balance of osmolytes in the vesicles via activating the ATP-driven glycine betaine transporter OpuA (Fig. 4E). To this end we co-reconstituted OpuA with the components of the metabolic network for ATP production. OpuA transports solutes into the vesicle lumen when the protein is oriented with the nucleotide-binding domains on the inside. It happens to be that we reconstitute OpuA for more than 90% in this desired orientation^{182,183}, but any protein in the opposite orientation is non-functional because ATP is only produced inside the vesicles. In vesicles with 13 mole% DOPG [1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol)] OpuA is constitutively active and imports glycine

Figure 5: ATP/ADP homeostasis and long-term transport. The internal composition of the vesicles at the start of the experiment is given in Figure 2A (enzymes) and Table 1 (ions, metabolites) and the preparation of vesicles is given in protocol A1, unless specified otherwise. **(A)** Effect of external glycine betaine (GB) on the ATP/ADP ratio measured by PercevalHR (protocol B3) in unshocked arginine-metabolizing vesicles (made with 13 mole% of DOPG; protocol A7), in the presence (black trace) and absence (blue trace) of 180 μ M GB, added at $t = -0.5$ h. 5 mM arginine was added at $t = 0$. Representative traces from biological triplicates are shown. **(B)** Schematic of the effect of osmotic upshift and partial volume restoration through glycine betaine uptake. **(C)** Comparison of glycine betaine uptake (protocol B1) driven by ATP formed in the arginine breakdown pathway from 400 nm extruded vesicles (blue circles), 200 nm extruded vesicles (protocol A4; black squares) and the creatine-phosphate/kinase system (protocol A5; pink triangles). Inset: zoom in of uptake during the first half hour. Representative traces from biological duplicates are shown.

betaine at the expense of ATP (Fig. 5A), albeit at a low rate¹⁵⁴. OpuA is ionic strength-gated and more active when sufficient levels of anionic lipids are present in the membrane^{154,182}, which is the physiologically more relevant situation; for this, we use 38 mole% DOPG and an internal ionic strength below 0.2 M to lock OpuA in the off state.

By increasing the medium osmolality with membrane-impermeant osmolytes (KPi or KCl), the vesicles shrink due to water efflux (Fig. 5B) until the internal osmotic balance is achieved, which occurs on the timescale of less than one second. Thus, the pressure exerted by the addition of KPi or KCl is dissipated by lowering the volume-to-surface ratio of the vesicles. The accompanying increase in internal ionic strength activates OpuA and glycine betaine is imported to high levels (Fig. 5C, blue circles). The vesicles now possess an interior that is a mixture of salts and glycine betaine. The consumption of ATP by the gated import of glycine betaine is shown in Figure 6A. Most remarkably, the gated import continues for hours when the internal ionic strength remains above the gating threshold and the pH is kept constant (Fig. 5C, blue circles). The open system, with arginine feed and product drain, performs at least an order of magnitude better than closed systems for ATP regeneration, where the substrate for ATP synthesis is present on the inside and cannot be replenished, as exemplified by the creatine-phosphate/kinase system (Fig. 5C, pink triangles)¹⁸³. Comparable results were obtained when smaller, yet more homogenous vesicles were formed by extrusion through 200 nm polycarbonate filters (Fig. 5C, black squares; also Fig. S5, p. 117).

To determine the fraction of vesicles with a fully functional arginine breakdown pathway, we compared the rates of transport of glycine betaine in our synthetic cell system with those of OpuA vesicles containing 10 mM of ATP. With our synthetic network transport only occurs when ATP is formed, which requires the presence of each of the enzymes well above the stochastic threshold. When the metabolic pathway reaches steady state, the ATP and ADP concentrations are about 3.3 and 1.7 mM, respectively (Table 1; ATP/ADP ratio of 2), and under these conditions we determined the rate of transport via OpuA. The K_M value for ATP is 3 mM and the K_I for ADP is 12 mM (taken from ref. 191). From these numbers we compute the V/V_{MAX} for transport in the synthetic

vesicles. For the OpuA vesicles with 10 mM ATP there is negligible formation of ADP when initial rates of transport are determined, and the V/V_{MAX} is calculated similarly. From the ratio of the V/V_{MAX} in the vesicles with full pathway over the V/V_{MAX} in the OpuA vesicles (Fig. 5C), we obtain a lower limit for the fraction of active vesicles of 70%.

Physicochemical homeostasis. Next, we tested how the physicochemical homeostasis is sustained when the vesicles are osmotically challenged. Figure 6A shows the evolution in time of the ATP/ADP ratio upon addition of arginine to vesicles that were exposed to an increased medium osmolality 30 min before the addition of arginine; FCCP was added to avoid pH effects on the readout of PercevalHR (Fig. S2B, p. 114). In the absence of glycine betaine the ATP/ADP ratio peaks at one hour and decreases over the next four to five hours (Fig. 6A, blue line), which indicates the presence of futile ATP hydrolysis when the internal salt concentration is high. Intriguingly, when glycine betaine was added 0.5 hour after arginine (Fig. 6A, black line), the ATP/ADP ratio drops instantly, but then remains stable for six hours. Glycine betaine therefore has two effects: its accumulation provides a ‘cytosol’ that is more compatible with enzyme function (Fig. 6B and 6C), but it also provides a metabolic sink for ATP through its OpuA-mediated transport.

The decrease in ATP/ADP ratio in the absence of glycine betaine can be explained by inhibition of enzymatic activity at high ionic strength (Fig. 6B), combined with futile hydrolysis of ATP. Accordingly, the decrease in ATP/ADP ratio is much less in vesicles in which the ionic strength is kept low (Fig. 5A); compare Figure 5A and 6A. We report ATP/ADP ratios because the absolute concentrations change when the vesicles are osmotically shrunk and subsequently regain volume. In most experiments, the initial adenine nucleotide (= ADP) concentration was 5 mM but increases when the vesicles are exposed to osmotic stress. In Figure 5A we report data of unshocked vesicles and here the ATP/ADP ratios of 2 to 3 correspond to 3.33 to 3.75 mM of ATP, respectively, which is in the range of concentrations in living cells.

Importantly, the introduction of an ATP-consuming process stimulates the full pathway at the expense of citrulline formation, and hence should stabilize the internal pH. Indeed, our results show that the system is

better capable of maintaining the internal pH relatively constant when ATP is utilized (Fig. 6D, black line). Under these conditions the arginine-to-citrulline conversion is diminished relative to full pathway activity. We therefore propose that the stabilizing effect of glycine betaine accumulation originates from a lowering of the ionic strength (partial restoration of the vesicle volume), a chaperoning effect on the proteins and the maintenance of the internal pH: hence reflecting what compatible solutes do in living cells¹⁸⁸.

Figure 6: Physicochemical homeostasis of arginine-metabolizing vesicles. The internal composition of the vesicles at the start of the experiment is given in Figure 2A (enzymes) and Table 1 (ions, metabolites) and the preparation of vesicles is given in protocol A1, unless specified otherwise. **(A)** Effect of glycine betaine (GB) on the ATP/ADP ratio measured by PercevalHR (protocol B3) inside arginine-metabolizing vesicles exposed to an osmotic upshift (addition of 250 mM KCl externally) in the presence (black trace) and absence (blue trace) of 180 μM GB, added at t = 0.5 h. 5 mM arginine was added at t = 0. Representative traces from biological triplicates are shown. **(B)** Activity of ArcA (left), ArcB (middle) and ArcC1 (right) in 50 mM KPi, pH 7.0 (black bar) and 300 mM KPi, pH 7.0 (blue bar) as determined from the production of citrulline (ArcA, ArcB) or ATP (ArcC1). The activities were normalized to those in 50 mM KPi, pH 7.0, see Table S1 (p. 113) for absolute activities. Data from biological duplicates, error bars indicate

Conclusions

In summary and perspective: we present the in vitro construction of a cell-like system that maintains a metabolic state far-from-equilibrium for many hours (Fig. 6E). This is one of the most advanced functional reconstitutions of a chemically defined network ever achieved, which allows the development of complex life-like systems with adaptive behavior in terms of lipid and protein synthesis, cell growth and intercellular communication. We show that ATP is used to fuel the gated transport of glycine betaine, which allows the synthetic vesicles to maintain a basic level of physicochemical homeostasis. We have recently postulated alternative mechanisms for metabolic energy conservation by coupling substrate/product antiporters to substrate decarboxylation, allowing the formation of a proton or sodium motive force¹⁹². Combining such a pathway with the here-developed network for ATP would allow an even greater control of the physicochemical homeostasis.

Maintenance of the ATP/ADP ratio, internal pH, and presumably ionic strength are crucial for any metabolic system in the emerging field of synthetic biochemistry^{27,179}. We expect that our metabolic network will find wide use beyond membrane and synthetic biology, as biomolecular out-of-equilibrium systems will impact the development of next generation materials (e.g. delivery systems) with active, adaptive, autonomous and intelligent behavior.

standard deviation. (C) Stability of ArcA (top), ArcB (middle) and ArcC1 (bottom) in 50 mM KPi, pH 7.0 (left) and 300 mM KPi, pH 7.0 (right) at 30 °C, in the presence (black squares) and absence (blue circles) of 200 mM GB. Data as in panel B. (D) Effect of GB on the internal pH measured by pyranine (protocol B4) inside arginine-metabolizing vesicles exposed to an osmotic upshift (250 mM KCl) in the presence (black trace) and absence (blue trace) of 180 μ M GB, added at $t = 0.5$ h. 5 mM arginine was added at $t = 0$. Representative traces from biological triplicates are shown. (E) Schematic of the synthetic metabolic network in the cell-like container with the intermediates: arginine (red squares), ornithine (blue circles), citrulline (orange triangles), carbamoyl-phosphate (pink triangles) and glycine betaine (yellow ovals). NH_3 and CO_2 are not shown.

Materials & Methods

Materials. Common chemicals were of analytical grade and ordered from Sigma-Aldrich Corporation, Carl Roth GmbH & Co. KG or Merck KGaA. The lipids were obtained from Avanti Polar Lipids, Inc. (>99% pure, in chloroform): 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-amine (DOPE) [850725C], 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) [850375C] and 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (DOPG) [840475C]. *n*-dodecyl- β -D-maltoside (DDM) [D97002] was purchased from Glycon Biochemicals GmbH and Triton X-100 [T9284] from Sigma-Aldrich Corporation. ^{14}C -glycine betaine was prepared enzymatically from ^{14}C -choline-chloride (American Radiolabeled Chemicals, Inc. [ARC 0208, 55 mCi mmol $^{-1}$]) as described in ref. 182. ^{14}C -L-arginine was purchased from Moravek, Inc. [MC-137, 338 mCi mmol $^{-1}$], ^{14}C -L-citrulline from American Radiolabeled Chemicals, Inc. [ARC 0508, 55 mCi mmol $^{-1}$] and ^3H -L-ornithine hydrochloride from PerkinElmer Health Sciences, Inc. [NET1212, 21.4 Ci mmol $^{-1}$].

Expression and purification of proteins. The construction of strains used for expression of ArcA, ArcB, ArcC1, ArcD2 and PercevalHR has been described in Chapter 2, together with the methods for expression, purification and storage of the proteins. OpuA was purified from *Lactococcus lactis* strain OPU401, which has the NZ9000 ΔopuA genotype and the pNZopuAHis vector, which was made in ref. 183. Additionally, ArcA, ArcB and ArcC1 have been purified in 50 mM NaPi instead of 50 mM KPi to allow reconstitutions devoid of potassium ions (see below under protocol A6); the system is also fully functional in sodium ion-based buffers (Chapter 2).

Expression of OpuA. *L. lactis* cells were grown in rich medium: 2% (w/v) Gistex from Brenntag AG, 65 mM NaPi, pH 7.0, 1% (w/v) glucose plus 5 $\mu\text{g mL}^{-1}$ chloramphenicol at 30 °C with stirring (200 rpm). The strain for OpuA was grown as a 2 L culture in a 3 L bioreactor stirred at 200 rpm with pH control (kept above pH 6.5 with 4 M KOH) and induced at an OD $_{600}$ of 2.0 with 0.05% (v/v) of culture supernatant from a nisin A-producing strain¹⁵⁰. After induction, the strain was grown for an additional 2 hours before harvesting. Harvesting and washing was done by centrifugation (15 min, 6.000 x g, 4 °C) and resuspension of the cells in ice-cold 100 mM KPi,

Table 2: Buffers used in this chapter

Buffer	Composition
A	100 mM KPi, pH 7.0
B	50 mM KPi, pH 7.0
C	50 mM KPi, pH 7.0 with 200 mM KCl
D	100 mM KPi, pH 7.0 with 0.5 mM L-ornithine
E	50 mM NaPi, pH 7.0
F	100 mM KPi, pH 7.0 with 250 mM KCl

pH 7.0 (buffer A, Table 2). Finally, cells were centrifuged again and resuspended to an OD₆₀₀ of 100 in ice-cold 50 mM KPi, pH 7.0 (buffer B), flash-frozen in liquid nitrogen in aliquots of 50 mL and stored at -80 °C.

Preparation of membrane vesicles for OpuA. The preparation of OpuA membrane vesicles was done as follows. After cells were thawed on ice, 100 µg mL⁻¹ DNase and 2 mM MgSO₄ were added. Cells were lysed by high-pressure disruption (Constant Systems, Ltd.) with two passages at 39 kpsi and 4 °C. After lysis, 5 mM Na₂-EDTA (pH 8.0) and 1 mM PMSF (100 mM stock in isopropanol) were added and cell debris was removed by centrifugation (15 min, 22.000 x g, 4 °C). Next, the supernatant was centrifuged for 90 min at 125.000 x g at 4 °C. The pellet (containing OpuA membrane vesicles) was resuspended in ice-cold buffer B with 20% (v/v) glycerol to a 10 mg mL⁻¹ protein concentration, before freezing in liquid nitrogen (2 mL aliquots) and storage.

Enzymatic assays for ArcA and ArcB. Activity of ArcA and ArcB was measured with the COLDER assay, originally described in ref. 143. We described this assay in Chapter 2 and it is repeated here, as it was used for the K_M and V_{MAX} data. Either 2 µg mL⁻¹ ArcA, or 0.25 µg mL⁻¹ ArcB was incubated in buffer B for 3 min at 30 °C, in a total volume of 275 µL. To start the reaction, varying concentrations of either L-arginine for ArcA (0 to 480 µM L-arginine), or L-ornithine plus carbamoyl-phosphate for ArcB (0 to 10 mM L-ornithine plus 0 to 5 mM carbamoyl-phosphate) were added and citrulline formation was analyzed every 30 sec over a 2.5 min period. 200 µL of COLDER solution (20 mM 2,3-butanedione monoxime, 0.5 mM thiosemicarbazide, 2.25 M phosphoric acid, 4.5 M sulfuric acid and 1.5 mM ammonium iron(III) sulfate) was pipetted into each well of a 96-well flat-bottom transparent polystyrene plate (Greiner Bio-One International

GmbH), to which 50 μL of reaction mixture was added to stop the enzymatic conversion. Additionally, a set of calibration samples with L-citrulline concentrations from 0 to 250 μM was added to the COLDER solution. To allow color development, the plate was sealed with thermo resistant tape (Nalge Nunc International) and incubated for 20 min at 80 $^{\circ}\text{C}$ in a block heater (Stuart). Afterwards, the plate was cooled down to room temperature for 30 min, the condensate was centrifuged (1 min, 1000 $\times g$, 20 $^{\circ}\text{C}$) and the absorbance was measured at 540 nm in a plate reader (BioTek Instruments, Inc.). Enzyme activity (in nmol L-citrulline $\times \text{min}^{-1} \times \text{protein}^{-1}$) was determined by the formula:

$$\text{Act}_{\text{enz}} = \frac{\Delta_{\text{enz}}}{\Delta_{\text{cal}}} * \frac{1}{c_{\text{enz}} * \text{vol}_{\text{rm}}} \quad (1)$$

where Δ_{enz} and Δ_{cal} are the slopes of the enzyme and calibration curves, respectively, in AU min^{-1} and $\text{AU} \times \text{nmol citrulline}^{-1}$; c_{enz} is the final concentration of enzyme in mg mL^{-1} and vol_{rm} is the volume of the reaction mixture in mL.

