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Department of Biotechnology

BioToP Biomolecular Technology of Proteins

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acib innovations from nature

Phospholipid vesicles to determine the transport functionality of mitochondrial carrier proteins

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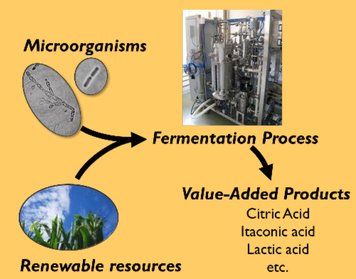
Research Group



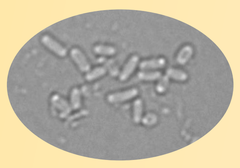
Introduction

Mitochondrial carrier proteins play a key role in transport mechanisms within many metabolic pathways. For the construction of more efficient cell factories to produce valuable metabolites like organic acids, a better understanding of the transport of these compounds is necessary [1]. A main objective of this project is the establishment of methods for the isolation of transporters from their native environment. Further, a second aim is the incorporation of fully functional transporters in artificial membrane-like systems for *in vitro* characterization. Therefore, we construct so called proteoliposomes [2]. In these spherical vesicles, membrane transporters are embedded in a phospholipid bilayer, surrounding an aqueous inner compartment. Thus, proteoliposomes allow simulation of distinct cellular compartments *in vitro* by varying the composition of phospholipids, membrane proteins and encapsulated substances. For the assembly of these vesicles we use and compare a cell-based and a cell-free system. Finally, we design assays to test the functionality and activity of these transporters with the constructed proteoliposomes.

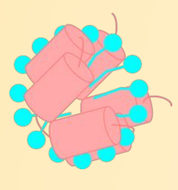
Golden Gate cloning is used to construct vector cassettes. This cloning strategy is based on type IIs restriction enzymes BsaI and BpiII (=BbsI), which cleave DNA outside of their recognition site [3].

Cell-based expression system

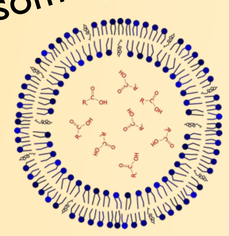
1. Expression in *E. coli* BL21



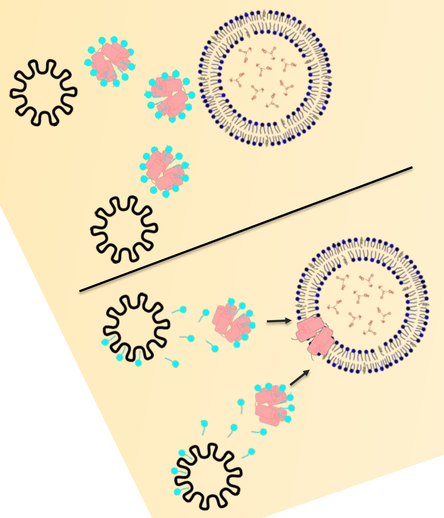
2. Protein solubilization and purification

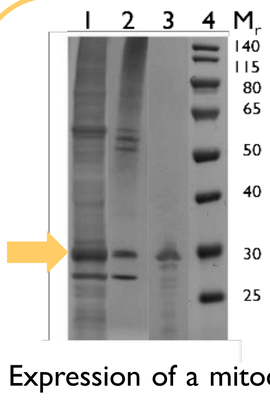


3. Liposome preparation



4. Reconstitution





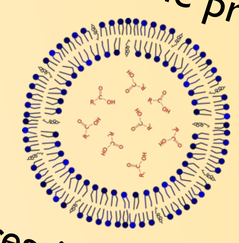
Expression of a mitochondrial citric acid transporter (M_r = 30 kDa) from *Aspergillus niger* can be seen in both expression systems. Western Blot evaluation of transporter expression in *E. coli* (lane 1), purified transporter (lane 2), cell-free expression system (lane 3) and molecular weight standard (lane 4).

