Chaperone/Usher Machinery: a Catalyst of Virulence Organelle Assembly

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Abstract

Many virulence organelles of Gram-negative bacterial pathogens are assembled via the periplasmic chaperone/usher (CU) pathway. The assembly process is a complex task, involving secretion of organelle subunits via the two membranes and periplasm, subunit folding and assembly. In this thesis, the mechanism of the organelle subunit trafficking and assembly via the CU pathway was investigated at different steps, subunit capture by the chaperone (**paper II)**, usher targeting (**paper III**), and transport through the usher (**paper I**), using the Caf1M/Caf1M CU system that assembles the *Yersinia pestis* F1 capsular antigen from Caf1 subunits.

In paper II, we performed mutagenesis of the binding motifs of the Caf1M chaperone and Caf1 subunit and analyzed the effect of the mutations on structure, stability, and kinetics of Caf1M-Caf1 and Caf1-Caf1 interactions. We show that a large hydrophobic effect combined with extensive main-chain hydrogen bonding enable Caf1M to rapidly capture/fold Caf1 subunit. The switch from the Caf1M-Caf1 contact to the Caf1-Caf1 contact occurs via the zip-out-zip-in donor strand exchange pathway with pocket 5 acting as the initiation site. Based on these findings, Caf1M with improved chaperone function was engineered. **In paper III**, we elucidated the mechanism of the usher-targeting step at atomic resolution. We show that a pair of conserved proline residues in free chaperone forms a 'proline lock', which blocks the usher binding. Binding of subunit to the chaperone opens the proline lock and allows the chaperone-subunit complex to bind to the usher. We show that this proline lock exists in other CU systems and represents a general allosteric mechanism for selective targeting of chaperone:subunit complexes to the usher and for release and recycling of free chaperone. **In paper I**, a novel middle domain of Caf1A usher (UMD) was isolated and its crystal structure was determined. We show that UMD and Caf1 fibre subunit displayed significant structural similarity. UMD did not bind Caf1M-Caf1 complexes, but its presence was shown to be essential for Caf1-fibre secretion. The study suggests that UMD may play the role of a subunit-substituting protein (dummy subunit), plugging or priming secretion through the channel in the Caf1A usher.

Keywords: assembly, chaperone/usher pathway, folding, secretion, allosteric regulation, protein structure, protein-protein interaction.

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"Learn from yesterday, live for today, hope for tomorrow."

Albert Einstein

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Yu, X.*, Visweswaran, G.R.*, Duck, Z.*, Marupakula, S., MacIntyre, S., Knight, S.D. & Zavialov, A.V. (2009). Caf1A usher possesses a Caf1 subunit-like domain that is crucial for Caf1 fibre secretion. *Biochem J* 418(3):541-51.

II Yu, X.*, Fooks, L.J.*, Moslehi-Mohebi, E., Tischenko, V.M., Askarieh, G., Knight, S.D., Macintyre, S. & Zavialov, A.V. (2012). Large Is Fast, Small Is Tight: Determinants of Speed and Affinity in Subunit Capture by a Periplasmic Chaperone. *J Mol Biol.* 471(4), 294-308.

III Yu, X.*, Dubnovitsky, A.P.*, Alex, F., MacIntyre, S., Knight, S.D. & Zavialov, A.V. Allosteric mechanism controls traffic in the chaperone/usher pathway (Submitted).

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Additional publications

Zavialov, A.V., Yu, X., Spillmann, D., Lauvau, G. & Zavialov, A.V. (2010). Structural basis for the growth factor activity of human adenosine deaminase ADA2. *J Biol Chem* 285(16), 12367-77

Saumendra, P.R.*, Mohammad, M.R.*, Yu, X.*, Tuittila, M.*, Knight, S.D. & Zavialov, A.V. Atomic resolution model for assembly,

architecture, and function of Enterotoxigenic Escherichia coli colonization factor CS6 (Submitted).

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Abbreviations

CU	Chaperone/usher
CD	Circular Dichroism
DASM	Differential adiabatic scanning microcalorimetry
DSC	Donor strand complementation
DSE	Donor strand exchange
E. coli	Escherichia coli
FGL	Long F_1G_1 loop
FGS	Short F_1G_1 loop
IM	Inner membrane
MS	Mass spectrometry
OM	Outer membrane
PDB	Protein Data Bank
PS	Periplasmic space
r.m.s	Root mean square
SC	Self complementate
SPR	Surface plasmon resonance
TM	Trans-membrane
UBS	Usher binding surface
UCD	Usher C-terminal domain
UMD	Usher middle domain
UND	Usher N-terminal domain

1 Background

1.1 General introduction

Bacteria, a biomass that exceeds that of all plants and animals, are present in most habitats on Earth. They can grow in soil, water and in extreme environments including acidic hot springs and even radioactive waste (Fredrickson et al., 2004) as well as in live bodies of plants and animals. We own a lot to the bacteria. Bacteria are vital in recycling nutrients, such as the fixation of nitrogen from the atmosphere and putrefaction. In industry, bacteria contribute to the production of cheese and yogurt. In human bodies, the vast majority of the bacteria are rendered harmless by the protective effects of the immune system and many bacteria are beneficial. However, a few species of bacteria are pathogenic and cause infectious diseases. Pathogenic bacteria produced devastating impact on human population. For example, in the Middle Ages, plague pandemics killed nearly one third of people in Europe (Drancourt & Raoult, 2002). Although the better sanitation, use of vaccines and antibiotics dramatically improved the situation, the bacterial infection diseases continue to represent a major health burden. For example, 2.2 million children lives are annually claimed by diarrheal diseases, which are mostly caused by enteropathogenic bacteria (WHO, 2000); about 2 million people a year are killed by tuberculosis (2002 WHO mortality data); 50% of the women in the world are affected by urinary tract infections, mostly caused by uropathogenic *E. coli* (UPEC), at some time in their life (Foxman, 2002; Ronald, 2002). Severe problems arise from emergence of antibiotic resistance in bacterial pathogens, growing numbers of vulnerable immunosuppressed patients, and the lack of new antibacterial agents to challenge the threat (Chopra et

al., 2008). The recent outbreak of Shiga-toxin-producing *E. coli* O104H4 infection in Germany illustrates the seriousness of this problem (RKI (2011) Report: Final presentation and evaluation of epidemiological findings in the EHEC O104:H4 outbreak. www.rki.de). The discovery of new drugs and vaccines against bacterial pathogens is essential to prevent future medical and social catastrophes. Achieving this goal obviously requires very detailed knowledge on the structure and function of major virulence factors and antigens of bacterial pathogens.

Most pathogenic bacteria express hair-like adhesive organelles known as adhesive pili or fimbriae on their outer surface. Adhesive pili are assembled from small protein subunits. In Gram-positive bacteria, adhesive pili are formed by covalent polymerization of subunits in a process that requires a dedicated sortase enzyme (Proft & Baker, 2009). In contrast, pili in Gram-negative bacteria are typically formed by non-covalent homo- or hetero- polymerization of subunits (Kline *et al.*, 2009; Waksman & Hultgren, 2009; Fronzes *et al.*, 2008; Zavialov *et al.*, 2007).

Based on their biosynthetic pathway, the pili of Gram-negative bacteria can be classified into five major classes: curli, type IV pili, Type IV secretion pili, Type III secretion needle and *chaperone/usher* (CU) pili (Fronzes et al., 2008). Of these five classes, the CU pili form the most abundant group of surface-exposed adhesive organelles and have been the most extensively studied. CU pili often constitute important virulence factors, responsible for specific host attachment and/or the evasion of host responses by triggering subversive signals that allow pathogens to evade immune defense and facilitate bacterial colonization or invasion (Wright *et al.*, 2007; Zavialov *et al.*, 2007). The classical CU pathway is used for assembly of these pili.

1.2 The chaperone/usher (CU) pathway

1.2.1 Introduction

The CU pathway has been extensively studied during the last two decades and a very detailed and beautiful molecular mechanism is emerging from these studies (Waksman & Hultgren, 2009; Zav'yalov *et al.*, 2009; Zavialov *et al.*, 2007). In this pathway (Fig. 1), subunits of pili (pilins), secreted into the periplasm, are captured by the periplasmic chaperone

and transported to the outer membrane usher, where they assemble into fiber and translocate to the cell surface. No external energy is required to drive these processes (Jacob-Dubuisson et al., 1994).

