Final Report

OTKA K104726: „Recognition of the flagellar export signal by the FliI ATPase”

Axial proteins of bacterial flagella are exported by the flagellum-specific export apparatus through the narrow central channel of the flagellum to the site of assembly at the distal end of filament. About a dozen different proteins are translocated by the flagellar export system which do not share significant sequence homology. Our work aimed at better understanding of the mechanism of substrate recognition by the export machinery.

Due to the obtained results the research plan had to be modified after accomplishing the first year of the project. The commonly believed starting hypothesis, that the FliI ATPase plays a central role in the recognition of protein subunits secreted by the flagellar export apparatus, was not justified by our experiments. We went on with exploring various aspects of the molecular mechanism of flagellar export and substrate recognition.

Summary of the most important results

1. The FliI ATPase is not the general recognition unit of the flagellar export system

(Sajó R, Liliom K, Muskotál A, Klein Á, Závodszky P, Vonderviszt F: Soluble components of the flagellar export apparatus, FliI, FliJ and FliH, do not deliver flagellin, the major filament protein, from the cytosol to the export gate. Biochim. Biophys. Acta – Mol. Cell Res 2014, 1843:2414-2413)

According to the general view, the FliI ATPase component of the export apparatus was supposed to bind and recognize the export substrates. Based on this hypothesis our work addressed the recognition mechanism of flagellar export substrates by the FliI ATPase. Flagellin, the main component of flagellar filaments, is the major export substrate. We studied the interaction between FliI and FliC by various biophysical techniques (quartz crystal microbalance, isothermal microcalorimetry, dual polarization interferometry) but could not observe any sign of interaction. We also demonstrated that in spite of previous reports, flagellin (FliC) had no effect on the enzymatic activity of FliI. Soluble components of the flagellar export system (FliH, FliJ, FliS) can bind to FliI and modify its functionality. In order to reveal whether FliI in complex with other export components can recognize FliC, we cloned His-tagged variants of all these proteins, overexpressed them in appropriate *E. coli* host strains and purified them by affinity chromatography. As reported by others, we also observed that FliH inhibited while FliJ increased the ATPase activity of FliI, respectively. However, addition of FliC to any of these complexes (FliI/FliH; FliI/FliJ; FliI/FliH/FliJ) did not influence the rate of ATP hydrolysis, not even in the presence of the FliS chaperone protein specifically helping the export of flagellin or phospholipid vesicles known to stimulate FliI.

The lack of interaction between FliI and FliC suggests that FliI is not the general recognition unit of the export system. FliI may be required for the recognition of the minor export substrates (rod proteins, hook protein, HAP proteins etc.) and for their efficient delivery to the export gate at the base of filaments. However, flagellin subunits are produced within the cells in a much larger (~100x) amount, and it seems that contribution of FliI is not essential for their transport to the membrane-embedded export machinery. Our results had been published and were confirmed soon by other groups (Erhardt et al., ATPase-Independent Type-III Protein Secretion in Salmonella enteric, PLoS Genet 10:e1004800, 2014; Minamino et al., The bacterial flagellar protein export apparatus processively transports flagellar proteins even with extreme infrequent ATP hydrolysis, Scientific Reports 4:7579, 2014).

2. Conformational properties of the flagellar export signal

In spite of intensive efforts, the nature of the signal directing flagellar protein secretion is still mysterious. A growing number of evidence indicates a critical role for the recognition signal located in the disordered N-terminal region of the secreted proteins. The identified signal sequences of different flagellar proteins, however, are highly diverse and share virtually no homology. It is an open question how such dissimilar signals interact with a single export apparatus. We believe that conformational properties of the signal sequence carry the clue for understanding the recognition mechanism.

