When synthetic cells and ABC-transporters meet

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Heterodimer formation of the homodimeric ABC transporter OpuA

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Abstract

Many proteins have a multimeric structure and are composed of one or more identical subunits. While this can be advantageous for the host-organism, it can be a challenge when targeting specific residues in biochemical analyses. In vitro splitting and re-dimerization to circumvent this problem is a tedious process that requires stable proteins. We present an in vivo approach to transform homodimeric proteins into apparent heterodimers, which then can be purified using a 2-step affinity-tag purification. We show that expression conditions are key for the formation of heterodimers and that the order of the differential purification and reconstitution of the protein into nanodiscs is important for a functional ABC-transporter complex. We demonstrate that the heterodimer obtained with this technique retains activity after reconstitution into nanodiscs.

4.1. Introduction

Most proteins exist as multimeric complexes, often as symmetric homomers with all subunits derived from the same gene, a feature that confers advantages for the cell as described by [1, 2]. First of all, large complexes have a smaller surface-area to volume ratio, which increases stability and may reduce protein denaturation and promiscuous interactions. Second, multiple small proteins are easier to fold than big ones, and these big complexes not only increase the chance for allosteric modulation of protein activity but also the frequency of substrate encounters. Moreover, homomultimeric proteins have additional advantages as error control in synthesis, and more efficient use of genomic space than heteromultimeric complexes, thereby saving metabolic energy [1, 2].

The study of homomultimeric proteins can be a challenge as any mutation will be replicated in the complex, which can be problematic if one aims to label specific sites with probes for fluorescence (e.g. single-molecule Förster Resonance Energy Transfer, smFRET) or electron spin resonance (e.g. Double Electron-Electron Resonance measurements, DEER). For smFRET a fluorescence donor and acceptor need to be introduced at specific sites, which is often done by introducing Cys or non-natural amino acids that can be labeled with the appropriate probe [3]. However, the introduction of two mutations already introduces four sites for labeling in homodimeric proteins, which complicates the spectroscopy. To circumvent the problem, one could purify the protein, separate the subunits and subsequently mix differentially labeled subunits and reassemble the protein complex. We have previously taken this approach in the study of the ATP-binding cassette (ABC) transporter OpuA from *Lactococcus lactis* [4]. The OpuA complex in the detergent-solubilized state falls apart when the glycerol concentration is less than 15-2% (v/v), but the dissociation of the complex is reversible. In this way we were able to create apparent heterodimeric complexes in which one of the membrane subunits was engineered and the other one not. Similar methods have been used for e.g. the membrane proteins Glt_{Ph}, VcINDY and BetP [5, 6]. However, this approach is not generally applicable, especially for proteins that are not stable in a detergent environment. We therefore sought for a more generic method to specifically label one subunit of homomultimeric protein complexes.

Here, we present a genetic approach to form apparent heteromultimeric from homomultimeric proteins. Again we use OpuA as a test case, which is an homodimeric membrane protein composed of two membrane subunits (OpuABC) and two nucleotide-binding subunits (OpuAA). OpuABC forms the transmembrane domain (TMD), surrounded by a scaffold and connected to the extracytoplasmic substrate-binding domain (SBD). We reasoned that it should be possible to form apparent heteromultimeric complexes by duplicating the *opuABC* gene and making constructs in which one of the subunits has a metal-affinity and the other a streptavidin tag for purification, named OpuABC-H and OpuABC-S, respectively. In vivo, the following protein complexes will form: $(OpuAA)_2$-$(OpuABC\text{-}H)_2$, $(OpuAA)_2$-$(OpuABC\text{-}S)_2$ and $(OpuAA)_2$-(OpuABC-H)-(OpuABC-S), theoretically in a ratio of 1:1:2 if the OpuABC-H and OpuABC-S subunits are formed in equal amounts (Fig. 4.1). Differential affinity chromatography can subsequently be used to enrich for $(OpuAA)_2$-OpuABC-H-OpuABC-S. In this paper we present the different strategies to form apparent heterooligomeric OpuA complexes in *L. lactis* and the methodology to purify the protein.

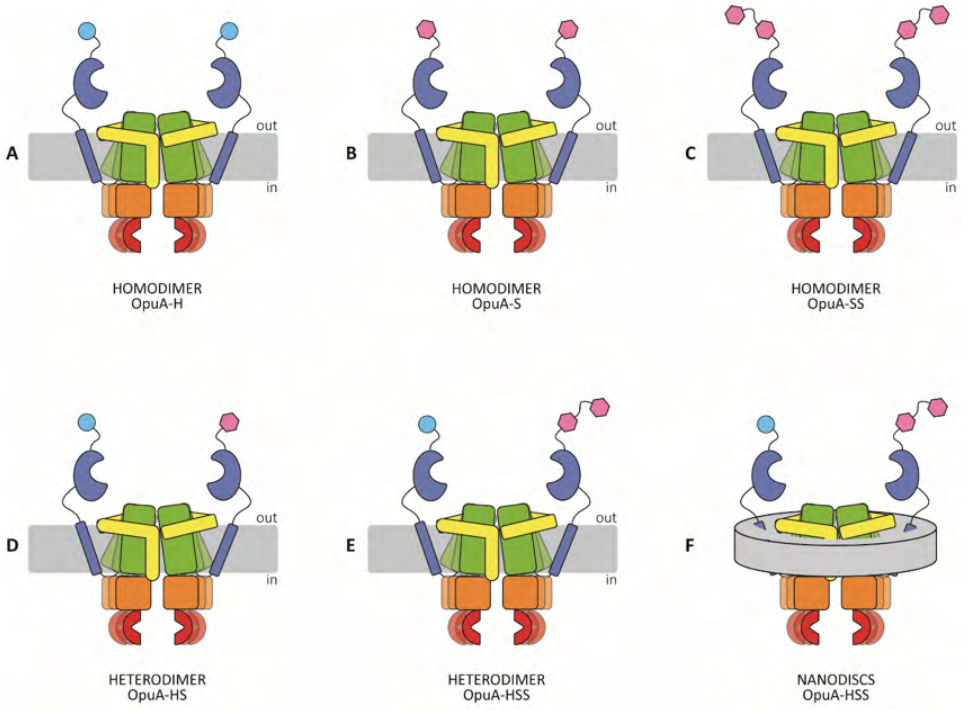

Figure 4.1 | Schematic representation of various OpuA constructs used in the present study. The wild type transporter OpuA is composed of two tandem cystathionine-β-synthase (CBS) domains (red), two copies of the ATP-binding subunit OpuAA (orange), two copies of the transmembrane domain (TMD) (green), including the scaffold domain (yellow) and two copies of the substrate-binding domain (SBD) (blue). (**A**) Homodimeric OpuA-H, the wild-type OpuA, with a His-tag (cyan circle) linked to the SBD; (**B**) Homodimeric OpuA-S, OpuA tagged with a StrepII-tag (pink hexagon) linked to the SBD; (**C**) Homodimeric OpuA-SS, OpuA containing a TwinStrepII-affinity tag (double pink hexagon) linked to the SBD; (**D**) Heterodimeric OpuA-HS, OpuA containing a His-tag in one SBD and a StrepII-tag in the second SBD; (**E**) Heterodimeric OpuA-HSS, OpuA composed of one SBD tagged with His-tag and another one with TwinStrepII-tag; (**F**) Schematic representation of OpuA-HSS in nanodiscs; lipids and MSP1D1 scaffolding protein are shown as grey disc.