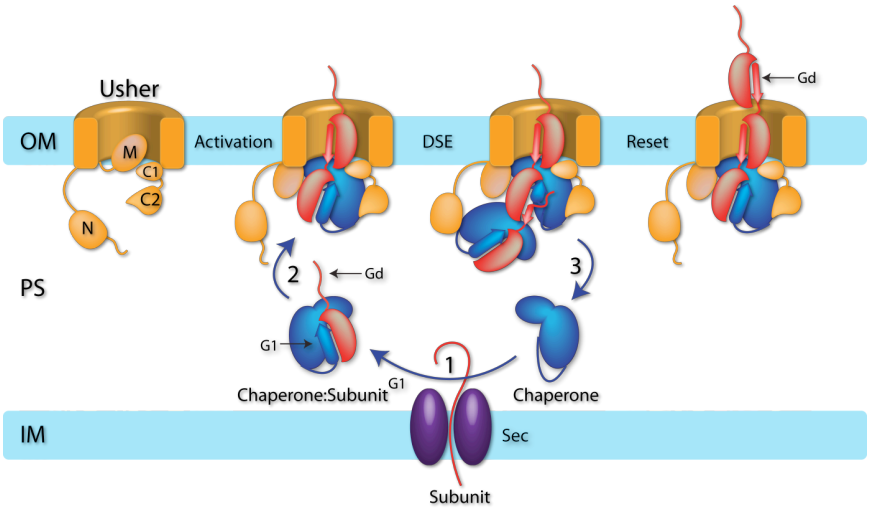

Figure 1. Schematic illustration of F1 fiber assembly by the chaperone/usher pathway. Major steps: (1) Chaperone capture/fold newly synthesis pilin subunit in the periplasmic space. (2) Chaperone:subunit complex binds to the activated outer membrane usher. (3) Fiber elongation involving multiple cycles of DSE where the chaperone G_1 strand is displaced by a subunit G_d strand to form each completely folded subunit module within the fiber and chaperone recycling. Chaperone, subunit, and usher are in blue, red, and orange, respectively. Donor strands are indicated by arrows. N, usher N-terminal domain; M, usher middle domain; C1 and C2, usher C-terminal domains.

1.2.2 Pilus subunits and structure of fibers

Newly synthesized pilus subunits are secreted into the periplasmic space through the inner membrane (IM) via the general secretary pathway SecYEG (Fig. 1). Each pilus subunit consists of incomplete immunoglobulin (Ig)-like domain (A-F strands) and an N-terminal extension. Since the domain lacks the last β-strand G in the canonical Ig fold (Zavialov *et al.*, 2003; Sauer *et al.*, 2002; Choudhury *et al.*, 1999; Sauer *et al.*, 1999), the pilus subunits are unstable in the periplasmic space and unable to self-assemble at the cell surface (Thanassi et al., 2002). In the periplasmic space, a periplasmic chaperone is required to capture/fold the pilus subunit by forming a chaperone:subunit complex, covering the exposed, due to the missing strand, hydrophobic core of

the subunit (Fig. 2). In the absence of functional chaperone, the pilus subunits form non-productive aggregation, which are targeted to the DegP protease for degradation (Vetsch *et al.*, 2004; Barnhart *et al.*, 2000; Jones *et al.*, 1997). At the cell surface, the hydrophobic core of the pilus subunit is covered by the N-terminal extension from the preceding subunit, which forms a *β*-strand (Zavialov *et al.*, 2003; Choudhury *et al.*, 1999). Since this *β*-strand plays structural role of the missing G strand, it was denoted G_d, G donor strand (Zavialov *et al.*, 2003) and this type of interaction was termed donor strand complementation (DSC) (Choudhury *et al.*, 1999; Sauer *et al.*, 1999).

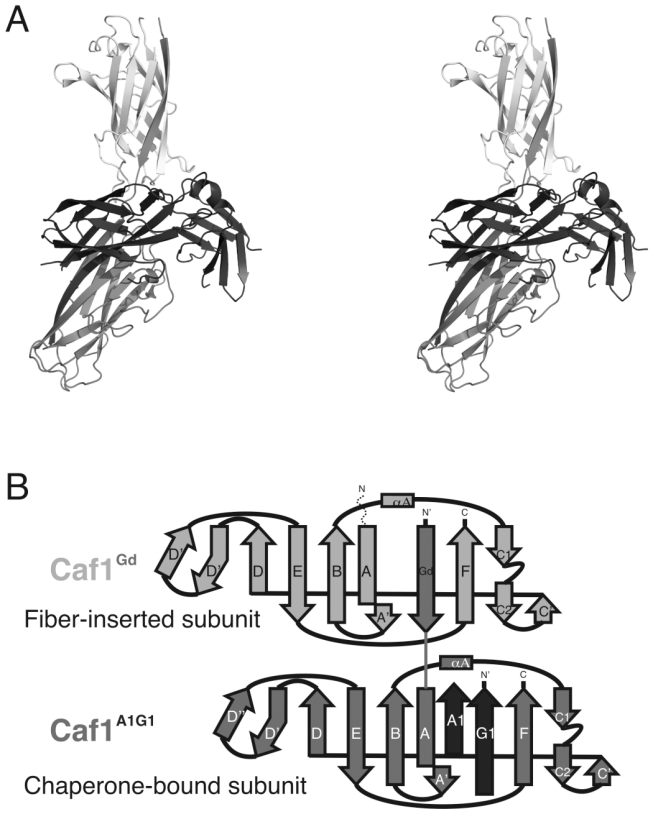

Figure 2. Caf1M:Caf1^{A1G1}:Caf1^{Gd} crystal structure. A. Stereo diagram of the Caf1M:Caf1^{A1G1}:Caf1^{Gd} complex. Caf1M is in dark gray. The chaperone bound Caf1^{A1G1} is in gray. The fiber inserted Caf1^{Gd} is in light gray. B. Caf1^{A1G1} (gray)-Caf1^{Gd} (light gray) topology diagram. The disordered G_d donor strand of Caf1^{Gd} is shown as a wavy, the ordered G_d donor strand of Caf1^{A1G1} and Caf1M A_1 and G_1 *β*-strands are shown as arrow (dark gray).

Some CU pili are simple in architecture, containing just one or two types of polymerizing pilus subunits (Fig. 3). Pili of this type are thin (~ 2-3 nm) and flexible (Keller et al, 2002). They frequently curl up into a capsule-like amorphous mass on the bacterial surface. For example, a large and dense capsule forms the F1 antigen (Chen & Elberg, 1977), which is made of polymers of Caf1 subunit (Zavialov *et al.*, 2002; MacIntyre *et al.*, 2001; Chapman *et al.*, 1999). Thick rod-like 'typical' pili form a different group. These pili are composed of multiple subunit types. For example, Type 1 pili, comprising 4 different subunit types (FimA, F, G, and H) forms a composite structure with a long helical rod connected to a short flexible distal tip. The rod, formed by a right-handed helical array of 500-3000 copies of the main structural pilus subunit FimA, is connected via one copy of FimF to the tip containing one copy of each FimG and FimH (Hahn *et al.*, 2002; Saulino *et al.*, 2000; Jones *et al.*, 1995). FimH consists of two immunoglobulin-like domains: the N-terminal lectin domain that is responsible for binding to mannosylated receptor, and the C-terminal pilin domain that forms interactions with either the FimC chaperone in the periplasm or the adjacent subunit FimG at the pili tip (Le Trong *et al.*, 2010a; Le Trong *et al.*, 2010b; Hung *et al.*, 2002; Choudhury *et al.*, 1999). In FimH subunit, the G_d strand is replaced by the lectin domain. Hence, FimH must be the first subunit recruited into the Type 1 pili in order to ensure display of the lectin domain at the pilus tip – as required for function.

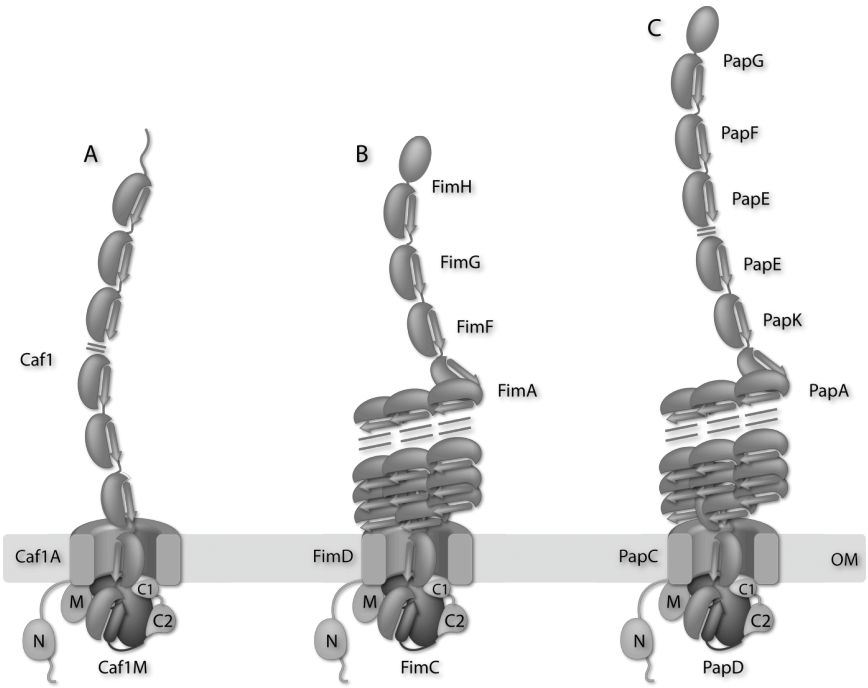

Figure 3. Schematic representation of the structure of FGL chaperone-assembled fimbrial polyadhesins (A, F1 fiber as example) and FGS chaperone-assembled thick rigid mono-adhesive pili (B, Type I pili and C, P pili as examples).