Stability measurements of ArcA and ArcB were performed as described above, with minor adjustments. In brief, ArcA and ArcB were diluted in either buffer B or 300 mM KPi, pH 7.0, with and without 200 mM glycine betaine, and incubated for 0, 1, 3 and 5 hours at 30 $^{\circ}\text{C}$. To start the reaction, either 150 μM L-arginine (for ArcA) or 5 mM carbamoyl-phosphate plus 5 mM L-citrulline (for ArcB) were added.

Enzymatic assays for ArcC1. The activity of ArcC1 was measured from changes in ATP/ADP ratio with the ratiometric fluorescent sensor PercevalHR¹⁴⁴. We described this assay in Chapter 2 and it is repeated here, as we used it for the K_{M} and V_{MAX} data. 3.3 $\mu\text{g mL}^{-1}$ ArcC1 was incubated in buffer B supplemented with 5 mM of MgSO_4 and 10 $\mu\text{g mL}^{-1}$ of purified PercevalHR in 105.250-QS cuvettes (Hellma Analytics) in a FP-8300 spectrofluorometer (Jasco, Inc.). ADP was added in varying concentrations (0.1 to 10 mM) and the mixture was incubated for 5 min at 30 $^{\circ}\text{C}$. To start the reaction, carbamoyl-phosphate was added in varying concentrations (0.2 to 10 mM). The fluorescence spectrum of PercevalHR was measured by excitation from $400 \pm 5 \text{ nm}$ to $510 \pm 5 \text{ nm}$, while the emission was recorded at $550 \pm 5 \text{ nm}$. As the pH of the reaction mixture changes in time and PercevalHR is sensitive to pH, it was

necessary to measure the pH changes and correct the Perceval HR readout accordingly. In the pH experiments PercevalHR was substituted with 0.1 μM pyranine and the fluorescence spectrum of pyranine was measured by excitation from $380 \pm 5 \text{ nm}$ to $480 \pm 5 \text{ nm}$, while the emission was recorded at $512 \pm 5 \text{ nm}$. Pyranine was calibrated as described under internal pH measurements with pyranine (see below).

The PercevalHR signal was calibrated at a sensor concentration of $10 \mu\text{g mL}^{-1}$ in buffer B with varying pH values (from 6.6 to 7.6), containing a mixture of ATP and ADP at a total concentration of 5 mM and a total MgSO_4 concentration of 5 mM. The ATP/ADP ratio was plotted against the ratio of the two excitation peaks at 430 nm and 500 nm and were fitted using the Hill equation:

$$\frac{F_{500}}{F_{430}} = \text{start} + (\text{end} - \text{start}) \frac{\left(\frac{\text{ATP}}{\text{ADP}}\right)^n}{k^n + \left(\frac{\text{ATP}}{\text{ADP}}\right)^n} \quad (2)$$

where k is the apparent affinity constant; n is the Hill coefficient; start and end refer to the y-values at the vertical asymptotes. The Hill coefficient was constrained to 1. When the parameters of datasets recorded at varying pH values were compared, it was evident that only start and end were affected by pH, k remained constant. The start and end values were plotted against the pH and were fitted by using the following logistic equations (Fig. S2C, p. 114):

$$\text{start} = y_0 + A * e^{R_0 * \text{pH}}, \text{end} = y_0 + A * e^{R_0 * \text{pH}} \quad (3)$$

where y_0 , A and R_0 are the fit parameters. The resulting equations were incorporated into equation 2 to yield a formula in which the ATP/ADP ratio is dependent on the pH of the solution and the ratio of the excitation peaks at 430 nm and 500 nm of PercevalHR:

$$\frac{\text{ATP}}{\text{ADP}} = \frac{4.11 * \left(\frac{F_{500}}{F_{430}} - (-0.021 + 2 * 10^{-6} * e^{1.76 * \text{pH}}) \right)}{(-1.15 + 3.5 * 10^{-3} * e^{0.96 * \text{pH}}) - \frac{F_{500}}{F_{430}}} \quad (4)$$

Stability measurements of ArcC1 were performed as described above, with minor adjustments. In brief, ArcC1 was diluted in buffer B or 300 mM KPi, pH 7.0, with and without 200 mM glycine betaine, and incubated for 0, 1, 3 and 5 hours at 30 °C. After incubation, 5 mM of ADP, 5 mM of MgSO₄ and either PercevalHR or pyranine were added. After 5 min of incubation at 30 °C, the reaction was started with the addition of 5 mM of carbamoyl-phosphate. For the experiments performed in 300 mM KPi, pH 7.0 a new calibration of both PercevalHR and pyranine was performed, resulting in the following equation:

$$\frac{\text{ATP}}{\text{ADP}} = \frac{6.59 * \left(\frac{F_{500}}{F_{430}} - (-0.214 + 8.2 * 10^{-5} * e^{1.29 * \text{pH}}) \right)}{(-0.52 + 6.3 * 10^{-4} * e^{1.15 * \text{pH}}) - \frac{F_{500}}{F_{430}}} \quad (5)$$

Co-purification of ArcD2 and OpuA. Membrane vesicles were quickly thawed and diluted to a total protein concentration of 3 mg mL⁻¹ for OpuA and 7 mg mL⁻¹ for ArcD2 in 50 mM KPi, pH 7.0 plus 200 mM KCl (buffer C) containing 20% (v/v) glycerol in case of OpuA. 0.5% (w/v) *n*-dodecyl- β -D-maltoside (DDM) was added to the vesicles for solubilization and the mixture was nutated for either 30 min (ArcD2) or 60 min (OpuA). Unsolubilized material was removed by centrifugation (20 min, 270.000 x g, 4 °C). Ni²⁺-Sephacryl resin (0.5 mL of Ni²⁺-Sephacryl resin per 20 mg total protein) was pre-equilibrated in buffer C with 10 mM imidazole plus 0.03% (w/v) DDM. The supernatant was diluted 1.6-fold (ArcD2) or 2.5-fold (OpuA) to reduce the DDM concentration and then added to the Ni²⁺-Sephacryl column material and nutated for 1 hour at 4 °C. The mixture was poured into a poly-prep column, after which the resin was washed with 20 column volumes of buffer C containing 50 mM imidazole plus 0.02% (w/v) DDM and 20% (v/v) glycerol in case of OpuA. Proteins were eluted in 3 column volumes of buffer C with 500 mM imidazole plus 0.02% (w/v) DDM and 20% (v/v) glycerol in case of OpuA.

Co-reconstitution of ArcD2 and OpuA. Synthetic lipids were mixed from chloroform stocks in the ratio of either 50 mole% DOPE, 12 mole% DOPC and 38 mole% DOPG or 50 mole% DOPE, 37 mole% DOPC and 13 mole% DOPG. Lipids were dried in a rotary vacuum setup (Büchi Labortechnik AG), dissolved in diethyl ether, dried again and rehydrated in buffer B to a final concentration of 20 mg of lipids x mL⁻¹. Dissolved lipids, cooled

with ice water, were sonicated with a tip sonicator (Sonics and Materials, Inc.) (15 sec on, 45 sec off, 70% amplitude, 16 cycles), subjected to three freeze-thaw cycles, alternating between liquid nitrogen and a water bath at room temperature, and extruded 13 times through a 400 nm pore size polycarbonate filter (Avestin Europe GmbH) to obtain liposomes.

Using an established protocol¹⁵⁴, ArcD2 and OpuA were co-reconstituted in preformed liposomes at a protein-to-lipid ratio of 1:2:400 (w/w), respectively. The liposomes were first diluted 5 times to a final concentration of 4 mg of lipids mL⁻¹ in buffer B with 25% (v/v) glycerol [final concentration 20% (v/v)] and then destabilized by a stepwise titration with 10% (v/v) Triton X-100, until the membrane was saturated with detergent (R_{sat} ; ref. 155), after which the purified membrane proteins were added. The proteins and destabilized liposomes were mixed for 15 min at 4 °C, after which detergent was removed by adding SM2 biobeads (600 mg per 20 mg of lipids) in three equal aliquots with 15 min incubation in between each addition. After the third addition, the mixture was incubated overnight, which was followed by one additional addition (200 mg per 20 mg of lipids) of SM2 biobeads and incubation for 1 hour.

Finally, the proteoliposomes were collected by centrifugation (2 hours for 38% (w/w) DOPG lipids or 4 hours for 13% (w/w) DOPG lipids, 125.000 x g, 4 °C) and resuspended in 200 µL buffer B per 20 mg of lipids, yielding a final concentration of 100 mg of lipids x mL⁻¹. Proteoliposomes were divided into three aliquots (of 6.6 mg of lipids each) and stored in liquid nitrogen. For the ¹⁴C-L-arginine transport assay (see below under transport assays), reconstitution was done similarly as above, except that ArcD2 was reconstituted at a protein-to-lipid ratio of 2:400 (w/w). Additionally, the proteoliposomes were diluted in buffer B without glycerol and centrifuged for 30 min, 325.000 x g at 4 °C.

Vesicles with a radius of 226 nm have 1.8×10^6 lipids per vesicle and 6.6 mg of lipids contain 5.2×10^{18} lipids, yielding 2.9×10^{12} liposomes (Appendix 1). Hence, a protein-to-lipid ratio of 1:400 (w/w) for ArcD2 gives 1.8×10^{14} molecules of ArcD2; and of 1:200 (w/w) for OpuA gives 9.1×10^{13} molecules of OpuA, which yields 61 molecules of ArcD2 and 32 molecules of OpuA per vesicle.

Encapsulation of the arginine breakdown pathway. Protocol A1: The ArcD2- and OpuA-containing vesicles used for most studies were composed of 50 mole% DOPE, 12 mole% DOPC plus 38 mole% DOPG. The proteoliposomes (66 μ L, 6.6 mg of lipids) containing ArcD2 and OpuA were mixed in buffer B with 1 μ M ArcA, 2 μ M ArcB, 5 μ M ArcC1, 5 mM ADP, 5 mM MgSO_4 , 0.5 mM L-ornithine and optionally 1.6 - 2.9 μ M PercevalHR or 100 μ M pyranine in a total volume of 200 μ L; the final liposome concentration is 33 mg of lipids $\times$ mL⁻¹. This yields a final buffer of 50 mM KPi, pH 7.0 plus 25 mM NaCl (carried over with the purified ArcA, ArcB and ArcC1) or 40 mM NaCl when PercevalHR is also included (Table 1). The enzymes, metabolites and dyes were encapsulated by five freeze-thaw cycles in a 0.5 mL Eppendorf tube, alternating between liquid nitrogen and a 10 °C ice water bath, with vortexing of the tube before freezing.

Next, the vesicles were extruded 13 times through a 400 nm pore size polycarbonate filter; the extruder was pre-washed in 100 mM KPi, pH 7.0 with 0.5 mM L-ornithine (buffer D). This procedure homogenizes the vesicles further and makes it likely that necessary components are present in all layers and compartments. The vesicles with encapsulated pyranine were then separated from free pyranine by running them over a 22 cm long Sephadex G-75 (Sigma) column in buffer D at 4 °C. To remove the residual external compounds, the vesicles were diluted to 6 mL in buffer D, collected by centrifugation (20 min, 325.000 $\times$ g, 4 °C) and washed with buffer D (6 mL), after which the vesicles were centrifuged and resuspended in 40 μ L per 6.6 mg of lipids, yielding a final concentration of 165 mg of lipids $\times$ mL⁻¹. Vesicles were kept on ice before subsequent measurements. Importantly, the ratio of the components inside the vesicles is very similar to the ratio in solution prior to encapsulation (Fig. S6, p. 118).

Protocol A2: Like protocol A1 except that the proteoliposomes were mixed with internal components in 60 mM KCl, yielding a final buffer of 15 mM KPi, pH 7.0 plus 25 mM NaCl and 40 mM KCl.

Protocol A3: Like protocol A1 except that the vesicles were loaded with 15 mM ADP plus equimolar concentrations of MgSO_4 .

Protocol A4: Like protocol A1 except that the vesicles were extruded through a 200 nm pore size polycarbonate filter.

Protocol A5: Like protocol A1 except that the vesicles were loaded with 10 mM ATP, 10 mM MgSO₄, 24 mM creatine phosphate and 2.4 mg mL⁻¹ creatine kinase in buffer B⁴⁰.

Protocol A6: Like protocol A1 except that the proteoliposomes containing ArcD2 and OpuA were first diluted in 6 mL of 50 mM NaPi, pH 7.0 (buffer E), collected by centrifugation (20 min, 325.000 x g, 4 °C) and resuspended in 66 µL of buffer E. They were then mixed in buffer E with the components mentioned above and the ArcA, ArcB and ArcC1 purified in 50 mM NaPi instead of 50 mM KPi. Additionally, the extruder was pre-washed and vesicles were resuspended in buffer E with L-ornithine. This encapsulation yields only sodium and no potassium ions inside the vesicles.

Protocol A7: Like protocol A1 except that the vesicles consisted of 50 mole% DOPE, 37 mole% DOPC plus 13 mole% DOPG.

Cryo-EM analysis of vesicles. The vesicles with encapsulated enzymes, metabolites (and sensors) were vitrified and imaging was done on a FEI Tecnai T20, 200 keV; Cryo-stage Gatan model 626. Samples were prepared under isosmotic conditions and images were recorded under low-dose conditions¹⁹³. Approximate diameters of the vesicles were measured in ImageJ. The diameters were converted to internal volume by assuming spherical vesicles, multiplication by abundance and re-normalization. The number of vesicles (n) used for analysis is given in the text and the figure legend.

Transport assays. *Protocol B1:* The vesicles with encapsulated enzymes and metabolites were diluted to a final concentration of 1.67 mg of lipids x mL⁻¹ in 100 mM KPi, pH 7.0 with 250 mM KCl (buffer F). Glycine betaine (GB) was added at a final concentration of 18 µM, of which 2% (mol/mol) was ¹⁴C-radiolabeled. The mixture was incubated for 30 min at 30 °C. The internal ATP production (and GB uptake) was then started by addition of 20 mM L-arginine and samples of 50 µL were taken at given time intervals (see Fig. 5C). For the vesicles encapsulated with protocol A5, GB uptake

was started with dilution of the vesicles. Samples were diluted in 2 mL of ice-cold buffer F and filtered over 0.45 μm pore size cellulose nitrate filters to stop the transport assay. The filter was then washed with another 2 mL of buffer F. Radioactivity on the filter was quantified by liquid scintillation counting using Ultima Gold MV scintillation fluid (PerkinElmer) and a Tri-Carb 2800TR scintillation counter (PerkinElmer). The pore size of the filters is larger than the diameter of the vesicles, but the filters retain more than 99% of the vesicles and allow for rapid filtration¹⁵⁴.

