Chapter 5

Investigation of possible interactions between amphipathic α-helix and CBS domains in ABC transporter OpuA

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1. Abstract

OpuA is an ABC transporter that couples ATP hydrolysis to glycine betaine uptake to cope with osmotic stress conditions. High-resolution crystal structures of the substrate-binding domain of OpuA are available but the full length transporter has so far been refractory to crystallization attempts. Therefore other strategies are required to investigate conformational states and interactions between protein domains, which are required to understand the translocation cycle of the transporter. The site-directed mutagenesis experiments described in chapter 4 illustrate the importance of the amphipathic α-helix (AH) in the OpuABC subunit for full transporter activity. Here, we report on the possible interaction between AH and the CBS domains of OpuA. A series of double cysteine mutants was created and dithiol cross-linking experiments were performed. On the basis of our preliminary cross-linking data, we find no evidence for an interaction of AH with the CBS module of OpuA.

2. Introduction

OpuA (osmoprotectant uptake system A), belongs to the OTCN subfamily within the ABC superfamily of transporters. Structurally, it is composed of an ATPase subunit OpuAA and a transmembrane (TMD) subunit OpuAB and a substrate binding protein OpuAC. The OpuAC moiety is fused to the TMD via a signal anchor sequence. The ATPase subunit of OpuA has a C-terminal extension, the tandem cystathionine β synthase (CBS) domain, which constitutes the ionic strength sensor, whose activity is modulated by the C-terminal 18-residue anionic tail (DIPDEDEVEEIEKEEENK) (Biemans-Oldehinkel, Mahmood & Poolman 2006, Mahmood et al. 2006). The CBS domains in OpuA form a dimeric structure with head-to-head interactions via CBS2, and the CBS2-CBS2 interface residues are critical for transport activity. The CBS1 domains are largely unstructured and merely serve as a linker between the NBD and CBS2 (Karasawa et al. 2011). The TMD core is composed of 6 transmembrane segments and an extension of 44 amino acids at N-terminus, which is for the greater forms an amphipathic α-helix (AH). The AH is necessary for high activity of OpuA but is not critical for the biogenesis of the protein or the ionic regulation of transport. Previously, we reported that not only an AH is important for the OpuA activity, rather a specific sequence is needed for full translocation activity. This suggests that the AH specifically interacts with another part of the protein, either the OpuAA or OpuABC subunit (Gul et al. 2012). To further characterize the role of AH, we made double cysteine replacement mutants to probe possible interactions between AH (S24C) and the CBS domains (various positions).

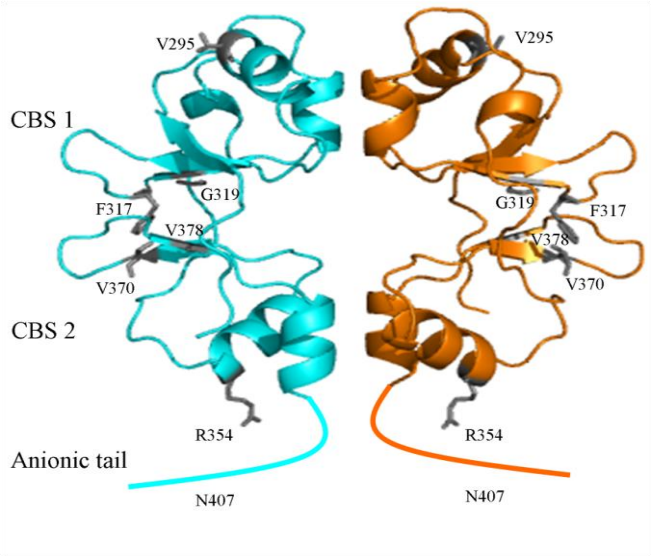

Figure 1: Dimeric structure of CBS domains showing position of residues replaced by cysteine. The homology model of the CBS domains of OpuA is based on the structure of MgtE (PDB entry:2YVX).

3. Materials and methods

3.1 Construction of OpuA single and double cysteine mutants

Plasmid pREBack OpuA S24Chis (Gul et al. 2012) having a single cysteine mutation in AH was used as template to create double cysteine mutants. Second-site mutations in the CBS domains or the anionic tail were constructed using the Quick change site-directed mutagenesis method (Zheng, Baumann & Reymond 2004). PCR amplification was done using *pfu* DNA polymerase (Promega), using primers specified in Table 1. Following digestion of the DNA by *Dpn*I, the DNA was transformed to *E. coli* MC1061. The exchange of the vector backbone of pRE-OpuA to pERL, containing a *L. lactis* origin of replication, was performed as described previously (Geertsma, Poolman 2007), resulting in pNZOpuA S24C+X###C his where (X= residue in CBS domain/anionic tail of OpuAA; # = position of the residue in the protein sequence; C= cysteine). In total seven double cysteine replacement mutants were created. Subsequently, the plasmid DNA was introduced into *L. lactis* Opu401 by electroporation.