1.2.3 Chaperone

The first structure of periplasmic assembly chaperone (PapD) was solved in 1989 (Holmgren & Branden, 1989). Later on, several other periplasmic chaperone structures have been determined (Van Molle *et al.*, 2009; Remaut *et al.*, 2006; Knight *et al.*, 2002; Choudhury *et al.*, 1999; Pellecchia *et al.*, 1998). The structures revealed two immunoglobulin-like domains joined together at an approximate 90° angle, with a large cleft between these two domains. Two invariant basic residues (Arg8 and Lys112 in PapD) are located in this cleft and were reported to be crucial for the chaperone function (Kuehn et al., 1993).

The F_1 and G_1 β-strands from the N-terminal domain are connected by a long and flexible loop. Based on the length of F_1G_1 loop, the periplasmic CU family could be classified into two groups: FGS (having a short F_1G_1 loop) and FGL (having a long F_1G_1 loop) (Zav'yalov *et al.*, 2009; Hung *et al.*, 1996).

Two groups of periplasmic chaperones assemble two distinct molecular architectures of adhesive organelles (Fig. 3). The classical FGS chaperones assemble thick rigid rod-like pili consisting of multiple different types of pilin subunits, with one specialized adhesive subunit located at the distal end, like P and type I pili (Sauer *et al.*, 2004; Knight *et al.*, 2000; Sauer *et al.*, 2000; Soto & Hultgren, 1999; Thanassi *et al.*, 1998). In contrast, the FGL chaperones assemble simple and flexible structures, e.g. the F1 antigen (Zavialov et al., 2007).

The function of the periplasmic chaperone is to capture nascent subunits as they emerge in the periplasm, and transport them across the periplasm to the usher (Fig. 1). The crystal structures of chaperone-subunit complexes revealed the basis for the chaperone-subunit interactions at the atomic resolution (Fig. 2) (Zavialov *et al.*, 2003; Sauer *et al.*, 2002; Sauer *et al.*, 2000; Choudhury *et al.*, 1999; Sauer *et al.*, 1999). The subunit is anchored between the two chaperone domains. The chaperone inserts its G_1 strand to the hydrophobic groove in the subunit, completing the incomplete Ig-like fold of the subunit. This prevents nonproductive aggregation and proteolytic degradation of the subunit in the periplasm and ensures its safe transportation to the assembly site on the usher.

During fibers assembly, the subunit G_d strand replaces the G_1 strand by a mechanism termed 'donor strand exchange' (DSE) (Choudhury *et al.*, 1999). Crystal structures of Caf1M:Caf1:Caf1 chaperone:subunit:subunit ternary complex from the F1 antigen and the PapE subunit complexes with a donor peptide provided the first structural evidences for DSE (Zavialov *et al.*, 2003; Sauer *et al.*, 2002) (Fig. 2). During DSE, the subunit undergoes a conformational change, switching from the chaperone-bound high-energy folding intermediate to a compacted, highly stable fiber-inserted state (Zavialov *et al.*, 2005; Zavialov *et al.*, 2003). The folding energy released in this process drives the DSE and probably also the fiber translocation to the cell surface (Yu *et al.*, 2012; Yu *et al.*, 2009; Zavialov *et al.*, 2003; Jacob-Dubuisson *et al.*, 1994). Multiple cycles of DSE results in formation of long fibers consisting of hundreds of globular modules, each linked by the G_d strand from the following subunit.

Two models for the process of DSE were proposed (Zavialov et al., 2003): in one model, chaperone:subunit complex was completely dissociated before the insertion of the subunit G_d strand; in the second model, the chaperone G_1 strand is replaced step by step by the subunit G_d strand via a mechanism termed zip-out-zip-in. Later, the zip-out-zip-in mechanism has been proved by using real-time electrospray ionization MS (Remaut *et al.*, 2006) and the P5 pocket was proposed to serve as the initiation site starting the DSE (Yu *et al.*, 2012; Remaut *et al.*, 2006).

DSE can take place *in vitro* in mixtures of chaperone:subunit complexes, but it proceeds only at very low speed (Rose *et al.*, 2008; Zavialov *et al.*, 2005). In contrast, *in vivo* or *in vitro* in presence of the usher DSE occurs very quickly (Nishiyama *et al.*, 2008; Remaut *et al.*, 2006; Vetsch *et al.*, 2006; Vetsch *et al.*, 2004).

1.2.4 Usher

Ushers are located at the outer membrane of Gram-negative bacteria. These are ~ 90kDa proteins consisting of five distinct functional domains: a N-terminal periplasmic domain (UND) (Henderson *et al.*, 2011; Eidam *et al.*, 2008; Nishiyama *et al.*, 2005; Nishiyama *et al.*, 2003), a trans-membrane domain (TMD) (Remaut et al., 2008), a middle domain (UMD) (Huang *et al.*, 2009; Yu *et al.*, 2009; Remaut *et al.*, 2008), and two C-terminal periplasmic domains (UCD1 and UCD2) (Phan *et al.*, 2011; Dubnovitsky *et al.*, 2010; Ford *et al.*, 2010) (Fig. 1).

The UND specifically binds chaperone:subunit complexes with high affinities (Nishiyama *et al.*, 2005; Ng *et al.*, 2004). The crystal structures of the UND of the FimD usher bound to the FimC:FimH chaperone:adhesin complex (Nishiyama *et al.*, 2005) and to the FimC:FimF chaperone:subunit complex (Eidam *et al.*, 2008) revealed the structural basis for these interactions. The binding is primarily mediated by the N-terminus sequence (residues 1-24) of the UND, which is completely unstructured in free UND. This sequence interacts primarily with the chaperone in the chaperone:subunit complexes. Deletion the sequence abolished the usher interaction with chaperone:subunit complex, creating nonfunctional usher (Eidam *et al.*, 2008; Nishiyama *et al.*, 2005). The UND is thought to represent the initial binding site for chaperone:subunit complexes in the usher (Ng *et al.*, 2004).

The TMD, consisting of 24 trans-membrane (TM) β-strands forming a β-barrel, is located at the outer membrane and it is the place where the fibers are assembled and trans-located from the periplasm to the surface of the bacteria. Crystal structures of this domain were determined in Pap and Fim FGS CU systems (Huang *et al.*, 2009; Remaut *et al.*, 2008). The structure is kidney-shaped with outer and inner diameters of 65Å×45Å and 45Å×25Å, respectively, which is big enough to allow the passage of growing fibers. Between the β6 and β7 strands, the polypeptide chain folds into a six-stranded β sandwich domain, sitting inside the β-barrel pore, UMD. The existence of this domain was predicted by the group of G. Capitani (Capitani *et al.*, 2006). UMD completely occludes the β-barrel, preventing the passage of periplasmic molecules. The UMD resembles pilin subunit (Yu *et al.*, 2009) and may work as a dummy subunit, serving as a plug (Huang *et al.*, 2009; Yu *et al.*, 2009; Remaut *et al.*, 2008). Hence, this form of the usher is referred to as a non-activated or apo form (Fig. 1). The plug domain is required for the fiber assembly, since the usher with a deletion of this domain is unable to assemble fibre *in vitro* or *in vivo* (Huang et al., 2009; Yu et al., 2009). Recent crystal structure of the FimD usher bound to its cognate FimC:FimH chaperone:subunit substrate (Phan *et al.*, 2011) revealed the active form of FimD usher. This structure shows that the UMD is positioned in the periplasm, underneath the TMD. Instead, the FimH lectin domain occupies the TMD channel.

The UCDs are thought to form the second binding site for the chaperone:subunit complex (Huang *et al.*, 2009; So & Thanassi, 2006). The exact role of the UCDs in fiber assembly is unclear. However the importance of UCDs in this process was reported for both FGL and FGS CU systems (Dubnovitsky *et al.*, 2010; Thanassi *et al.*, 2002; Harms *et al.*, 1999). The structure of FimD:FimC:FimH complex shows that UCD1 forms extensive contacts with both the chaperone and the subunit, while UCD2 interacts only with the chaperone (Phan *et al.*, 2011).

How these usher domains cooperate to facilitate the DSE during fibre assembly? Superposition using the structures of $FimD_N$:FimC:FimF and FimD:FimC:FimH revealed that the usher can accommodate two chaperone:subunit complexes, one bound to the N-terminal domain and the other to the C-terminal domains, at the same time without steric

clashes. In this model, the G_d strand of subunit FimF (representing the incoming subunit) lies above the hydrophobic cleft of the pilin domain of FimH (representing the subunit at the base of the growing fiber) and is perfectly positioned to initiate the DSE reaction. Hence, it is likely that the usher works as a steric catalyst of DSE. The following functional cycle was proposed for the usher (Fig. 1). After activating the usher, the chaperone:subunit complex is bound to the usher's UCDs, with the UND lying idle; new chaperone:subunit complex is recruited to the UND and brought close to the penultimate subunit located at the UCDs, positioning them for DSE; upon DSE, the chaperone is displaced from the penultimate subunit and released from the UCDs; in order to reset the assembly platform for a new incorporation cycle, usher has to hand-over the chaperone:subunit complex from the UND to UCDs; during this step the penultimate subunit translocates into the TMD channel.

1.3 Plague and F1 antigen

Yersinia pestis is the etiologic agent of pneumonic, septicemic, and notorious bubonic plagues, causing a number of high-mortality epidemics throughout human history. *Yersinia pestis* has been classified by the CDC (Centers for Disease Control and Prevention) as a category A pathogen and is requiring preparation for a possible terrorist attack.

