Doctoral Thesis

Probing the type 1 pilus assembly platform FimD and its interactions with chaperone-subunit complexes

Author(s):
Nishiyama, Mireille

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Probing the type 1 pilus assembly platform FimD and its interactions with chaperone-subunit complexes

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Mireille Nishiyama

Eidg. dipl. Apothekerin

born on July 13, 1975

citizen of Ingenbohl, Schwyz, Switzerland

accepted on the recommendation of:

Prof. Dr. R. Glockshuber, examiner

Prof. Dr. L. Thöny-Meyer, co-examiner

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SUMMARY

A broad variety of pathogenic bacteria assemble adhesive surface organelles termed pili that mediate binding to host tissue. These fibers are highly oligomeric, filamentous protein complexes, which are anchored to the outer bacterial membrane. Type 1 pili are found in more than 80% of uropathogenic *Escherichia coli* strains that cause cystitis. Their quaternary structure is characterized by a pilus rod consisting of a right-handed, helical array of the main structural subunit FimA, and a linear tip fibrillum composed of subunits FimF, FimG and the adhesin FimH. The biogenesis of type 1 pili is governed by the conserved chaperone/usher pathway: the periplasmic chaperone FimC forms stoichiometric complexes with pilus subunits, catalyzes their folding, and transports them to the assembly platform (also termed usher) FimD in the outer membrane where the chaperone dissociates and subunits are incorporated into the pilus.

In contrast to the well-characterized interplay between the chaperone and pilus subunits, the assembly process taking place at the outer membrane has not been well characterized. The focus of my PhD work was therefore the closer investigation of the type 1 pilus assembly platform FimD. The first approach was to identify domains of FimD that are involved in the initial step in type 1 pilus assembly, i.e. the recognition of chaperone–subunit complexes by the assembly platform. For this purpose, it was attempted to produce predicted N- and C-terminal periplasmic domains of FimD as isolated proteins in *E. coli*. A N-terminal, periplasmic domain comprising the first 139 residues of FimD (FimDN) was thereby identified as a soluble, autonomously folding unit with a high thermodynamic stability ($\Delta G = -33.0 \text{ kJ mol}^{-1}$). This domain selectively binds FimC–subunit complexes with micromolar affinities, while neither FimC nor pilus subunits alone are recognized. FimDN has the highest affinity towards the FimC–FimH complex, ensuring that the FimH pool in the periplasm is exploited efficiently, and that the majority of type 1 pili bear the adhesin FimH at their tip.

In order to gain a deeper insight into the interactions of FimDN with FimC–subunit complexes, structural information was required. The subsequent approach was therefore the structure determination of isolated FimDN by NMR spectroscopy and that of the ternary complex between FimDN and FimC–FimHP (FimHP denotes the pilin domain of FimH) by X-ray crystallography. Using a C-terminally truncated FimDN variant (residues 1–125), which
exhibits the same affinity as full-length FimD towards the FimC-FimHp complex, crystals of the ternary complex could be obtained. The 1.8 Å X-ray structure of the ternary complex revealed that the key element of FimD for recognition of chaperone-subunit complexes is an “arm” (residues 1–24), which is completely unstructured in isolated FimD as observed by NMR. Upon ternary complex formation, however, the arm adopts a well-defined structure and specifically interacts with both the chaperone FimC and the bound pilin domain FimHp. Since the arm is the only FimD region that forms contacts with the chaperone-bound subunit, the ability of FimD to selectively bind different chaperone-subunit complexes resides in the arm of FimD. Furthermore, the arm acts as cargo sensor, in that it enables FimD to discriminate between chaperone molecules loaded with pilus subunits and free chaperones. This prevents that the assembly platform is occupied by an unloaded chaperone that would stall pilus assembly. The X-ray structure further revealed that the common element of the interactions of FimD with the four different FimC-subunit complexes is a specific surface complementarity between the N-terminal FimC domain and the structured “core” of FimD (residues 25–125). This defines the specificity of the FimC-FimD contact, making FimC and FimD a functional chaperone/usher pair in which neither FimC nor FimD can be replaced by a chaperone or an assembly platform from another pilus system. An unexpected role of the C-terminal hinge segment (residues 126–139), which was not present in the crystal structure, was revealed by a TROSY-NMR experiment, in which [\(^{15}\text{N},^{1}\text{H}\)]-chemical shift variations between isolated full-length FimD (residues 1–139) and full-length FimD in the ternary complex were mapped. Even though this C-terminal segment is not directly involved in binding of the FimC-subunit complexes, a number of amino acid residues in this segment showed strong chemical shift variations suggesting a movement of this segment upon binding of the FimC-FimHp complex. In conjunction with biochemical data, this hints at an intramolecular association/dissociation equilibrium between the FimD core and the C-terminal hinge segment that is related to a movement of residues 1–125 relative to the transmembrane domain of FimD upon binding of FimC-subunit complexes.

Towards the goal to elucidate the entire pilus assembly process at the outer membrane, initial work on full-length FimD was taken up. An expression system for full-length FimD was established that allows the production of the protein at high levels. Preliminary results obtained in this study provide the basis for further biochemical and structural characterization of the assembly platform.
ZUSAMMENFASSUNG


Im Vergleich zum gut charakterisierten Zusammenspiel von Chaperon und Pilusuntereinheiten ist der Assemblierungsprozess, der sich an der äußeren Membran abspielt, weniger gut verstanden. Meine Doktorarbeit befasste sich deshalb mit der Untersuchung der Assemblierungsplattform FimD. Das erste Ziel war, Domänen von FimD zu identifizieren, die am ersten Schritt des Assemblierungsprozesses beteiligt sind, nämlich der Erkennung von FimC-Pilusuntereinheit Komplexen. Es wurde versucht, vorausgesagte N- und C-terminale, periplasmatische Domänen als isolierte Proteine in *E. coli* zu produzieren. Eine N-terminale periplasmatische Domäne bestehend aus 139 Aminosäuren (FimD\textsubscript{N}) wurde dabei als eigenständig faltende Einheit identifiziert, die eine hohe thermodynamische Stabilität aufweist ($\Delta G = -33.0$ kJ mol$^{-1}$). Diese Domäne bindet selektiv die FimC-Pilusuntereinheiten Komplexe mit mikromolaren Affinitäten, während weder FimC noch Pilusuntereinheiten allein gebunden werden. FimD\textsubscript{N} bindet FimC–FimH Komplexe mit der höchste Affinität, was gewährleistet, dass das FimH-Repertoire im Periplasma effektiv ausgenutzt wird, und dass die Mehrheit der Pili an der Spitze das Adhesin FimH trägt.