3.2 Expression of cysteine mutants

For nisin A-inducible expression of single and double cysteine mutants of OpuA, *L.lactis* Opu401 (Biemans-Oldehinkel, Mahmood & Poolman 2006) was used as host strain. All single and double cysteine mutants were cultivated semi-anaerobically in 2% (w/v) gistex LS (Eemnes, NL) and 65 mM sodium phosphate (NaPi) pH 6.5, supplemented with 1 % (w/v) glucose plus 5 µg/ml chloroamphenicol, using 2 liter pH- and temperature controlled bioreactors. Cells were cultivated to OD_{600} of ~ 2, after which 0.1% (v/v) nisin A (filter-sterilized culture supernatant from *L. lactis* NZ9700) (de Ruyter, Kuipers & de Vos 1996) was added to induce the nisin A promoter. After 2 h, the cells were harvested by centrifugation, washed with 50 mM potassium phosphate, pH 7, resuspended in the same buffer, and stored at -80˚C after flash-freezing of 40 ml aliquots of cells in liquid nitrogen. Membrane vesicles were prepared by lysing the cells in a cell disruption system (high pressure Constant Systems cell disrupters, UK) at a pressure of 39,000 psi at 4˚C. Subsequently, differential (ultra)centrifugation was done to separate membrane vesicles from cell debris and cytosolic fractions as described (van der Heide, Poolman 2000).

3.3 Crosslinking of OpuA cysteine mutants in membrane vesicles

The accessibility of the thiol groups in the double cysteine mutants was assessed by 1,3 propanedithiol bismethanesulfonate (MTS-3-MTS), 3,6-Dioxaoctane-1,8-diyl bismethanethiosulfonate (MTS-8-02-MTS) and 3,6,9,12,15-pentaoxaheptadecane-1,17-diyl bis-methanethiosulfonate (MTS-17-05-MTS) crosslinkers (Toronto chemicals Inc. approximate S-S distance are 5, 11 and 22 Å for MTS-3-MTS, MTS-8-02-MTS and MTS-17-05-MTS, respectively). Membrane vesicles at 1 mg/ml in 50 mM KPi pH 7.0 were pretreated with 1 mM dithiothreitol (DTT; freshly prepared) for 1 h at 20˚C, followed by washing by centrifugation (15 min at 80,000 rpm) to remove excess of DTT. The membranes were then incubated with 5 µM MTS cross-linker for 2 h at 4˚C. The reaction was stopped by addition of 5 mM NEM and the extent of crosslinking was estimated from the mobility shift of OpuAA and OpuABC on SDS-PAGE and Western blots.

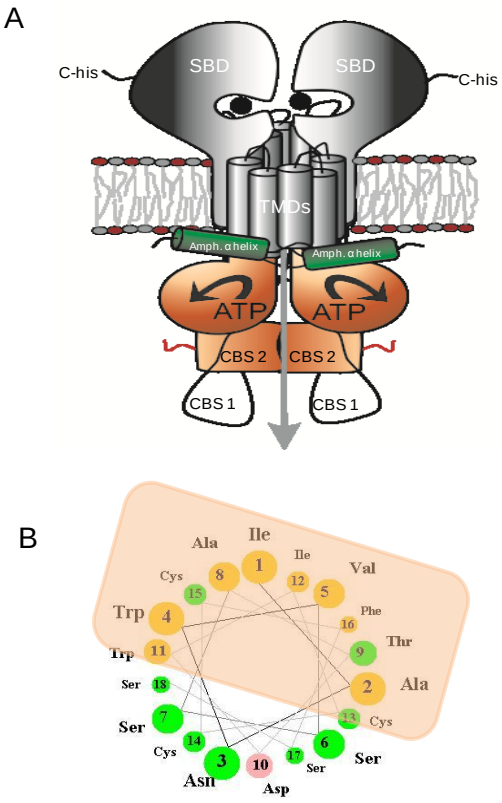

Figure 2:
(A) Organization of the dimeric OpuA complex
The OpuA system consists of a cytoplasmic membrane-associated ATPase (OpuAA) with two tandem CBS domains 1 and 2, the membrane-spanning substrate translocator protein OpuAB, and the extracellular ligand-binding protein OpuAC. OpuABC contains the predicted N-terminal amphipathic α-helix. The TMDs create the channel through which the substrate passes during translocation.
B) Helical wheel presentation of amphipathic *α-helix* of OpuA.