The 26-47 segment of *Salmonella* flagellin contains the recognition signal for the flagellar export system. This segment lies within the disordered N-terminal region of the molecule. It is unclear what the essential conformational features of this signal sequence are. We created a fusion construct in order to facilitate exploration of the essential properties of the export signal. Our construct involved an N-terminal His6-tag, the 22-residue-long flagellin export signal, followed by the GFP protein and finally the C-terminal disordered region of flagellin. The C-terminal disordered part of flagellin was included to study the role of the FliS flagellin-specific chaperone in the export process. We hoped that this construct would have allowed monitoring of the efficiency of the export process by measuring the fluorescence intensity of the intracellular and cell culture supernatant fractions. Unfortunately, the encoded fusion protein was not efficiently secreted by the flagellum-specific export system. It seems that unfolding of GFP by the flagellar export machinery was problematic. A new construct has been made in which GFP is replaced by the CCP2 domain of the human complement protein C1r. A plasmid library has been constructed whose elements encode export signal variants mutated in highly conserved positions which are fused to the CCP2 reporter protein. This plasmid library is used to transform the flagellin deficient SJW2636 Salmonella host strain. Mutant colonies are checked for the efficiency of the export process. Experiments are still in progress to identify residues which are critically important for defining the essential conformational characteristics of the export signal.

Amphipatic helical ordering of the flagellin signal sequence

(Tőke O, Vonderviszt F: Amphipathic helical ordering of the flagellar secretion signal of Salmonella flagellin. Biochem. Biophys. Res. Commun. 2016, accepted for publication; doi: 10.1016/j.bbrc.2016.06.012.)

We investigated the conformational properties of the FliC export signal sequence by NMR spectroscopy under various conditions. In aqueous solution the signal sequence was highly flexible lacking a well-defined structure. However, it became stabilized and assumed a folded structure in solvents mimicking amphipathic environments. Mostly the N-terminal half of the peptide chain showed a significant propensity to form an α-helical structure with an amphipathic character. In addition, a short segment in the C-terminal region (A16-D18) also preferentially adopted a helical conformation.

It is not known how the flagellar export signal is recognized by the flagellar export machinery. The membrane-associated FlhA-FlhB platform is supposed to be responsible for substrate recognition, but the molecular details of this process remain elusive. N-terminal targeting signals (TSs) are typical in eukaryotes for protein import into different organelles, including mitochondria, chloroplasts, peroxisomes or the endoplasmic reticulum. Although the particular import mechanisms are remarkably different, the TSs appear to have similar conformational characteristics. Typically, they are structurally disordered but all involve a sequence element with a high propensity to form an α-helical segment in which hydrophobic residues cover one side of the helix. Upon interaction with membrane-bound receptor proteins these targeting signals radically change their conformation adopting an amphipathic α-helical structure which is recognized by the receptor.

Our results reveal that the flagellar export signal of *Salmonella* flagellin is capable of acquiring a partially α-helical amphipathic structure under appropriate conditions. This observation raises the possibility that amphipathic helical ordering may play an important role in recognition of the export substrates by the flagellum-specific export machinery, resembling the mechanism observed in the case of protein translocation into intracellular organelles.

3. The FlhA, FlhB and FliK components of the export system

Available experimental data suggest that the cytosolic domains of the FlhA and FlhB membrane components of the export system form a docking platform for the complexes of soluble components and exported protein subunits, and they may also play an important role in export substrate recognition. First, we aimed at exploring this possibility.

We cloned and overexpressed the His6-tagged variants of the C-terminal cytosolic portions of the FlhA and FlhB proteins, $FlhA_C$ and $FlhB_C$, respectively. $FlhB_C$ is known to undergo autocatalityc cleavage at position Asn-269/Pro-270 which is essential for proper functioning. The cleavage products of $FlhB_C$, subdomains $FlhB_{CN}$ and $FlhB_{CC}$, were also cloned and overexpressed. It took a lot of efforts to overcome aggregation and refolding problems during purification of these protein samples. Finally, preparation protocols were successfully developed for $FlhA_C$, $FlhB_C$ and $FlhB_{CC}$. $FlhB_{CN}$ consisting of only 68 amino acids was found to be readily degraded during overexpression.

Purified $FlhA_C$ exhibited a CD spectrum characteristic for alpha-helical proteins indicating that our sample contained well-folded molecules. The interaction between $FlhA_C$ and the major export substrate, flagellin (FliC) was studied by various biophysical methods. Preferentially immobilization-free techniques (including isothermal titration calorimetry, microscale thermophoresis and analytical gel filtration) were applied but we could not observe any significant interaction. While ITC experiments demonstrated that the FliS flagellin-specific export chaperone bound strongly to FliC with a dissociation constant of ~20 nM, addition of $FlhA_C$ to the FliC-FliS complex did not produce any measurable heat effect. Similarly, calorimetric investigations did not show any interaction between $FlhB_C$ and flagellin. While highly sensitive immunoblotting experiments by other groups clearly demonstrate the interaction between FlhAC/FlhBC and export substrates, our direct biophysical techniques failed to detect binding. It seems that these interactions are rather weak and several export components may be involved in forming the transient functional recognition complex. Further experiment are needed to reveal the related molecular events.