Um die Wechselwirkung zwischen FimD\textsubscript{N} und den FimC–Pilusuntereinheiten Komplexen besser zu verstehen, war strukturelle Information erforderlich. Der anschließende Ansatz war
Zusammenfassung
Zusammenfassung

Um den Assemblierungsprozess von Typ-1 Pili, der an der äusseren Memran ablauft, vollständig aufklären zu können, wurde schliesslich die Arbeit an Vollängen FimD aufgenommen. In dieser Doktorarbeit wurde ein Expressionssystem etabliert, das die Produktion von FimD in grösserer Menge ermöglicht. Die hier erworbenen Kenntnisse über FimD dienen als Basis für weitere Untersuchungen an dieser Assemblierungsplattform.
Introduction

1. INTRODUCTION

1.1 Type 1 pili and P pili: Prototypes of bacterial adhesive surface organelles

Biological relevance

Gram-negative bacteria assemble a diverse array of multi-subunit organelles on their surfaces. They serve to accomplish important biological processes like host tissue adhesion, transfer of genetic information by conjugation, secretion of virulence factors, and cellular motility (Fernandez and Berenguer, 2000). The adhesion to host tissues is achieved by filamentous surface organelles, called pili or fimbriae (Mol and Oudega, 1996). They contain adhesins that bind to specific receptors on host cells. The interaction between the adhesin and the receptor is critical to the ability of many pathogenic bacteria to colonize host tissues and cause diseases (Hultgren et al., 1993). Virtually all Gram-negative bacteria that have been examined so far were found to be able to produce one or more types of pili (Nowicki et al., 1984). They differ in their morphological properties, their assembly pathways, and their receptor specificities. Two of the best characterized adhesive organelles are the type 1 pili and P pili from uropathogenic Escherichia coli. P pili bind specifically to the Galα(1-4)Gal moieties of glycolipids present in the human kidney and have been shown to be required for the establishment of pyelonephritis (Lund et al., 1987; Roberts et al., 1994). Type 1 pili are important virulence determinants for the development of bladder infections including cystitis (Connell et al., 1996; Langermann et al., 1997). A FimH (adhesin of type 1 pili)-based vaccine has been shown to elicit IgG antibodies that block colonization in vivo and protect mice against a mucosal infection of the bladder (Langermann et al., 1997). The receptor binding event activates complex signal transduction cascades in the host cell (Mulvey et al., 2000). It has been shown that bladder epithelial cells exfoliate in response to infection by type 1-piliated E. coli strains via a rapid apoptosis-like mechanism involving host DNA fragmentation and the activation of proteolytic enzymes known as caspases (Mulvey et al., 1998). The exfoliation and clearance of infected bladder cells is proposed to act as one of the host defense mechanisms. Besides mediating attachment to host tissues, type 1 pili are responsible for bacterial invasion and persistence within bladder epithelial cells (Martinez et al., 2000; Mulvey et al., 2001). This allows the pathogen to evade host defenses and antibiotic treatment. Even more, Mulvey et al. observed that intracellular bacteria are able to replicate,
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leading to the establishment of quiescent bacterial reservoirs within the bladder tissue (Mulvey et al., 2001). The existence of intracellular bacteria may help explain the recurrent cystitis seen in a large proportion of women (Schilling and Hultgren, 2002).

Assembly pathways

Adhesive pili of Gram-negative bacteria are assembled by four distinct pathways. The first pathway, the chaperone/usher pathway, is employed for the assembly of more than 30 surface organelles found in pathogenic strains (reviewed in Hung and Hultgren, 1998; Sauer et al., 2000; Thanassi et al., 1998a). Type 1 pili and P pili represent the prototypic organelles assembled by this pathway. The assembly machinery is composed of two specialized classes of proteins: a periplasmic chaperone and an outer membrane assembly platform, also called usher (Figure 1). This pathway will be discussed in detail in the following paragraphs. A second pathway is used for type IV or bundle-forming pili (Parge et al., 1995). These long, polarly localized pili are expressed by a number of bacteria, such as enteropathogenic E. coli (EPEC), Pseudomonas aeruginosa and Vibrio cholerae. Type IV pilus assembly requires 14 or more assembly components which are homologous to many accessory proteins of the type II secretion pathway in Gram-negative bacteria and of the flagellum biogenesis machinery of archaea (Peabody et al., 2003; Sauvonnet et al., 2000). A third assembly pathway is employed to assemble curli, thin, irregular, and highly aggregated surface structures expressed by many clinical E. coli strains and Salmonella enteritidis (Olsen et al., 1989). The subunits of curli are thought to be secreted as monomers to the cell surface where accessory proteins promote their polymerization to fibers (Chapman et al., 2002). A fourth pathway is proposed for the biogenesis of CS1 pili and other related fimbrial structures from enterotoxigenic E. coli (ETEC) (Sakellaris et al., 1996). Like the chaperone/usher pathway, this pathway also employs a specialized set of periplasmic chaperones and outer membrane assembly platforms. However, they share no sequence similarity with the components of the chaperone/usher pathway (Smyth et al., 1996).
Figure 1: Schematic model of type 1 pilus assembly by the "chaperone/usher" pathway. The periplasmic chaperone FimC binds newly translocated pilus subunits (FimA, FimG, FimF, FimH) and accelerates their folding. In these complexes, FimC donates its G_{i} donor strand to the individual subunits and completes the immunoglobulin-like fold of the subunits. FimC−subunit complexes diffuse to the assembly platform ("usher") FimD, which specifically recognizes FimC−subunit complexes via its periplasmic, N-terminal segment. Subsequently, FimC is released to the periplasm, and the subunit is delivered to the translocation pore of FimD where it is supposed to interact with the previously incorporated subunit via donor strand exchange. The pilus rod, which is composed of FimA subunits, assembles spontaneously into its helical quaternary structure on the cell surface. IM, inner membrane; OM, outer membrane.
Introduction

Architecture of bacterial pili

Pili assembled via the chaperone/usher pathway can be divided in two classes according to their morphological properties (Hung et al., 1996). The first class (fimbrial pili) comprises pili with a rigid rod-like structure (e.g. type 1 and P pili) consisting of a thick helical rod that is joined to a thin tip fibrillum (Jones et al., 1995; Kuehn et al., 1992). Most structural and functional studies of adhesive organelles have been done with pili belonging to this first class. The second class (non-fimbrial pili) comprises pili, which are very thin fibers (<2 nm) and tend to coil up into an amorphous mass on the surface of the bacterium (Ahrens et al., 1993; Le Bouguenec et al., 1993). The best studied member of these non-fimbrial pili is the F1 capsular antigen of the plague pathogen *Yersina pestis* (MacIntyre et al., 2001; Zavialov et al., 2003).