10 days

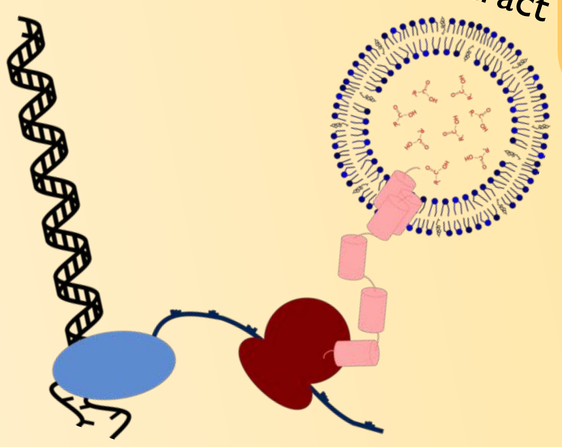
1 day

Cell-free expression system

1. Liposome preparation

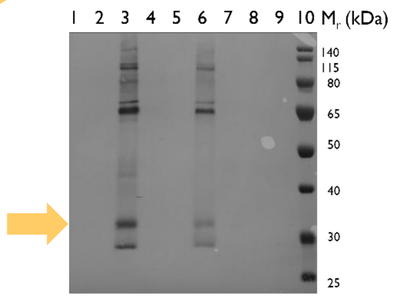


2. Expression in *E. coli* Extract

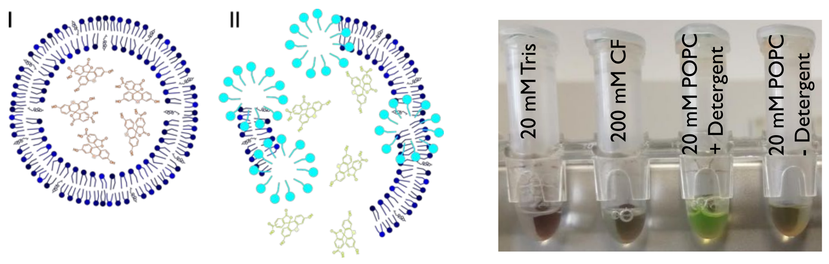


Cell-free system advantages:

- Fast batch reaction
- Direct incorporation in liposomes
- Working with cytotoxic peptides and proteins
- Usage of isotopic and non-natural amino acids



Evaluation by Western Blot shows ultrafiltrated reconstituted proteoliposomes with incorporated citric acid transporters (M_r = 30 kDa) in lanes 3 and 6. In the other lanes intermediate filter samples (lanes 1, 2, 4 and 5), a reconstitution approach without liposomes (lanes 7 to 9) and a molecular weight standard (lane 10) were plotted.



To enable the determination of the transporter activity (substrate flux across a membrane) the substrate is encapsulated into the vesicles. Incorporation of biotinylated phospholipids into the membranes allows separating the vesicles from the supernatant with help of magnetic streptavidin beads. Here, a fluorophore is used to evaluate this system: carboyxfluoresceine (CF) is encapsulated into the vesicles (I). The concentration is high, which leads to self-quenching (the vesicle suspension appears red). The biotinylated vesicles can be bound to magnetic beads and washed. They still appear reddish. By dissolving the bilayer of vesicles with detergents, the fluorophore is released, the solution appears bright yellow (II). Experimental set-up: test tubes filled with magnetic beads and either 20 mM biotinylated POPC liposomes or 20 mM Tris-HCl and 200 mM CF as references. Detergent is added into all sample (exception of the last test tube on the right). The release of CF can be clearly seen and proves that vesicles are sufficient loaded with substrate and bound to the streptavidin beads. No addition of detergent shows that vesicle stay intact on the beads.

Testing of proteoliposomes

Conclusion

- Establishment of cell-based and cell-free proteoliposome preparation processes
- Transporter expression in the cell-based system yields higher protein amounts
- The cell-free system significantly reduces the proteoliposome preparation time

Outlook

- Set-up of substrate flux analysis using radioactive labeled substrates
- Develop new strategies for metabolic engineering considering details about transport processes

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www.biotop.boku.ac.at/

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This Work has been supported by the Austrian BMWFW, BMVIT, SFG, Standortagentur Tirol, Government of Lower Austria and Business Agency Vienna through the Austrian FFG-COMET-Funding Program.