3.4 Purification of crosslinked OpuAA-OpuABC

For purification of OpuA, membrane vesicles bearing single and double cysteine mutants were untreated or crosslinked with MTS crosslinkers as described above. The membrane vesicles (5 mg of protein/ml) were treated with 1% SDS in buffer A (50 mM KPi, 200 mM KCl) for 15 min at 25 ˚C to dissociate non-crosslinked domains. Samples were then diluted to 0.1% SDS with buffer A plus 0.05% DDM

plus 15 mM imidazole and incubated with Ni-sepharose (GE Healthcare) for 1 h. Following thorough washing of Ni-sepharose to get rid of OpuAA subunit, OpuABC was eluted with buffer A plus 200 mM imidazole.

3.5 Western Blotting

Protein samples were analyzed by 10% polyacrylamide, non-reducing SDS-PAGE electrophoresis. Subsequently, the gels were submitted to semi-dry electroblotting and immunodetection with a primary antibody raised against OpuAA and OpuABC. Chemiluminescence detection was done using the Western-Light kit (Tropix Inc.) and quantified using the Fujifilm LAS-3000 imaging system.

4. Results and Discussion

4.1 Engineering of single and double cysteine mutants

According to our FRET analysis (Gul et al. 2012), the position of AH is close to the membrane surface. The accessibility experiments with single cysteine mutants suggests that the polar face of AH might specifically interact with another part of the protein, either the TMD, NBD or CBS domains (chapter 4). To unravel the possible interaction between AH and the CBS domains, we created single and double cysteine mutants. In all constructs with Cys pairs, Ser-24 of AH is changed to Cys and the second substitution is made in the CBS domains. The AH helix S24C mutant (chapter 4) was used as template to create second-site cysteine mutations in the CBS domains. Figure 1 shows the positions of cysteine mutations in OpuA. V295, F317 and G319 are located in the CBS1 domain, whereas R354, V370 and G378 are in the CBS2, and N407 is in the anionic tail. The respective positions were selected to cover a significant part of the surface of the CBS domains. The double and single cysteine mutants were expressed in *L. lactis* Opu401. The expression level of all cysteine replacement mutants was comparable to that of wildtype OpuA.

4.2 Crosslinking of double cysteine mutants

To determine the interaction between AH and CBS domains, membrane vesicles were treated with MTS crosslinkers, followed by non-reducing SDS-PAGE and Western blotting, using a polytopic anti-OpuAA antibody. OpuA is assembled as dimeric complex with two OpuABC and two OpuAA subunits (figure 2). Thus, a total of 4 cysteine residues are present in the protein. Three types of cross-linked species can be expected in double cysteine mutants: OpuAA-OpuAA, OpuABC-OpuABC, OpuAA-OpuABC. As the molecular weight of OpuAA is ~ 45 kDa and OpuABC is ~ 62 kDa, the OpuAA-OpuAA and OpuAA-OpuABC crosslinks are expected to migrate at 90 and 107 kDa. However, depending on the position of the crosslinks, the linked species may migrate faster than expected for a single

polypeptide of the same size. Inter-OpuA cross-links can be discriminated from intramolecular cross-links by including single cysteine mutants of AH and CBS domains in the analysis. The crosslinking profile in figure 3 shows a similar size of the cross-linked species in the double and single cysteine mutants, suggesting that inter OpuA OpuAA-OpuAA crosslinks are formed. We find no evidence for crosslinks between the AH and the CBS domains of OpuA.