The plague is transmitted to people from rodents via fleas. It has been estimated that 11,000 to 24,000 non-encapsulated *Yersinia pestis* bacterial can be injected into the blood by a single flea (Perry & Fetherston, 1997). During the early stages of infections, the Type III secretion system (T3SS) protects *Yersinia pestis* from phagocytosis (Viboud & Bliska, 2005; Cornelis & Wolf-Watz, 1997). After several hours of incubation inside of the host, *Yersinia pestis* expresses a large capsule-like structure composed of aggregating F1 antigen (Chen & Elberg, 1977) that helps *Yersinia pestis* to resist uptake by new phagocytes (Du *et al.*, 2002). The bacteria grow to high numbers and cause the formation of a bubo (bubonic plague) at lymph nodes. Later, the infection spread into the blood stream where bacteria are removed in the liver and spleen. Continual growth of the bacteria in the liver and spleen will cause septicemic plague. Occasionally, the infection proceeds to the respiratory system, causing the pneumonic plague. Pneumonic plague can be transmitted from human to human without involvement

of fleas or animals (Cleri *et al.*, 1997; Perry & Fetherston, 1997). At this stage, the fragments of F1 antigen can provoke unproductive proinflammatroy response, contributing to a toxic shock and death of the host. Without antibiotic treatment pneumonic plague is an invariably fatal disease. The antibiotic treatment is effective only during the first 24 h following infection, because the disease develops rapidly (Titball & Williamson, 2001).

Both live attenuated and killed whole cells vaccines have been used in human. However, the safety of live attenuated vaccines in human is questionable. The killed cells vaccines do not show effective protection against pneumonic plague, and side effects, such as malaise, headaches elevated temperature and lymphadenopathy occur (Jefferson *et al.*, 2000). Vaccines consisting of purified antigens have been shown to provide effective protection against bubonic and pneumonic plague in animal models. Especially recombinant vaccines composed of a fusion protein of the F1 antigen and V antigens or mixtures of the surface purified F1 antigen and recombinant V antigen protected monkey against pneumonic as well as bubonic plague (Titball & Williamson, 2001; Heath *et al.*, 1998).

F1 antigen consists of many thousand copies of identical Caf1 subunits linked into a flexible fiber (Soto & Hultgren, 1999; Hung *et al.*, 1996) with nonpilus, amorphous or capsule-like morphology (Chen & Elberg, 1977). The capsule is assembled by the FGL chaperone:usher pair Caf1M:Caf1A, and the expression is regulated by *Caf1R* (Zav'yalov *et al.*, 2009; Zavialov *et al.*, 2007; Zavialov *et al.*, 2002). Electro micrographs of *Yersinia pestis* demonstrates that F1 antigen is maximally expressed on the surface of the bacterial at 37 °C after 72h of cultivation *in vitro* (Chen & Elberg, 1977). There is no expression of F1 antigen below 22°C (Chen & Elberg, 1977). The short nonaggregated F1 fragments can specifically bind to human IL-1β, a cytokine that is an important mediator of the inflammatory response (Zav'yalov *et al.*, 1995). Thereby, the pathway which IL-1β stimulate macrophages to produce various proinflammatory mediators could be suppressed by its binding to the fragments of the F1 antigen (Kida *et al.*, 2005). In addition, like other capsules or capsule-like antigens, F1 antigen is involved in the antiphagocytic effect by reducing the number of bacteria that interacts with the macrophages (Du *et al.*, 2002).

1.4 Aim and outline of this thesis

The aim of this thesis was to elucidate the mechanisms of the three consecutive steps of the CU pathway: subunit capture by the chaperone, usher targeting by the chaperone:subunit complex, and subunit transport through the usher. The specific objectives of the study were

- To elucidate properties of the chaperone that enable the chaperone function;
- To understand the basis for discrimination between free chaperone and pre-assembly complex by the usher;
- To unveil the structure the middle domain (UMD) of the usher and the role of this domain in fiber assembly and secretion though the usher.

The results and discussions of the study are presented in chapter 2, which is subdivided into three subchapters, each devoted to one of the three specific objectives of the study.

2 Results and discussions

2.1 Determinants of Speed and Affinity in Subunit Capture by a Periplasmic Chaperone

2.1.1 Introduction

Caf1 fibers are highly stable structures. Yet, these fibers are not able to assemble in the absence of the Caf1M chaperone. Previous studies suggested that subunit self-assembly is a very slow process and the chaperone helps to prevent detrimental aggregation of non-complemented subunits by quickly forming chaperone:subunit complexes. The chaperone-subunit and subunit-subunit interactions are both based on donor strand complementation. So why is Caf1M able to bind the non-complemented subunit more efficiently than the G_d strand of a fellow Caf1 subunit? What properties help it in this process and why isn't the G_d donor strand of Caf1 able to promote subunit self-assembly?

The binding motifs of Caf1M and Caf1 are remarkably different (Fig. 4). The G_1 donor strand of Caf1M is significantly more hydrophobic than the G_d strand of Caf1. In addition, Caf1M uses the second strand A_1 to bind the subunit, which is missing in the Caf1-Caf1 contact. Hence, to answer to the questions above, we performed convergence mutagenesis of the binding motifs of the chaperone and subunit donor strands and analyzed the effect of the mutations on stability, structure, and kinetics of subunit-subunit and chaperone-subunit interactions.

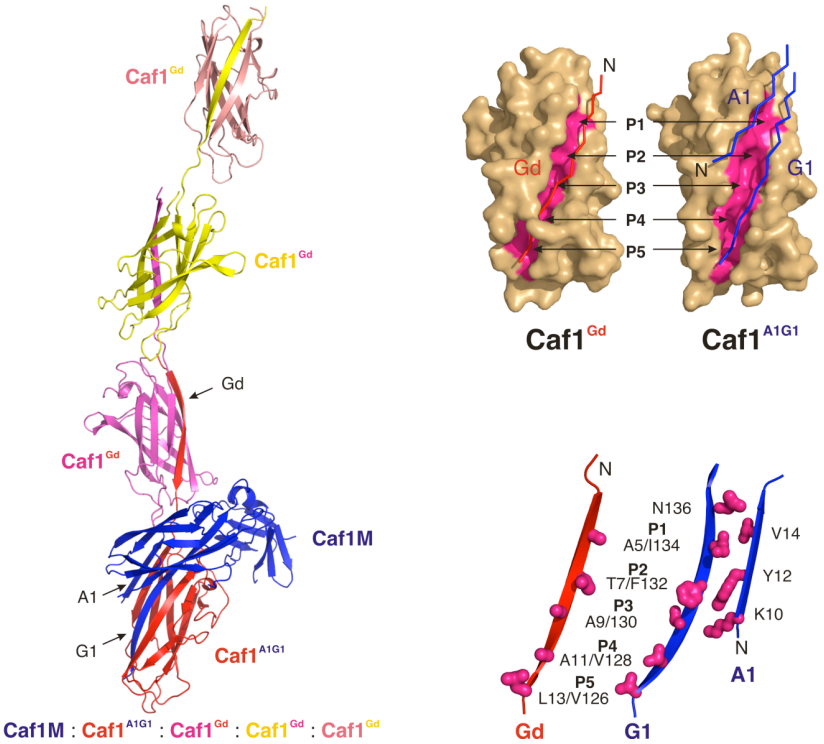

Figure 4. Chaperone:subunit and subunit:subunit binding sites. A. Chaperone bound Caf1^{A1G1} subunit and fiber inserted Caf1^{Gd} subunits (Cartoon diagrams). B. Comparison of the acceptor cleft in Caf1^{Gd} (left) and Caf1^{A1G1} (right) subunits. The molecular surface of the subunit is in beige except for the hydrophobic surface of the cleft, which is in violet. Positions of subunit G_d and chaperone A_1 and G_1 donor strands in the groove are indicated with ribbons. C. Comparison of the chaperone and subunit binding motifs (ribbon diagrams). Donor residues from G_d and G_1 strands and residues forming contacts between A_1 and G_1 strands are shown. Caf1M is in blue; Caf1^{A1G1} is in red; Caf1^{Gd} are in magenta, yellow and pink.

2.1.2 Large G_d donor residues destabilize Caf1 fiber

We first tested the influence of G_d-to-G_1 donor residue substitutions on the assembly and thermostability of the Caf1 fibers. Caf1 G_d donor strand residues at positions P1-4 were replaced individually or in combination with the large hydrophobic residues. The amount and length of assembled fibers at different temperatures were studied using varies gel systems. Multiple substitutions greatly decreased polymerization. Single mutations apparently did not effect polymerization, but decreased the thermostability of the Caf1 fibers.

To quantify the effect of mutations on the thermodynamic stability of Caf1 fibers, we used self-complementated (SC) construct of Caf1, Caf1sc (Zavialov A.V. et al., 2005). In this construct, the G_d strand, was genetically moved to the C-terminus of the subunit, enabling self-complementation of Caf1sc and formation of a monomeric fiber module with a classical immunoglobulin fold. Caf1sc provides a simple *in vitro* model to estimate thermodynamic properties of F1 fibers. The same single or multiple mutations were introduced into the G_d strand of Caf1sc and analyzed using differential adiabatic scanning microcalorimetry (DASM). The experiments revealed a stepwise drop in free energy of unfolding with each additional small-to-large donor residue substitution.