There is a highly curved segment, called the hook, which connects the helical filament to the membrane-embedded flagellar motor. The length of the hook is controlled by the FliK protein which is supposed to work as an internal molecular ruler. Interaction between FliK and $FlhB_C$ upon completion of the hook is critical to substrate specificity switching of the export apparatus from rod/hook type to filament type substrates. To get a better understanding of FliK function, the full length protein as well as its various fragments, were cloned, overexpressed and successfully purified. Our proteolytic and calorimetric experiments demonstrated that the N-terminal half of FliK, containing residues 1-205, lacks ordered structure and does not show cooperative melting behavior. On the other hand the C-terminal 258-359 segment is well-folded and stabilized by a substantial binding energy. Recent models on hook-length-control assume that the folding of N-terminal part of FliK exiting the distal end of the hook acts to pull the FliK molecule through the central channel of the hook rapidly. Our results show that the N-terminal part of FliK has no internal stability suggesting that its folding cannot provide the driving force for fast secretion/translocation. (manuscript in preparation)

4. Conformational adaptability of FliS export chaperone

(Sajó R, Tőke O, Hajdú I, Jankovics H, Micsonai A, Dobó J, Kardos J, Vonderviszt F: Structural plasticity of the Salmonella FliS flagellar export chaperone. FEBS Lett. 2016, 590:1103-1113.)

FliS is the specific export chaperone for the major flagellar filament component protein, flagellin (FliC). It is a small protein, consisting of 135 amino acids, which has multiple functions within the flagellar export system. Besides facilitating flagellin export, it has been demonstrated that FliS also interacts with components of the flagellar export systems and plays a role in the transcriptional regulation of flagellar biosynthesis, too.

The 3D structure of Salmonella FliS has not been determined yet. Based on X-ray structures available for FliS from different species we have constructed the homology model of FliS from Salmonella typhimurium which suggested a highly α-helical structure consisting of a 4-helix bundle. Proteolytic experiments indicated that it has a compact core part. However, scanning calorimetric measurements revealed that FliS does not show a cooperative melting transition. Monitoring the heat-induced structural changes by CD spectroscopy demonstrated that the ordered secondary structure is lost over a very broad temperature range (40-100ºC) which may be indicative of gradual unfolding of the native structure. This was further supported by the analysis of the measured CD spectra which revealed that temperature increase resulted first in the shortening of helices and their number decreased only at higher temperatures. Molecular dynamics simulations showed relatively large fluctuations in the α-helix content compared to that of stable proteins exhibiting cooperative unfolding transitions with similar temperature midpoints. Fluorescence monitoring of the local environment of the lone W122 residue also showed a somewhat different unfolding behavior as compared to the overall characteristics revealed by CD spectroscopy. These observations suggest that various parts of the molecule unfold in a non-cooperative manner.

Substantial amount of secondary structure but lack of cooperativity in unfolding are typical characteristics of the molten globule (MG) state of proteins. MGs are believed to have fluctuating tertiary structure, while our NMR measurements indicated a folded core for FliS. A similar situation was observed for the MG state of α-lactalbumin and the NCBD protein. Their internal dynamics is caused by interconversion between discrete folded conformations. Despite populating compact folded conformations, they still have a slowly fluctuating tertiary structure thus adhering to the definition of a MG. FliS may also readily assume different conformations as indicated by the structural heterogeneity of subunits even within the unit cell of the available FliS crystal structures. Our experiments suggest that FliS belongs to the family of atypical MG proteins.

The ability to sample different conformations may facilitate binding to various partners. FliS is a component of the flagellar export machinery which has several binding partners within the cell. Conformational adaptability seems to be an essential requirement in order to fulfill its various functional roles. A molten globule ensemble of interconverting folded structures may provide a convenient target for conformational selection by diverse partners.

