Minimal metabolic pathways for ATP and generation of a proton motive force in synthetic cells

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Summary, Appendix and References

Summary

This thesis describes the development of new metabolic routes for synthetic cells. A pathway is presented that generates a proton motive force, as well as a pathway that provides the cell with energy in the form of ATP. Alongside, methods are presented to monitor these pathways with sensors and external methods.

Chapter 1 introduces the concept of a synthetic cell. Moreover, it considers how much energy in the form of ATP is required for a synthetic cell to maintain itself and to reproduce. To generate this amount of energy, several methods are discussed to yield ATP and energy in the form of electrochemical gradients. As a result of the small size of these vesicles, monitoring of these pathways is challenging. Therefore, a list is provided with several protein sensors and chemical probes that enable the measurement of several parameters inside a synthetic cell.

Chapter 2 describes the development of an enzymatic pathway that generates a proton motive force in proteoliposomes. It consists of a membrane transporter, which transports the substrate L-malate to the inside of the vesicles, and an internal enzyme which converts it into lactate which subsequently can leave the vesicles either by passive diffusion or transport by the membrane transporter. This process consumes a proton, thereby increasing the internal pH. We show that the import of L-malate versus the export of lactate is electrogenic. This generates the second component of the proton motive force, a membrane potential with a negative charge on the inside of the vesicles. The electrical potential that is achieved is approximately -30 mV, which is lower than the membrane potential of some bacteria (like *E. coli*), but could be sufficient for the generation of ATP via the F_1F_0-ATPsynthetase.

In **Chapter 3** two methods for the measurement of amino acids are compared. While amino acids are routinely quantified by many labs, the basic amino acids L-arginine, L-citrulline and L-ornithine proved to be difficult to measure. We have tried GC-MS, which is commonly used for amino acids. This method requires volatile compounds, therefore derivatization of the amino acids is necessary. This proved to be troublesome, as the yields for arginine, citrulline and ornithine are low. Moreover, upon derivatization, arginine can convert into citrulline, making quantification nearly impossible. Reversed phase HPLC proves to be a much better method with simple and fast derivatization. Despite the structural similarities between L-arginine and L-citrulline, separation is sufficient and interpretation of the data is straightforward. As a bonus, this method enables the measurement of NH_3, which makes HPLC the method of choice.

In **chapter 4** a pathway is presented that is able to provide ATP for synthetic cells. It consists of a membrane transporter and three internal enzymes. The pathway is

started by adding L-arginine to the outside of the vesicles. L-arginine is imported by the membrane transporter and converted into L-citrulline and subsequently L-ornithine plus carbamoyl phosphate. L-ornithine is subsequently exported by the transporter. In the last step of the pathway, the phosphate group of carbamoyl phosphate is transferred to ADP, yielding ATP. As L-arginine import and L-ornithine export is coupled in a 1-to-1 ratio, there is no build-up of waste products inside of the synthetic cells and the pathway can run for extended amounts of time. We monitor both the ATP to ADP ratio and pH inside the vesicles, as well as the total amount of L-arginine, L-citrulline, L-ornithine and ammonia. Additionally, we show that the generated ATP can be utilized to import glycine betaine from the external medium by introduction of another membrane transporter. When our vesicles are hyperosmotically challenged, import of glycine betaine appears to stabilize the ATP-to-ADP ratio over longer periods of time.

A method to monitor the membrane potential of vesicles is presented in **chapter 5**. Electrical probes cannot be used on the scale of our liposomes. Instead, fluorescent molecules are used. These so-called Nernstian probes are notoriously difficult to work with as they readily stick to surfaces and are sensitive to temperature and the lipid-to-dye ratio. We present a protocol that provides a solid basis for experiments and we were able to calibrate the signal of the probe to negative inside membrane potentials ranging from 0 to -162 mV.

For the monitoring of the ATP concentration inside synthetic cells, multiple sensors were evaluated. The ATeam ATP sensors were tested and the results can be found in the **Appendix**. These are interesting sensors, but they were found to be unsuitable for use in artificial cells.