4.2. Results

We first constructed a series of vectors and evaluated the expression and purification of homodimeric OpuA variants with three different affinity tags (Fig. 4.1): His-tag, StrepII-tag and TwinStrepII-tag. Each of the tags is present at the C-terminus of the OpuABC subunit. The genes are present in an operon in the order *opuAA-opuABC* and they are cloned under the control of the tightly regulated nisin-inducible p_{NisA} promoter, using the medium-copy number vector pNZ8048 (Cm^{Res}, pSH71 origin of replication; Fig. 4.2A) and pIL253 (Ery^{Res}, pAMβ1 origin of replication; Fig. 4.2A). The pNZ8048 vector has a so-called pSH71 rolling-circle type of replication [7], whereas the pAMβ1-derived vector pIL253 is a theta-replicating plasmid [8]. Both vectors are compatible with each other and can thus be used for co-expression of proteins in the same host [9].

4.2.1. Verification of activity with different affinity tags

To avoid recombination with wild-type *opuA* genes, the plasmids were transformed into *L. lactis* Opu401, an *opuA* deletion strain that was derived from *L. lactis* NZ9000 [10]. SDS-PAGE analysis shows the successful purification of OpuA with the three different affinity tags. Both subunits (OpuAA and OpuABC are present in an approximate 1:1 ratio) (Fig. 4.2B) [4, 10–12]. We obtained approximately 110 mg of membrane vesicles per 2L culture for all the three strains. However, the OpuA yield was very different depending on the affinity tag used, the His-tag being the most efficient with a yield of 18% of purified OpuA per total amount of protein, followed by TwinStrepII-tag with a 9% yield and StrepII-tag with a 2.5% yield.
The ATP hydrolysis activities of homodimeric OpuA with either His-tag, StrepII-tag or TwinStrepII-tag were verified in the nanodisc environment. OpuA was reconstituted in MSP1D1 nanodiscs with the lipid composition of 38 mol % DOPG, 12 mol % DOPC and 50 mol % DOPE and a reconstitution ratio of OpuA/lipids/MSP1D1 of 1:20:2000 [11]. The size-exclusion chromatography profiles of the three homodimeric OpuA complexes are very similar (Fig. 4.2C). SDS-PAGE analysis (Fig. 4.2D) illustrates the presence of the OpuAA (47 kDa) and OpuABC (63 kDa) subunits and the scaffold protein MSP1D1 (25 kDa). The ATPase activity with and without the substrate glycine betaine was determined using a coupled enzyme assay consisting of limiting amounts of OpuA in nanodiscs and an excess of pyruvate kinase and lactate dehydrogenase activity. The glycine betaine-dependent hydrolysis of ATP by OpuA was not significantly influenced by the different affinity tags (Fig. 4.2E).

4.2.2. Heterodimer formation

Next, we transformed pNZ*opuAHis* in combination with either pIL*opuAS* or pIL*opuASS* into *L. lactis* Opu401, to obtain heterodimers with different affinity tags. Theoretically this approach yields three different species: His-tagged homodimeric OpuA (OpuA-H), Strep-tagged homodimeric OpuA (OpuA-S) and heterodimeric OpuA containing both a His and a Strep-tagged subunit (OpuA-HS; Fig. 4.3A). To solely select and purify the desired heterodimeric protein we apply a two-step affinity chromatography (summarized in Fig.

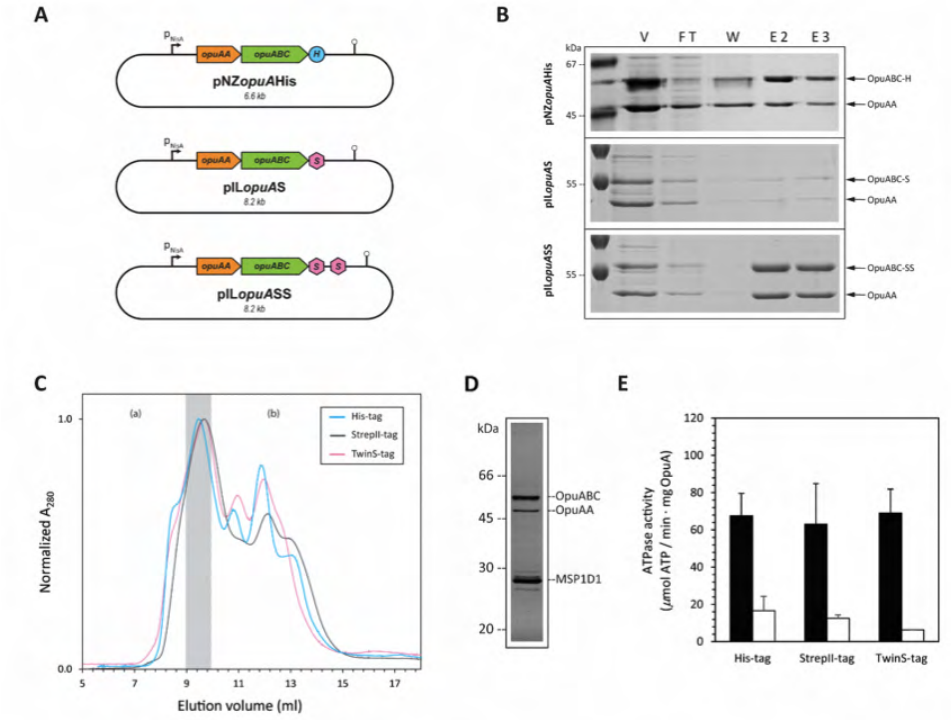

Figure 4.2 | Characterization of three differently tagged homodimeric OpuA constructs. (**A**) Schematic plasmid maps of the expression vectors. *opuAA*, gene encoding the ATPase subunit and CBS domains of OpuA; *opuABC*, gene encoding the TMD and SBD of OpuA; P_{NisA}, nisin inducible promoter; H, His-tag; S, StrepII-tag; double S, TwinStreptII-tag; bent arrows and lollipop symbols represent the promoters and terminators, respectively. (**B**) SDS-PAGE analysis (12.5% polyacrylamide) of affinity purifications of the three homodimeric OpuA constructs (OpuA-H, OpuA-S and OpuA-SS). The indicated proteins were purified from crude membrane extracts as explained in the text. The fractions tested were membrane vesicles (V), column flow through (FT), wash (W) and elution fractions (E). (**C**) Size-exclusion chromatography profiles of homodimeric OpuA nanodiscs, using a Superdex 200 increase 10/300 GL column. The chromatograms were normalized to the highest peak. The peak fractions used for further analysis are indicated in gray. (a) and (b) represent the peak fractions of aggregated and empty nanodiscs, respectively. (**D**) Typical peak fraction of nanodiscs analyzed by 12.5% SDS-PAGE, showing the presence of OpuAA, OpuABC and the scaffold protein MSP1D1. (**E**) ATPase activity in the presence (black bars) and absence (white bars) of 62 μM glycine betaine. Error bars represent the standard deviation of independent triplicates.