In contrast to the non-fimbrial pili that are built up of only one or sometimes two types of subunits, the fimbrial pili are composed of several different subunits that form a 1-2 μm long rigid pilus rod and a short and flexible tip fibrillum (Jones et al., 1995) (Figure 1). In type 1 pili, the rod consists of 500-3000 FimA subunits, arranged in a right-handed helix with an external diameter of 6.9 nm and 3.4 subunits per turn (Hahn et al., 2002). The interior of the rod contains a channel with a diameter of 2.0-2.5 nm. The tip fibrillum contains two adaptor proteins FimF and FimG, and the adhesin FimH at the tip (Jones et al., 1995). FimH mediates binding to mannose units of the glycoprotein receptor uroplakin Ia on the surface of urinary epithelia (Zhou et al., 2001). FimH has also been implicated in attachment to abiotic surfaces in a model biofilm system (Pratt and Kolter, 1998). Deletion mutants of FimF and FimG showed aberrant numbers and lengths of pili indicating that they might be involved in the proper pilus assembly (Russell and Orndorff, 1992). However, their exact functions are not known to date.

Compared to type 1 pili, P pili have a longer tip fibrillum, but otherwise similar dimensions (Kuehn et al., 1992). The rod of P pili is formed by the main structural subunit PapA, and the tip fibrillum is mainly made up of the subunit PapE. The minor subunit PapK links the tip fibrillum to the rod, while PapF serves as an adaptor between the tip fibrillum and the adhesin PapG (Jacob-Dubuisson et al., 1993; Lindberg et al., 1987).
**Introduction**

**Regulation of biosynthesis**

The expression of type 1 pili and P pili is controlled by phase variation. In case of type 1 pili, this correlates with the orientation of a short, invertible DNA segment (so-called fim switch) located immediately upstream of fimA that contains the promoter for the fimA, fimI and fimC genes (Abraham et al., 1985) (Figure 2).

**fim operon**

**pap operon**

![Diagram](image)

**Figure 2.** Genetic organisation of the fim gene cluster coding for type 1 pili and the pap operon coding for P pili. The expression of both pili is controlled by phase variation. Whereas the fim gene cluster is regulated by the site-specific inversion of the the fim switch, the pap operon is modulated by specific methylation/demethylation of two GATC sites located within the pap regulatory region. Promotors are depicted as arrows. For the expression of the fim genes five promoters are required, while only two promoters control the expression of the pap genes.

The orientation of this fim switch therefore directs the transcription of fimA, fimI and fimC and consequently the bacterial phenotype. Switch inversion requires the regulatory proteins FimB and FimE that act as site-specific recombinases (Klemm, 1986). FimB acts in both directions (on-to-off and off-to-on), whereas FimE acts independently to only turn off expression. Recent work has shown that PapB, one of the regulatory proteins of P-pili, prevents expression of type 1 pili by inhibition of FimB activity at the fim switch and by increasing the expression of FimE (Xia et al., 2000). This suggests that the expression of type 1 pili and P pili is mutually exclusive within a single bacterium and prevents simultaneous expression of
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multiple adhesins that may otherwise be induced under similar environmental conditions. Multiple adhesin expression would be disadvantageous to bacteria, since they are exposed to a stronger host immune pressure due to simultaneous expression of several surface antigens. This hypothesis is supported by the finding that pyelonephritogenic E. coli containing operons for different fimbrial types seldom express more than one fimbrial type at once (Nowicki et al., 1984). In addition to FimB and FimE, global regulators including the histon-like protein (H-NS) or DNA binding proteins (leucine-responsive regulatory protein Lrp and integration host factor IHF), and also environmental conditions like temperature and medium are reported to affect the expression of type 1 pili (Dorman and Higgins, 1987; Gally et al., 1993; Olsen et al., 1998; Roesch and Blomfield, 1998).

Despite their similarities in pilus architecture and genetic organisation (Figure 2), phase variation of P pili occurs differently as in type 1 pili, namely without DNA rearrangement. It rather involves the specific methylation status of two GATC sites located within the pap regulatory region. The expression of P pili is positively controled by the local regulators PapI and PapB in concert with the global regulators Lrp and catabolite gene activator protein (CAP). Deoxyadenosine methylase (Dam) is also required for pap transcription (Hernday et al., 2002; van der Woude et al., 1996).

1.2 The chaperone/usher pathway — Structural and functional aspects

Overview

Pilus subunits enter the periplasm via the SecYEG translocon (Dodd et al., 1984; Van den Berg et al., 2004). During translocation of the subunits or shortly thereafter, signal peptidase cleaves off their signal sequence and DsbA introduces a disulfide bond, which is conserved among all subunits (Jacob-Dubuisson et al., 1994a). In the periplasm, each subunit interacts with the chaperone to form a stable chaperone-subunit complex (Jones et al., 1993). Interaction with the chaperone (i) facilitates the efficient folding of the subunit (Vetsch et al., 2004), (ii) stabilizes it, preventing its aggregation and/or degradation (Vetsch et al., 2002), (iii) caps its interactive surfaces, inhibiting premature fiber formation (Kuehn et al., 1991), and (iv) primes it for assembly by maintaining its activated conformation (Sauer et al., 2002; Zavialov et al., 2003). In the absence of the chaperone, subunits go off-pathway, resulting in misfolding and aggregation (Hung et al., 2001). The built-up of these off-pathway subunits
has been shown to activate the Cpx two-component signaling system that responds to periplasmic stress by inducing the expression of a variety of genes including those encoding periplasmic protein folding factors, such as DsbA, and periplasmic proteases, such as DegP (Jones et al., 1997; Lee et al., 2004). These factors in turn act to relieve the stress caused by misfolded or aggregated subunits.

Chaperone-subunit complexes diffuse to the outer membrane assembly platform (discussed in detail in subsequent sections). In the absence of the assembly platform, chaperone-subunit complexes accumulate in the periplasm but no pili are assembled or secreted (Klemm and Christiansen, 1990; Norgren et al., 1987). The assembly platform is thought to facilitate chaperone uncapping to expose the interactive surfaces on the subunits that drive their polymerization (Barnhart et al., 2003). It forms a pore 2 nm in diameter, wide enough to allow passage of folded subunits assembled into a linear fiber, such as the tip fibrillum, but not the helical pilus rod (Li et al., 2004; Saulino et al., 2000; Thanassi et al., 1998b). A solution to this problem was revealed by experiments demonstrating that P and type 1 pilus rods can be unwound into linear fibers with a diameter of 2 nm without depolymerization (Abraham et al., 1992; Thanassi et al., 1998b). Therefore, it is believed that the pilus rod traverses the pore in a linear form, and only winds into its final helical form upon reaching the external surface. In the P pilus system, the PapH subunit terminates rod assembly and has been proposed to function as an anchor (Baga et al., 1987). In case of type 1 pili, however, no such protein was identified so far (Valenski et al., 2003). The formation of the final quaternary structure is thought to be a spontaneous process that takes place in the absence of ATP or electrochemical gradients (Jacob-Dubuisson et al., 1994b).