To understand why substitutions of donor residues in the G_d strand to larger hydrophobic amino acids destabilized Caf1 fibers, we determined the crystal structures of the Caf1M:Caf1^{A1G1}:Caf1^{Gd} chaperone:subunit:subunit complexes, carrying selected mutations. Structures were solved by molecular replacement with the wild-type ternary complex (PDB accession code 1Z9S) as a search model. Compared to the wild-type Caf1^{Gd}, the mutant Caf1^{Gd} revealed a less tightly packed β sandwich and a more exposed cavity, suggesting that larger hydrophobic residues, such as those present in the G_1 strand of the chaperone, arrest folding of the subunit.

2.1.3 Functional chaperone requires large hydrophobic G_1 strand donor residues

Why do the chaperones posses such relatively large hydrophobic donor residues? To address this question, we investigated the importance of hydrophobicity of the Caf1M chaperone donor residues for the initial capture of Caf1 subunit. We used real time surface plasmon resonance (SPR) measurements to study the kinetics between the Caf1M and the Caf1. The association (k_{on}), dissociation (k_{off}) rate constants and the equilibrium constant (Kd) of binding were determined. We found that unlike subunit-subunit interactions having dissociation halftimes in the order of billions of years (Piatek *et al.*, 2009; Puorger *et al.*, 2008), Caf1M-Caf1 binding is highly dynamic and not particularly tight.

Mutations decreasing hydrophobicity on the G_1 strand dramatically decreased the k_{on} value. Conversely, mutations increasing the hydrophobic surface increased the k_{on} value. The strong correlation

between the association rate and hydrophobicity of Caf1M G_1 strand, demonstrates that the hydrophobic effect plays a paramount role in subunit binding. To understand why this is important, we monitored the secondary structure changes during Caf1M-Caf1 association using circular dichroism (CD) in a temperature shift experiment. The result suggested that Caf1M binds largely unstructured subunit, and then assists in its folding. It is likely that the high hydrophobicity of the binding motif of the chaperone is required to capture the unfolded intermediate of the subunit. The G_d strand of the fellow subunit is significantly less hydrophobic and hence cannot perform this function (Fig. 5).

2.1.4 The A_1 strand of Caf1M is crucial for chaperone-subunit association

The structure of Caf1M:$Caf1^{A1G1}$:$Caf1^{Gd}$ complex shows that the A_1 strand adds significant interaction area to the acceptor pockets 1-3 of $Caf1^{A1G1}$ subunit, and forms 11 main chain hydrogen bonds with the A strand of $Caf1^{A1G1}$ (Fig. 2 and 4). These interactions are absent in the Caf1-Caf1 contact in fibers, where only Caf1 G_d strand is used for covering the hydrophobic cleft of Caf1 subunit. To study the importance of the Caf1M A_1 strand, we created deletions in the A_1 strand, which removed different amounts of hydrogen bonds. The Caf1M mutants revealed significant defects in the ability to assist in the Caf1 assembly. The SPR study demonstrated that the deletions dramatically decreased the Caf1M-Caf1 association rate. Hence, the A_1 strand in the chaperone plays an important role in the subunit capture (Fig. 5). The chaperone double stranded structure probably acts as a preformed platform for rapid capture and folding of secreted Caf1. The fast binding of the unfolded subunit probably relays on both fast zipping of edge strands via hydrogen bonding and stabilization of the folding nucleus by donation of large hydrophobic donor residues.

2.1.5 The DSE initiates at the P5 site

DSE proceeds via several steps of partial displacement of the chaperone G_1 and A_1 strands by the subunit G_d strand (Remaut *et al.*, 2006) in a 'zip-out-zip-in' mechanism (Zavialov *et al.*, 2003). In the Caf1 CU system pocket 5 is occupied by the G_1 strand residue Val126 and later displaced by the slightly larger G_d strand residue Leu13 (Fig. 4). Our studies showed that substitutions decreasing the hydrophobicity at the P5 residue (Leu13Val, Leu13Ala) in the G_d strand greatly inhibited fimbrial assembly, which can be explained by a reduced potential of the subunit. However, mutation that increases the hydrophobicity of the P5

residue (Val126Ile) in the G_1 strand also efficiently inhibited fimbrial assembly. This could be due to an increased protection of the chaperone. Although the P5 mutations change the stability of polymer or Caf1M:Caf1 complex, it does not significantly affect the overall stability compared mutations in the other sites (P1-4). It is likely that these mutations cause such a dramatic effect on DSE not simply by changing the thermodynamic processes, but rather by increasing the kinetic barrier. Here, our results for the Caf1 CU system shows that pocket 5 also serves as the initiation site for DSE (Fig. 5).

2.1.6 Engineering chaperones with enhanced chaperone function

While elucidating the mechanism of chaperoning by Caf1M, we created Caf1M constructs that were able to bind and fold Caf1 significantly faster than the wild-type protein. E.g. the Val130IleVal128Ile mutant had a k_{on} value that is 2.3 times higher than that of wild-type Caf1M. We also demonstrated that it is possible to modulate the rate of the final step of fiber assembly by introducing changes in the chaperone P5 site, the initiation site for DSE. Hence, our study provides a basis for the rational design of assembly chaperones with improved chaperone function.

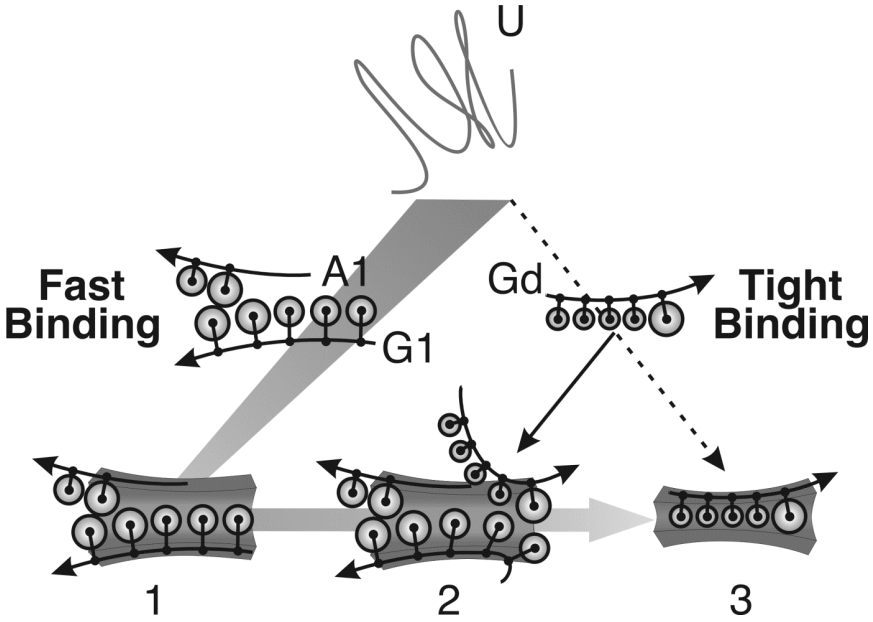

Figure 5. Mechanism of Chaperone-catalyzed biogenesis of fiber polymerization. Newly synthesized Caf1 subunit (U) is largely unstructured. There is little of the folded

precursor into which the subunit G_d strand would perfectly fit, making the self-assembly an unlikely event. Large hydrophobic binding motifs of Caf1M on a 2-strand platform (A_1 and G_1 strands) shift donor strand affinity to the predominant semi-folded conformation of subunit (U), enhancing probability of binding. The ensuing chaperone:subunit complex (1) represents a high-energy transition intermediate due to intercalation of the bulky donor residues of the chaperone between the two β sheets of the subunit with maximal opening of the cleft retained by the 'folding platform'. Chaperone G_1 and A_1 strands are displaced by a progressive zip-out-zip-in mechanism initiated at pocket 5 with replacement of Val126 for the G_d strand Leu13 of a second subunit (2), to produce the fully compacted, highly stable fiber module (3). Donor residues are shown as spheres with radii reflecting the size. Continuous gray arrow, pathway of fiber assembly catalyzed by chaperone; dashed arrow, pathway for direct insertion of G_d strand.

2.2 Chaperone assisted transport is allosterically regulated

2.2.1 Introduction

The chaperone transports organelle subunits across the periplasm to the outer membrane usher, where they are released and incorporated into growing fibers (Fig. 1). At each functional cycle, the usher binds one molecule of subunit-loaded chaperone and release one molecule of free chaperone. To drive assembly in the right direction, the usher must distinguish between subunit-loaded and free chaperone. Previously, it was suggested that the usher could do this by detecting subunit in the chaperone:subunit complex (Nishiyama *et al.*, 2005). However, structures of chaperone:subunit complexes bound the usher N-terminal domain revealed only very small interfaces between usher and subunit, seriously weakening this hypothesis (Eidam *et al.*, 2008; Nishiyama *et al.*, 2005). In paper II, we investigated the usher-targeting step in assembly of the *Yersinia pestis* F1 capsule and discovered an allosteric mechanism that determines selective targeting of chaperone:subunit complexes to the usher as well as the release and recycling of free chaperone.