4.3B,C).
The initial protocol to purify hetero OpuA-HS mutant (Fig. 4.1) was based on a Ni^{2+}-Sepharose purification to remove Strep-tagged homodimers (OpuA-S) followed by a Strep-tactin purification to remove His-tagged homodimers (OpuA-H) or vice versa. Finally, size-exclusion chromatography was used for further purification and quality control (degree of monodisperse protein). However, no or little protein was obtained after the two purification steps, therefore either the heterodimeric species was not formed or lost in the purification process.

4.2.3. Homologous recombination

The pNZ*opuAHis* and pIL*opuAS* vectors are compatible and have different antibiotic markers, but both carry homologous *opuA* sequences (Fig. 4.2A) that may recombine and jeopardize heterodimer formation. RecA is the major protein involved in homologous recombination and DNA repair in *L. lactis* [13–15]. We therefore constructed a *L. lactis* Opu401 Δ*recA* strain (Table 4.1). We transformed both plasmids and applied the same purification protocol as described above, but the yield of heterodimeric OpuA was negligible (data not shown), suggesting that RecA homologous recombination is not a main problem.

4.2.4. Optimization of induction

Overproduction of proteins can activate stress responses, which has been shown to influence protein expression in *L. lactis* [16] and other (micro)organisms [17–19]. One strategy to minimize this effect is to slow down the protein production by decreasing the amount of inducer or lowering the post-induction temperature. To optimize the induction conditions we performed small-scale (50 ml) induction tests. After reaching a cell density of $OD_{600} = 0.5$, we induced with 0.05%, 0.02%, 0.01% or 0.002% of nisin A, and the post-induction temperature was either kept at 30 °C or lowered to 21 °C for 2, 4 or 8 hours. For each condition membrane vesicles were obtained and OpuA was purified by a single Ni^{2+}-Sepharose purification step, obtaining a mixture of His-tagged homodimers (OpuA-H) and heterodimers (OpuA-HS) (Fig. 4.3 and 4.4). The fraction of OpuA-HS was then quantified by Western-blotting with monoclonal antibodies raised against the StrepII-tag. Indeed, a lower induction temperature led to higher amounts of heterodimer, albeit at the expense of cell biomass to purify protein from.
Based on the induction test we selected two conditions for further experiments: (i) induction with 0.05% (v/v) culture supernatant of a nisin producing strain for 2 hours and (ii) induction with 0.001% (v/v) culture supernatant of a nisin producing strain for 4 hours, both with a post-induction temperature of 21 °C. Large-scale cultures were induced and membrane vesicles were prepared as described in materials and methods. The decrease in the post-induction temperature reduced the final membrane vesicle protein yield from 55 to 37.5 mg/L but the amount of heterodimeric OpuA was increased by at least 2-fold. The purification of OpuA-HS from the best induction condition (0.01% nisin; 21 °C; 4 hours) was analyzed by SDS-PAGE and Western-blotting (Fig. 4.5). We also observed that the yield of heterodimeric OpuA is dependent on the order of the purification steps. A strep-tactin purification followed by a Ni^{2+}-Sepharose purification yields a recovery of 0.008% (75 mg of total protein yields 6.4 μg of heterodimer). A Ni^{2+}-Sepharose purification followed by a Strep-tactin purification