**Periplasmic chaperones**

Periplasmic chaperones of the chaperone/ usher pathway consist of two domains arranged in a boomerang-like shape (Holmgren and Branden, 1989; Knight et al., 2002; Pellecchia et al., 1998; Zavialov et al., 2003). Each domain has an immunoglobulin (Ig)-like fold with seven primary β-strands (strands A–G). An extended loop in the N-terminal domain that connects the F and G strands (the F1 and G1 strands, hence the F1–G1 loop) contains a conserved motif of solvent-exposed alternating hydrophobic residues that are involved in chaperone-subunit complex formation. Chaperones can be classified based on the length of their F1–G1 loops (Hung et al., 1996). FGS chaperones including FimC and PapD have shorter loops with 10–20
residues, whereas FGL chaperones including Cfa1M of Y. pestis have longer loops with 21-29 residues. Interestingly, FGS chaperones specifically assemble fimbrial pili, while FGL chaperones specifically assemble non-fimbrial pili (cf. p.9). The F₁-G₁ loop comprises a motif of alternating hydrophobic and hydrophilic residues, which play a critical role in subunit binding (Choudhury et al., 1999; Kuehn et al., 1993; Sauer et al., 1999). The motif contains 3 hydrophobic residues in FGS chaperones and 4 to 5 hydrophobic residues in most FGL chaperones. A comparison of an isolated chaperone and a chaperone with bound pilus subunit shows that upon binding of a subunit, the conformation of the F₁–G₁ loop alters significantly while the overall structure of the chaperone is not affected (Choudhury et al., 1999; Sauer et al., 2002).

**Donor strand complementation**

Most subunits consist of a single so-called pilin domain that interacts with the chaperone (Choudhury et al., 1999; Sauer et al., 1999). Adhesin subunits generally have an additional domain, a receptor-binding domain, that interacts with a specific receptor (Dodson et al., 2001; Merckel et al., 2003). Each subunit pilin domain has an incomplete Ig-like fold. The pilin domain thereby possesses strands A–F of the Ig-like fold but lacks a C-terminal G strand, resulting in a groove on the surface and leaving its hydrophobic core incomplete — hence the instability of subunits when expressed without the chaperone (Vetsch et al., 2002). In the chaperone-subunit complex, the chaperone G₁ strand and a portion of the F₁–G₁ loop, which has become part of the extended G₁ strand, are inserted between the A- and the F-strand of the subunit completing its Ig-like fold (Choudhury et al., 1999; Sauer et al., 1999) (Figure 3). This donor strand complementation interaction stabilizes the subunit by shielding its hydrophobic core. Indeed, the alternating hydrophobic residues of the extended G₁ strand reach deep into the hydrophobic core of the bound subunit. Donor strand complementation, however, produces an atypical Ig fold in the subunit, since the chaperone G₁ strand runs parallel to the subunit F strand, whereas in a canonical Ig fold, the G strand runs anti-parallel to the F strand.

**Driving force for pilus assembly**

Recent structural studies of pilus assembly intermediates provide a more detailed insight into fiber assembly (Sauer et al., 2002; Zavialov et al., 2003). The structures revealed that donor
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strand complementation also governs subunit-subunit interactions in the quaternary structure of pili. All subunits except the adhesin have an N-terminal extension of about 15 amino acids that in the chaperone-subunit complex is disordered and does not contribute to the subunit Ig-like fold (Choudhury et al., 1999; Sauer et al., 2002; Zavialov et al., 2003). Like the chaperone G1 strand, the N-terminal extension of subunits contains a conserved motif of alternating hydrophobic residues (Hung et al., 1996). These N-terminal extensions serve as donor strands for a preceding subunit in the pilus and replace the chaperone donor strand in the subunit (Sauer et al., 2002; Zavialov et al., 2003). However, unlike the chaperone G1 strand, the N-terminal extension is inserted anti-parallel to the F-strand of the neighboring subunit. The adhesin at the tip of a pilus lacks an N-terminal extension. It has instead its N-terminal receptor binding domain, but still has a groove that can interact with the N-terminal extension of an adjacent subunit (Choudhury et al., 1999). This ensures that the adhesin is incorporated at the tip of the mature pilus. The mature pilus thus consists of an array of canonical Ig-like domains, each of which contributes its N-terminal extension to complete the Ig-like fold of its more distal neighbor. Moreover, the crystal structures revealed that the chaperone traps the subunit in an activated folding state by preventing the compact packing of its hydrophobic core (Sauer et al., 2002; Zavialov et al., 2003). In the chaperone-subunit complex, the chaperone G1 strand holds the groove in an open conformation by pulling the parallel subunit F strand away from the subunit A strand (Figure 3). In the subunit-subunit complex, the subunit donor strand is inserted anti-parallel to the subunit F strand and shifted in register relative to the chaperone G1 strand. This donor strand exchange allows the ends of the subunit A and F strands to move towards each other, leading to the closure of the groove, and the subunits adopt a more ordered and compact overall state. The reported extraordinary stability of type 1 pili, their dissociation into monomers requires incubation at 37 °C in saturated guanidine chloride for several hours or boiling at pH values below 1.8, is possibly a consequence of this tight subunit-subunit interaction (Eshdat et al., 1981).

By holding the groove open and preventing the compact packing of the subunit hydrophobic core, the chaperone traps the subunit in an activated folding state. Vetsch et al. reported that the FimC–FimG complex is formed quantitatively and very rapidly in vitro when folding of FimG is initiated in the presence of both FimC and the assembly-competent subunit FimF, even though the FimC–FimG complex is thermodynamically less stable than the FimF–FimG complex (Vetsch et al., 2004).
Figure 3. Structural basis of donor strand complementation in subunit-subunit interactions. (A) Ribbon diagrams and (B) topology diagrams of the subunit PapE with complementing strands before (left, from the PapD-PapE complex) and after (right, from the PapE-PapK peptide complex) donor strand exchange. To prevent self-polymerization a truncated PapE variant lacking the N-terminal extension was used for the crystallization. PapE is shown in cyan, the chaperone G strand in yellow, the PapK peptide in red, and the N-terminal extension of PapE (not present in the crystal structures) in blue. Ribbon diagrams show the positions of the N- and C-termini of PapE before (open groove) and after (close groove) donor strand exchange. Upon donor strand exchange, the G strand of the chaperone, running parallel to the subunit F strand, is replaced by the N-terminal extension of the preceding subunit, now running anti-parallel to the F strand. This leads to the closure of the pilin core and the subunit adopts a more ordered and compact fold.

Here, the question arises why the spontaneous formation of more stable subunit-subunit interaction does not occur in vitro or in the periplasm. The most likely reason would be a high activation energy barrier for the donor strand exchange reaction. Since fiber formation is a spontaneous process, it was proposed that the free energy difference between the chaperone-bound subunit and a subunit in the context of the fiber may be the driving force for pilus
assembly (Jacob-Dubuisson et al., 1994b; Zavialov et al., 2003). The assembly platform, where the donor strand exchange takes place in vivo, potentially lowers the activation energy of this reaction, thus facilitates the chaperone dissociation and the formation of subunit-subunit complexes. The assembly platform thus may work as a catalyst in the donor strand exchange reaction. The elucidation of how the assembly platform manages this catalysis is currently one of the biggest challenges in the field of pilus assembly.