2.2.2 Caf1A discriminates between free and Caf1 loaded chaperone without recognizing Caf1

The N-terminal domain of Caf1A ($Caf1A_N$) was identified based on the sequence homology between the Caf1A and FimD ushers, as well as functional studies of Caf1A. Isolated $Caf1A_N$ was expressed and crystallized. $Caf1A_N$ was also co-crystallized with the Caf1M:Caf1 preassembly complex. Crystal structures of free $Caf1A_N$ and

Caf1A_N:Caf1M:Caf1 complex were determined to 2.0 and 1.8 Å resolution, respectively. The core structure of Caf1A_N consists of a small five-stranded β barrel sealed at the top and bottom with connective loops and helices α1 and α2 (Fig. 6).

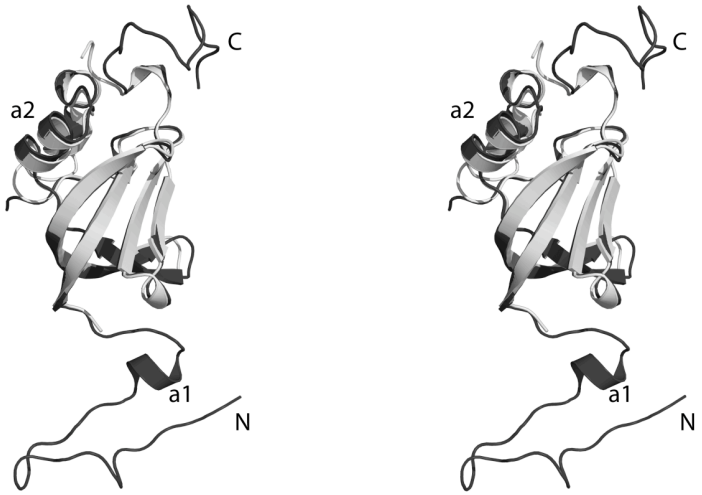

Figure 6. Stereo ribbon diagram of free Caf1A_N (light gray) superimposed with Caf1A_N from the Caf1A_N:Caf1M:Caf1 complex (dark gray).

A 22 amino acid N-terminal sequence of Caf1A_N is unstructured in free Caf1A_N. In the Caf1A_N:Caf1M:Caf1 complex, this sequence interacts with seven closely situated residues in Caf1M (Pro41, Leu43, Leu67, Ile102, Pro103, Pro104, and Arg69), which form a hydrophobic usher-binding surface (UBS) (Fig. 7). The residues involved in the binding are highly conserved, in particularly, Pro103 (invariant) and Pro104 in Caf1M and Phe4 in Caf1A. Substitutions of these residues to Ala dramatically affected the binding and capsule assembly. To our surprise, the interactions between Caf1A_N and the Caf1 subunit were almost negligible (less than 3% of the total binding area) and decreasing this contact area by half using mutagenesis did not affect the capsule formation. This finding was puzzling, because our binding experiments revealed high affinity of Caf1A_N to Caf1M:Caf1 interaction (Kd=2.42±0.24 μM) and no affinity to Caf1M. Moreover, the binding of Caf1A_N to Caf1M:Caf1 was not inhibited by subunit-free Caf1M even at 500-fold. How dose Caf1A_N distinguish free and Caf1-bound Caf1M without recognizing Caf1?

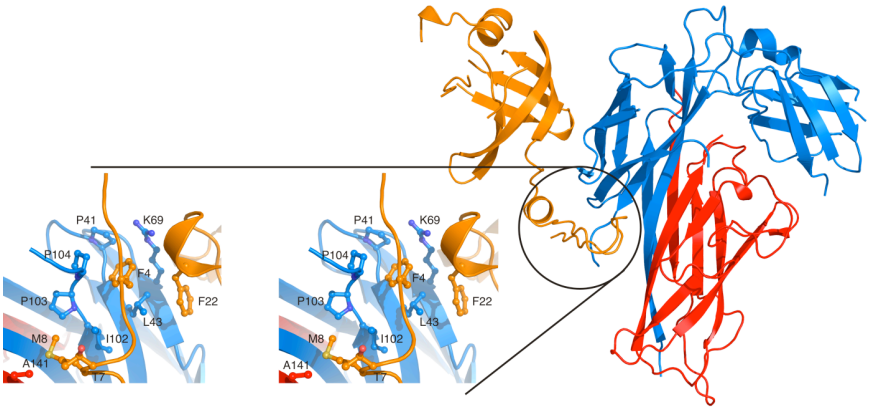

Figure 7. Crystal structure of Caf1A$_N$:Caf1M:Caf1 complex and the binding interface between Caf1A$_N$ and Caf1M:Caf1 (stereo view). Major residues participate the binding are labeled and shown as sticks and spheres. Caf1A$_N$, Caf1M and Caf1 are colored in orange, blue and red, respectively.

2.2.3 Caf1M binding to Caf1A is allosterically controlled by Caf1

To exclude a possible effect of the Caf1M tetramerization on Caf1A$_N$-Caf1M association, we examined binding of Caf1A$_N$ to Caf1M mutants incapable of tetramerization. None of these mutants showed specific binding. Hence, we hypothesized that free Caf1M takes on a conformation that prevents it from efficiently binding to Caf1A$_N$. To investigate this hypothesis, we determined a 1.8Å crystal structure of Caf1M-Δ2-12, Δ113-129 mutant, which represents a good model for the monomeric subunit-free Caf1M (Fig. 8).

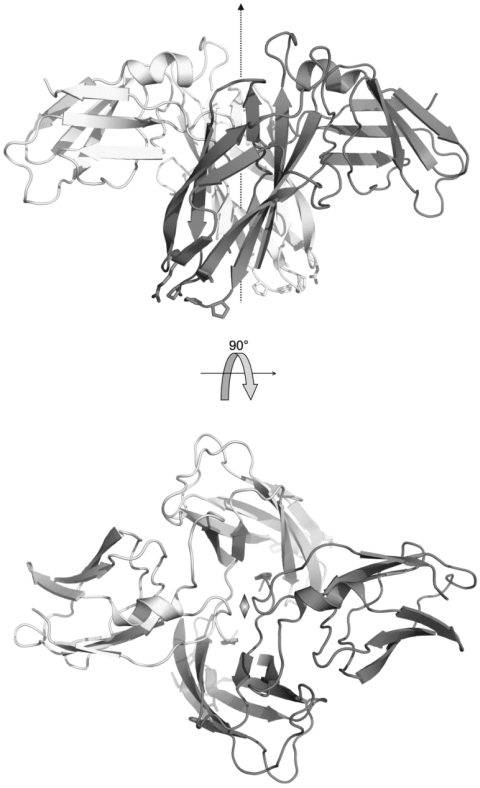

Figure 8. Crystal structure of monomeric Caf1-free Caf1M in the asymmetric unit. Two monomers in the dimer are shown in different colors. The UBS residues are shown with sticks.

Structural comparison of free and Caf1-bound Caf1M revealed significant conformational differences (Fig. 9). In the Caf1-bound Caf1M the G_1 donor strand is extended and firmly docked into the subunit acceptor cleft. In this conformation, it forms additional hydrogen bonds to the end of the F_1 strand, causing the F_1 strand to twist (Fig. 11C). This effects positions of several residues at the end of the strand and, most importantly, P103 and P104 at the beginning of the F_1G_1 loop. In free Caf1M, Pro104 occupies a hydrophobic pocket between the side chains of Pro41, Leu43, Ile102 and the aliphatic part of the side chain of Arg69, which all belong to the UBS. Hence, in the free Caf1M, the UBS is in a closed or collapsed conformation that prevents binding to the usher (Fig. 9). In contrast, in Caf1-bound Caf1M, Pro103 and P104 are rotated to move Pro104 out of the hydrophobic pocket (Fig. 9).

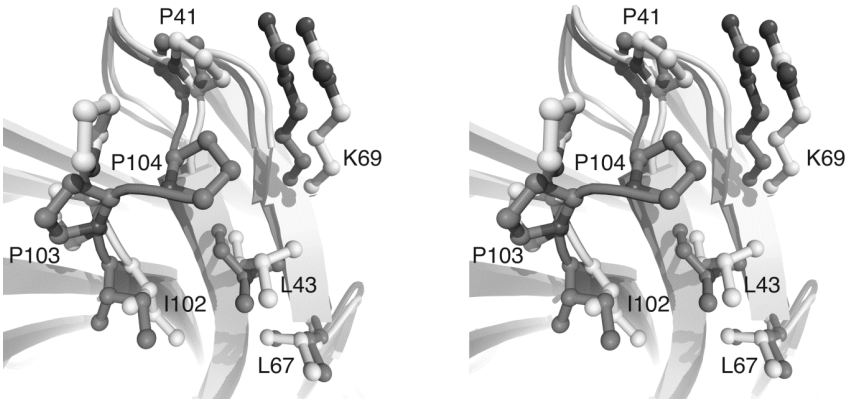

Figure 9. Superposition of the Caf1A binding site in Caf1-free (dark gray) and Caf1-bound (light gray) Caf1M (stereo view). The UBS residues are labeled and shown as sticks and spheres.