Protocol B2: The transport of ^{14}C -L-arginine was measured similarly as in protocol B1, except that proteoliposomes (66 μL , 6.6 mg of lipids) with only ArcD2 in the membrane, and L-ornithine or L-citrulline (100 μM , 1 mM or 10 mM) in buffer B in the vesicle lumen, were used (encapsulation was done by five freeze-thaw cycles [liquid nitrogen, 10 °C ice water] in a total volume of 200 μL). The proteoliposomes were first extruded 13 times through a 400 nm pore size polycarbonate filter, then 13 times through a 200 nm filter and diluted to 6 mL in buffer B with or without the same concentration of L-ornithine or L-citrulline as on the inside. Proteoliposomes were collected by centrifugation (20 min, 225.000 $\times g$, 4 °C) and either washed with buffer B (6 mL), centrifuged again and resuspended in 30 μL buffer B per 6.6 mg of lipids, or directly resuspended in buffer B, yielding a final concentration of 220 mg of lipids $\times \text{mL}^{-1}$. For the transport assay, proteoliposomes were diluted to a final concentration of 2.2 mg of lipids $\times \text{mL}^{-1}$, in buffer B with 10 μM L-arginine, of which 10% (mol/mol) was ^{14}C -radiolabeled, and 100 μL samples were taken at given time intervals (see Fig. 2D and 2E).

To impose a membrane potential, proteoliposomes in buffer B, were diluted 100-fold in 50 mM NaPi, pH 7.0 ($\Delta\Psi = -120 \text{ mV}$); 48.15 mM NaPi plus 1.85 mM KPi, pH 7.0 ($\Delta\Psi = -80 \text{ mV}$); or 39.6 mM NaPi plus 10.4 mM KPi, pH 7.0 ($\Delta\Psi = -40 \text{ mV}$), each supplemented with 1 μM of the potassium ionophore valinomycin. Samples were diluted in 2 mL of ice-cold buffer B and filtered as above to stop the transport assay. Additionally, the filter was washed with buffer B before scintillation counting.

Internal ATP:ADP ratio measurements with PercevalHR. *Calibration:* Purified PercevalHR and nucleotides (ATP and ADP) were encapsulated in liposomes. The encapsulation mixture contained liposomes at 7.5 mg of lipids x mL⁻¹, 1.6 - 2.9 μM PercevalHR, 5 mM of nucleotides in varying ratios and 5 mM MgSO₄ in buffer B. The samples were subjected to five freeze-thaw cycles [liquid nitrogen, 10 °C ice water], extruded 13 times through a 400 nm pore size polycarbonate filter, centrifuged twice (20 min, 225.000 x g, 4 °C) and finally resuspended in buffer B to a concentration of 167 mg of lipids x mL⁻¹. The liposomes were diluted in buffer F with 10 μM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) to a final concentration of 3.34 mg of lipids x mL⁻¹ in 105.250-QS cuvettes (Hellma Analytics) in a FP-8300 spectrofluorometer (Jasco, Inc.) and incubated for 3 min at 30 °C. The fluorescence spectrum of PercevalHR was measured by excitation from 400 ± 5 nm to 510 ± 5 nm, while the emission was recorded at 550 ± 5 nm. The encapsulated ATP/ADP ratio was plotted against the ratio of the peaks at 500 nm and 430 nm. Equation 2 was re-written with $n = 1$, to obtain the following equation:

$$\frac{\text{ATP}}{\text{ADP}} = \frac{k * (\frac{F_{500}}{F_{430}} - \text{start})}{\text{end} - \frac{F_{500}}{F_{430}}} \quad (6)$$

By fitting equation 6 to the data points (Fig. S2D, p. 114) the following values were obtained: $k = 3.02$, $\text{start} = 0.46$, $\text{end} = 1.30$.

Protocol B3: The vesicles with encapsulated enzymes, metabolites and PercevalHR were diluted in buffer F with 10 μM FCCP to a final concentration of 3.34 mg of lipids x mL⁻¹ in 105.250-QS cuvettes (Hellma Analytics) in a FP-8300 spectrofluorometer (Jasco, Inc.) and incubated for 30 min at 30 °C. To start ATP production, 5 mM L-arginine was added, and after 30 min of incubation glycine betaine (0, 180 μM or 3.6 mM) was added. The fluorescence spectrum of PercevalHR was measured continuously, as described above. The ATP/ADP ratio was obtained from the ratio of the peaks at 500 nm and 430 nm, using equation 6 and the values obtained under calibration.

Internal pH measurements with pyranine. *Calibration:* 100 μM pyranine was encapsulated in liposomes in 50 mM KPi at varying pH between 6.0 and 8.0. The samples were subjected to five freeze-thaw cycles [liquid nitrogen, 10 °C ice water], extruded 13 times through a 400 nm pore size polycarbonate filter and run over a 22 cm long Sephadex G-75 (Sigma) column. The vesicles were collected by centrifugation (20 min, 325.000 x g, 4 °C), washed once, centrifuged and resuspended to a concentration of 167 mg of lipids x mL⁻¹. The liposomes were diluted in 50 mM KPi at varying pH to a final concentration of 3.34 mg of lipids x mL⁻¹ in 105.250-QS cuvettes (Hellma Analytics) in a FP-8300 spectrofluorometer (Jasco, Inc.) and incubated for 3 min at 30 °C. The fluorescence spectrum of pyranine was measured by excitation from 380 $\pm$ 5 nm to 480 $\pm$ 5 nm, while the emission was recorded at 512 $\pm$ 5 nm. The fluorescence spectrum of 0.1 μM pyranine in solution in 50 mM KPi at varying pH between 6.0 and 9.0 was also measured. The data points from encapsulated pyranine in unshocked vesicles overlapped perfectly with the in-solution data, therefore the pH in solution was plotted against the ratio of the peaks at 450 nm and 405 nm and fitted using the following logistic function:

$$y = \frac{L}{1 + e^{-k * (x - x_0)}} \quad (7)$$

where L is the curve's maximum value; k is the logistic growth rate and x_0 is the x -value of the sigmoid's midpoint. Equation 7 was re-written with the following parameters: $x = \text{pH}$ and $y = F_{450\text{nm}}/F_{405\text{nm}}$ to obtain the following equation:

$$\text{pH} = \frac{\ln \left(L * \frac{F_{405}}{F_{450}} - 1 \right)}{-k} + x_0 \quad (8)$$

By fitting equation 8 to the data points in 50 mM KPi (Fig. S4B, p. 116), the following values were obtained: $L = 3.60$, $k = 2.38$ and $x_0 = 7.84$.

When vesicles are exposed to an osmotic upshift by the addition of 250 mM KCl, the internal KPi concentration increases to ~300 mM. The high salt concentration shifts the calibration of pyranine, and therefore additional calibration curves were made with pyranine in 300 mM KPi at

pH values ranging from 6.0 and 9.0. This data was fitted to equation 8 (Fig. S4B, p. 116), as above, to obtain the following values: $L = 3.70$, $k = 2.21$ and $x_0 = 7.57$. The calibration is also shifted when the vesicles were encapsulated with 15 mM Mg-ADP instead of 5 mM Mg-ADP (see above under protocol A3). Therefore, we also made calibration curves with pyranine in 300 mM KPi plus 15 mM Mg-ADP at pH values ranging from 6.0 to 7.5 (with 15 Mg-ADP and 300 mM KPi the salts precipitated above pH 7.5). This data was fitted to equation 8 (Fig. S4C, p. 116) to obtain the following values: $L = 3.22$, $k = 2.16$ and $x_0 = 7.56$.

Protocol B4: The vesicles with encapsulated enzymes, metabolites and pyranine were diluted in buffer F to a final concentration of 3.34 mg of lipids $\times$ mL⁻¹ in 105.250-QS cuvettes (Hellma Analytics) in a FP-8300 spectrofluorometer (Jasco, Inc.) and incubated for 30 min at 30 °C. To start the ATP production, 5 or 20 mM L-arginine was added and after 30 min of incubation, glycine betaine was added at a concentration of 0 or 180 μ M. The fluorescence spectrum of pyranine was measured as indicated above, and the internal pH was obtained from equation 8 and the values obtained under calibration.

External pH measurements with pyranine. *Calibration:* The fluorescence spectrum of 0.1 μ M pyranine in 10 mM KPi plus 355 mM KCl was measured at varying pH between 6.0 and 8.5 in 105.250QS cuvettes (Hellma Analytics) in a FP-8300 spectrofluorometer (Jasco, Inc.). The fluorescent spectrum was measured by excitation from 380 $\pm$ 5 nm to 480 $\pm$ 5 nm, while the emission was recorded at 512 $\pm$ 5 nm. The pH in solution was plotted against the ratio of the peaks at 450 nm and 405 nm and fitted using equation 8 (Fig. S4D, p. 116), from which the following values were obtained: $L = 3.27$, $k = 2.23$ and $x_0 = 7.48$.

Protocol B5: The vesicles with encapsulated enzymes and metabolites were diluted in 10 mM KPi, pH 7.0 plus 355 mM KCl to a final concentration of 3.34 mg of lipids $\times$ mL⁻¹ in 105.250-QS cuvettes (Hellma Analytics) in a FP-8300 spectrofluorometer (Jasco, Inc.) and incubated for 30 min at 30 °C. To start the ATP production, 5 mM L-arginine was added. The fluorescence spectrum of pyranine was measured as indicated above, and the internal pH was obtained from equation 8 and the values obtained under calibration.

Amino acid and ammonia analysis. Protocol B6: The vesicles with encapsulated enzymes and metabolites were diluted in buffer F, with or without glycine betaine, to a final concentration of 3.34 mg of lipids $\times$ mL⁻¹ and incubated for 30 min at 30 °C. The assay was started by addition of 5 mM L-arginine and samples of 75 μ L were taken at regular time intervals. Vesicles were removed from the samples by centrifugation (20 min, 225.000 $\times$ g, 4 °C) to stop the conversion of external amino acids. Subsequently, 50 μ L of supernatant was mixed with 87.5 μ L of 1 M boric acid, pH 9.0 (adjusted with 4 M KOH) and kept on ice before derivatization. 37.5 μ L of 99.8% methanol and 1.5 μ L of 42 mM diethyl ethoxymethylenemalonate (DEEMM) were added to the samples for derivatization, after which the samples were incubated for 30 min in a sonication bath at room temperature, followed by 2 hours of incubation in a heat block at 70 °C.

The protocol for reverse-phase high-performance liquid chromatography (HPLC) was adapted from ref. 194. Amino acid samples were analyzed on a Shimadzu prominence HPLC system, containing a DGU-20A5R degassing unit, a LC-30AD solvent delivery unit, a SIL-30AC autosampler, a CTO-20AC column oven and an SPD-M20A UV-VIS/Photodiode Array detector. A Shimadzu XR-ODS 3 $\times$ 75 mm C18 column was used to run the binary gradient with a flow rate of 0.9 mL min⁻¹ and an injection volume of 5 μ L. Eluent A was 25 mM acetate, pH 5.8, supplemented with 0.02% (w/v) Na-azide. Eluent B was an 8:2 (v/v) mixture of acetonitrile and methanol. The gradient was as follows (all percentages are volume-based): start was at 94% eluent A and 6% eluent B; 87% A and 13% B at 2 min; 83% A and 17% B at 10 min; 71% A and 29% B at 11 min; 67% A and 33% B at 16 min; 40% A and 60% B at 16.1 min to 18 min; 94% A and 6% B at 18.1 min towards the end of the protocol at 20 min. The compounds were identified based on retention times and quantified using the external standard method (Fig. S7, p. 119).

Membrane permeability with stopped-flow fluorescence. Principle of the method: To determine the permeability of solutes through the vesicle membrane, two independent and complementary fluorescence-based kinetic assays were used as described in ref. 24. The first assay reports volume changes of vesicles by means of calcein self-quenching fluorescence³¹. The second assay monitors the pH variation in the vesicle

lumen using the ratio-metric fluorophore pyranine¹⁹⁵. Both assays exploit the out-of-equilibrium relaxation kinetics of the vesicles after the increase of the external osmotic pressure (osmotic upshift), i.e. the addition of a solute to the vesicle solution. After the osmotic upshift, the thermodynamic equilibrium is re-established by water efflux (to re-equilibrate the chemical potential of water) and/or solute influx (to dissipate the solute concentration gradient)¹⁹⁶. The contribution of the two fluxes to the recovery kinetics depends on the relative permeability of water and the solute as described in ref. 24.

Osmolyte solutions: The 1 M stock solutions of Na-acetate, NH₄Cl and NH₄-acetate were prepared by dissolving the salts into 100 mM KPi; the pH was adjusted to 7.0 using 4 M NaOH. An empirical linear relation between concentration and osmolality for each solution was determined as described in ref. 24. The stock solutions were then diluted to an osmolality of ca. 300 mosmol kg⁻¹ by mixing with the liposome solution (yielding 95 mM Na-acetate, 110 mM NH₄Cl and 110 mM NH₄-acetate).

Liposome preparation: Liposomes were prepared as described under internal pH measurements with pyranine (see above), with minor adjustments. Calcein was added to the vesicle solution (2 mg of lipids in buffer A in a total volume of 1 mL) at a self-quenching concentration of 10 mM and enclosed by three freeze-thaw cycles, alternating between liquid nitrogen and a 40 °C water bath. Pyranine was encapsulated similarly (final concentration of 300 μM). Next, the liposomes were extruded 13 times through a 200 nm pore size polycarbonate filter and run over a 22 cm long Sephadex G-75 (Sigma) column in buffer A. Vesicles were collected and diluted to a total volume of 12 mL in buffer A. Empty liposomes for blank correction were prepared using the same procedure without addition of calcein or pyranine.

Stopped-flow fluorescence measurements: The permeability of the liposomes for KPi, KCl, Na-acetate, NH₄Cl and NH₄-acetate was assessed by monitoring the quenching of the fluorescence of calcein and by determining the changes in the internal pH using pyranine as a probe. A stopped flow apparatus (SX20, Applied Photophysics Lim., Leatherhead, Surrey, UK) was used to measure fluorescence intensity kinetics upon imposition of an osmotic upshift to the liposomes filled with calcein or

pyranine. The osmolyte (ca. 300 mosmol kg⁻¹ after mixing) and the liposome solutions (pre-equilibrated with 250 mM KCl) were each loaded in two distinct syringes, forced through the mixer (1:1 mixing ratio and 2 ms dead time) and into the optical cell (20 µL volume and 2 mm path length). The temperature was set at 20 °C using a water bath.

The white light emitted by a Xenon arc lamp (150W) was passed through a high precision monochromator and directed to the optical cell via an optical fiber. The band pass of the monochromator was optimized and set to 0.5 nm (for calcein) or 1.4 nm (for pyranine) to prevent fluorophore photobleaching during the experiment. The fluorophores were excited at 495 nm (for calcein) or at both 405 nm and 453 nm (for pyranine). 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 (3 for slow kinetics and 9 for fast kinetics) were performed for each experimental condition. Complete stopped-flow settings and acquired data processing are described in ref. 24.