**Outer membrane assembly platforms**

Assembly platforms are large, integral outer membrane proteins (Kuehn et al., 1994). Mature FimD comprises 833 amino acid residues and has a molecular mass of 91.4 kDa (Klemm and Christiansen, 1990). Bacterial outer membrane proteins with known structures span the membrane via a series of transmembrane antiparallel β-strands arranged to form a β-barrel (Schulz, 2000; Wimley, 2003). Proteins found in the outer membrane of mitochondria and chloroplasts also adopt similar β-barrel folds (Paschen et al., 2003). Besides the recently solved structure of the N-terminal domain of FimD (cf. chapter 2.2), no structural information at atomic level is available for bacterial assembly platforms. A topology study of K88 pilus assembly platform FaeD predicts that the assembly platform consists of a large central transmembrane domain with 22 β-strands and two, N- and C-terminally located, periplasmic domains (Harms et al., 1999). Thanassi et al. recently proposed that PapC comprises an N-terminal β-barrel domain with 26 β-strands and a C-terminal domain located within the barrel channel, in a fashion analogous to the plug domains found in outer membrane iron-siderophore uptake proteins (Henderson et al., 2004). According to this prediction, the N-terminal domain of the assembly platform is part of the transmembrane domain. The topology prediction of Thanassi et al. is based on a fluorescence labeling study, in which PapC cysteine variants were labeled sequentially by two thiol-reactive dyes differing in their outer membrane permeability. The interpretation of the data is, however, rather controversial. In addition, the possibility that some cysteines may not be easily accessible due to structural hindrance was not considered. Taking into account that the N-terminal 139 residues of FimD forms a soluble, autonomously folding domain (cf. chapter 2.1), the predicted topology model of Harms et al. with two periplasmic domains seems to be more conceivable at present.
Introduction

Earlier electron microscopy studies of assembly platforms revealed ring-shaped oligomers composed of 6–12 subunits with a central pore of 2 nm in diameter (Saulino et al., 2000; Thanassi et al., 1998b). In contrast to these studies, which were performed with His-tagged proteins, a recent study with natural PapC revealed that it forms a dimer with a twin-pore configuration, similar to the TOM protein import complex of the mitochondrial outer membrane (Ahting et al., 1999; Li et al., 2004). In this study, large ring-like particles were observed only for His-tagged PapC. This indicates that the previously reported large oligomeric rings are rather an artefact caused by protein aggregation. Furthermore, gel filtration analysis showed that His-tagged PapC was prone to aggregation. Interestingly, a previous study on PapC reconstituted into proteoliposomes revealed that the pore-forming activity of PapC increased upon addition of outer membrane lipid lipopolysaccharide (LPS) (Thanassi et al., 1998b). Since the addition of LPS did not increase incorporation of PapC into the liposomes or the pore size of PapC, LPS could have affected the conformation of PapC in the liposome membrane. This indicates that the oligomeric state of assembly platforms in vitro might differ from that in vivo in dependence of experimental conditions. Some membrane proteins were even reported to have tendencies to denature in detergent solution through both disassembly and aggregation (Engel et al., 2002). Considering that there is no detailed study regarding the oligomeric state of assembly platforms to date, there is a need to elucidate the influence of various experimental parameters, e.g. detergent type, protein concentration or even cofactors, on the assembly of usher subunits. The accurate determination of the oligomeric state is also of fundamental importance for understanding the physiological mode of action of the assembly platform in pilus assembly.

The assembly platforms have been reported to selectively bind their target chaperone-subunit complexes (Dodson et al., 1993; Saulino et al., 1998). In contrast, isolated chaperones alone are not bound to the assembly platform. Assembly platforms are reported to have the highest affinity \( K_D \sim 9 \text{ nM} \) for their cognate chaperone-adhesin complexes. All other chaperone-subunit complexes are bound to the assembly platform with 10- to 100-fold lower affinities (Saulino et al., 1998). Because the adhesin is the first subunit to become incorporated into the pilus, this observation has led to the hypothesis that the relative affinities of the assembly platform for the various chaperone-subunit complexes determine the order of the subunits within the pilus. At least, this mechanism would ensure that a substantial fraction of pili bear an adhesin at their tip. Besides the specific affinities of the assembly platforms for the various
Introduction

Chaperone-subunit complexes, the relative affinities of the subunits towards each other are most likely another critical factor determining the final quaternary structure of pili.

Specificity of the assembly machinery

Each fimbrial gene cluster, or operon, codes for its own chaperone and assembly platform, suggesting that the assembly machinery is specific for its own fimbrial system only. This is consistent with the observation that the K88 and K99 chaperones, FaeE and FanE, were unable to complement each other (Bakker et al., 1991). However, another complementation experiment has shown that some chaperones can partly function in the biosynthesis of other fimbrial systems (Jones et al., 1993). In this study, FimC was unable to complement a papD null mutant, but was able to stabilize the P pilus major subunit PapA in the periplasm. This indicates that the inability of FimC to mediate P pilus assembly was not due to the lack of interaction with PapA. In contrast, the chaperone PapD was able to complement a fimC null mutant, resulting in a normal level of type 1 pili production. However, these pili were hardly capable to agglutinate erythrocytes, indicating that the presentation of the adhesin FimH was different from pili assembled by the native chaperone.

When the adhesins of type 1 and F1C pili were reciprocally exchanged in both pilus systems, phenotypically normal pili with converted receptor specificities were assembled, suggesting that heterologous minor components were accepted by the non-parental assembly machinery (Klemm et al., 1994). Moreover, it has been reported that the FGS chaperone PapD was able to bind peptides corresponding to the carboxyl terminus of subunits that are assembled by FGL chaperones (Hung et al., 1996). These results indicate that the subunit recognition by the chaperone is not stringently restricted to subunits of its own pilus system. The specificity of the assembly machinery might reside rather in the interaction of the assembly platform with the chaperone-subunit complexes. This hypothesis is supported by the observation that the reciprocal exchange of single elements of the assembly machinery of type 1 and F1C pili, i.e. either the chaperones or the assembly platforms, resulted in severe reduction or complete abolishment of fimbriation and adhesion. However, the reciprocal exchange of both the chaperone and the assembly platform resulted in both cases in the production of functional pili (Klemm et al., 1995).
1.3 Outline of the thesis

Despite their fundamental role in pilus assembly, only limited information is available on the molecular mechanism underlying the functions of assembly platforms. The main goal of this thesis is to gain more detailed insight into the type 1 pilus assembly process at the outer membrane. Towards this goal, it was first tried to identify segments of FimD that are important for the recognition of chaperone-subunit complexes. The results presented in chapter 2.1 demonstrate that the N-terminal 139 residues of FimD (FimD_N) can be stably expressed as a soluble, monomeric, autonomously folding unit. FimD_N selectively binds FimC-subunit complexes and has the highest affinity towards the FimC-FimH complex. To obtain snapshots of the initial step in pilus assembly, high-resolution structures of FimD_N before and after binding of a chaperone-subunit complex were determined (cf. chapter 2.2). Finally, for detailed understanding of the function of FimD in the entire pilus assembly process, initial work for studying the full-length FimD was taken up (Chapter 2.3).
2. RESULTS

2.1 Identification and Characterization of the Chaperone-Subunit Complex-binding Domain from the Type 1 Pilus Assembly Platform FimD

Nishiyama M, Vetsch M, Puorger C, Jelesarov I and Glockshuber R.