Samenvatting

Dit proefschrift beschrijft de ontwikkeling van nieuwe metabolische routes voor synthetische cellen. De eerste route die beschreven wordt genereert een proton motive force, oftewel een elektrisch membraan potentiaal en een verschil in pH aan beide kanten van het membraan. De tweede route voorziet de synthetische cel van energie in de vorm van ATP, de universele energiedrager die in alle levende cellen, dus ook in ons lichaam, een grote rol speelt in vele metabolische processen. Tegelijkertijd worden ook werkwijzen beschreven om deze routes te monitoren met (eiwit) sensoren en andere externe methodes.

Hoofdstuk 1 introduceert het concept van een synthetische cel. Deze begint bij een lege cel, oftewel een klein blaasje afgesloten door een membraan. Aan dit blaasje worden eiwitten toegevoegd, zowel binnenin de cel als in het membraan. Met deze eiwitten worden functies toegevoegd en met elke functie lijkt de synthetische cel steeds meer op een echte (bacteriële) cel. In dit hoofdstuk wordt ook beschreven hoeveel energie zo'n cel uiteindelijk nodig zal hebben om zichzelf in stand te kunnen blijven houden en zich te kunnen vermenigvuldigen. Deze energie kan op verschillende manier gegenereerd worden, bijvoorbeeld in de vorm van ATP en elektrochemische gradiënten. Het kleine formaat van een synthetische cel maakt het moeilijk om de processen binnenin cel te volgen. Om dit makkelijker te maken hebben we een lijst opgesteld van chemische en eiwitsensoren die daarbij van pas kunnen komen en de verschillende parameters kunnen monitoren.

In **hoofdstuk 2** wordt een enzymatische route beschreven die een proton motive force kan opwekken in liposomen. Deze route bestaat uit een membraaneiwit dat L-malaat (appelzuur) van buiten de cel naar binnen kan transporteren en een intern eiwit dat het getransporteerde L-malaat omzet naar lactaat (melkzuur). Lactaat kan de cel weer verlaten door zelf passief over het membraan te diffunderen of met behulp van het membraaneiwit. Bij de omzetting van L-malaat naar lactaat wordt een proton opgenomen. Dit zorgt er voor dat de pH aan de binnenkant stijgt. Daarnaast laten we ook zien dat het transport van L-malaat over het membraan elektrogeen is, er wordt een elektrisch potentiaal over het membraan gegenereerd (de binnenkant wordt negatief geladen). Het potentiaalverschil dat opgewekt wordt is ongeveer -30 mV. Dit is lager dan bijvoorbeeld het membraanpotentiaal dat *E. coli* heeft, maar kan onder omstandigheden genoeg energie leveren om bijvoorbeeld ATP te genereren via het F_1F_0-ATPsynthetase membraaneiwit.

Hoofdstuk 3 gaat over methodes om de concentraties van aminozuren te meten. Hoewel dit voor menig lab routinewerk is, blijken de basische aminozuren arginine, citrulline en ornithine lastig te meten. Als eerste hebben we gaschromatografie

gekoppeld aan massaspectrometrie geprobeerd. Voor deze methoden moeten de moleculen vluchtig zijn. Om de aminozuren vluchtig te maken worden de moleculen eerst chemisch aangepast. Voor vele aminozuren is dit eenvoudig, maar dit proces loopt zeer slecht bij arginine, citrulline en ornithine. Daar komt bij dat arginine na deze stap uiteen kan vallen in citrulline, wat de kwantificatie praktisch onmogelijk maakt. Een tweede methode, vloeistofchromatografie met omgekeerde polariteit, zorgt voor betere resultaten. Ook hier moeten de aminozuren chemisch worden aangepast, maar deze stap is snel en eenvoudig. Ondanks dat arginine en citrulline erg op elkaar lijken weet deze methode ze te scheiden en de concentraties te meten. Samen met het feit dat ook ammonia met deze methode gemeten kan worden, maakt dat vloeistofchromatografie de voorkeur krijgt.