Structural comparison of free Caf1M and $Caf1A_N$:Caf1M:Caf1 revealed that the closed UBS conformation cannot form optimal interactions with the binding residues of $Caf1A_N$. Most strikingly, Phe4 of Caf1A, which is essential for the binding, cannot insert into its local binding pocket, because in the closed UBS conformation this pocket is occupied by Pro104 of Caf1M (Fig. 7 and 9).

Hence, our structural study suggested an allosteric mechanism in which Pro103 and Pro104 act in concert as a 'structural lock' that prevents or allows the usher binding depending on whether subunit is bound or not (Fig. 10). In free Caf1M, the lock keeps the UBS closed, preventing Caf1M from binding to Caf1A. Caf1 binding unlocks the proline lock and opens the UBS, allowing the Caf1M:Caf1 complex to target and bind to Caf1A (Fig. 10).

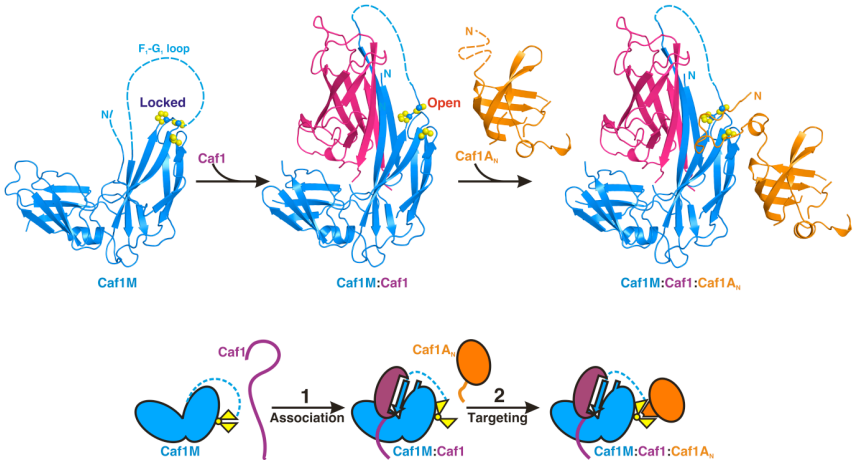

Figure 10. Caf1-induced changes in Caf1M enable Caf1M binding to Caf1A. Upper panel, crystal structures of the Caf1M monomer, Caf1M:Caf1, Caf1A$_N$, and Caf1A$_N$:Caf1M:Caf1 complex (cartoon diagrams). Side chains of the UBS residues are shown as spheres. The disordered part of the N terminus and the F_1G_1 loop is indicated with dashed lines. Lower panel: Schematic representation of structures shown in upper panel. The Caf1, Caf1M, and Caf1A$_N$ are shown in magenta, blue, and orange, respectively.

2.2.4 Communication between the Caf1 and Caf1A binding sites in Caf1M

How does Caf1 binding switch the UBS conformation from closed to open? Caf1 binding changes the local environment of the proline lock (Fig. 11).

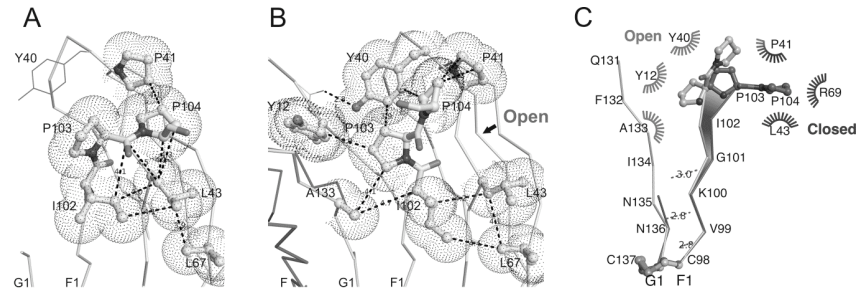

Figure 11. Caf1 binding opens the proline lock by generating an alternative hydrophobic pocket and F_1 strand twisting. A. The binding sites for proline lock in Caf1-free Caf1M. B. The binding sites for proline lock in Caf1-bound Caf1M. C. F1 strand twisting due to the binding of Caf1. The closed conformation is shown in dark gray. The open conformation is shown in light gray. Residues involved in the binding and UBS are labeled and shown as balls and sticks. Atomic raddi are indicated with mesh. Distances between interacting atoms are indicated in Å. The main chains are shown as ribbon.

In the Caf1M:Caf1 complex, Tyr12 and Ala133 from A_1 and G_1 strands respectively form van der Waals interactions with Pro103. In addition, the side chain of Tyr40 which in Caf1M:Caf1 is linked to the main chain of the A_1 strand via a hydroxyl-amide hydrogen bond, forms extensive interactions with both Pro103 and Pro104. These interactions are absent in the free Caf1M, because the concerned Caf1-binding segments are unstructured. To examine whether these interactions are sufficient to maintain an open UBS, we replaced Tyr12 by Gly and Tyr40 by Ala residues. The mutations led to a dramatic effect: both Caf1A_N-(Caf1M:Caf1) binding and assembly were practically abolished. Moreover, the structure of the Tyr40Ala mutation revealed closed UBS. These results suggest that the switch of UBS conformation from opened to closed is facilitated by the formation of the pocket Tyr12-Tyr40-Ala133 after Caf1 binding. The twist in the F_1 strand may also facilitate opening the UBS as it contributes to rotation of the proline lock residues (Fig. 11). MD simulations of Caf1M provided additional support to these conclusions.

2.2.5 The proline lock exists in other CU systems

To investigate if a similar allosteric proline lock mechanism exists in other CU systems, we examined available structures of subunit-free and subunit-bound chaperones in other CU systems, including those from the FGS CU pathway. Superposition of available subunit-bound chaperone structures, demonstrated that regardless of chaperone type they all have an open UBS. Superposition of available subunit-free chaperone structures (excluding those in self-capping form) revealed a unique closed UBS. Hence, the proline lock exists in other CU systems and represents a general allosteric mechanism for selective targeting of chaperone:subunit complexes to the usher and for release and recycling of free chaperone (Fig. 12).

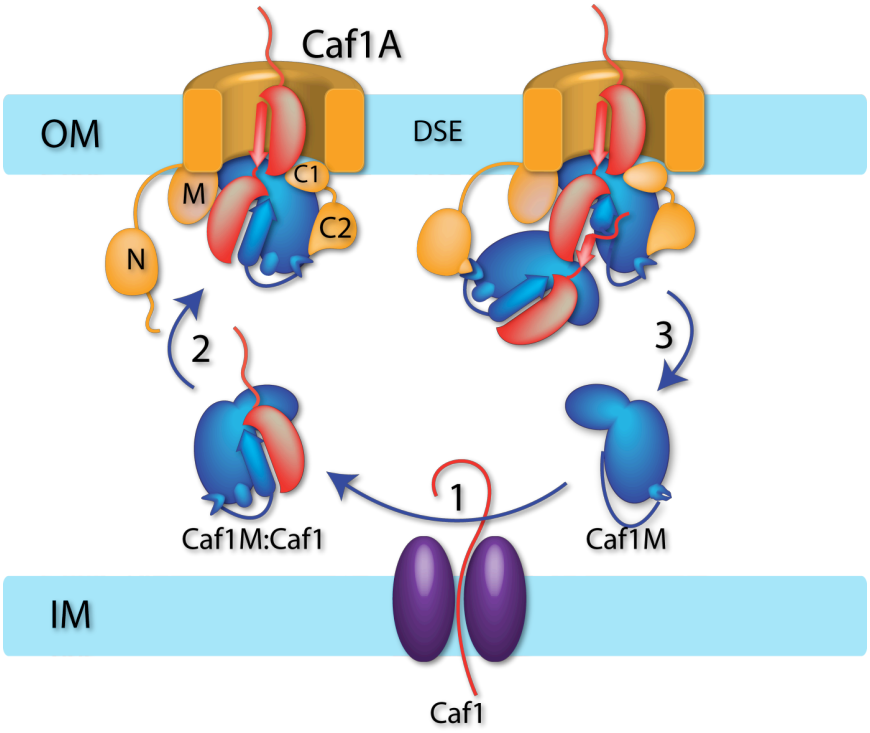

Figure 12. The chaperone-subunit association establishes the subunit trafficking. Step 1, Caf1M with a collapsed UBS captures/binds a newly synthesis Caf1 subunit. Caf1 binding opens the UBS in Caf1M. Step 2, the Caf1M:Caf1 complex with the open UBS binds to $Caf1A_N$ with high affinity and the DSE starts. Step 3, Caf1M dissociates from the usher C-terminal domains after the DSE, the incoming Caf1 is incorporated into the base of the fiber, and the fiber translocates to the cell surface.

2.3 Usher middle domain (UMD), a dummy fibre subunit

2.3.1 Introduction

The final steps of fibre assembly and translocation to the cell surface are coordinated by the outer membrane usher protein. Sequence analysis of the Caf1A usher with the HMM-B2TMR program (Martelli *et al.*, 2002) suggested that it consists of a transmembrane (TM) β-barrel and several soluble domains: usher N-terminal domain (UND), usher C-terminal domain (UCD), and a domain, which sequence locates within the TM β-barrel segment, usher middle domain (UMD) (Fig. 13). The sequence of the hypothetical UMD was highly conserved, suggesting and important

function for this domain. In paper I, we experimentally identified UMD and investigated its structure and function. The study suggested that UMD plays the role of a subunit-substituting protein (dummy subunit), plugging or priming secretion through the channel in the Caf1A usher.