Table S1: Enzyme activities for data presented in Figure 6B

Buffer	ArcA	ArcB	ArcC1
50 mM KPi	2.9 ± 0.3	233.3 ± 52.3	362.9 ± 22.8
300 mM KPi	4.8 ± 2.9	128.9 ± 49.7	135.0 ± 76.8

All values are given in $\mu\text{mol min}^{-1} \text{mg}^{-1}$. Errors indicate standard deviation.

Figure S1: Representative CryoTEM images used for size analysis. Representative micrographs showing vesicles extruded through a (A) 400 nm polycarbonate filter and (B) 200 nm polycarbonate filter; the yellow lines indicate the size measurements. Some of the shapes are somewhat distorted, most likely due to the interaction of the vesicles with the grid.

Figure S2: Fluorescence-based ATP/ADP ratio sensor PercevalHR. Fluorescence spectrum, calibration and pH dependency of PercevalHR measured in a FP-8300 spectrofluorimeter (Jasco, Inc.). (A) Representative excitation spectrum from 400 nm to 510 nm of PercevalHR encapsulated in the lipid vesicles with an emission wavelength of 550 nm, at equimolar ATP to ADP (2.5 mM each). The spectrum was corrected for background fluorescence. (B) Effect of pH on the readout of PercevalHR fluorescence. The ratio of the excitation peaks at 500 nm and 430 nm was measured for different ATP to ADP ratios, measured in 50 mM KPi, pH 7.6 (blue circles), pH 7.4 (black squares), pH 7.2 (pink triangles), pH 7.0 (yellow diamonds), pH 6.8 (green circles), and pH 6.6 (red squares), each supplemented with 5.5 mM $MgSO_4$. Representative traces from biological duplicates are shown. (C) Plot of the start and end values against pH in 50 mM KPi, as fitted with equation 3. (D) Calibration of PercevalHR inside the lipid vesicles. The ratio of the excitation peaks at 500 nm and 430 nm changes when the ATP to ADP ratio is varied. Data from biological duplicates, error bars indicate standard deviation. The data points were fit with the Hill equation (black line), as described in materials & methods ($n = 1$, $k = 3.02$, start = 0.46, end = 1.30).

Figure S3: pCMBS does not inhibit arginine/ornithine antiport by ArcD2 ΔC . Radiolabeled arginine uptake by (A) wild-type ArcD2 and (B) cysteine-less ArcD2 (ArcD2 ΔC) in the absence (blue circles) and presence (black squares) of 25 μM of pCMBS. The pCMBS was added 45 minutes prior to the start of the measurement to allow for the binding reaction to occur. The proteoliposomes were loaded with 0.5 mM L-ornithine and the final ^{14}C -L-arginine concentration was 20 μM for the wild-type and 10 μM for the cysteine-less ArcD2. Data from a single experiment, but similar measurements (optimization of assay and labeling) were done multiple times.

Figure S4: Calibration of pyranine inside lipid vesicles. (A) Representative excitation spectrum from 380 nm to 480 nm of pyranine inside the lipid vesicles with an emission wavelength of 512 nm, at pH 7.0. (B) Pyranine was measured at varying pH in 50 mM KPi (blue circles), in 300 mM KPi (pink triangles) and inside the lipid vesicles containing 50 mM KPi (green circles), using a FP-8300 spectrofluorimeter (Jasco, Inc.). The ratio of the excitation peaks at 450 nm and 405 nm changes as a function of pH. The data points in 50 and 300 mM KPi were fit with a logistic function (black and yellow traces), as described in materials & methods (50 mM KPi: $L = 3.60$, $k = 2.38$, $x_0 = 7.84$; 300 mM KPi: $L = 3.70$, $k = 2.21$, $x_0 = 7.57$). The data points inside the unshocked lipid vesicles perfectly match those in 50 mM KPi. Representative traces from biological duplicates are shown. (C) Similar to panel B, pyranine was measured at varying pH in 300 mM KPi with 15 mM Mg-ADP (blue circles) and 300 mM KPi (pink triangles). Data points in 300 mM KPi plus 15 mM Mg-ADP were fit (black trace) to obtain: $L = 3.22$, $k = 2.16$ and $x_0 = 7.56$. (D) Similar to panel B, pyranine was measured at varying pH in 10 mM KPi plus 355 mM KCl (blue circles). Data points in 10 mM KPi plus 355 mM KCl were fit (black trace) to obtain: $L = 3.27$, $k = 2.23$ and $x_0 = 7.48$.

Figure S5: ATP production and pH changes in arginine-metabolizing vesicles obtained by extrusion through 200 nm polycarbonate filters. (A) Analogous to Figure 6A, the effect of glycine betaine (GB) import on ATP production as measured by PercevalHR fluorescence (protocol B3) in arginine-metabolizing vesicles exposed to an osmotic upshift (addition of 250 mM KCl) in the presence (black trace) and absence (blue trace) of 180 μ M GB (added at $t = 0.5$ h); 5 mM arginine was added at $t = 0$. Representative traces from biological duplicates are shown. (B) Analogous to Figure 6D, the effect of GB on the internal pH measured by pyranine (protocol B4) in arginine-metabolizing vesicles exposed to an osmotic upshift (250 mM KCl) in the presence (black trace) and absence (blue trace) of 180 μ M GB (added at $t = 0.5$ h); 5 mM arginine was added at $t = 0$. Representative traces from biological duplicates are shown.

Figure S6: SDS-Polyacrylamide gel electrophoresis of purified and encapsulated proteins. (A) On the left, encapsulation mixture containing 0.94 μg of ArcA, 2.4 μg of ArcB and 1.8 μg of ArcC1. On the right, the vesicles with encapsulated proteins and co-reconstituted ArcD2 and OpuA. OpuA consists of two separate proteins: OpuABC, containing the transmembrane domain fused to the substrate-binding domain; and OpuAA, the nucleotide-binding domain fused to a regulatory domain. Although ArcB and ArcD2 have a different monomeric molecular weight (40.9 and 56.7 kDa respectively), ArcD2 migrates at a similar position as ArcB, which has been observed for many other membrane proteins¹³⁹. The purification of his-tagged ArcC1 (ArcC1-His) always yields a small amount of wild type ArcC1 (ArcC1-WT), because the cell expresses ArcC1-WT at a basal level and heterooligomers are formed; the two proteins do migrate at a different position. (B) Quantification of the ratio of the ArcA, ArcB and ArcC1 before (encapsulation mixture) and after reconstitution (inside the vesicles) as analyzed with ImageJ (standard deviation from analyzing 4 independent loadings is 0.1). The ratio inside the vesicles does not differ significantly from that of the encapsulation mixture.

Figure S7: Raw spectra from HPLC chromatograms. Representative chromatograms of amino acids and ammonia produced by the vesicles containing the arginine breakdown pathway. Chromatograms were recorded at 269 nm. As expected, at $t = 0$ only 5 mM arginine is detected (at retention time = 4 min). Citrulline, NH_3 and ornithine (at retention time 3.8; 6.8 and 14 min, respectively) appear at the expense of arginine in the chromatograms of samples taken at 2 and 6 hours. Inset: truncated chromatograms of standard solutions containing 5 mM of a single amino acid or NH_3 . Such chromatograms were used to determine the retention time and to calibrate the signals.

Chapter 4

Overview of current synthetic cells & the challenges in their construction

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Introduction

In this chapter I discuss three subjects. I start by giving an overview of modules that are important for building synthetic cells, continue with possible improvements to our system for metabolic energy conservation and finally discuss a number of challenges we faced in constructing our system. I will start off by defining what synthetic cells are and explain the purpose of making them.

There are two categories of synthetic cells, which are subdivided by the way they are constructed (Fig. 1). In the first place we have the minimal cells, which follow the ‘top-down’ synthetic biology approach: the aim is to get to a minimal set of biochemical functions that still form a functioning cell by knocking out genes in existing organisms. A functioning cell is defined as one that can sustain itself, grow and divide. In the top-down approach a minimal genome is transplanted into an empty cell (devoid of DNA) to create a new cell. This approach has been pioneered by the Venter group and resulted in the creation of the first synthetic organism JCVI-syn1.0¹⁹⁷ and subsequent improved versions^{160,198}. While this approach can quickly lead to a minimal cell, the disadvantage is that these cells still contain a lot of genes that have no known function (the latest iteration, JCVI-syn3A, still has 91 out of 493 genes with unclear function)¹⁹⁸. On the other hand we have the synthetic cells that follow the ‘bottom-up’ approach. In this case, the aim is to build a functioning cell from scratch by implementing and combining different functional modules, like e.g. compartmentalization, information processing or metabolism^{157,178,199}. The big advantage here is that every module can be studied in detail before it is implemented (and modified where needed), but getting to a functioning cell will take much longer. In the rest of this chapter we will focus on these bottom-up constructed synthetic cells.

Why are we interested in building synthetic cells? Richard Feynman had a quote on his blackboard that said “What I cannot create, I do not understand.” This argument has been applied to building synthetic cells, in the sense that once we know how to build a cell from scratch, we also understand how a cell works. While this argument has some validity, I feel it oversimplifies things: a simple recipe for a working cell may not tell us all about the intricacies happening inside a cell. Studying these

intricacies and implementing them successfully into synthetic cells is a difficult task but will lead to a better understanding of how a cell works. A second argument is that building synthetic cells will give us information about the origin of life, since contemporary cells are always created from a parent cell (as far as we know), but at some point in the past an ‘ancestral cell’ had to be formed from molecular components. While I partially agree with this argument, I am not sure whether building a synthetic cell from DNA, proteins and lipids will add much information, especially since those compounds may not have been around when the ancestral cell formed²⁰⁰. I also think that convincing theories for the origin of life have been proposed^{201,202}, and while we should keep working on validating these theories, they do not require the building of cells from scratch in the lab. I have personally always seen the building of synthetic cells as a technical challenge: to test and push the limits of current and future methods in synthetic biology. Any information that is gathered along the way is a bonus.

Figure 1: Schematic of synthetic cell construction. While the ‘top-down’ approach (left) starts from an existing microbe and develops into a minimal cell, the ‘bottom-up’ approach (right) starts from molecular components and predefined modules, which are combined into an ‘initial’ synthetic cell. The initial synthetic cell can be functionalized further with additional modules, leading to a cell that can sustain itself and ultimately grow and divide.

Current progress towards synthetic cells

The following overview is not exhaustive as I focus on four modules that I think are most important to build an ‘initial’ synthetic cell. This initial synthetic cell should be able to maintain itself, but it does not necessarily need to grow or divide. To this end, I focus on the modules of (i) compartmentalization, to keep the cell’s inside separated from the outside; (ii) information processing, to renew components of the cell; (iii) metabolism, to provide energy and building blocks; and (iv) homeostasis, to regulate and balance the components of the cell (Fig. 1). Once these modules have been implemented in an initial synthetic cell, it can serve as a basis for the addition of further modules such as: signaling, communication between cells, motility, stress response, cell growth and (unregulated) cell division. I will not discuss those modules here, but they have been recently reviewed in ref. 203 and 204.

Compartmentalization. The formation of diffusion barriers, also known as compartmentalization, is not only essential for separating the controllable inside of a cell from the changing outside, but also for partitioning different catalysts within a cell. This partitioning of reaction networks allows the enzymes to run optimally, even if they require e.g. a different pH, provided the barrier can maintain a pH gradient. While the compartments of prokaryotic and eukaryotic cells are usually made out of lipid bilayers, prokaryotic cells also use polyhedral shells composed entirely of protein²⁰⁵. Compartmentalization of enzymes and small molecules has received a lot of attention, since findings from these studies have potential in medical applications, like drug delivery or the creation of artificial organelles²⁰⁶⁻²¹⁰. Internal compartmentalization has also been realized in synthetic cells, e.g. by capturing nanoreactors into a larger polymersome²¹¹. In this section I will focus on the membranes that separate the inside and outside of a cell, for which three parameters are especially important: the material the membrane is made of, the permeability of that membrane and the eventual size of the compartment.

For the membrane material, we already have quite some choice: vesicles have been made from fatty acids, (phospho)lipids, oil-water interfaces, polymers and combinations of these components (Fig. 2)²⁰⁶. The clear

advantage of using (phospho)lipids is that they are also used natively and therefore have excellent compatibility with proteins purified from living cells. In addition, methods have been developed to synthesize phospholipids *de novo, in vitro*^{166,167,212}. A disadvantage of (phospho)lipid membranes is that they are impermeable for large and charged solutes and need (specialized) membrane proteins to allow for solute translocation²¹³. Vesicles made out of polymers have gained a lot of interest lately, as they tend to be tougher, stabler and more malleable than (phospho)lipid membranes²¹⁰. Their membranes are thicker and tend to be less permeable to small molecules, and it is possible to insert membrane channels (like OmpF, gramicidin or DNA nanopores) into them to control transport²¹⁴⁻²¹⁶. Furthermore, hybrid vesicles composed of (phospho)lipids and polymers were used to reconstitute e.g. cytochrome *bo*₃, proteorhodopsin and ATP binding cassette transporters²¹⁷⁻²¹⁹.

Figure 2: Schematic of different membrane materials. The simplest membranes are made out of a fatty acid bilayer (top-left). Living cell membranes utilize a bilayer consisting of (phospho)lipids (top-right), which are made of two fatty acid chains combined with a hydrophilic headgroup. Oil-water interfaces (bottom-left) can form vesicles, but are usually functionalized with a monolayer of phospholipids or surfactants. Polymer membranes (bottom-right) are synthesized from hydrophilic and hydrophobic polymer chains that spontaneously assemble into a membrane structure.

Fatty acids have been extensively studied, as they are hypothesized to form the building blocks for the compartmentalization of the first primitive cells^{220,221}. Fatty acid membranes are significantly more permeable than phospholipid membranes²¹³, which allows for passive transport of solutes, but decreases the protection of encapsulated content. A major disadvantage of fatty acid membranes is that they can precipitate with a small concentration of divalent cations (~ 1.5 mM of Mg^{2+} or Ca^{2+})²²² and inhibit the activity of many enzymes (like DNA and RNA polymerases)²⁰⁶, which makes them less suitable for an initial synthetic cell. Oil-water interfaces can also be used for compartmentalization¹⁷², especially in combination with phospholipids or surfactants, so that a monolayer is formed at the interface^{223,224}. While these interfaces are promising to study certain processes in the aqueous compartment, it is hard to imagine an initial synthetic cell maintaining itself in organic oil, as it will not be possible to introduce membrane proteins for e.g. the uptake of (water soluble) solutes or membrane-bound energy-transducing machineries.

The size of vesicles can be tuned for the different types of membranes, which is done in bulk by sonication or extrusion or with microfluidics. Whereas bulk methods are easier to perform, since they do not need highly-specialized equipment, they lead to vesicles that are more heterogeneous in size²²⁵. Vesicles created with microfluidics tend to be very homogeneous in size but have the disadvantage that, with current methods, they retain some organic solvent in the membrane, which is deleterious for proteins²²⁵. For both liposomes and polymersomes, vesicles have been created with sizes classified as small (SUV, 20-100 nm diameter), large (LUV, 100-1000 nm) or giant unilamellar vesicles (GUV, 1-200 μm)²¹⁰.