5. Development of flagellin-based fusion constructs and their cost-effective production exploiting the flagellar export machinery

We are making efforts to utilize the knowledge acquired from basic research of the flagellar export systems in bio-nanotechnological applications. We developed flagellin-based fusion proteins possessing various catalytic and molecular recognition functionalities. By preserving the export signal, these constructs are secreted from the host cells by the flagellar export apparatus in large amounts allowing easy and cost-effective production.

Integrin-binding flagellin variants

(Kovacs B, Patko B, Szekacs I, Orgovan N, Kurunczi S, Sulyok A, Khanh NQ, Toth B, Vondervisztf F, Horvath R: Flagellin based biomimetic coatings: from cell-repellent surfaces to highly adhesive coatings. Acta Biomaterialia 2016, accepted for publication)

The ability to control the adhesion of living cells with engineered surfaces is of high importance today. In the present work, we demonstrated that oriented layers of wild-type flagellin and flagellin variants displaying the integring-binding RGD motif can be used as biomimetic coatings to create cell-adhesion-regulating surfaces. Previously we showed that the bacterial flagellar protein, flagellin, adsorbs through its terminal segments to hydrophobic surfaces, forming an oriented monolayer and exposing its variable D3 domain to the solution. This nanostructured layer is highly cell-repellent since it mimics the surface of the flagellar filaments. In this study we created genetically engineered flagellin mutants by replacing the variable D3 domain with one or more RGD motifs flanked by various oligopeptide linkers modulating flexibility and accessibility of the inserted segment. These flagellin variants are secreted by the flagellar export system allowing easy purification and can be applied to create monolayer surface coatings. The RGD-containing mutants induced different levels of cellular adhesion, depending on the employed linkers, and the number of peptide motifs inserted, while wild-type flagellin was shown to form a surface layer with strong anti-adhesive properties. Quantitative adhesion data were obtained by employing label-free optical waveguide lightmode spectroscopy.

Application of flagellin-based fusion proteins for fabricating tunable surfaces opens up new avenues. Other peptide sequences can be easily introduced into flagellin in a similar manner. Flagellin variants exhibiting specific recognition properties offer the possibility to create a large variety of coatings with multiple functionalities. Since flagellin does not adsorb at all on hydrophilic surfaces, by patterning the hydrophobicity of the surfaces and exposing them to the solutions of functionalized flagellin, patterned functional surfaces could be easily created in a straightforward and cost-effective manner.

Catalytic FliC-XynA nanorods

(Klein Á, Szabó V, Kovács M, Patkó D, Tóth B, Vondervisztf F (2015) Xylan-degrading catalytic flagellar nanorods. Mol. Biotechnol. 57:814-819.)

In this work we created a fusion construct of xylanase A from *B. subtilis* (XynA) and *Salmonella* flagellin (FliC) which is applicable to build xylan-degrading catalytic nanorods of high stability. The XynA enzyme was introduced into the middle part of flagellin replacing the variable D3 domain. Unfortunately, bacterial overexpression of the fusion protein in standard *E.coli* host strains was problematic because the disordered terminal regions of the fusion construct were readily degraded during the purification steps. However, we realized that the FliC-XynA fusion protein contains the N-terminal part of flagellin involving the flagellum-specific export signal. To exploit flagellar export, the plasmid encoding for FliC-XynA was transformed into the flagellin deficient SJW2536 Salmonella host strain which is unable to produce flagellin, and only the base and the short hook part of the filament is formed. The FliC-XynA chimera when overexpressed in SJW2536 was secreted into the culture medium by the flagellar export system allowing its easy purification in a functional form. Filamentous assemblies displaying high surface density of catalytic sites were produced by ammonium-sulfate-induced polymerization. The XynA enzymes retained their catalytic activity when the fusion protein assembled into flagellar nanorods. FliC-XynA nanorods were resistant to proteolytic degradation and preserved their enzymatic activity for a long period of time. Furnishing enzymes with self-assembling ability to build catalytic nanorods offers a promising alternative approach to enzyme immobilization onto nanostructured synthetic scaffolds.

The results of our work have been published in 5 papers with a total IF of 18.65. We paid special attention for training students and young researchers. These efforts are reflected in a bachelor's thesis and a master's thesis. In addition, two of our students obtained 1st prize at the local Conference of Scientific Students' Associations.