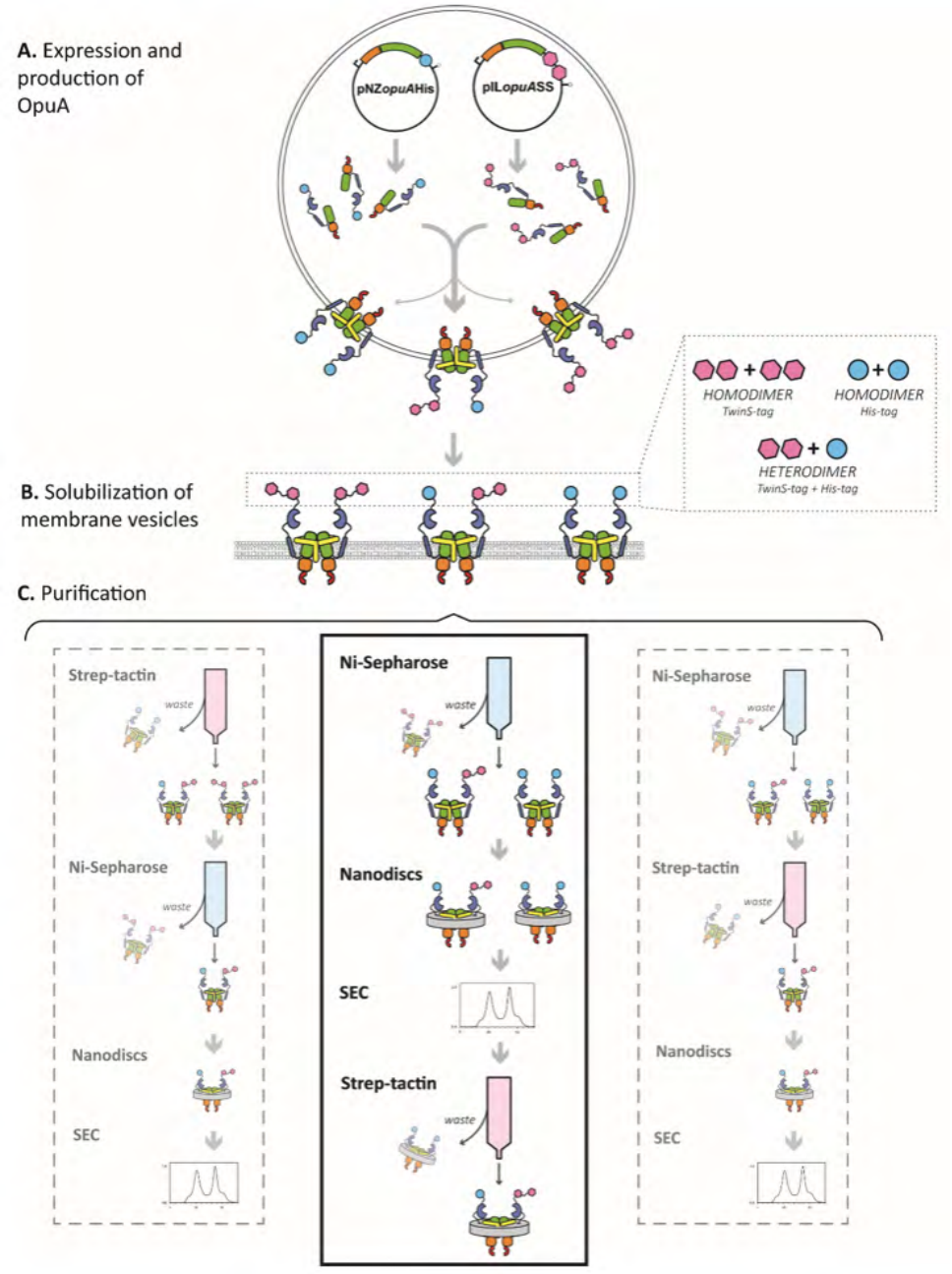

Figure 4.3 | Schematic representation of the protocol for the purification of the heterodimeric OpuA. (**A**) *L. lactis* Opu401 strain carrying plasmids pNZ*opuAHis* and pIL*opuASS* was grown in glucose-M17 broth and the genes were expressed under optimal conditions (0.05% nisin A; 21 °C; 4 hours). Three possible OpuA variants are formed in the cell: OpuA-H, OpuA-S and OpuA-HSS. (**B**) Solubilization of membrane vesicles were carried out as described in the Methods section. (**C**) Three different purification methods were tested by varying the order of the different purification steps. Dotted light grey squares frame the less efficient protocols.

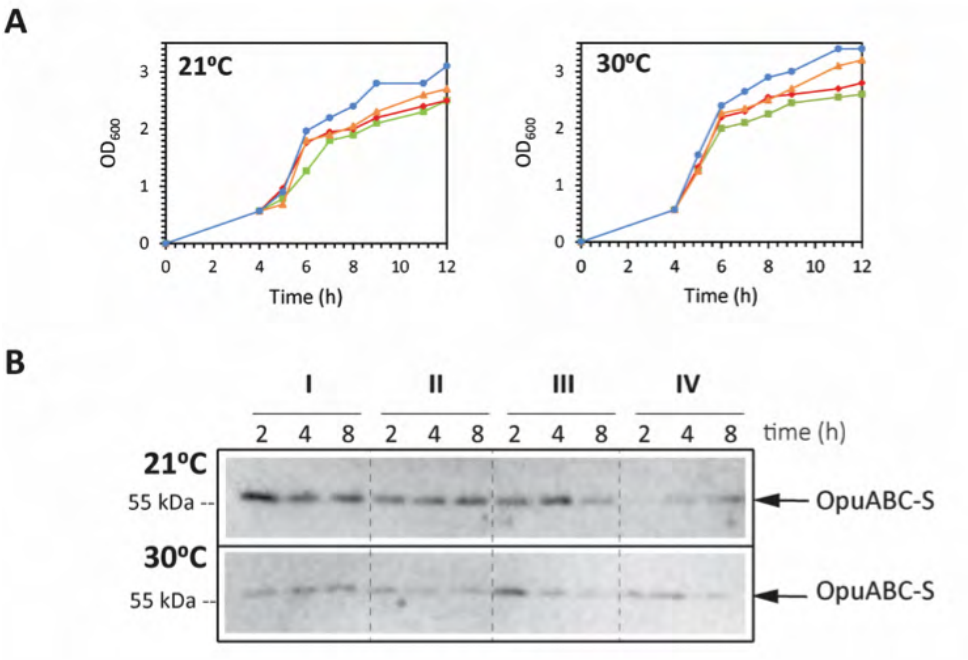

Figure 4.4 | Optimization of the heterodimer formation under different induction conditions. (**A**) *L. lactis* Opu401 strain, harboring plasmids pIL*opuAS* and pNZ*opuAHis*, was propagated at 30 °C in glucose-M17 broth as described in the text. When cultures reached an OD_{600} of 0.5, they were induced at four different nisin A concentrations: 0.05% (I, green square), 0.02% (II; red diamond), 0.01% (III; orange triangle) and 0.002% (IV; blue circle). Cultures (50 ml) were then incubated at two different temperatures, 21 °C or 30 °C, and induction times of 2, 4 and 8 hours were tested. (**B**) Membrane vesicles were obtained and proteins were purified with Ni^{2+}-Sepharose resin. To check the presence of the heterodimeric OpuA variant, final elution fractions were analyzed by Western blot analysis, using monoclonal antibodies directed against the StrepII-tag.

yields a recovery of 0.12% (75 mg of total protein yields 88 μg of heterodimer). Despite the improvements in the conditions, the low efficiency and recovery yield prohibited further studies, e.g. reconstitution of heterodimeric OpuA for functional analyses.

4.2.5. TwinStrepII-tag

Fusion proteins containing two copies of StrepII-tag, i.e. TwinStrepII-tag, have higher affinity for Strep-tactin compared to those containing only a single StrepII-tag, thus allowing more efficient protein purification, as we showed for the homodimeric complex (Fig. 4.2B). To increase the yield of heterodimeric OpuA, we switched from pIL*opuAS* to pIL*opuASS*, which contains the C-terminal TwinStrepII-tag sequence. After two steps of purification, the yield of heterodimeric OpuA was indeed higher and increased to approximately 0.4% recovery (19 mg of total protein yielded 80 μg of heterodimer).
The four times higher recovery yield, now allowed for continuation with further experiments. Moreover, the TwinStrepII-tagged OpuA subunit (OpuABC-SS) not only allowed obtaining

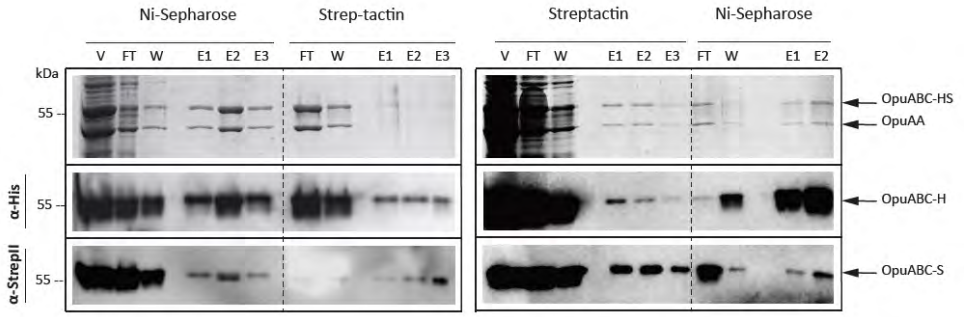

Figure 4.5 | SDS-PAGE (upper panel) and Western blot (two lower panels) analysis of the 2-step affinity purification of OpuA. *L. lactis* Opu401 (pNZ*opuAHis*, pIL*opuAS*) was grown and induced under optimal conditions (0.01% nisin; 21 °C; 4 hours). Membrane vesicles were obtained as described in the Methods section, and after solubilization of the membranes with 0.5% DDM, the lysate was subjected to two affinity purification steps: Ni-Sepharose followed by Strep-tactin (left panel) or *vice versa* (right panel). The following fractions were tested: vesicles (V), flow through (FT), wash (W), and elution fractions (E). Monoclonal antibodies directed against the His-tag and StrepII-tag were used, as indicated in the left side of the immunoblots.

a higher protein yield, but also migrates differently than the his-tagged subunit (OpuABC-H) on a SDS-PAGE (see example on Fig. 4.7), allowing easy visualization of the extent of heterodimer formation on SDS-PAGE. Heterodimeric OpuA-HSS was reconstituted into nanodiscs, however, SDS-PAGE analysis of SEC chromatograms profiles showed dissociation of the nucleotide-binding domain (OpuAA) from the OpuA complex, explaining the lack of ATP hydrolysis activity (data not shown).