Information processing. The transcription and replication of DNA, as well as the translation of RNA into proteins, all fall under information processing. Ever since the encapsulation of a cell-free expression system in phospholipid vesicles¹⁷⁸ and the subsequent development of the PURE system (a purified expression system, see Table 1)^{175,226}, this topic has seen lots of developments. On the one hand, *in vitro* replication systems have been made with peptides, DNA, RNA and proteins²²⁷. Other studies looked at applications of cell-free extracts, ranging from the biosensing

Table 1: Components of the PURE system

Buffers	Enzymes	Aminoacyl-tRNA synthetases	
HEPES-KOH (pH 7.6) Potassium glutamate Magnesium acetate Spermidine Putrescine Dithiothreitol 10-Formyltetrahydrofolate 20 amino acids 46 tRNAs ATP, GTP, CTP, UTP	Transcription T7 RNA polymerase Translation Ribosome Methionyl-tRNA transformylase Initiation factors: IF1, IF2, IF3 Elongation factors: EF-G, EF-Tu, EF-Ts Release factors: RF1, RF3, RRF Energy regeneration Creatine phosphate Creatine kinase Myokinase Nucleoside-diphosphate kinase Pyrophosphatase	AlaRS ArgRS AsnRS AspRS CysRS GlnRS GluRS GlyRS HisRS IleRS	LeuRS LysRS MetRS PheRS ProRS SerRS ThrRS TrpRS TryRS ValRS

of small molecules (like mercury or fluoride) or disease-causing bacteria and viruses to the production of glycoproteins and therapeutic proteins^{228,229}. Three important parameters for building an initial synthetic cell are the self-replication of the expression system (in particular of the ribosome), the proper folding of cytosolic proteins and the proper insertion of membrane proteins into a membrane.

Although the ribosome probably was a self-replicating entity in earlier days, nowadays it is always encoded in the genome²³⁰. Since the ribosome is a key component for translation of messenger RNA into proteins, it needs to replicate (and thus maintain) itself. Despite the complexity of the ribosome, the 30S ribosomal subunit has already been fully synthesized and assembled *in vitro* and retains 21% activity compared to the native subunit²³¹. Only the 23S part of the 50S subunit has been synthesized *in vitro*, while the rest was purified from *Escherichia coli* cells²³². Reconstitution of functional 50S from *in vitro* synthesized 23S is difficult and seems to require a high concentration of salt. Self-replication of the expression system also depends on self-replication of

the DNA that codes for the system, a feat that has recently been shown by using the $\Phi 29$ virus replication proteins¹⁶². Assembly of the ribosome has been studied in living cells by combining mass spectrometry with cryo-electron microscopy²³³. These techniques revealed the presence of 13 structural intermediates and multiple assembly pathways, indicating the dynamic character of ribosome assembly²³³.

Improper protein folding can lead to the formation of macromolecular aggregates, which are a waste of energy and can be deleterious to the cell²³⁴. Most cell-free expression systems lack chaperones, which prevent improper folding *in vivo*. This led to the identification of 792 cytosolic *E. coli* proteins that are aggregation prone (of > 3,000 proteins studied)²³⁵. A follow-up study showed that in many cases the proper folding could be rescued by addition of the molecular chaperones GroEL/GroES or DnaK/DnaJ/GrpE to the PURE system²³⁶. The insertion of membrane proteins on the other hand is in many cases dependent on the SecYEG translocon membrane channel or on YidC (Fig. 3), which need to be inserted into the membrane itself²³⁷. SecYEG has already been expressed with the PURE system on the outside and inside of vesicles and was shown to be functional as it translocated e.g. OmpA, YidC and EmrE into the membrane^{238,239}. The studies supplemented the PURE system with either SecA and SecB or SRP and FtsY, which all help in bringing the membrane protein into the SecYEG channel (Fig. 3)²³⁷⁻²³⁹.

Metabolism. All life-sustaining chemical reactions that happen in an organism, ranging from converting fuel into metabolic energy or building blocks to the excretion of waste products, form what is known as its metabolism. These enzyme-catalyzed reactions are numerous in even the simplest bacteria, as e.g. 161 genes (of the 688 genes in total) in *Mycoplasma pneumoniae* code for known metabolic enzymes¹⁹⁸. Reconstituting the entire metabolism of a bacteria into a synthetic cell may be unrealistic as it would require the purification and functional reconstitution of too many components. However, parts of metabolism, like the generation of ATP and NADPH, fixation of carbon dioxide or turning glucose into desirable chemicals, have been realized *in vitro*.

The generation of ATP has been accomplished by using light or chemical energy, as described in Chapter 1. To recap: the F_0F_1 -ATP synthase has

been reconstituted together with either bacteriorhodopsin²⁶ or photosystem II and proteorhodopsin (Fig. 3 in Chapter 1)²⁵ to harvest light for ATP production. Additionally, we used the breakdown of arginine to generate ATP with chemical energy (Chapter 3)¹⁵³. Although these systems are fundamentally different, as e.g. ATP is produced on either the outside (light-based) or inside (chemical-based) of vesicles, we can compare two fundamental characteristics: the ATP synthesis rate and the duration of ATP synthesis. The bacteriorhodopsin study²⁶ reports a maximum turnover number for ATP synthesis of 8.3 s^{-1} , while the photosystem II plus proteorhodopsin system²⁵ achieves a rate of 4.3 s^{-1} . We calculated an ATP synthesis rate of 0.19 s^{-1} for the arginine breakdown

Figure 3: Schematic of protein targeting to the Sec translocon and YidC. (A) Proteins destined for the periplasm are targeted to the SecYEG translocon via a signal sequence that is recognized by the motor protein SecA. The molecular chaperone SecB keeps the protein in a translocation competent state. (B) Most membrane proteins contain information that is recognized by the signal recognition particle (SRP; a complex of Ffh and 4.5S RNA), after which the entire complex is targeted to the SecYEG translocon via the membrane-associated signal-particle receptor FtsY. (C) Some membrane proteins are targeted to YidC instead of the SecYEG translocon, and inserted into the membrane by YidC. It is unclear if SRP is involved in this pathway. Figure adapted from ref. 237.

pathway from the amount of ATP produced in one hour in unshocked vesicles (Fig. 5A in Chapter 3 and Appendix 2). While this rate is lower than the rates from the light-based systems, it is of the same order of magnitude as the turnover of the arginine/ornithine antiporter (Fig. 6B in Chapter 2), which suggests that arginine/ornithine antiport is rate-determining for arginine breakdown. The duration of ATP synthesis is reported as 4 to 6 hours at 30 °C for the bacteriorhodopsin system and 3 days at room temperature for the photosystem II plus proteorhodopsin system^{25,26}. We show ATP synthesis for at least 6 hours at 30 °C (Chapter 3), but also performed a stability experiment (see below, Fig. 7), which shows activity up to 26 hours.

While the generation of ATP is crucial, as it is one of the main energy sources for a cell, the generation of cofactors like NADPH is critical for the proper function of many enzymes. Baeyer-Villiger monooxygenases can e.g. be used for the synthesis of steroids, antibiotics, pheromones and polymerizable monomers, but need to be reduced by NADPH to sustain their activity²⁴⁰. The simplest way to generate NADPH is from glucose-6-phosphate via glucose-6-phosphate dehydrogenase, which has already been shown in polymersomes²⁴¹. A recent study combines NADH/NAD⁺ cycling with ATP generation, by using mitochondrial respiratory complex I, a ubiquinone oxidase, F₀F₁-ATP synthase and a dehydrogenase²⁴². Complex I generates an electrochemical proton gradient (used for ATP generation) while converting NADH to NAD⁺, which is recycled to NADH by alcohol or lactate dehydrogenase²⁴². The system could be supplemented with a membrane-bound, proton translocating or a soluble, energy-independent NAD(P)⁺ transhydrogenase to obtain NADPH²⁴³. A simpler system, using formate dehydrogenase, NAD(P)⁺ transhydrogenase and glutathione reductase, is currently being developed in our lab, which would allow for the production, as well as the recycling of NADH and NADPH.

Once ATP and cofactor generation has been realized, one can use these molecules for other processes, like the fixation of carbon dioxide or to turn glucose into desirable chemicals. Most carbon fixation studies focus on improving the enzyme responsible for carbon dioxide capture (the carboxylase, e.g. RuBisCo) or on making photorespiration more efficient. A recent synthetic biology study designed a carbon fixation pathway from

scratch, using 17 enzymes from 9 different organisms¹⁷⁹. The pathway's carbon fixation rate was optimized by including enzymes for ATP and NADPH regeneration and is comparable to the rate found in cell extracts¹⁷⁹. Glycolysis has been used for the *in vitro* conversion of glucose into desirable chemicals, by using the ATP generated in the second half of glycolysis and either NAD(P)H recycling or “purge valves” to get rid of excess NAD(P)H^{244,245}. In this way, glucose was converted into isoprene²⁴⁴, polyhydroxybutyrate bioplastic²⁴⁵, monoterpenes²⁴⁶ and isobutanol²⁷. The production of isobutanol is especially interesting, as it employs a rheostat that responds to and maintains ATP levels (Fig. 4 in Chapter 1)²⁷.

Homeostasis. The definition of homeostasis is a state of internal stability in an organism or cell that is maintained while continuously adjusting to internal and external changes. To achieve homeostasis inside a synthetic cell, the modules of compartmentalization, information processing and metabolism need to be combined and energetically balanced. To do this properly, the energy generation pathways need to have a higher capacity than the energy demanding pathways, and the production and consumption of energy need to be controllable²⁴⁷. The presence of positive and negative feedback loops (preferably interacting between different pathways) is essential to maintain an out-of-equilibrium stability²⁴⁷. In my view, the importance of cellular homeostasis is often overlooked in studies on building of synthetic cells. Many studies focus on creating one module, like information processing, signaling or cell division, without considering e.g. the energy, pH, ionic strength and crowding requirements of these modules.

How can a synthetic cell achieve homeostasis? In living cells, homeostasis is achieved by controlling their internal pH, ionic strength, volume (or macromolecular crowding, osmolarity) and metabolic energy²⁴⁸. The presence of a membrane that separates the controllable inside from the changing outside is obviously essential, as well as the existence of membrane proteins that can transport nutrients, waste and information signals. Multiple pathways and mechanisms exist in living cells to achieve homeostasis, which could be implemented in a synthetic cell, as recently discussed in ref. 248 and initiated in our studies (Chapter 3). In the following we focus on implementing homeostasis in the arginine breakdown pathway.

The three main issues for homeostasis in the arginine breakdown pathway are the changes in internal pH, the stability of the enzymes and the buildup of the waste products ammonia, carbon dioxide and ornithine. The internal pH in the arginine breakdown pathway is affected by (i) the consumption of three protons by ornithine transcarbamoylase and carbamate kinase; (ii) the production of protons through ATP hydrolysis; (iii) the outward diffusion of ammonia, leaving behind a proton for every ammonium ion produced; (iv) the fate of carbon dioxide, either diffusing out or producing protons in the formation of bicarbonate; and (v) the balance between futile hydrolysis of arginine to citrulline and full pathway activity (Fig. 4). The first two points are determined by the chemistry of the reactions: for every molecule of ATP formed, three protons are consumed, while the hydrolysis of ATP produces one proton. The internal pH should thus increase, if we ignore the production of ammonium ions and carbon dioxide. On the other hand, the internal pH stays stable if both ammonia and carbon dioxide diffuse out and even decreases if bicarbonate is formed and only ammonia diffuses out.

Figure 4: Schematic of the arginine breakdown pathway. The futile hydrolysis of arginine and arginine/citrulline exchange are depicted by bold and dashed black arrows, respectively. The yellow dashed arrows indicate passive diffusion of ammonia and carbon dioxide. The protons that are consumed and/or produced are highlighted in pink. The net charge is shown of all compounds at neutral pH. ADI = arginine deiminase; OTC = ornithine transcarbamoylase; CK = carbamate kinase; AOA = arginine/ornithine antiporter.

In Chapter 3 we have seen that after an initial increase in pH after arginine addition, the pH decreases (Fig. 3E, 4D and 6D in Chapter 3). While the initial increase is mainly influenced by the starting concentration of Mg-ADP, the decrease in pH is caused by the futile hydrolysis of arginine to citrulline, producing an excess of ammonium ions (Fig. 4 in Chapter 3). Consumption of ATP helps to stimulate the full pathway over the futile hydrolysis of arginine, but a small decrease in the internal pH is still observed (Fig. 6D in Chapter 3). To achieve homeostasis, a pathway or mechanism needs to be added that gives control over either the pH or the futile hydrolysis of arginine. To control the pH, we could add a decarboxylation reaction like malolactic fermentation, which imports and decarboxylates malate while consuming an internal proton (Fig. 2B in Chapter 1). Malolactic fermentation thus increases the internal pH, counteracting the decrease caused by arginine breakdown. Importantly, the activity of this fermentation increases at lower internal pH levels, thereby providing feedback control^{13,15}. However, malolactic fermentation also generates a membrane potential (inside negative relative to the outside), as the substrate and product carry a different net charge. This membrane potential favors the futile hydrolysis of arginine to citrulline because arginine/citrulline antiport is accelerated by a membrane potential that is inside negative (Fig. 6C in Chapter 2; Fig. 2E in Chapter 3). Thus, even the combination of two simple pathways already introduces an enormous complexity that needs to be modeled and tested in order to control the internal pH.

As an alternative, the F_0F_1 -ATP synthase/hydrolase could be added, which can export protons at the expense of ATP¹. The F_0F_1 -ATP synthase/hydrolase thus increases the internal pH by using ATP, and importantly the enzymes of *Enterococcus hirae*, *Streptococcus pneumoniae*, *S. sanguis* and *S. mutans* have been shown to be feedback-regulated by the internal pH²⁴⁹⁻²⁵¹. pH control can also be implemented by introducing a sodium/proton antiporter, which exchanges external sodium for internal protons or *vice versa*, depending on the ion gradients²⁵². The outside buffer for the arginine breakdown pathway would have to contain sodium ions, and ideally a secondary sodium-dependent transporter should be added to make use of the sodium gradient and to prevent internal sodium buildup.

Instead of adding control over the pH, we could add control over the futile hydrolysis of arginine. The simplest way to do this is to use an arginine/ornithine antiporter that does not transport citrulline or has a very low affinity for it. Since citrulline is very similar to both arginine and ornithine (Fig. 1B in Chapter 1), it is unlikely that any arginine/ornithine antiporter exists that does not transport citrulline at all. However, the affinity for citrulline (as measured by its inhibition constant) does vary between the different antiporters studied so far (Table 5 in Chapter 1), so an antiporter with very low affinity for citrulline probably exists. Alternatively, we could add a membrane potential that is inside positive relative to the outside, so that arginine/citrulline antiport is inhibited. Arginine/ornithine antiport is unaffected since both compounds carry the same net charge. The ultimate synthetic cell however will require a membrane potential that is inside negative (and thus favor arginine/citrulline antiport), as numerous processes in living cells are dependent on an electrochemical proton and/or sodium gradient that is inside negative.

While the homeostasis of internal pH is a challenge that asks for further study, the issues of enzyme stability and buildup of waste products might be easier to solve. The stability of the individual cytosolic enzymes has been studied in low and high ionic strength solutions (Fig. 6C in Chapter 3). Arginine deiminase is fairly stable at a low ionic strength at 30 °C, but ornithine transcarbamoylase and carbamate kinase lose 50% activity over a period of 5 hours. The same observations are made at a high ionic strength, except that carbamate kinase loses 100% activity after 3 hours when there is no glycine betaine present. The stability of the entire arginine breakdown pathway was also studied by incubating the vesicles in a high ionic strength solution for several hours at 30 °C (see below, Fig. 7), which showed 60% remaining activity after 4 hours. Apparently the stability of carbamate kinase is increased when encapsulated in the vesicles as opposed to being in solution. Furthermore, the second stability experiment showed a remaining activity of 20% after 24 hours. Although this remaining activity is fairly impressive, it could be improved further with a more (thermo)stable carbamate kinase (Chapter 2). An organism like *Haloferax volcanii*, that has a high internal ionic strength and lives at higher temperatures^{253,254}, could provide a stabler enzyme.