Hoofdstuk 4 gaat over een metabolische route die ATP kan genereren in synthetische cellen. Deze route bestaat uit een membraantransporter en drie intracellulaire eiwitten. De productie van ATP start met het toevoegen van L-arginine aan de buitenkant van de cellen, waarna het door de membraantransporter naar binnen wordt gebracht. L-arginine wordt omgezet in L-citrulline en vervolgens in L-ornithine plus carbamoyl fosfaat. L-ornithine is een restproduct en wordt weer naar buiten gebracht door het membraaneiwit. In de laatste stap van de keten wordt de fosfaat groep van carbamoyl fosfaat overgezet op ADP, met als eindproduct ATP. Doordat de import van L-arginine en de export van L-ornithine gekoppeld zijn met een 1 op 1 ratio is er geen ophoping van het restproduct en kan de productie van ATP voor langere tijd door blijven gaan. In het proces meten we de ATP tot ADP ratio, de interne pH, de concentraties van L-arginine, L-citrulline, L-ornithine en als laatste ammoniak. Daarbij kunnen we ook aantonen dat de gegenereerde ATP daadwerkelijk gebruikt kan worden voor het importeren van glycine betaine door een tweede membraan transporter. Deze import van glycine betaine kan zelfs de cellen stabiliseren als we ze resuspenderen in een oplossing met een hoog zoutgehalte.

In **hoofdstuk 5** wordt een methode gepresenteerd om een membraanpotentiaal te meten. In grote cellen kan dit gedaan worden met elektrodes, maar dit is niet mogelijk in onze synthetische cellen omdat ze te klein zijn. Om het membraanpotentiaal te meten maken we gebruik van fluorescente moleculen, in dit geval zogenaamde Nernstian probes. Het werken met deze moleculen is niet eenvoudig aangezien ze snel blijven plakken aan oppervlakken en zeer gevoelig zijn voor de temperatuur en de ratio tussen membraanlipiden en de fluorescente moleculen zelf. Met deze methode kunnen we membraanpotentialen meten tussen 0 en -162 mV (de negatieve lading zit aan de binnenkant van de cellen) en is daarmee een goede basis voor toekomstige experimenten.

Om ATP concentraties binnen synthetische cellen zijn meerdere eiwitsensoren getest. In hoofdstuk 4 hebben we PercevalHR gebruikt, maar ook de ATeam sensoren zijn interessant. De resultaten van de drie ATeam sensoren zijn te vinden in de **Appendix**.

Appendix

ATeam ATP sensor evaluation

The ATeam sensors were among the first fluorescent probes for detecting ATP[86]. Three versions are available: ATeam 1.03, which utilizes the ε subunit of the F_0F_1 ATP synthase from *Bacillus subtilis* to bind ATP. This ATP binding domain is replaced by the ε subunit of the F_0F_1 ATP synthase from *Bacillus* sp. PS3 in ATeam 3.10, which resulted in a much higher affinity of the sensor for ATP. Lastly, DTeam is identical to ATeam 1.03 but has two mutations, which abolishes its capacity to bind ATP.

The ATeam sensors are FRET-based sensors and exploit the fluorescent proteins CFP and mVenus. The ε subunit adopts a compact conformation when bound to ATP, which draws the two FPs closer together. This results in an increased FRET efficiency. As a readout, the emission intensities of CFP and mVenus are recorded at 475 and 527 nm, respectively, upon excitation of CFP at 435 nm.

These sensors were evaluated for their use in vesicles containing the arginine breakdown pathway (see chapter 4). The experiments were conducted with the sensors in solution and revealed that ATeam 1.03 has a K_D for Mg-ATP of 1 mM. The 3.10 variant has an affinity for Mg-ATP of 1 µM that is approximately a 1000-fold higher. However, this sensor is also sensitive towards ADP and AMP, albeit with lower affinities (K_D values of 57 µM and 1 mM for ADP and AMP, respectively). As anticipated, DTeam does not bind ADP and AMP in the concentration range from 0.1 µM to 10 mM.

The effect of NaCl on the binding of Mg-ATP to the Ateam sensors was also studied. The affinity towards ATP of ATeam 3.10 was decreased at higher salt concentrations (Fig. 2). At a concentration of 1 M NaCl, the K_D was an order of magnitude higher than without added salt. The salt effect was not observed with ATeam 1.03, as its affinity to ATP was virtually unaffected by NaCl.