Results  

chaperone-subunit complex binding domain of FimD

Summary

The outer membrane protein FimD represents the assembly platform of adhesive type 1 pili from *Escherichia coli*. FimD forms ring-shaped oligomers of 91.4 kDa subunits that recognize complexes between the pilus chaperone FimC and individual pilus subunits in the periplasm and mediate subunit translocation through the outer membrane. Here, we have identified a periplasmic domain of FimD (FimD\(_N\)) comprising the N-terminal 139 residues of FimD. Purified FimD\(_N\) is a monomeric, soluble protein that specifically recognizes complexes between FimC and individual type 1 pilus subunits, but neither binds the isolated chaperone, nor isolated subunits. In addition, FimD\(_N\) retains the ability of FimD to recognize different chaperone-subunit complexes with different affinities, and has the highest affinity towards the FimC-FimH complex. Overexpression of FimD\(_N\) in the periplasm of wild type *E. coli* cells diminished incorporation of FimH at the tip of type 1 pili, while pilus assembly itself was not affected. The identification of FimD\(_N\) and its ternary complexes with FimC and individual pilus subunits opens the avenue to structural characterization of critical type 1 pilus assembly intermediates.
Results

chaperone-subunit complex binding domain of FimD

Introduction

Attachment of bacteria to host cell tissues represents an important initial event during the establishment of bacterial infections. In the case of uropathogenic *Escherichia coli* strains, this process is mediated by adhesive surface organelles termed pili or fimbriae that are attached to the outer bacterial membrane. Type 1 pili from *E. coli* specifically bind to mannose units of uroplakin Ia, a surface glycoprotein of urinary epithelial cells. Moreover, type 1 pili mediate internalization of uropathogenic *E. coli* strains into eukaryotic host cells, where they are protected from rapid clearance and a variety of host defense mechanisms.

Type 1 pili are composed of up to 3000 structural protein subunits that form filaments with a diameter of about 7 nm and a typical length of 1-2 μm. Each pilus consists of a right-handed helical rod that is composed of the main structural subunit FimA, and a short tip fibrillum joined to the rod. The tip fibrillum is believed to be a linear array of 3 different subunits: the two adaptor proteins FimG and FimF, and the adhesin FimH, which is supposed to be located at the tip of the fibrillum. All structural pilus subunits are thought to be one-domain proteins with immunoglobulin-like fold that share substantial sequence similarity. The only exception is the adhesin FimH: In addition to a pilin domain that is homologous to the other structural subunits, it contains a second, N-terminal domain that binds mannose units of receptor glycoproteins.

Assembly of type 1 pili in vivo depends on two proteins that are not part of the final pilus structure: the periplasmic pilus chaperone FimC and the outer membrane assembly platform FimD. The underlying assembly mechanism, termed chaperone/usher pathway, has been postulated for more than 30 different adhesive pili from Gram-negative bacteria. FimC is a boomerang-shaped protein composed of two domains with immunoglobulin-like fold. FimC forms complexes with folded type 1 pilus subunits in the periplasm and stabilizes them by donating a missing β-strand to the subunits which have incomplete immunoglobulin-like folds. This interaction, termed donor strand complementation, prevents premature subunit polymerization in the periplasm, and protects subunits from proteolytic degradation and nonspecific aggregation. Chaperone-subunit complexes are then targeted to the outer membrane assembly platform FimD, where the chaperone-subunit complexes dissociate and the subunits are incorporated into the growing pilus. Donor strand complementation is also the mechanism underlying the interaction between subunits in the intact pilus.

The assembly platform FimD represents the most important, but so far least understood component in the pilus assembly machinery and possesses multiple, important functions.
Results

The assembly platform FimD represents the most important, but so far least understood component in the pilus assembly machinery and possesses multiple, important functions. FimD consists of 833 residues and shares 20-30 % sequence identity with the known bacterial pilus assembly platforms\(^{16}\). Data on the related P pilus assembly platform PapC indicate that assembly platforms are ring-shaped oligomers of 6-12 subunits forming a central pore in the outer membrane\(^{14}\). This pore, about 2 nm in diameter, is not large enough to accommodate the approximately 7 nm thick pilus rod. It is therefore believed that pilus subunits are translocated to the cell surface as monomers or in a linear array of subunits that assemble into the final pilus structure outside the cell\(^{14,15}\). Moreover, FimD does not only anchor the pilus to the outer \textit{E. coli} membrane, but specifically recognizes FimC-subunit complexes with different affinities\(^{17,18}\). Kinetic measurements performed by surface plasmon resonance revealed that both PapC and FimD show the fastest association rates and 20- to 150-fold higher affinities towards chaperone-adhesin complexes relative to other chaperone-subunit complexes\(^{18}\). This is consistent with the localization of the adhesins at the tip of the mature pili. In addition, dissociation of chaperone-subunit complexes from the assembly platform was found to be slow. This argues that pilus subunits are committed to incorporation into the pilus after binding to the assembly platform.

Despite of their fundamental role in pilus assembly, little is known about the molecular mechanism underlying the functions of assembly platforms. For example, it is not known which part of the assembly platform interacts with the chaperone-subunit complexes, and how the different chaperone-subunit complexes are discriminated. Towards the goal of a more detailed characterization of FimD, we have made use of a structure prediction for the subunits of the assembly platform FaeD\(^{19}\), which shares about 25 % sequence identity with FimD. This study predicted that assembly platforms have a porin-like, central transmembrane domain, and that the N- and C-terminal regions of the protein form periplasmic domains (Figure 1A).