Finally, the buildup of the waste products ammonia, carbon dioxide and ornithine can be solved by making use of (microfluidic) flow cells²⁵⁵, as the waste products either diffuse out or are exported out of the vesicles performing arginine breakdown (Fig. 4). A flow system, making use of polycarbonate filters that are permeable for small molecules but not for vesicles, is currently being developed in our lab. This flow cell can ‘refresh’ the external medium of the vesicles through constant delivery of arginine and the removal of ammonia, carbon dioxide and ornithine, while keeping the vesicles trapped in a compartment. The performance of our system for metabolic energy conservation is already impressive, but we also look forward to seeing the proposed improvements being implemented.

Building ATP-regenerating systems

In the final part of this chapter I discuss a number of challenges we faced in constructing our system for metabolic energy conservation. Some of these challenges, like removing external arginine hydrolysis and stability of the arginine breakdown pathway, were mentioned before in this thesis. Other challenges involve optimization of the enzyme ratios and volume measurements, which could be important for future research.

Removing external arginine hydrolysis. As described in Chapter 3, we found that in the initial design of the arginine breakdown pathway, a substantial amount of citrulline was formed on the outside of the vesicles. Even though the vesicles were washed twice with large amounts of buffer (6 ml on a 40 μ l pellet), arginine deiminase was still associated with the surface of the vesicles. While the cellular localization of the *Lactococcus lactis* arginine deiminase has not been determined, the proteins from *Trichomonas vaginalis* and *Tritrichomonas foetus* have been reported to be partly membrane-associated²⁵⁶, hinting at a possible explanation. Since arginine deiminase (ArcA) from *L. lactis* contains cysteines, we inactivated external enzyme by modification with the membrane-impermeable sulfhydryl reagent *p*-chloromercuribenzenesulfonate (pCMBS) (Fig. 5A, left).

Even though the reaction of ArcA does not change the pH (assuming ammonium ions do not dissociate in solution), we made use of an unspecific effect of arginine on pyranine, which increases the

fluorescence ratio of pyranine, and thus the pH readout by 0.1 pH unit (Fig. 5B; see Fig. 8 for pyranine calibration). This effect was not observed with citrulline, ornithine or carbamoyl phosphate and the added arginine was properly buffered. We assume the effect of arginine on pyranine is unspecific, although this has not been reported in literature.

Figure 5: Inhibition of arginine hydrolysis with pCMBS. Standard conditions are: 0.2 μ M ArcA, 0.2 μ M ArcB, 0.5 μ M ArcC1, 5 mM Mg-ADP, 0.1 μ M pyranine in 50 mM KPi, pH 7.0 at 30 °C (protocol B6). Representative traces from biological duplicates are shown. **(A)** Inhibition by different concentrations of pCMBS on ArcA (left) and ArcB (right). 10 mM arginine or 10 mM citrulline was added at t = 0; 1 mM DTT (black trace) was added after 25 min. **(B)** Unspecific effect of arginine on pyranine. After addition of 10 mM arginine at t = 0, the pH increases by 0.1 pH unit (black trace). Conversion to citrulline by ArcA (blue trace) removes the effect on pyranine.

We also checked if pCMBS could inactivate *L. lactis* ornithine transcarbamoylase (ArcB), which also has cysteines. Although ArcB does not convert citrulline plus inorganic phosphate to carbamoyl phosphate plus ornithine on its own, as the equilibrium constant of the reaction is very small, we forced the reaction in the direction of carbamoyl phosphate by adding carbamate kinase (ArcC1). ArcC1 has no cysteines, so it cannot be inactivated by pCMBS and thus the reaction runs from citrulline degradation to Mg-ATP formation (Fig 5A, right). Both reactions consume protons and thus the pH increases. Interestingly, while dithiothreitol (DTT) reverses the inactivation of ArcB by pCMBS, the inhibition of ArcA is more permanent. The chemical modification of enzymes by membrane-impermeable reagents can thus be used to get rid of unwanted external breakdown of substrates. Care should be taken however that unwanted side-effects are avoided, like the inhibition of membrane proteins (we designed a cysteine-less variant of ArcD2 for this reason).

Optimization of the enzyme ratios. In the first iterations of the arginine breakdown pathway, we chose an ArcA:ArcB:ArcC1 ratio of 1.25:1:0.8 (w/w). Next, we lowered the concentration of ArcA to a ratio of 0.75:1:0.8 (w/w), as we saw a lot of citrulline production in high-performance liquid chromatography (HPLC) measurements and thus reasoned that ArcB might be limiting the pathway activity. The new ratio showed less citrulline production, while the ATP/ADP ratio under load stayed the same. To optimize this ratio even further, the pH changes caused by either ArcC1, ArcB plus ArcC1 or all three enzymes were assayed in solution (Fig. 6). ArcC1 takes up two protons, while ArcB takes up one proton and we assumed the produced ammonium ion does not dissociate, nor does the produced carbon dioxide form bicarbonate. This assumption would not hold in the arginine breakdown pathway, where the internal (localized) concentrations stimulate ammonia diffusion and bicarbonate formation (Chapter 3; ref. 257).

With only ArcC1 in solution, a sudden, rapid increase in pH is seen (Fig. 6A). The increase in pH is more gradual with ArcB added and varying concentrations of ArcC1 do not seem to change the initial reaction rate, suggesting that the reaction of ArcB is rate-determining (Fig. 6B). With all three enzymes, the initial reaction rate does seem to increase with

higher concentrations of ArcB plus ArcC1 (Fig. 6C). Although these initial reaction rate are partly obscured by the unspecific effect of arginine on pyranine, the data suggest that the reaction of ArcB (plus ArcC1) is rate-determining. From these experiments we tentatively conclude that an

Figure 6: Optimization of the enzyme ratios. Standard conditions are: 5 mM Mg-ADP, 0.1 μ M pyranine in 50 mM KPi, pH 7.0 (protocol B7). Representative traces from biological duplicates are shown. (A) pH increase from different concentrations of ArcC1 in solution, as measured with pyranine. 10 mM carbamoyl phosphate was added at $t = 0$. (B) Increasing the concentration of ArcC1 while keeping ArcB constant does not change the initial reaction rate, indicating that ArcB catalyzes a rate-determining step. 10 mM citrulline was added at $t = 0$. (C) Keeping the concentration of ArcA constant, while increasing ArcB and ArcC1 leads to a fast and higher increase in pH. Combined with the data in panel B, this indicates that ArcB catalyzes the rate-determining reaction. The large increase in pH at $t = 0$ is due to an unspecific effect of arginine on pyranine (see Fig. 5B). 10 mM arginine was added at $t = 0$.

ArcA:ArcB:ArcC1 ratio of 0.5:1:5 (w/w) is the most optimal under our experimental conditions of 50 mM KPi at pH 7.0. In most experiments presented in this thesis we used an ArcA:ArcB:ArcC1 ratio of 0.5:1:2.5 (w/w) for ATP regeneration inside the vesicles, and the salt concentration is higher (50 mM KPi plus 25 mM NaCl in unshocked vesicles, ~230 mM KPi plus ~110 mM NaCl in shocked vesicles).

Stability of the arginine breakdown pathway. In addition to the stability of the individual ArcA, ArcB and ArcC1 enzymes in low and high ionic strength solutions (Fig. 6C in Chapter 3), the stability of the full pathway was also measured (Fig. 7A). While measurements on the individual enzymes showed that ArcC1 is fully inactivated in high ionic strength solution after 3 hours, the full pathway still shows 60% activity after 4 hours, and even 20% remaining activity after 24 hours (Fig. 7B). Apparently being encapsulated in vesicles enhances the stability of

Figure 7: Stability of the arginine breakdown pathway. Standard conditions are: ArcD2:OpuA:lipid ratio is 1:2:400 (w/w), using 50:38:12 (w/w) DOPE:DOPG:DOPC lipids extruded through 400 nm polycarbonate filters. In the transport assays we used vesicles corresponding to 44.1 nM ArcD2 and 23.2 nM OpuA, and we added 18 μ M 14 C-glycine betaine (GB) plus 20 mM arginine in 50 mM KPi, pH 7.0 plus 250 mM KCl at 30 °C. Data from a single experiment. (A) Effect of incubation time at 30 °C on the arginine breakdown pathway as inferred from the uptake of GB, which depends on the ATP produced by the pathway. Vesicles were incubated for 1, 2, 4, 8, 24 and 26 hours before uptake was started through addition of 10 mM arginine. (B) The data from panel A, plotted as the percentage of remaining total uptake (blue bars) and the initial uptake rate measured over the first 1.5 hours (green bars).

ArcC1, an observation we also made for the effect of freeze-thaw cycles on ArcB (Fig. 4D in Chapter 2). As mentioned above, the stability of the pathway could be improved by using more (thermo)stable enzymes.

Volume measurements. Since we found that the osmotically upshifted vesicles imported glycine betaine to a high level (Fig. 5C in Chapter 3) and glycine betaine partially restores the volume of shocked vesicles¹⁸⁸, we tried to perform direct volume measurements. Experiments were done with static (90 ° angle) light scattering, dynamic light scattering, calcein dequenching and an ionic strength sensor²⁵⁸, but we did not find conclusive answers on the restoration of volume. While we did see an effect of the addition of salt to the vesicles (osmotic upshift) with static light scattering and calcein dequenching, no clear restoration was seen upon the addition of arginine. This could be because the effect of shocking is fast (seconds), while the volume restoration by the arginine breakdown pathway takes much longer (hours).

There are other reasons why the chosen techniques might be unsuitable for measuring the volume restoration of vesicles. The light scattering techniques are mostly used to determine the size or molecular weight of small globular particles. The analysis of vesicles that change shape upon osmotic upshift and restoration is way more complex, and the changes may go unnoticed in the light-scattering measurements. Calcein dequenching requires a fairly high internal concentration of calcein (at least 1 mM), which could inhibit the activity of the proteins. The ionic strength sensor was tested in solution and *in vivo*²⁵⁸, but not in synthetic vesicles. Perhaps the sensor binds to the membrane of synthetic vesicles, preventing it from working properly. Time constraints have not made it possible to complete the volume measurements and properly evaluate the different methods.

Materials & Methods

Materials. Common chemicals, lipids, radiolabeled compounds and detergents were obtained as described in Chapter 2 and/or 3. The expression, purification, reconstitution and storage of proteins has also been described in Chapter 2 and/or 3.

External pH measurements with pyranine. *Calibration:* The fluorescence spectrum of 0.1 μM pyranine in 50 mM KPi was measured at varying pH between 6.8 and 7.6 in a 96-well flat-bottom black polystyrene plate (Greiner Bio-One International GmbH) in a plate reader (Tecan Group Ltd.). The fluorescence was measured by excitation from 380 ± 20 nm to 470 ± 20 nm, while the emission was recorded at 512 ± 20 nm. The pH in solution was plotted against the ratio of the peaks at 460 nm and 400 nm and fitted using an exponential function:

$$y = a * e^{b * x} \quad (1)$$

where a and b are fitting parameters. By fitting equation 1 tot the data points in 50 mM KPi (Fig. 8), the following values were obtained: $a = 8.0 \times 10^{-6}$ and $b = 1.64$.

Protocol B6: Either $38.4 \mu\text{g mL}^{-1}$ ArcA or $47.5 \mu\text{g mL}^{-1}$ ArcB plus $35.0 \mu\text{g mL}^{-1}$ ArcC1 were diluted in 50 mM KPi pH 7.0 plus 0.1 μM pyranine, 5 mM ADP and 5 mM MgSO_4 in a total volume of 100 μL . Additionally, either 2, 0.2, 0.02 μM or no *p*-chloromercuribenzenesulfonate (pCMBS) was added to the mixture. The mixture was incubated in a 96-well flat-bottom black polystyrene plate at 30°C for 20 min before either 10 mM L-arginine for ArcA or 10 mM L-citrulline for ArcB plus ArcC1 was added to start the reaction. The fluorescence emission at 512 ± 20 nm was measured at the peak excitations of 460 ± 20 nm and 400 ± 20 nm every 1 min over a 60 min period. To reverse the pCMBS inhibition, 1 mM dithiothreitol (DTT) was added after 30 min. The pH of the solution was obtained from equation 1 and the values obtained under calibration.

Protocol B7: Like protocol B6 except that lower concentrations of enzymes were used (see figure legends); no pCMBS was used; and the mixture was incubated for 5 min before addition of 10 mM carbamoyl phosphate (for ArcC1), 10 mM L-citrulline (for ArcB plus ArcC1) or 10 mM L-arginine (for ArcA, ArcB and ArcC1).

Transport assays. The vesicles were created as described in protocol A1 in Chapter 3, transport assays were performed as described in protocol B1 in Chapter 3.

Figure 8: Calibration of pyranine in solution on a plate reader. (A) Representative excitation spectra from 380 nm to 480 nm of pyranine in 50 mM KPi with an emission wavelength of 512 nm, at varying pH. The excitation spectrum for pyranine shows a clear peak at 460 nm but not at 400 nm (see Fig. S4 in Chapter 3 for comparison with the spectrofluorometer), which limits the sensitivity of the plate reader in these measurements. We used the plate reader for medium-throughput screening of enzymatic conditions but did not convert the pH values into reaction rates. (B) The ratio of the excitation peaks at 460 nm and 400 nm from panel A were plotted as a function of pH. The data points were fitted with an exponential function (black trace), as described in materials and methods: $a = 8.0 \times 10^{-6}$ and $b = 1.64$.

Liposome calculations

To calculate the number of liposomes, the internal volume per liposome and the number of proteins per liposome, we used the following parameters and assumptions:

Parameters:

- Liposome radius: 226 nm ± 113 nm. This is the average radius based on internal volume, obtained from CryoTEM micrographs with liposomes that were extruded with 400 nm filters (Fig. 2C in Chapter 3).
- Surface area and molecular weight of lipids: see Table 1.
- Amount of lipids per batch: 6.6 mg (see materials & methods in Chapter 3).
- Molecular weight and amount of proteins: see Table 2.
- Encapsulation volume for 6.6 mg of lipids: 200 µl (see protocol A1 in Chapter 3).

Assumptions:

- All proteins added to the liposomes were incorporated in the membrane or encapsulated in the vesicles. The number of enzymes and membrane proteins in the vesicles is probably not correct, as e.g. freeze-thaw encapsulation efficiencies are around 45% (see e.g. ref. 259 or 260).
- All liposomes have the same radius of 226 nm. This is not correct (Fig. 2B and 2C in Chapter 3) but simplifies the explanation of our calculations. We comment on the distribution of the vesicle radii at the end of the appendix.