The design of the ATeam sensors is similar to that of the crowding sensors published by Boersma *et al.*[102], except that the ε subunit is replaced by a flexible domain composed of two α-helices. To test whether the ATeam sensors are sensitive to macromolecular crowding, we conducted an experiment using Ficoll PM70 to simulate a high degree of excluded volume (Fig. 3). All three sensors behaved similar towards crowding by Ficoll PM70. The FRET ratio increased with Ficoll concentration, implying that the sensors are not solely responding to ATP.

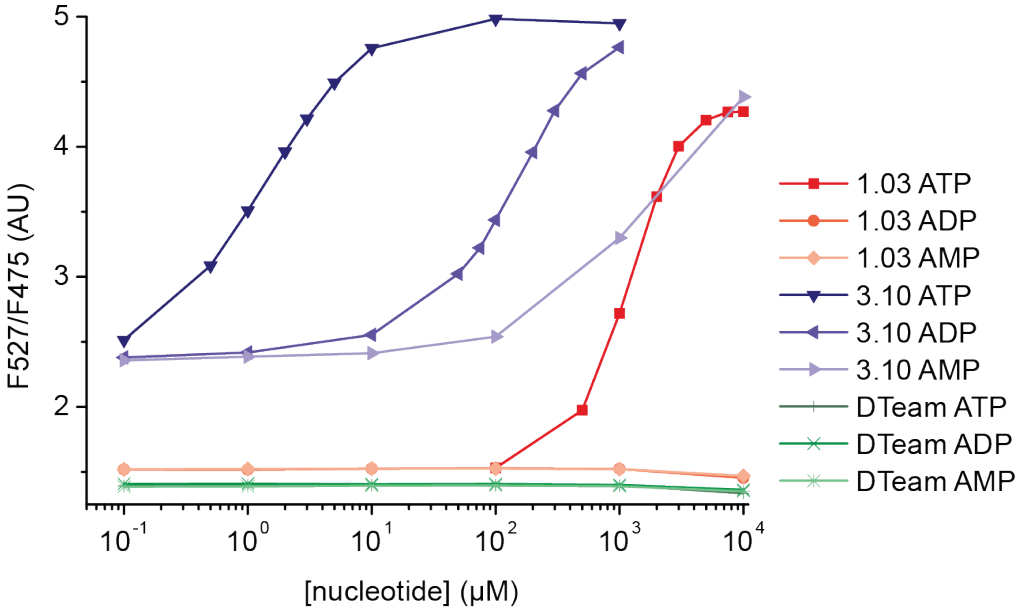

Figure 1. Changes in the fluorescent ratios of the ATeam sensors upon addition of nucleotides in 50 mM KPi pH 7.4, 150 mM NaCl plus 0.2 % BSA. All nucleotides were added with equimolar amounts of $MgCl_2$.

Based on the data presented in Figure 1, ATeam 3.10 is not suitable for the use in vesicles containing the arginine deiminase pathway. This sensor has the highest affinity towards ATP and should theoretically be able to report the production of even tiny amounts of ATP. In reality it will already be fully saturated at the start of an experiment, as the ADP concentration inside the vesicles is ten times higher than the K_D for ADP. Ateam 1.03 does not have this problem and could be used to measure ATP inside of vesicles. The linear range is however limited (approximately from 0.1 to 2 mM of ATP), which may lead to an on or off behaviour in liposomes. This impedes the quantification of ATP inside vesicles. Moreover, the effect on ionic strength and crowding of osmotically shocking vesicles as shown in Chapter 4, Figure 5 will further hamper the usefulness ATeam sensors inside proteoliposomes.

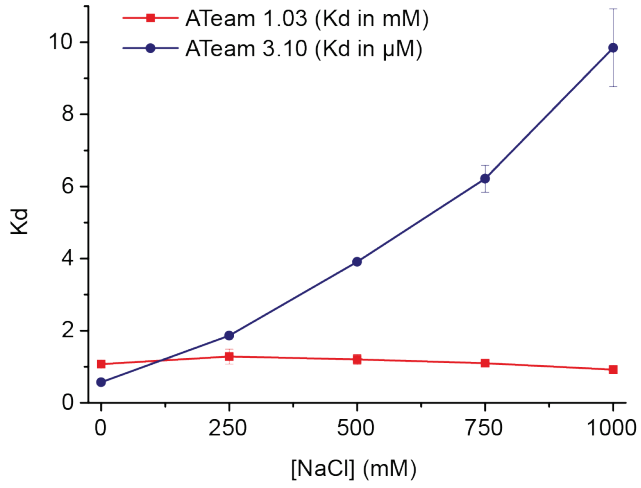

Figure 2. Sensitivity of the ATeam sensors to NaCl in 50 mM KPi pH 7.4. The K_D of ATeam 1.03 and 3.10 as a function of NaCl concentration.