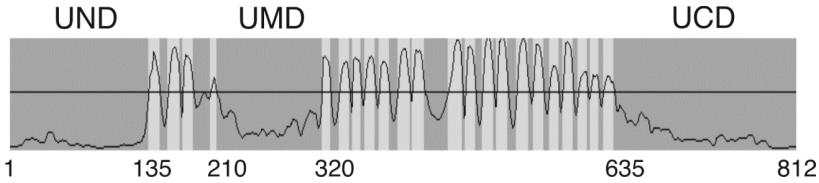

Figure 13. Prediction of the middle domain of Caf1A. Light-gray bars represent predicted TM β-strands. Regions of the sequence that are involved in formation of soluble domains are indicated.

2.3.2 Isolation and characterization of UMD of Caf1A

Since boundaries of UMD could not be predicted accurately, we initially expressed the entire sequence located between the TM β-strands. Analysis of chemical and temperature denaturation of the purified protein using circular dichroism (CD), showed that it is capable of autonomous folding and has a typical for single domain globular proteins stability. However, analytical size exclusion chromatography indicated that the protein has an abnormally large gyration radius in solution. To study the cause of this abnormality, the protein was subjected to limited proteolysis with trypsin and chymotrypsin. Both enzymes quickly truncated the protein to single species. Mass spectrometry analysis of the truncated species suggested that the construct contained an unstructured sequence at its N-terminus. Therefore, the shorter version $UMD_{232\text{-}320}$ was expressed and purified. Analysis of the stability of purified $UMD_{232\text{-}320}$ indicated that it contains an intact core structure.

2.3.3 Structure of UMD of Caf1A

The structure of the $UMD_{232\text{-}320}$ was determined by single anomalous dispersion phasing (SAD) using an Au derivative data set. The asymmetric unit contains a symmetric domain-swapped dimer of similar UMD monomers (Fig. 14). The $UMD_{232\text{-}320}$ monomer has an s-type immunoglobulin (Ig) like fold (Bork *et al.*, 1994), consisting of a seven stranded β-sandwich with the first strand formed by the N-terminal sequence of the neighboring monomer. No electron density was found for the first seven and the last four residues of $UMD_{232\text{-}320}$, suggesting that these sequences are not structured. UMD contains nearly 50% of the

most conserved residues of usher proteins (Capitani *et al.*, 2006). Most of these residues have a clear structural role. However, four highly conserved residues (Ile252, Arg263, Glu291, and Asp300) are situated on the surface of the protein and have no apparent structural role, but may instead be important for UMD functionality (Fig. 14).

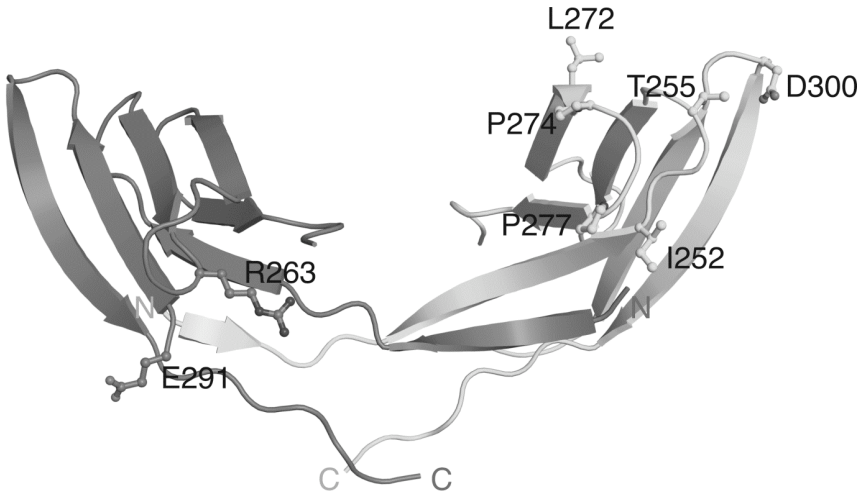

Figure 14. Crystal structure of $UMD_{232-320}$ dimer (Cartoon diagram). Two monomers in the dimer are shown in different colors. The invariant residues and residues contributing to the hydrophobic patch on the surface are shown as sticks and spheres.

2.3.4 UMD is essential for Caf1 secretion

To investigate the requirement of UMD for F1 assembly, a series of internal deletions were created within the *caf1A* gene. Immunoblotting analysis revealed that the deletion of UMD did not affect the formation of the usher in the outer membrane. The ability of deletion mutants to catalyze the surface assembly of F1 polymers was assessed in an *in vivo* quantitative immunofluorescence assay. The deletions completely abolished the surface assembly of Caf1. Instead, Caf1 accumulated in the periplasm. In order to assess the functional role of UMD, we analyzed its possible interaction with chaperone-subunit complexes. However, no binding between UMD and Caf1M:Caf1 or Caf1M:Caf1:Caf1 complexes could be detected in the Ni-NTA affinity assay or ion-exchange-based binding experiments.

2.3.5 UMD functions as a 'dummy subunit' capping/uncapping of the usher channel

Superposition of structures of UMD and the fiber inserted Caf1 revealed significant structural similarity: of 71 core Cα atoms of UMD, 60 were superimposed with the corresponding atoms on Caf1 with an r.m.s.d. of 1.78 Å. (Fig. 15). This finding and the fact that in the PapC usher the UMD is locates inside the TM β-barrel (Remaut *et al.*, 2008), strongly suggested that UMD may function as a dummy subunit, mimicking a fiber-incorporated subunit and plugging the usher translocation pore prior to initiation of fibre assembly and secretion. Plugging the pore might be important to prevent leakage of large periplasmic components through the outer membrane. However, if this was the only role of UMD, we might expect the Caf1A with deleted UMD to be functional in assembly and secretion. As this was not the case (Caf1 accumulated in the periplasm, but not on the surface), UMD appears to fulfill some other functions. These could possibly be the maintaining the very large usher β-barrel in a translocation-competent conformation and initiation of secretion process.

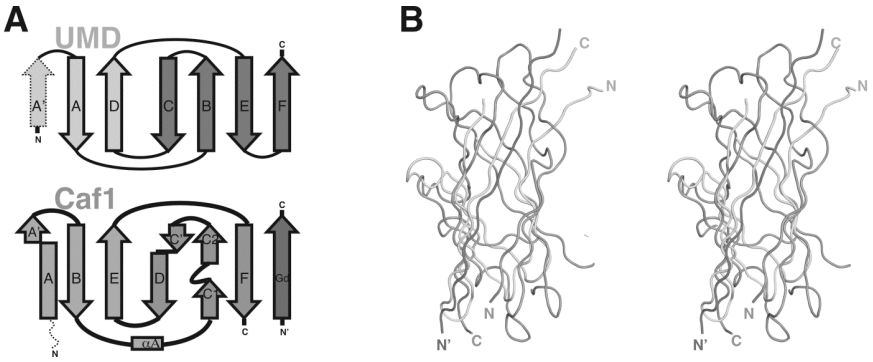

Figure 15. UMD and fiber inserted Caf1 subunit display significant structural similarity. A. Topology diagrams of UMD and Caf1. Arrows indicate β-strands; the single helix (αA) in Caf1 is shown as rectangle. B. Superposition of UMD and fiber inserted Caf1 (PDB accession code 1P5U) structures (stereo view). UMD and Caf1 are shown in dark and light gray respectively.

3 Conclusion and future perspectives

The results of this thesis represent only a small part of the massive work performed by many researches from different laboratories to elucidate the detailed mechanism of the CU pathway. Thanks to these studies, the CU pathway is now probably the best understood secretion mechanism. Nevertheless, many questions remained unanswered. Bellow, I list some points of particular interest:

(1) *To determining structure of the full-length apo usher and mechanism of its activation.* There is no structure of full-length apo usher available. Hence, it is not known how various domains of the usher interact in this form. Comparison of the non-activated or apo FimD pore domain structure and that in the complex with FimC:FimH, shows that FimD undergoes large conformational changes, including reposition of the UMD domain out from the TM channel (Fig. 1) and enlarging the channel, upon activation. How are these changes triggered?

(2) *To determining structure of the full-length usher from an FGL CU system and mechanism of its activation*. FGL CU systems assemble fibers, which contain no analogs of two-domain adhesive subunits (like FimH or PapG in FGS system). If the binding to the adhesive subunit triggers the activation of usher in FGS CU system, how the usher from FGL system can be activated? Does the chaperone:subunit:subunit ternary complex have to be pre-formed in order to activate the FGL usher?

(3) *To determine functional mechanism of UCDs*. UCDs are believed to act as the second chaperone:subunit binding site. Upon DSE, usher has to reset the assembly platform for a new incorporation cycle (Fig. 1),

which includes release of the chaperone capping the penultimate subunit, hand-over the chaperone:subunit complexes from the UND to UCDs, and translocation of one fiber subunit into the TMD channel. UCDs are involved in all these processes. Hence, information related to the conformation change of UCDs and kinetic studies will help to unveil the usher resetting process.

The CU pathway is an excellent drug target. The future applied studies will help to exploit the wealth of structural information to design new efficient inhibitors of the pathway to treat infection diseases.

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