Here, we present the first biochemical characterization of an individual domain of a pilus assembly platform. We demonstrate that the predicted, N-terminal periplasmic domain of FimD (FimD\(_{N}\)) comprising the first 139 residues of the full-length protein, is a stable, soluble protein that exclusively interacts with chaperone-subunit complexes \textit{in vitro}. Furthermore, we show that overexpression of soluble FimD\(_{N}\) in the periplasm of \textit{E. coli} wild type cells competitively diminishes incorporation of FimH at the tip of type 1 pili. Our data indicate that the ability of FimD to discriminate between individual pilus subunits resides in its N-terminal, periplasmic domain.
Results

Expression and purification of the soluble N-terminal periplasmic domain of FimD

Figure 1A shows the predicted topology of a FimD subunit in the outer E. coli membrane, based on an epitope tagging analysis and computer modeling study on the related assembly platform FaeD from the K88 pilus system\textsuperscript{19}. Sequence alignment of FimD with FaeD predicts that FimD is composed of an N-terminal and a C-terminal domain oriented towards the periplasm, and a central, porin-like transmembrane domain. Based on this model, we attempted to express the predicted N-terminal FimD domain with its natural signal sequence, and the predicted C-terminal FimD domain with the *dsbA* signal sequence, as isolated proteins in the periplasm of *E. coli*. While we failed to produce the C-terminal FimD segments by periplasmic expression, three different constructs of the predicted N-terminal domain, ending at residues 126, 134, or 139 of the mature FimD protein (Figure 1B), were obtained in high quantities as soluble proteins in the periplasm. To ensure that no functionally important residues of the N-terminal FimD domain are missing, we focussed on the longest, expressed construct (C-terminus at residue 139) and termed this fragment FimDN. FimDN was then overproduced in the periplasm of the protease-deficient *E. coli* strain HM125 and purified to homogeneity from the periplasmic fraction by ion exchange and hydrophobic chromatography with yields of 2.3 mg per liter of bacterial culture and OD\textsubscript{600} (Figure 2A, inset). Edman sequencing and MALDI-TOF mass spectrometry showed that FimDN has an unusually long signal sequence of 45 residues. Cleavage of the signal sequence after residue 45 is however in agreement with the consensus sequence of *E. coli* signal peptidase\textsuperscript{20}. Based on this result, we numbered the residues of FimD according to the sequence of the mature FimDN (Figure 1A and 1B).

FimDN is a monomeric, autonomous folding unit

Far-UV circular dichroism (CD) spectra indicated that purified FimDN predominantly consists of β-sheets (Figure 2B). The two highly conserved cysteine residues within the N-terminal region of assembly platforms (Cys63 and Cys90 in FimD) were shown to quantitatively form a disulfide bond in purified FimDN through Ellman’s assay after unfolding (data not shown). When FimDN was unfolded by 5 M guanidinium chloride (GdmCl) (Figure 2B), reduced by dithiothreitol, and refolded under reducing conditions, a far-UV CD spectrum almost identical to that observed for the oxidized protein was obtained (data not shown), indicating that the disulfide bond is not strictly required for folding of FimDN.
Results

**chaperone-subunit complex binding domain of FimD**

to that observed for the oxidized protein was obtained (data not shown), indicating that the disulfide bond is not strictly required for folding of FimD$_N$.

We next investigated the oligomerization state of purified FimD$_N$ by analytical ultracentrifugation. Equilibrium sedimentation analysis at pH 8.0 and 20 °C (Figure 2A) yielded a monodisperse species with a molecular mass of 13020 ± 182 Da, which is in good agreement with the calculated mass of 15179 Da for the mature FimD$_N$ monomer. This suggests that the N-terminal domain of FimD alone is not sufficient to trigger association of FimD subunits to ring-shaped oligomers of full-length FimD in the outer membrane.

To test whether purified FimD$_N$ represents a cooperative folding unit with intact tertiary structure, denaturant- and heat-induced unfolding transitions were recorded (Figure 3A and 3B). Unfolding and refolding of FimD$_N$ in GdmCl, followed by far-UV CD at 218 nm, yielded cooperative, fully reversible transitions (Figure 3A). Evaluation of the data according to the two-state model of folding revealed a free energy of folding of $-33.0 ± 2.4$ kJ mol$^{-1}$ at pH 7.4 and 25 °C. The cooperativity of folding (m-value) of $14.3 ± 1.1$ kJ mol$^{-1}$ M$^{-1}$ is in good agreement with the m-value expected for a 139 residue protein (13.9 kJ mol$^{-1}$ M$^{-1}$)$^{21}$. Thermal unfolding of FimD$_N$ was followed by differential scanning calorimetry and also proved to be reversible, as two successive scans of the same sample were almost superimposable (Figure 3B). The thermogram is characterized by a single cooperative, endothermic transition with a thermal transition midpoint ($T_m$) at $66.8 ± 0.2$ °C and a calorimetric enthalpy ($\Delta H_{cal}$) of $254.5 ± 3.0$ kJ mol$^{-1}$ (Figure 3B). The van't Hoff enthalpy ($\Delta H_{vH}$) of $264.0 ± 3.1$ kJ mol$^{-1}$ was calculated from the same data set assuming a two-state transition equilibrium, and the obtained ratio $\Delta H_{cal}/\Delta H_{vH}$ of 0.96 is in good agreement with a two-state thermal unfolding process of FimD$_N$.$^{22}$ Overall, these data clearly demonstrate that FimD$_N$ forms a stable, autonomously folding domain and possesses a well-defined tertiary structure. This is further confirmed by the pronounced chemical shift dispersion in the one-dimensional $^1$H-NMR spectrum of FimD$_N$ (Figure 3C).

**FimD$_N$ selectively recognizes FimC-subunit complexes**

In order to test whether FimD$_N$ interacts with the type 1 pilus chaperone FimC, individual type 1 pilus subunits, or chaperone-subunit complexes, we first performed experiments with the adhesin FimH, as previous surface plasmon resonance experiments had revealed that wild type FimD has the highest affinity to FimH among all chaperone-subunit complexes$^{18}$. In addition to full-length FimH, we used its isolated pilin domain, FimH$_C$, because FimH$_C$ is the
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*chaperone-subunit complex binding domain of FimD*

only part of FimH that interacts with FimC, has the same overall fold as all other type 1 pilus subunits\(^6\), and a lower tendency than the full-length protein to aggregate non-specifically\(^23\). The potential affinity of FimD\(_N\) towards assembly intermediates of type 1 pili was first tested by analytical gel filtration. For this purpose, FimD\(_N\) was incubated either with equimolar amounts of isolated FimC, the isolated pilin domain FimH\(_C\), or the FimC-FimH\(_C\) complex for one hour at pH 7.4 prior to gel filtration. Figure 4A shows that FimD\(_N\) forms a stable, ternary complex with FimC and FimH\(_C\). In contrast, neither isolated FimC nor FimH\(_C\) is recognized by FimD\(_N\). (Figure 4B). Analysis of the eluted fraction corresponding to the FimD\(_N\)-FimC-FimH\(_C\) ternary complex by SDS-PAGE confirmed the presence of all three proteins in the complex (Figure 4A, inset).