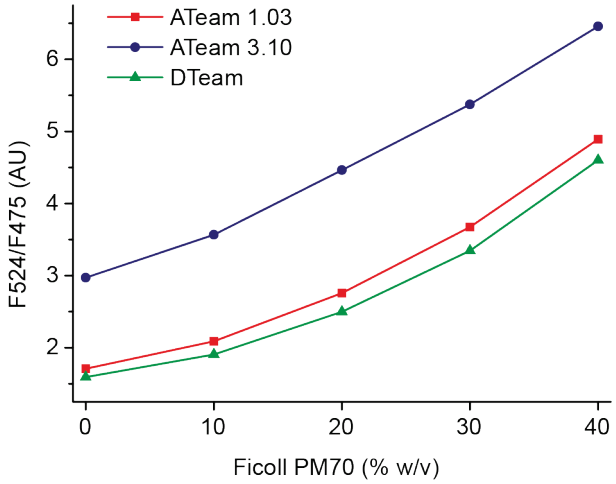

Figure 3. The effect of macromolecular crowding on the ATeam 1.03 (red), ATeam 3.10 (blue) and DTeam (green) in 50 mM KPi pH 7.4. Ficoll PM70 was used as crowding agent.

Methods

E. coli BL21-DE3 cells containing either pRset_A1.03, pRset_A3.10 or pRset_DTeam were grown in lysogeny broth with 100 µg ml^{-1} ampicillin. 0.5L cultures were grown at 30 °C, 200 rpm to an OD_{600} of 0.6, after which they were cooled to 20 °C. 100 µg of IPTG was added to start the transcription of the sensor gene from the plasmid. The cultures were incubated for 24 hours at 20 °C, 200 rpm before being harvested by centrifugation (6000 x g, 4 °C, 15 min) and washed with 600 ml of 50 mM NaPi pH 7.4, 100 mM NaCl. After centrifugation (6000 x g, 4 °C, 15 min), the cells were resuspended in approximately 30 ml of 20 mM NaPi PH 7.4, 100 mM NaCl and stored at -20 °C. After thawing 100 µg mL^{-1} of DNAse was added and the cells were lysed by high-pressure disruption in a single passage at 25 kpsi and 4 °C. Directly after lysis 0.1 mM of PMSF was added. The suspension was spun at 145.000 x g for 60 minutes at 4 °C to remove cell debris. The supernatant was flash frozen in LN_2 and stored at -80 °C.

All purification steps were performed at 4 °C or on ice, unless specified otherwise. Ni^{2+}-sepharose resin was pre-equilibrated with wash buffer (50 mM NaPi pH 8, 200 mM NaCl) supplemented with 10 mM imidazole. Cell lysate was thawed on ice, added to the equilibrated resin and nutated for 1h. The mixture was transferred to a polyprep column (Bio-Rad Laboratories, Inc.) and washed with 20 column volumes of wash buffer supplemented with 25 mM Imidazole. The protein was eluted with a maximum of 3 column volumes of was buffer supplemented with 250 mM Imidazole. Subsequently, the elution fractions were run on a Superdex 200 Increase 10/300 GL size-exclusion column (GE Healthcare) in 10 mM NaPi pH 7.4, 150 mM NaCl. Fractions with sensor were aliquoted in volumes of 50 µl, flash frozen in LN_2 and stored at -80 °C.

To measure the fluorescence of the ATeam sensors in solution, sensor was diluted to a concentration of 5 µg mL^{-1} in 50 mM KPi pH 7.4, 150 mM NaCl and 0.2 % (w/v) of BSA in 105.250-QS cuvettes (Hellma Analytics). The spectrum of the ATeam sensors was measured by a Jasco FP-8300 spectrofluorometer at 37 °C. Excitation was set at 435 ±5 nm, emission was captured from 460 ±5 nm to 560 ±5 nm.

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