We calculated the number of liposomes, by first determining the surface area of a 226 nm radius sphere, using the following equation:

$$A = 4 * \pi * r^2 \quad (1)$$

This gives a surface area of $6.42 \times 10^5 \text{ nm}^2$ for a 226 nm radius sphere. By multiplying the surface area of the sphere by two (since the liposome is made of a lipid bilayer) and then dividing it by the average surface area

Table 1: Lipid properties

Type of lipid	Presence (%)	Surface area per lipid (nm ²)	Molecular weight per lipid (g mol ⁻¹)	References
DOPE	50	0.65	744	261
DOPC	37	0.82	786	261
DOPG	13	0.69	797	262
Weighted average		0.72	766	

Table 2: Protein properties

Protein	Native molecular weight (g mol ⁻¹)	Amount per 6.6 mg of lipids (μg)	Amount per 6.6 mg of lipids (molecules)
ArcD2	56,700	16.7	1.77×10^{14}
OpuA	215,600	33.3	9.30×10^{13}
ArcA	190,800	37.5	1.18×10^{14}
ArcB	245,400	96.0	2.36×10^{14}
ArcC1	72,400	72.0	5.99×10^{14}

per lipid (0.72 nm²), we obtain 1.79×10^6 lipids per liposome. Since 6.6 mg of lipids were used per batch, we divide that number by the average molecular weight per lipid (766 g mol⁻¹) and multiply by the Avogadro constant to obtain the total number of lipids available: 5.18×10^{18} . Division of the total lipids available by the lipids per liposome gives the total number of liposomes: 2.90×10^{12} .

ArcD2 was reconstituted at a lipid-to-protein ratio of 1:400 (w/w), meaning 16.7 μg of ArcD2 was added to 6.6 mg of lipids. This gives 1.83×10^{14} molecules of ArcD2, divided by the total number of liposomes gives 63 molecules of ArcD2 per liposome. For OpuA a lipid-to-protein ratio of 1:200 (w/w) was used, meaning 33.3 μg of OpuA was added to 6.6 mg of lipids. This translates into 9.30×10^{13} molecules of OpuA, which gives 32 molecules of OpuA per liposome.

To determine the internal volume per liposome, we calculated the volume of a 226 nm radius sphere, using the following equation:

$$V = \frac{4}{3} * \pi * r^3 \quad (2)$$

This gives a volume of $4.84 \times 10^7 \text{ nm}^3$ (or $4.84 \times 10^{-11} \text{ }\mu\text{l}$) per liposome. 37.5 μg of ArcA was added to the encapsulation volume (200 μl), which translates the 1.18×10^{14} molecules into a concentration of 5.92×10^{11} molecules μl^{-1} . Multiplying the volume per liposome with this concentration gives 29 molecules of ArcA per liposome. The same calculations were done for ArcB and ArcC1, giving 57 and 145 molecules per liposome, respectively.

Note that when multiplying the total number of liposomes with the internal volume per liposome gives an internal volume of 140 μl , while 6.6 mg of lipids can be resuspended in 40 μl (see protocol A1 in Chapter 3). Clearly, the internal volume per liposome is lower, because a large fraction of the vesicles has a smaller radius (see the distributions in Fig. 2B and 2C in Chapter 3). Instead of taking the average radius based on internal volume, we can divide the radii obtained from the cryo-EM micrographs in 10 intervals and calculate the same parameters (Table 3). The table shows that the internal volume for most vesicles is (much) smaller than 40 μl , except for the 10% largest vesicles, which have an average internal volume of 118.8 μl . The average radius of 84 nm (equal to Fig. 2B in Chapter 3) gives an internal volume of only 18 μl , which matches the experimental observation. Such vesicles would have 8 molecules of ArcD per liposome, 4 of OpuA, 1 of ArcA, 3 of ArcB and 7 of ArcC1.

Table 3: Parameter calculation for 10 equal intervals

Interval	Radius (nm)	Total number of liposomes	Volume per liposome	Internal volume (μl)
1	26.2	2.2×10^{14}	7.6×10^{-14}	0.2
2	38.3	1.0×10^{14}	2.4×10^{-13}	0.7
3	46.8	6.8×10^{13}	4.3×10^{-13}	1.2
4	55.3	4.8×10^{13}	7.1×10^{-13}	2.1
5	64.1	3.6×10^{13}	1.1×10^{-12}	3.2
6	74.4	2.7×10^{13}	1.7×10^{-12}	5.0
7	86.2	2.0×10^{13}	2.7×10^{-12}	7.8
8	102.2	1.4×10^{13}	4.5×10^{-12}	13.0
9	131.6	8.6×10^{12}	9.6×10^{-12}	27.7
10	213.8	3.2×10^{12}	4.1×10^{-11}	118.8
Average	83.9	5.4×10^{13}	6.2×10^{-12}	18.0

ATP synthesis rate

To calculate the ATP synthesis rate of the arginine breakdown pathway, we first focused on ATP/ADP ratio data (obtained with PercevalHR) for vesicles with a lipid composition of 50:13:37 (w/w) DOPE:DOPG:DOPC. We used the following parameters and assumptions:

Parameters:

- Starting ATP/ADP ratio: 0.5. This ratio is obtained at the start from experiments with unshocked vesicles, without an ATP drain on the pathway (Fig. 5A in Chapter 3).
- Final ATP/ADP ratio: 3. This ratio is obtained after one hour from experiments with unshocked vesicles, without an ATP drain on the pathway (Fig. 5A in Chapter 3).
- Mg-ADP concentration at start of experiment: 5 mM.
- Encapsulation volume for 6.6 mg of lipids: 200 μ l (see protocol A1 and A7 in Chapter 3). Only 0.33 mg of lipids were used for the experiments (see protocol B3 in Chapter 3), giving an 'experimental encapsulation volume' of 10 μ l.

Assumptions:

- All added Mg-ADP is encapsulated in the vesicles. This is probably not correct, as e.g. freeze-thaw encapsulation efficiencies are around 45% (see e.g. ref. 259 or 260).
- The encapsulation volume is equal to the internal volume of the liposomes. This is not correct, but as our calculation of the internal volume per liposome does not match with experimental observations (see Appendix 1), we prefer to work with the encapsulation volume here.
- The ATP/ADP ratio can be converted into concentrations of Mg-ATP and Mg-ADP directly. This is a fair assumption, as the protonophore FCCP was added to the experiments to negate any pH effects on the readout of PercevalHR (Chapter 3; ref. 144).
- There is Mg-ATP present at the start of the experiments. The ATP/ADP ratio at the start of the experiments is 0.5, indicating that there is some Mg-ATP present, either due to incomplete purification of the

used Mg-ADP stock or because adenylate kinase is present, which converts 2 molecules of Mg-ADP into Mg-ATP and Mg-AMP.

- There is no endogenous ATP hydrolysis. This is not correct as we have observed some futile ATP hydrolysis in Chapter 3.

The starting ATP/ADP ratio of 0.5 indicates the presence of 1.66 mM Mg-ATP and 3.33 mM Mg-ADP at the start of the experiment. After one hour, an ATP/ADP ratio of 3 is reached, which translates into 3.75 mM Mg-ATP and 1.25 mM Mg-ADP. 2.09 mM Mg-ATP is thus produced in one hour. 2.09 mM in an encapsulation volume of 10 μl equals 2.09×10^{-8} mol Mg-ATP. Multiplying by the Avogadro constant gives 1.26×10^{16} molecules of Mg-ATP produced in one hour, or 3.50×10^{12} molecules produced in one second.

From Appendix 1, we obtained the total number of liposomes (2.90×10^{12}) and the number of ArcD2 molecules per liposome (63). This total number of liposomes however was calculated for 6.6 mg of lipids, while only 0.33 mg of lipids were used for the experiments. This thus reduces the number of liposomes to 1.45×10^{11} . Dividing the molecules produced in one second by this number of liposomes and the number of ArcD2 molecules per liposome gives an ATP synthesis rate of 0.38 s^{-1} .

Alternatively, the ATP synthesis rate can be calculated with external amino acid concentration data, obtained with high-performance liquid chromatography (HPLC), and glycine betaine (GB) uptake data, obtained with radiolabeled GB, for vesicles with a lipid composition of 50:38:12 (w/w) DOPE:DOPG:DOPC. For this we used the following additional parameters and assumptions:

Parameters:

- From HPLC experiments: 0.04 mM ornithine is exported in one hour with shocked vesicles, without an ATP drain on the pathway (Fig. 4A in Chapter 3).
- External volume for HPLC experiments: 50 μl (see protocol B6 in Chapter 3).
- From GB uptake experiments: $2.3 \mu\text{mol GB} \times \text{mg OpuA}^{-1}$ is taken up in one hour with shocked vesicles, with an ATP drain on the pathway (Fig. 5C in Chapter 3).

- 0.17 mg of lipids was used for the GB uptake experiments (see protocol B1 in Chapter 3), giving an ‘experimental encapsulation volume’ of 5 μl .
- Uptake of one molecule of GB requires two Mg-ATP molecules¹⁹¹.

Assumptions:

- The concentration of exported ornithine is equal to the produced concentration of Mg-ATP. This is a fair assumption, as the conversion of one molecule of arginine to ornithine yields one Mg-ATP (but it ignores endogenous ATP hydrolysis and adenylate kinase activity; see previous assumptions).
- The concentration of imported GB is half the produced concentration of Mg-ATP. This is a fair assumption if the turnover number of OpuA-mediated GB import ($k_{\text{cat}} = 1 \text{ s}^{-1}$, see Fig. 2A in Chapter 3) is faster than the ATP synthesis rate of the arginine breakdown pathway.
- The arginine breakdown pathway works at a similar speed in unshocked vesicles with a lipid composition of 50:13:37 (w/w) DOPE:DOPG:DOPC and in shocked 50:38:12 (w/w) DOPE:DOPG:DOPC vesicles. This is probably not correct, as the lipid composition has an effect on at least ArcD2 (Fig. 6D in Chapter 2), and shocking the vesicles has an effect on ArcA, ArcB and ArcC1 (Fig. 6B in Chapter 3).

According to the HPLC experiments, 0.04 mM ornithine is exported in one hour in an external volume of 50 μl , which equals 2.0×10^{-9} mol ornithine. The same amount of Mg-ATP is produced inside the vesicles, which is ~10 times less Mg-ATP than with the ATP/ADP ratio data in unshocked vesicles (see above). Following the same calculations as above, the HPLC experiments thus give an ATP synthesis rate of 0.04 s^{-1} .

The GB uptake experiments show that 2.3×10^{-6} mol GB is taken up in one hour by vesicles with 1 mg of OpuA. As 0.17 mg of lipids were used for the experiments, the vesicles only had 0.85 μg of OpuA. Correcting for OpuA concentration gives an uptake of 1.95×10^{-9} mol GB, which requires the consumption of 3.91×10^{-9} mol Mg-ATP, which is about 5 times lower than with the ATP/ADP ratio data in unshocked vesicles. Following similar calculations as above, but reducing the number of liposomes to 7.25×10^{10} as only 0.17 mg of lipids were used, the GB uptake experiments give an ATP synthesis rate of 0.14 s^{-1} .

The rate calculated from ATP/ADP ratio experiments (0.38 s^{-1}) seems an outlier when compared to the other rates. This is probably due to the assumption that the encapsulation volume is equal to the internal volume of the liposomes. It could very well be that the internal volume is only 10% of the encapsulation volume, which would give a rate of 0.04 s^{-1} , corresponding to the rate from HPLC experiments. In any case, the calculated rates are probably an underestimation of the actual ATP synthesis rate, as not all vesicles are active (a lower limit of 70% active vesicles was calculated in Chapter 3) and because we ignore endogenous ATP hydrolysis.

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Summary

Whenever someone asked about what I was doing in the lab, I would usually explain my project as: “I am trying to generate and conserve energy inside of small soap bubbles.” While this might oversimplify four years of research, it summarizes the main goal of this thesis well. The energy in this case is not the 230 volts that we plug our appliances into, but so called ‘metabolic energy’ that is used in cells. Two forms of metabolic energy are used: electrochemical ion gradients and adenosine triphosphate (ATP), the universal cellular energy currency. Cells can generate (and conserve) metabolic energy with complex pathways, like oxidative phosphorylation, or with simpler pathways, like deamination of amino acids, oxidation of carboxylic acids or by using light (see Chapter 1). The focus in this thesis is on the deamination of arginine to create ATP. Coming back to the analogy: the small soap bubbles are a good visual representation, but the synthetic vesicles in this thesis have a much smaller size (hundreds of nanometers instead of tens of millimeters) and are made out of phospholipids instead of detergent molecules. All cellular membranes make use of phospholipids and, importantly, phospholipid vesicles (liposomes) are more stable and less permeable than soap bubbles.

Arginine breakdown. The deamination of arginine in cells is carried out by the arginine deiminase (ADI) pathway, which consists of three cytosolic proteins and one membrane transporter (Fig. 1). After arginine is imported, it is deaminated to citrulline and ammonium ion by ADI. Subsequently, phosphorolysis of citrulline by ornithine transcarbamoylase (OTC) yields ornithine plus carbamoyl phosphate (carbamoyl-Pi). This reaction is thermodynamically unfavorable, but proceeds when the reaction products are consumed. Carbamoyl-Pi plus adenosine diphosphate (ADP) is then converted into ATP, carbon dioxide and ammonium ion by carbamate kinase (CK). Finally, the arginine/ornithine antiporter (AOA) exports the (by)product ornithine and imports the substrate arginine, while carbon dioxide and ammonia (formed from the ammonium ions) can diffuse out. Details on the ADI pathway, like how it is used and regulated in cells, are described in the second part of Chapter 1. These details include the structural studies on the individual proteins from various organisms and their kinetic

constants: the speed of the enzyme-catalyzed reactions and the affinity of the enzymes for their substrates.

In Chapter 2 the ADI pathway proteins from *Lactococcus lactis* are studied, to determine their kinetic constants and the effect of varying several parameters on their activity (summarized in Table 1). These parameters (pH, temperature, freeze-thaw cycles, pathway intermediates and different salts) are important for the control of ATP generation inside liposomes (see below; Chapter 3). Since the reaction of OTC is thermodynamically unfavorable, it was studied in the opposite direction: from ornithine plus carbamoyl-Pi to citrulline. The enzymes perform their reactions as expected and work relatively well in the tested conditions. Additionally, the most important findings from Chapter 2 are: the AOA is relatively slow and possibly the rate-determining reaction of the pathway and the CK only works in a very narrow pH and temperature range. These two proteins are thus targets for increasing the speed and robustness of the arginine breakdown pathway.

ATP generation inside liposomes. After confirming that the individual proteins from *L. lactis* work properly, the arginine breakdown pathway was reconstituted in liposomes, to create ATP inside of synthetic vesicles (see Chapter 3). This reconstitution shifts the focus from the individual proteins to measuring properties of the entire pathway. For this reason, the fluorescent sensors pyranine and PercevalHR were added to monitor

Figure 1: Schematic of arginine breakdown. ADI = arginine deiminase; OTC = ornithine transcarbamoylase; CK = carbamate kinase; AOA = arginine/ornithine antiporter. Water, protons and inorganic phosphate are not shown.