4.2.6. Optimization of reconstitution

Two sequential affinity tag purifications require the protein to be stable in the detergent-solubilized state for up to 12 hours, which is problematic for OpuA as the complex readily dissociates, especially in low glycerol concentrations [4]. We therefore proceeded by performing the reconstitution in between the two purification steps. Thus, the OpuA complexes are purified by metal-affinity chromatography and then immediately incorporated into MSP1D1-based nanodiscs, which yields a population of homodimeric OpuA-H and heterodimeric OpuA-HSS nanodiscs. Within the membrane environment of the nanodiscs OpuA is much more stable and the glycerol concentration can be lowered to 4%, which increased the strep-tactin purification efficiency. Furthermore, to improve the quality of the OpuA nanodiscs, we varied the lipid:protein stoichiometry during the self-assembly process (Fig. 4.6). We found that a combination of higher concentration of OpuA (4.32 μM) and a ratio of 1:20:1000, yields a more separated peak fraction during size-exclusion chromatography and a higher ATPase activity (Fig. 4.6B).

4.2.7. Purification of the OpuA-HSS heterodimer

Cells were induced under optimal condition (21 °C, 4 hours, 0.01% nisin) and 24 mg of membrane vesicles were subjected to Ni^{2+}-Sepharose purification, yielding a total of 3.8 mg

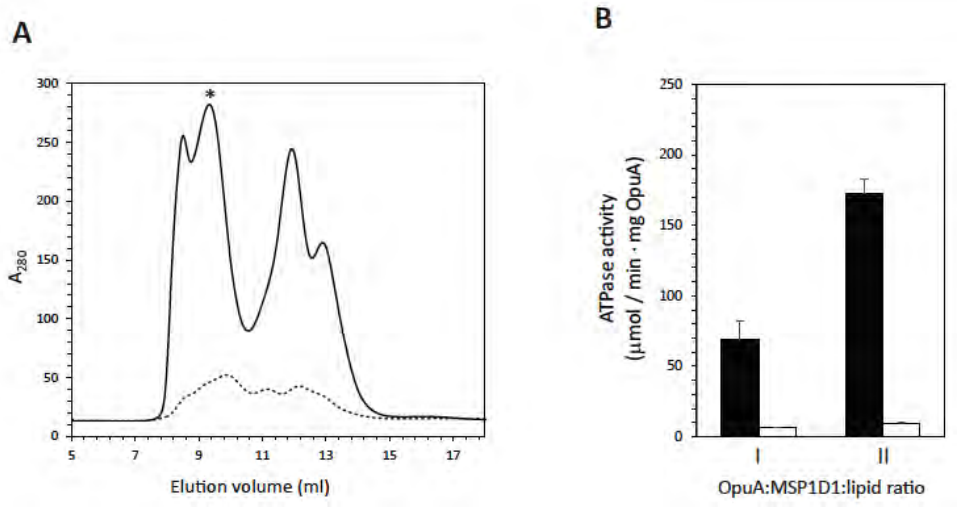

Figure 4.6 | Optimization of OpuA reconstitution in nanodiscs. Homodimeric OpuA-SS was purified and reconstituted in nanodiscs formed at different molar ratios and concentrations. (**A**) Size-exclusion chromatography profile of nanodiscs formed at a OpuA:MSP1D1:lipid ratio of 1:20:2000 (dotted line) and 1:20:1000 ratio; in the latter case we used a 6-times higher concentration of OpuA. Star represents the peak fraction with active OpuA-SS nanodiscs. (**B**) ATPase activity of OpuA-SS reconstituted in nanodiscs formed at a ratio of 1:20:2000 ratio (I) and 1:20:1000 (II). Black and white bars represent activity in the presence and absence of 62 μM glycine-betaine, respectively. Error bars represent the standard deviation of triplicates.

of his-tagged homodimers and heterodimers (OpuA-H and OpuA-HS). This mixture was reconstituted into nanodiscs and purified by SEC. The chromatogram showed a well-separated peak fraction (Fig. 4.7B) and the consequent SDS-PAGE analysis showed a comparable intensity for both subunits (Fig. 4.7A), suggesting that both OpuAA and OpuABC were present in the same concentration. Next, the selected peak fraction was purified on a Strep-tactin resin, yielding a total of 50 μg of heterodimeric OpuA in nanodiscs, which corresponds to a recovery of 0.2%. Moreover functionality of the protein was demonstrated by ATPase activity measurements (Fig. 4.7C). We attribute the lower activity of the heterodimer to the loss of a fraction of the OpuAA subunit as can be seen on SDS-PAGE gels (Figure 4.7A).

4.3. Discussion

One of the crucial steps in the in vivo formation of heterodimers is for the two differently tagged subunits to meet and dimerize. As mentioned before, there is also the probability of identical subunits to encounter each other and form homodimers. It is important to know what factors influence this process to tweak the dimerization in favor of heterodimers.

4.3.1. Spatial separation

Spatial separation is one of the most trivial factors that can influence multimer formation. For luciferase in *E. coli* it was shown that the efficiency of dimerization of its subunits (LuxA and