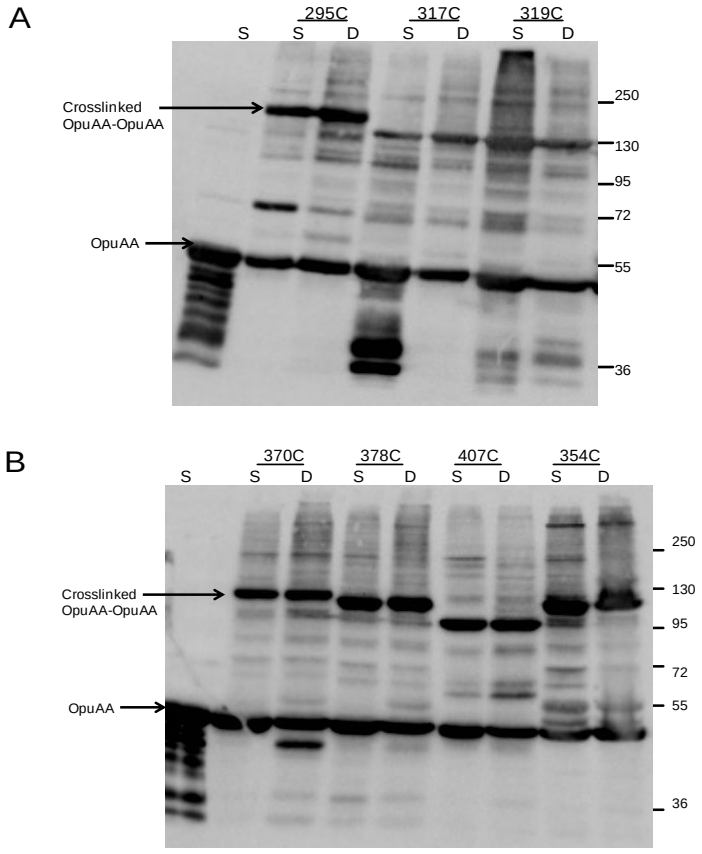

Figure 3: Crosslinking results of OpuA cysteine mutants. Membrane vesicles expressing OpuA and treated with MTS-17-05-MTS crosslinker at a final concentration of 5 µM. Samples were analyzed by non-reducing 10% SDS-PAGE and anti-OpuAA immunodetection. 5 µg of total protein/lane was loaded. **S**: single cysteine mutants **D**: double cysteine mutants. Position of monomeric and crosslinked subunits are indicated.

4.3 Purification of cross-linked OpuAA-OpuABC

To determine whether or not the cross-linked species originate from intermolecular OpuAA-OpuAA or intramolecular OpuAA-OpuABC species, the membrane vesicles with single R354C and double S24C+R354C cysteine mutants were treated with 1% SDS to dissociate the OpuA complex. Although 1% SDS was used to dissociate the OpuA complex prior to purification of OpuABC via its C-terminal His-tag, small amounts of OpuAA were present in all samples. Also, some OpuAA-OpuAA cross-links were observed in R354C. The amount of crosslinked species was highest in S24C+R354C (figure 4).

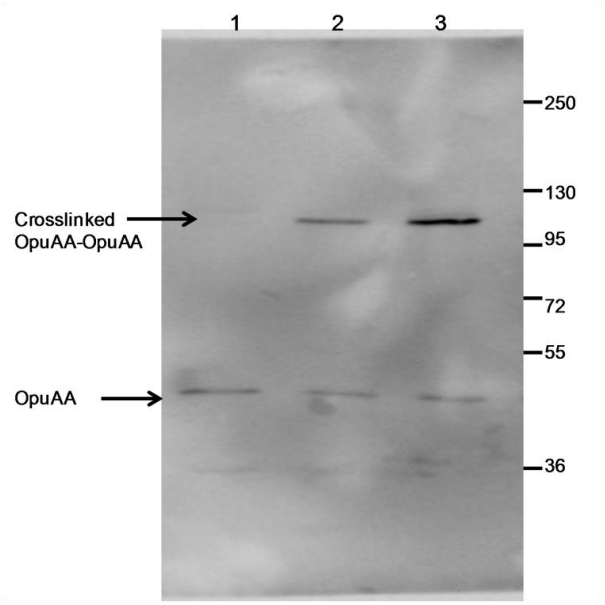

Figure 4: Purified OpuAA-OpuABC complex. MTS-8-MTS cross-linked membrane vesicles of OpuA S24C (lane 1), OpuA R354C (lane 2) and OpuA (S24C+R354C) (lane 3) were treated with 1% SDS, and purification was done in the absence of glycerol to dissociate OpAA from OpuABC. His-tagged OpuABC was purified and visualized by non-reducing 10% SDS PAGE and anti-AA antibody immunodetection.

5. Conclusion

Our cross-linking experiments provide no indications for specific interactions between the amphipathic α-helix on OpuABC and residues on the surface of the CBS module of OpuAA.

Table 1: List of OpuA primers used in the study

Mutant	Primer Sequence
V295CFw	GACGGACCAAGCTGTGCCTTGAAGAAGATGAAGACTG
V295CRv	CTTCTTCAAGGCACAGCTTGGTCCGTCAACATCAATG
F317C Fw	AAAAGCGTCAATGTCGTGGTGTTGTTACGAGTG
F317C Rv	ACAACACCACGACATTGACGCTTTTTGTCAACAG
G319C Fw	GTCAATTCCGTTGTGTTGTTACGAGTGAACAAG
G319C Rv	CTCGTAACAACACAACGGAATTGACGCTTTTTG
R354CFw	GAAATGCTTGTATGTGATATCTTGCCAATTATCTATG
R354CRv	GGCAAGATATCACATACAAGCATTTCTTTAGAAAC
V370C Fw	CTTTAGCAGTTTGTGATGATAATGGATTCCTTAAAG
V370C Rv	CCATTATCATCACAAACTGCTAAAGGCGTTGGG
G378C Fw	GATTCCTTAAATGTGTATTGATTCGAGGAAGTG
G378C Rv	CGAATCAATACACATTTAAGGAATCCATTATCATC
N407CFw	GAGGAGGAGTGTAAATGATTGATTTAGCTATTG
N407CRv	CAATCATTTACACTCCTCCTCTTTTTCAATC