Table 1: Overview of arginine breakdown proteins

Protein	K_M			
	Arginine	Ornithine	Carbamoyl-Pi	Mg-ADP
Arginine deiminase	34.5 μ M			
Ornithine transcarbamoylase		0.7 mM	2.7 mM	
Carbamate kinase			0.6 mM	1.6 mM
Arginine/ornithine antiporter	6.0 μ M	2.5 mM		

^a Average of the determined k_{cat} values. ^b Simplified as the severity of the effect on the activity of the protein: + = low effect; ~ = medium effect; - = strong effect; ND = not determined. FT = freeze-thaw cycles; PI = pathway intermediates.

the internal pH and ATP/ADP ratios, respectively. The internal pH rises when ATP is generated, as the reactions of OTC and CK consume protons. *Vice versa*, the internal pH decreases when ATP is consumed and when (diffusible) ammonia is formed from ammonium ion, as both reactions produce protons. The internal pH is a good indicator of what is happening inside the liposomes, while the ATP/ADP ratios show the general performance of the pathway. This general performance was also assayed with high-performance liquid chromatography (HPLC), which measures the concentrations of arginine, citrulline, ornithine and ammonia. These techniques were all used to observe the pathway's potential for metabolic energy generation, as described in Chapter 3.

Addition of external arginine causes an initial increase in internal pH (as ATP is generated), followed by a decrease in pH over longer timescales. HPLC data reveals that the decrease in pH is due to the formation of ammonia, as external ammonia levels increase substantially. Additionally, HPLC data shows that the pathway exports citrulline, while only ornithine should be exported when arginine is converted into ATP (Fig. 1). If the pathway runs out of ADP however, the reactions of CK and OTC stop working, since CK needs ADP to convert carbamoyl-Pi into ATP and OTC needs carbamoyl-Pi consumption to perform the thermodynamically unfavorable reaction. The reaction of ADI still works and the AOA can export citrulline instead of ornithine, thus arginine is (futilely)

k_{cat} (s^{-1})	Stability in ^b				Salt compatibility ^b			
	pH	Temp.	FT	PI	NaPi	KCl	NaCl	Tris
3.3	+	~	+	+	+	+	+	-
490 ^a	~	~	-	+	~	+	ND	~
200 ^a	~	-	+	~	-	~	~	~
0.1 ^a	ND	ND	ND	ND	ND	ND	ND	ND

hydrolyzed into citrulline and ammonium ion in the absence of ADP. Futile hydrolysis of arginine was not anticipated on the basis of *in vivo* studies on arginine deamination, but reconstitution of the pathway in liposomes enabled the discovery of this intricacy.

Metabolic energy conservation. How can the futile hydrolysis of arginine be controlled, since it is a wasteful side reaction that hinders the conservation of metabolic energy? The simplest way is to introduce an ATP-consuming process, so that the pathway does not run out of ADP. In Chapter 3 the ATP-driven transporter OpuA is added to the vesicles, which imports the compatible solute glycine betaine for volume regulation in (osmotically stressed) vesicles. An osmotic upshift increases the activity of OpuA, but also raises the concentrations of proteins, pathway intermediates and salts inside the vesicles, which can be deleterious for protein function. The experiments indeed show that the pH decreases less after adding OpuA and, interestingly, the pathway can maintain a higher ATP/ADP ratio over longer timescales, even though the vesicles are osmotically stressed. The addition of OpuA does not only help as an ATP-consuming process, but the presence of glycine betaine protects the proteins from osmotic stress, leading to a higher ATP/ADP ratio and thus metabolic energy conservation. This protection is a property of high concentrations of compatible solutes (like glycine betaine) that is also seen in living cells.

To advance the conservation of metabolic energy in the arginine breakdown pathway even further, it is useful to consider pathways and mechanisms that can implement control over certain parameters, like the internal pH and ionic strength. In this way, a basic level of homeostasis could be achieved over the pathway. Chapter 4 starts with an overview of current synthetic cells, focusing on the topics of compartmentalization, information processing, metabolism and homeostasis. The three main issues for homeostasis in the arginine breakdown pathway are then highlighted, namely: the changes in internal pH, the stability of the proteins and the buildup of waste products. While these issues are not solved in this thesis, suggestions are made for mechanisms that can be implemented in the synthetic vesicles. These mechanisms can improve the vesicles further and can engineer properties, akin those of living cells. Finally, a number of challenges in building synthetic vesicles for metabolic energy conservation are discussed at the end of Chapter 4.

Samenvatting

Wanneer iemand mij vroeg wat ik allemaal aan het doen was in het lab, dan legde ik mijn project meestal uit als: “ik probeer energie op te wekken en te behouden in kleine zeepbelletjes.” Dit is natuurlijk een sterke vereenvoudiging van vier jaar onderzoek, maar het vat het hoofddoel van dit proefschrift goed samen. De energie is in dit geval niet de 230 volt waar we onze apparaten van voorzien, maar zogeheten ‘metabolische energie’ die door cellen gebruikt wordt. Er bestaan twee vormen van metabolische energie: elektrochemische ion-gradiënten en adenosinetrifosfaat (ATP), de universele cellulaire energiedrager. Cellen kunnen metabolische energie opwekken (en bewaren) met complexe routes, zoals oxidatieve fosforylering, of met simpelere routes, zoals de deaminatie van aminozuren, oxidatie van carbonzuren of door middel van licht (zie Hoofdstuk 1). De focus in dit proefschrift ligt op het creëren van ATP uit de deaminatie van arginine. Terugkomend op de analogie: de kleine zeepbelletjes zijn een goede visuele weergave, maar de synthetische vesikels in dit proefschrift hebben een veel kleiner formaat (honderden nanometers in plaats van tientallen millimeters) en zijn gemaakt van fosfolipiden in plaats van zeepmoleculen. Alle membranen van cellen gebruiken fosfolipiden en, nog belangrijker, fosfolipide vesikels (liposomen) zijn stabiel en minder permeabel dan zeepbelletjes.

Afbraak van arginine. De deaminatie van arginine wordt in cellen door de arginine deiminase (ADI) route uitgevoerd, die uit drie cytosolische eiwitten en één membraantransporter bestaat (zie Figuur 1 in de Summary). Zodra arginine geïmporteerd is, wordt het gedeamineerd tot citrulline en ammonium ion door ADI. Vervolgens geeft de fosforolyse van citrulline door ornithine transcarbamoylase (OTC) de producten ornithine plus carbamoylfosfaat (carbamoyl-Pi). Deze reactie is thermodynamisch gezien ongunstig, maar vindt plaats als de reactieproducten geconsumeerd worden. Carbamoyl-Pi plus adenosinedifosfaat (ADP) wordt daarna omgezet tot ATP, koolstofdioxide en ammonium ion door carbamaatkinase (CK). Tenslotte exporteert de arginine/ornithine antiporter (AOA) het (bij)product ornithine en importeert hij het substraat arginine, terwijl koolstofdioxide en ammonia (gevormd uit de ammonium ionen) de cel uit kunnen diffunderen. Kenmerken van de ADI-

route, zoals hoe de route gebruikt en gereguleerd wordt in cellen, staan beschreven in het tweede deel van Hoofdstuk 1. Deze kenmerken omvatten de structurele studies over de individuele eiwitten van verschillende organismen en hun kinetische constanten: de snelheid van de enzym-gekatalyseerde reactie en de affiniteit van de enzymen voor hun substraten.

In Hoofdstuk 2 worden de eiwitten van de ADI-route uit *Lactococcus lactis* onderzocht, om hun kinetische constanten en het effect van het variëren van verschillende parameters op hun activiteit te bepalen (samengevat in Tabel 1 in de Summary). Deze parameters (pH, temperatuur, vries-dooi cycli, tussenproducten van de route en verschillende zouten) zijn belangrijk om de ATP generatie in liposomen te reguleren (zie hieronder; Hoofdstuk 3). Aangezien de reactie van OTC thermodynamisch ongunstig is, werd deze reactie in de tegengestelde richting onderzocht: van ornithine plus carbamoyl-Pi naar citrulline. De enzymen voeren hun reacties uit zoals verwacht en werken relatief goed in de geteste condities. Daarnaast zijn de meest belangrijke vindingen in Hoofdstuk 2 als volgt: de AOA is relatief langzaam en mogelijk de snelheidsbepalende stap van de route en de CK werkt alleen in een erg klein pH- en temperatuurbereik. Deze twee eiwitten zijn dus doelwitten om de snelheid en robuustheid van de arginine-afbraakroute te verbeteren.

ATP generatie in liposomen. Nadat bevestigd was dat de individuele eiwitten uit *L. lactis* goed werken, werd de arginine-afbraakroute gereconstitueerd in liposomen, om ATP binnenin synthetisch vesikels te creëren (zie Hoofdstuk 3). Deze reconstitutie verlegt de focus van de individuele eiwitten naar het meten van eigenschappen van de gehele route. Hiervoor werden de fluorescente sensoren pyranine en PercevalHR toegevoegd om respectievelijk de interne pH en ATP/ADP ratio te meten. De interne pH stijgt wanneer ATP wordt gegenereerd, aangezien de reacties van OTC en CK protonen verbruiken. *Vice versa*, de interne pH gaat omlaag wanneer ATP wordt verbruikt en wanneer (diffundeerbaar) ammonia uit ammonium ion wordt gevormd, omdat beide reacties protonen produceren. De interne pH is een goede indicator van wat er gebeurt in de liposomen, terwijl de ATP/ADP ratio de algemene prestaties van de route laat zien. De algemene prestaties zijn ook geanalyseerd met high-performance liquid chromatography (HPLC), wat

de concentraties van arginine, citrulline, ornithine en ammonia meet. Deze technieken zijn allemaal gebruikt om de potentie van de route voor het opwekken van metabolische energie te observeren, zoals beschreven staat in Hoofdstuk 3.

Toevoeging van externe arginine zorgt voor een initiële stijging in de interne pH (omdat ATP gegenereerd wordt), gevolgd door een afname in pH op langere termijn. HPLC-data laat zien dat de afname in pH te wijten is aan de vorming van ammonia, aangezien de externe niveaus van ammonia aanzienlijk stijgen. Bovendien laat de HPLC-data zien dat de route citrulline exporteert, terwijl alleen ornithine geëxporteerd zou moeten worden als arginine in ATP omgezet wordt (zie Figuur 1 in de Summary). Als in de route echter de ADP opraakt, stoppen de reacties van CK en OTC, omdat CK ADP nodig heeft voor de omzetting van carbamoyl-Pi naar ATP en OTC het verbruik van carbamoyl-Pi nodig heeft voor de uitvoering van de thermodynamisch ongunstige reactie. De reactie van ADI werkt nog wel en de AOA kan citrulline exporteren in plaats van ornithine, dus arginine wordt (zinloos) gehydrolyseerd naar citrulline plus ammonium ion in de afwezigheid van ADP. Zinloze hydrolyse van arginine werd niet verwacht op basis van *in vivo* onderzoek naar arginine-deaminatie, maar door reconstitutie van de route in liposomen kwam deze complexiteit aan het licht.

Conservatie van metabolische energie. Hoe kan de zinloze hydrolyse van arginine gereguleerd worden, aangezien het een verspillende zijreactie is die de conservatie van metabolische energie belemmert? De simpelste manier is om een ATP-verbruikend proces te introduceren, zodat de ADP niet opraakt. In Hoofdstuk 3 wordt de ATP-gedreven transporter OpuA aan de vesikels toegevoegd, die de compatibele oplosstof glycinebetaïne importeert ten behoeve van volume regulatie in (osmotisch gestreste) vesikels. Een osmotische verhoging vergroot de activiteit van OpuA, maar de concentraties van eiwitten, route-tussenproducten en zouten binnen de vesikels nemen ook toe, wat schadelijk kan zijn voor eiwitfunctie. De experimenten laten inderdaad zien dat de pH minder daalt na toevoeging van OpuA en, interessant genoeg, dat de route op de langere termijn een hogere ATP/ADP ratio kan volhouden, ook al zijn de vesikels osmotisch gestrest. De toevoeging van OpuA helpt dus niet alleen als ATP-verbruikend proces, maar de

aanwezigheid van glycinebetaine beschermt de eiwitten tegen osmotische stress. Dit leidt tot een hogere ATP/ADP ratio en dus conservatie van metabolische energie. Deze bescherming is een eigenschap van hoge concentraties van compatibele oplosstoffen (zoals glycinebetaine) die ook in levende cellen optreedt.

Om de conservatie van metabolische energie in de arginine-afbraakroute verder te bevorderen, is het zinvol om over routes en mechanismen na te denken die bepaalde parameters (zoals interne pH en ionische sterkte) reguleren. Op deze manier kan een basaal niveau van homeostase in de route worden bereikt. Hoofdstuk 4 begint met een overzicht van huidige synthetische cellen, met de focus op de onderwerpen compartimentering, informatieverwerking, metabolisme en homeostase. De drie hoofdproblemen voor homeostase in de arginine-afbraakroute worden daarna aangeduid, namelijk: de veranderingen in interne pH, de stabiliteit van eiwitten en de opbouw van afvalproducten. Hoewel deze problemen niet opgelost worden in dit proefschrift, worden er suggesties gedaan voor mechanismen die aan de synthetische vesikels toegevoegd kunnen worden. Deze mechanismen kunnen de vesikels verder verbeteren en eigenschappen inbouwen vergelijkbaar met die van levende cellen. Tenslotte worden aan het einde van Hoofdstuk 4 een aantal uitdagingen besproken in het bouwen van synthetische vesikels voor de conservatie van metabolische energie.

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Ed en Anita: waar Groningen mijn eerste thuis is, en Mildam mijn tweede, kan ik met zekerheid zeggen dat Joure mijn derde thuis is geworden. Jullie zorgzaamheid, gastvrijheid en nuchtere blik heeft veel voor mij gedaan, en ik hoop dat ik daar nog lang van mag genieten (en nog vaak van het groene ei mag eten)!

Jan en Nathalie, Hanneke en Duncan: aan de ene kant in Heerenveen, aan de andere kant op het altijd zonnige Malta. Ik blijf het bijzonder vinden hoe verschillend we zijn geworden, maar het toch altijd met elkaar kunnen vinden. Ik kijk er naar uit om oom te worden en hoop dat ik binnenkort ook weer eens naar Malta mag!

Heit en Mem: ik soe dit stikje graach yn it Frysk skriuwe wolle, mar dan bin ik noch wol even dwaande. Ook al liet ik tijdens mijn studie en PhD niet altijd zo veel van mij horen, toch kon ik altijd op jullie leunen. Jullie zeiden dan wel dat jullie niet zo veel snapten van waar ik aan werkte, maar ik kon altijd wel met veel interesse over mijn project vertellen, zodat ik lekker mijn ei kwijt kon. Ik vond het ook heerlijk om de drukte van stad te vervangen door de rust van Mildam, en ik hoop dat ik hier nog jaren van kan genieten. Dankewol!

Jeske: ‘last but not least’ is hier een groot understatement. Zonder jou was dit boekje waarschijnlijk nooit afgekomen: bedankt voor al die schoppen onder mijn kont. Jouw doorzettingsvermogen is een inspiratiebron geweest, ik zou daar eigenlijk wel wat van willen stelen. Ik hou ervan dat ik alles met jou kan delen, van de hoogtepunten: banden lekrijden in Moremi, een hele dag regen in Snæfellsnes, nep sigaren kopen in Havana, tot de dieptepunten: onze cheese nights, katbeest ophalen en jouw heerlijke baksels. Mijn frustraties en mijn passies: ik zou nog wel honderd dingen kunnen noemen, maar dan komt dit boekje weer niet af. Ik hoop dat jij net zo hebt genoten van de afgelopen 10 jaar, als dat ik dat heb, en ik hoop dat wij nog vele mooie dingen samen mogen beleven. Ik hou jou.