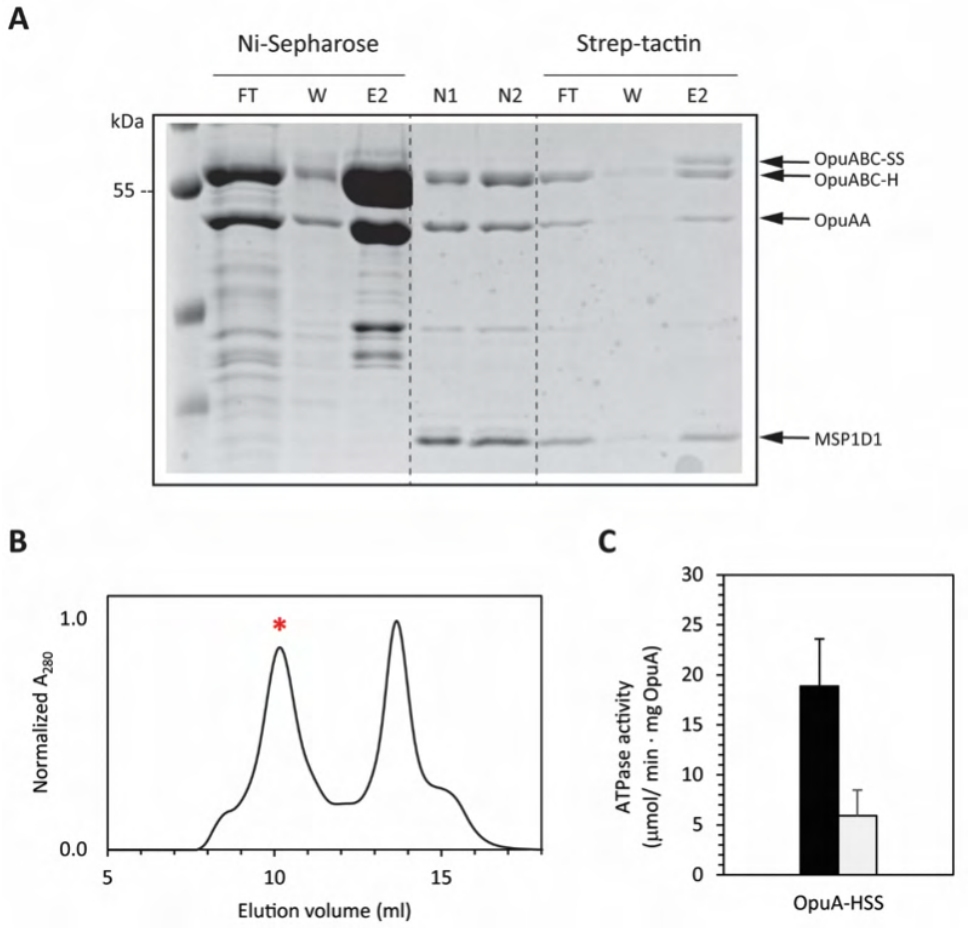

Figure 4.7 | Purification and characterization of the heterodimeric OpuA-HSS. (A) Membrane vesicles containing a mixture of OpuA-H, OpuA-SS and OpuA-HSS were obtained as described in the Methods section and subjected to a series of purification steps: (i) Ni-sepharose purification; (ii) Nanodisc reconstitution: (iii) SEC: and (iv) Strep-tactin purification. (**A**) Comassie-stained 12.5% SDS-PAGE samples of the different stages of the purification process. The fractions tested were flow through (FT), wash (W), elutions (E) and the peak fraction containing nanodiscs (N). Note that OpuABC-H and OpuABC-SS can be distinguished by their different migration in 12.5% SDS-PAGE gels. (**B**) Size-exclusion chromatography profile of the OpuA-H and OpuA-HS nanodiscs. Star represents the peak fraction used for further studies. (**C**) ATPase activity in the presence (black bar) and absence (white bar) of 62 μM glycine-betaine. Error bars represent the standard deviation of independent triplicates.

LuxB) decreases when transcribed from distant chromosomal sites [20]. In a different study [21] it was found that 9 out of 12 hetero-oligomeric protein complexes from *S. cerevisiae* assemble cotranslationally and the other 3 make use of chaperones, illustrating that assembly of oligomers is not a trivial process. Membrane proteins have a slower diffusion [22] and are restricted to a two dimensional space. It is very well possible that dimerization for membrane proteins has an even stronger dependence on location in the cell where the polypeptide is synthesized.

4.3.2. Multimerization interface

Many homomeric proteins have an enrichment for protein contacts towards the C-terminus [23]. We reason that, provided a structure is available, the formation heterodimers can enhanced by modification of the interaction region. Alternatively, expressing the protomers in close proximity (perhaps on the same mRNA) could enhance the heterodimer formation.

4.3.3. Expression conditions

Overproduction of membrane proteins induces stress (*vide supra*). And many interactions are temperature dependent. Moreover, diffusion scales linearly with temperature, thus, temperature directly or indirectly influences many processes in the cell. Where there are countless factors potentially influencing the formation of heterodimers, we chose a systematic approach to vary temperature and production conditions and found the best conditions to be at lower temperature (21 °C) and a long induction period (4h).
We have paved the way to in vivo heterodimer formation and show an efficient way of purifying the formed heterodimers. This *in vivo* approach is compatible with instable proteins by the possibility of keeping the protein stable in a native environment in the form of nanodiscs. Further research may lead to a further increase in the ratio of hetero- over homodimers.

4.4. Materials and methods

4.4.1. Materials

Common chemicals were ordered from Sigma-Aldrich or Merck. The pMSP1D1 plasmid was purchased from Addgene [20061]. The lipids: 1,2-dioleoyl-*sn*-glycero-3- phosphatidylcholine (DOPC) [850375C], 1,2-dioleoyl-*sn*-glycero-3-phos- phatidylethanolamine (DOPE) [850725C], and 1,2-dioleoyl-*sn*-glycero- 3-phosphatidylglycerol (DOPG) [840475C] were purchased from Avanti Polar Lipids, Inc (>99% pure, in chloroform). *n*-dodecyl-β-D-maltoside (DDM) [D97002] was purchased from Glycon Biochemicals GmbH.

4.4.2. Construction of strains and growth conditions

The bacterial strains, plasmids and primers used in the present study are listed in 4.1. Plasmids were propagated in *Lactococcus lactis* strain Opu401 [10] (which is *L. lactis* NZ9000 with the OpuA genes deleted). To construct the OpuA Strep-tagged homodimer, the P_{NisA} promoter [24] and the *opuABC* genes were PCR-amplified from pNZ*opuAHis* [10] with primers 6428

Strain	**Reference**
L. lactis Opu401	[10]
L. lactis NZ9700	[25]
L. lactis 401ΔrecA	This work
E. coli MG1655	[26]
plasmid	
pNZ*opuAHis*	[10]
pIL*OpuAS*	This work
pIL*OpuASS*	This work
pCS1966	[27]
pCS1966-RecA	This work
pIL253	[8]
pMSP1D1	Addgene
Oligonucleotide	**Sequence**
6494	AATCGATAAGCTTGGCTGCAG
6493	AACGAAGTGAGGGAAAGGCTAC
6429	AAACTGCGGAUGAGACCAAGCAGAACGACCCTCAATGGATCC
6428	AGCTCCAAGAUCTAGTCTTATAAC
6430	ATCCGCAGTTUGAAAAATAATAATTGGATTAGTTCTTGTGGTTACG
6431	ATCTTGGAGCUTCCATGTAATCGGGTTCTTC
7288	AATCAATCAUGAACCTGCTCCTC
7287	ATGATTGATUGGATTAGTTCTTGTGGTTACG
7234	AATTCCCAAGUTAGTCATTCTGACTG
7233	ACTTGGGAATUCGTCAAGTTTCAACGGAATTAG
7038	AGGCTACACTAGUTCTAGAGCG
7039	AGGTTGTCCACUCGGTACCCAG
7037	ACTAGTGTAGCCUTCAAGATCCTAGTCAGCATTCC
7036	AGTGGACAACCUATAGAAGCCACTTATCCAAG

Table 4.1 | Strains, plasmids and oligonucleotides used in the present study

and 6429. The backbone of the pIL253 vector [8] was amplified with primers 6430 and 6431, which contained the StrepII-tag sequence. The two amplified fragments were ligated to create the pIL*opuAS* vector. The OpuA TwinStrepII-tagged homodimer was constructed using the pIL*opuAS* vector as a template, where the StrepII-tag polypeptide sequence (SA-WSHPQFEK) was exchanged for the TwinStrepII-tag (WSHPQFEKGGGSGGGSGGS-SAWSHPQFEK), yielding the pIL*opuASS* vector. As a result, the three vectors contained the *opuABC* genes under the control of the nisin-inducible P_{NisA} promoter and the recombinant genes produce proteins with an affinity tag at the C-terminus of the OpuABC subunit. The strains producing the heterodimeric OpuA contained two possible combination of plasmids: pNZ*opuAHis* with pIL*opuAS* or pNZ*opuAHis* with pIL*opuASS*.

To construct the recA deletion strain *L. lactis* 401Δ*recA*, the flanking regions of *opuA* were amplified using primers 7036 and 7037. The pCS1966 vector [27] was amplified with primers 7038 and 7039. These two fragments were ligated obtaining the pCS1966-RecA vector. The pCS1966 derivative was obtained and maintained in *Escherichia coli* K-12 strain MG1655

[26]. The pCS1966-RecA vector was introduced in *L. lactis* 401 cells and positive colonies were selected in SA medium plates [28] supplemented with 20 μg/ml 5-fluoroorotic acid hydrate.
All the constructs were engineered by the ligation-free uracil-excision based-technique USER cloning method [29]. The PCR-amplifications were carried out by the PfuX7 DNA polymerase [30] with uracil-containing primers and the amplified fragments were ligated with USER enzyme (New England Biolabs, Inc). Plasmid DNA was isolated using the Macherey-Nagel NucleoSpin Plasmid QuickPure Kit (Thermo Fisher Scientific, Netherlands). DNA clean-up was performed with the Macherey-Nagel NucleoSpin Gel and PCR Clean-up Kit (Thermo Fisher Scientific). Constructs were checked by PCR amplification and subsequent sequencing analysis by Eurofins Scientific (Netherlands).
The strains were routinely cultivated semi-anaerobically at 30 °C in M17 broth (Oxoid, Germany) supplemented with 1% (w/v) glucose (Sigma-Aldrich, Nederland). When needed, the media were supplemented with 5 μg/ml of erythromycin and/or chloramphenicol.

4.4.3. Expression of opuABC genes

L. lactis OpuA-producing strains were grown in 2L or 10L pH-controlled bioreactors. Cells were propagated in M17 broth supplemented with 1% (w/v) glucose plus 5 µg/ml of the appropriate antibiotic at 30 °C with stirring (200 rpm). A constant pH of 6.5 was kept by titrating the culture with 4 M KOH. Unless specified otherwise, cultures were induced at an OD_{600} of 2 with 0.05% (v/v) of culture supernatant of the nisin A producing strain *L. lactis* NZ9700 [24] to initiate the transcription of the nisA promoter and the addition of additional 1% (w/v) glucose to obtain higher growth yields. To promote heterodimer formation the temperature of the culture was gradually decreased to 21 °C by cooling the fermentor with ice-cold water, simultaneously with addition of the inducer. Cells were harvested by centrifugation (15 min, 6,000 x g, 4 °C) after 2 hours of induction, washed twice and resuspended to an OD_{600} of 100 in ice-cold 50 mM KPi pH 7.5 buffer, flash-frozen and stored at -80 °C.

4.4.4. Optimization of the induction conditions

In order to promote the heterodimer formation in the cell, different induction conditions were tested, that is, nisin A concentration, the post-induction temperature and induction time were varied. For this purpose, *L. lactis* Opu401, carrying pNZ*opuAHis* and pIL*opuAS* was grown in a 2L flask containing M17 broth, supplemented with 1% (w/v) glucose, 5 μg/ml erythromycin plus 5 μg/ml chloramphenicol, at 30 °C with stirring (200 rpm). When the culture reached an OD_{600} of 0.5, it was divided into smaller cultures of 50 ml each, and induced with 0.05%, 0.02%, 0.01% or 0.002% (v/v) of nisin A. The cultures were then incubated at 21 °C or 30 °C for induction times varying from 2 to 8 hours. Cells were harvested by centrifugation (15 min, 6,000 x g, 4 °C), washed twice and resuspended to an OD_{600} of 34 in ice-cold 50 mM KPi, pH 7.5. Samples of 1.5 ml were mixed with 400 mg of 0.1 mm glass beads (Sigma-Aldrich) and lysed with a TissueLyser LT (Qiagen) for 5 min at high speed. Glass beads and cellular debris were removed by centrifugation (15 min, 25,000 x g, 4 °C). Pellets were discarded and membrane vesicles were collected by centrifugation (20 min, 267,000 x g, 4 °C) and resuspended in 1.8 ml of ice-cold 50 mM Kpi pH 7.5

supplemented with 20% glycerol. Then, membrane vesicles were solubilized with 0.5% (w/v) DDM and nutated for 2 hours at 4 °C, after which Ni^{2+}-Sepharose purification was carried out as described below. The presence of the heterodimer was analyzed by immunoblotting the elution samples with antibodies against StrepII-tag.

4.4.5. Isolation and preparation of membrane vesicles

The isolation and preparation of membrane vesicles was performed as described in [31] with minor changes. Cell pellets were thawed on ice and supplemented with 2 mM $MgSO_4$ plus 100 μg/ml DNAse. Cells were lysed by double passage through a cell disruptor (Constant Systems Ltd.) at 39 KPsi. After lysis, 1 mM PMSF plus 0.05 M EDTA (pH 8.0) were immediately added to avoid protein degradation. The cell debris was removed by centrifugation (15 min, 12,000 x g, 4 °C), and the membrane vesicles were collected by ultracentrifugation (1 hour, 267,000 x g, 4 °C) and resuspended in ice-cold buffer A (50 mM KPi, pH 7.5, 20% (v/v) glycerol). Aliquots were flash frozen and stored at -80 °C. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

4.4.6. Purification of OpuA

The purification process of OpuA was divided in a series of steps, of which the order was determined by the type and number of affinity tags of the final construct. Hence, the homodimeric forms of OpuA were subjected to a single affinity purification depending on the affinity tag, that are Nickel-Sepharose or Strep-tactin purification, followed by reconstitution in nanodiscs and size-exclusion chromatography on a Superdex 200 increase 10/300 GL column. However, the heterodimeric forms of OpuA required a two-step purification process that could be conducted in different order as described in the "Results" section. Below, we independently described all the required steps for the purification of any of the OpuA constructs, but note that the order may vary for each of them.

Solubilization of membrane vesicles

Prior to Ni^{2+}-Sepharose affinity purification, membrane vesicles were quickly thawed and diluted to a final protein concentration of 5 mg/ml in 50 mM KPi pH 7.0, 200 mM KCl, 20% (v/v) glycerol plus 10 mM imidazole. When Strep-tagged protein samples were intended to be purified, membrane vesicles were harvested (20 min, 267,000 x g, 4°C) and pellets were dissolved in 50 mM Tris-HCl pH 8.0, 150 mM NaCl plus 20% (v/v) glycerol at a protein concentration of 5 mg/ml. Then, membrane vesicles were solubilized with 0.5% (w/v) DDM and nutated for 1 hour at 4 °C. Supernatant was collected by ultracentrifugation (20 min, 267,000 x g, 4 °C).

Ni^{2+}-Sepharose affinity purification of his-tagged proteins

Ni^{2+}-Sepharose resin (GE Healthcare) (0.5 ml of resin per 10 mg total protein) was pre-equilibrated with 12 column volumes (CV) of distilled water followed by 4 CV of wash buffer (50 mM KPi pH 7.0, 200 mM KCl, 0.02% (w/v) DDM plus 20% (v/v) glycerol) supplemented with 10 mM imidazole (pH 7.5). To decrease the detergent concentration, solubilized membrane vesicles were diluted five-fold in ice-cold buffer (50 mM KPi pH 7.0, 200 mM KCl, 20% (v/v) glycerol plus 10 mM imidazole), and then incubated with the

Ni^{2+}-Sepharose resin under rotation for 2 hours at 4 °C. The mixture was poured into a column, and the resin was washed with 20 CV of wash buffer supplemented with 50 mM imidazole. Proteins were eluted with 2.5 CV of wash buffer supplemented with 500 mM imidazole. Protein concentration in the elution fractions was determined by absorbance measurements at 280 nm.

Reconstitution in lipid bilayer nanodiscs

Reconstitution was performed as described previously [11], with some modifications. Synthetic lipids were mixed in a ratio of 50 mol % DOPE, 12 mol % DOPC and 38 mol % DOPG and the preformed liposomes were prepared as described by [32]. The mixture was then extruded 13 times through a 400 nm polycarbonate filter (Avestin Europe GmbH) to obtain large unilamellar vesicles and then solubilized with 12 mM DDM followed by heavy vortexing. The standard procedure for the reconstitution of OpuA in nanodiscs was at an OpuA/MSP1D1/liposomes ratio of 1:20:2000 (w/w), respectively, in a final volume of 700 μL giving the following composition: 50 mM KPi pH 7.0, 12 mM DDM, 4% (w/v) glycerol, 0.72 /muM OpuA, 14.3 μM MSP1D1 plus 1.43 mM lipid. To optimize the method, we tested an OpuA/MSP1D1/lipid ratio of 1:20:1000 (w/w), starting with a 6-times higher concentration of OpuA, having a final composition of: 50 mM KPi pH 7.0, 12 mM DDM, 4% (v/v) glycerol, 4.32 μM OpuA, 85.8 μM MSP1D1 plus 4.29 mM lipid. When needed, OpuA samples were concentrated in 0.5 ml 30,000 kDa concentrators (Vivaspin). The reconstitution mixture was nutated for 1 hour at 4 °C, after which detergent was removed by adding 500 mg of SM2 Biobeads and incubating overnight at 4 °C with gentle agitation. Biobeads and protein aggregates were carefully removed by transferring the sample with a syringe to a new Eppendorf tube and subsequent centrifugation (25,000 x g, 10 min, 4 °C).

Size-Exclusion chromatography

The reconstitution mixture was fractionated by size exclusion chromatography (SEC), using a Superdex 200 Increase 10/300 GL column (GE Healthcare) equilibrated with 50mM KPi pH 7.0 supplemented with 200 mM KCl. Protein-containing fractions were pooled and stored at 4 °C until further use.

Purification of strep-tagged OpuA proteins

Strep-Tactin superflow high capacity resin (IBA LifeSciences) (1 ml resin per 10 mg/ml protein) was pre-equilibrated with 4 CV of buffer W1 [50 mM Tris-HCl pH 8.0, 150 mM NaCl supplemented with 20% (v/v) glycerol plus 0.02% (w/v) DDM, for membrane vesicles samples] or with buffer W2 [50 mM Tris-HCl pH 8.0, 150 mM NaCl supplemented with 4% (v/v) glycerol, for samples containing OpuA nanodiscs]. Samples were incubated with the pre-equilibrated resin and nutated for 1 hour at 4°C. The flow through was slowly passed twice through the column by gravity, and the column was then washed 5-times with 3 CV of buffer W1 or W2. Proteins were eluted with 0.5 CV of buffer W1 or W2, both supplemented with 10 mM of d-Desthiobiotin. After 5 minutes of incubation the elution was collected and the procedure was repeated 4 times more. Protein concentration was determined in the elution fractions by absorbance measurements at 280 nm.

4.4.7. SDS-PAGE and Western blotting analysis

Samples from all the steps of the purification process were collected and analyzed by SDS-PAGE using 12.5% poly-acrylamide gels. Pictures of the Coomassie-stained gels were taken by a Fujifilm LAS 3000 Imaging system (Fujifilm). To confirm correct subunit composition, Western blot analysis were carried out. Samples were resolved in a 12.5% SDS-PAGE and transferred to a PVDF membrane with primary antibodies against StrepII-tag or 6xHis-tag (Qiagen). Transfer of the proteins was done in 40 minutes at 0.08 Amp in a Trans-Blot SD Semi-Dry Transfer system (Bio-Rad). Proteins were visualized by inducing chemiluminescence with the CDP-star kit (tropix, inc) in the LAS-3000 imaging system.

4.4.8. ATPase activity assay

The ATPase activity of OpuA reconstituted in nanodiscs was analyzed using a coupled enzyme assay as described previously [11, 33]. In brief, the measurements were performed at 30 °C in a 96-well plate using a Spark 10 M 96-well plate reader (Tecan). A standard measurement solution of 200 μl/well contained 50 mM KPi (pH 7.0), 0.3 M KCl, 57 nM OpuA reconstituted in nanodiscs, 4 mM sodium phosphoenolpyruvate, 0.3 mM NADH and 3.5 μl of pyruvate kinase/lactic dehydrogenase enzyme mixture from rabbit muscle in 50% glycerol, with or without 62 μM glycine betaine. After incubation for 3 min at 30 °C, 10 mM MgATP pH 7.0 was added to each well and the absorbance of NADH at 340 nm was monitored over a period of 15 min. The oxidation of NADH is stoichiometrically coupled to the amount of ATP consumed, and the ATPase activity was expressed as the moles of ATP hydrolyzed per min per mg of OpuA.

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