Binding of FimD\(_N\) to the FimC-FimH\(_C\) complex at pH 7.4 and 25 °C was then determined quantitatively by isothermal titration calorimetry (Figure 5). Titration data revealed a 1:1 stoichiometry of binding and a dissociation constant (K\(_D\)) of 1.2 \(\mu\)M (Figure 5, Table 1). Moreover, the obtained thermodynamic parameters show that binding of FimD\(_N\) to the FimC-FimH\(_C\) complex is enthalpy-driven (\(\Delta H = -50.2 \pm 11.1 \text{ kJ mol}^{-1}\)) and entropically unfavorable (\(\Delta S = -57.0 \text{ J mol}^{-1} \text{ K}^{-1}\)) (Table 1). An identical titration experiment was performed for binding of FimD\(_N\) to the complex between FimC and full-length FimH. The thermodynamics of binding were indistinguishable within experimental error from those obtained for the FimC-FimH\(_C\) complex (Table 1), proving that FimD\(_N\) interacts exclusively with FimC and the pilin domain of FimH in the ternary complex.

We next tested the ability of FimD\(_N\) to interact with complexes between FimC and the subunits FimF, FimG or FimA. Besides full-length FimF and FimG, we used the variants FimF\(_t\) and FimG\(_t\), in which the N-terminal donor strands of the subunits were truncated so that spontaneous oligomerization of non-bound or chaperone-bound subunits was suppressed\(^11\). As we did not detect differences in binding between FimC-bound pilus subunits with and without donor strand (data not shown), we conclude that the donor strand segment of the subunits does not form contacts with FimD\(_N\) in the ternary complex. In gel filtration experiments and subsequent calorimetric titrations, we observed selective recognition of the FimC-FimF\(_t\) complex by FimD\(_N\), with slightly weaker binding (K\(_D\) = 3.4 \(\mu\)M) compared to FimC-FimH\(_C\), but otherwise very similar, enthalpy-driven binding characteristics (Figure 4C, Table 1). In contrast to the FimC-FimH and FimC-FimF\(_t\) complexes, we could not detect binding of FimD\(_N\) to the FimC-FimG\(_t\) or the FimC-FimA complex in gel filtration experiments that were performed under identical conditions (cf. Figure 4D for the experiment with FimC-FimG\(_t\)).
Thus, a potential affinity of FimD$_N$ for the FimC-FimG$_t$ or FimC-FimA complexes, must be at least 10- to 100-fold lower than that observed for the FimC-FimH and FimC-FimF$_t$ complexes.

**Expression of FimD$_N$ competitively diminishes incorporation of FimH at the tip of type 1 pili**

As FimD$_N$ formed soluble, ternary complexes with FimC and the adhesin FimH, we reasoned that overexpression of soluble FimD$_N$ in the periplasm of an *E. coli* wild type cell should compete with the natural FimD assembly platform in the outer membrane for binding of FimC-FimH complexes and thus prevent incorporation of FimH at the tip of type 1 pili. To test this hypothesis, we analyzed *E. coli* W3110 wild type cells transformed with the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible FimD$_N$ expression plasmid for their ability to agglutinate with yeast cells when grown in the presence and absence of IPTG. This assay is based on the specific recognition of mannose units on the cell wall of yeast by FimH, which leads to agglutination of *E. coli* cells expressing intact type 1 pili with yeast cells. Figure 6A shows that agglutination of *E. coli* W3110 with yeast was significantly diminished when FimD$_N$ expression was induced by IPTG, while a wild-type like agglutination phenotype was observed for non-induced cells. As positive and negative controls, agglutination assays with non-transformed W3110 cells and the fimH deletion strain W3110ΔfimH, respectively, were performed under identical conditions (Figure 6A). In another control experiment, it was verified that IPTG had no inhibitory effect on the agglutination assay (data not shown). Analysis of non-induced and induced cells by electron microscopy revealed that overexpression of FimD$_N$ had no influence on the phenotype of piliated W3110 cells with regard to the overall number of pili per cell and the average length distribution of type 1 pili (Figure 6B). This suggests that overexpression of FimD$_N$ in the periplasm competitively and selectively diminished incorporation of FimH at the tip of type 1 pili, but does not affect the overall pilus assembly, and in particular not the incorporation and oligomerization of FimA.

To examine the influence of the overexpression of FimD$_N$ on the relative distributions of the four pilus subunits FimA, FimF, FimG and FimH in type 1 pili directly, we generated polyclonal rabbit antibodies against all four subunits. Type 1 pili from IPTG-induced and non-induced W3110/pFimD$_N$ cells were purified by precipitation with magnesium ions and analyzed for their content of individual subunits by immunoblotting after separation of the subunits by SDS-PAGE. Figure 6C shows that incorporation of FimH into type 1 pili is indeed about 3-fold decreased upon overexpression of FimD$_N$ in the periplasm, while FimD$_N$
Results

Chaperone-subunit complex binding domain of FimD had no significant effect on the incorporation and relative distribution of FimF and FimA. However, even though we could not detect binding of FimD\(N\) to FimC-FimG complexes in vitro, we found that the amount of FimG was decreased in pili isolated from bacteria overexpressing FimD\(N\) (Figure 6C), indicating that FimH favors incorporation of FimG into pili. This is also supported by the reduced relative amount of FimG in pili isolated from the fimH deletion strain W3110\Delta fimH (Figure 6C).
Results

chaperone-subunit complex binding domain of FimD

Discussion

The type 1 pilus assembly platform FimD of *E. coli* is a multifunctional, outer membrane protein complex that is required for a series of critical steps in type 1 pilus assembly. Besides its functions as a translocation pore for folded pilus subunits and membrane anchor for type 1 pili, FimD has been shown to bind the different FimC-type 1 pilus subunit complexes with different affinities. Surface plasmon resonance experiments revealed that FimD possesses the highest affinity for the FimC-FimH complex, while it bound the other FimC-subunit complexes with 20- to 150-fold lower affinity. The donor-strand hypothesis of subunit-subunit interactions within type 1 pili predicts that FimH is either incorporated into type 1 pili as terminal subunit in the tip fibrillum, or not at all. Thus, the high affinity of FimD towards the FimC-FimH complex guarantees that the FimH repertoire in the periplasm is exploited efficiently, and that the majority of type 1 pili bear the adhesin FimH at the tip. FimD is thus a critical regulator of pilus stoichiometry and order of subunit incorporation.

Towards a detailed mechanistic understanding of the functions of FimD, we have succeeded in identifying a periplasmically oriented domain of FimD comprising the N-terminal 139 residues of mature FimD, in which the specificity of FimD for binding of the FimC-FimH complexes is retained. Our finding that residues 1-139 of FimD are responsible for the recognition of FimC-subunit complexes is in agreement with a recent functional characterization of C-terminally truncated variants of the P pilus assembly platform PapC, which also indicated that N-terminal regions of PapC are required for the recognition of chaperone-subunit complexes. Moreover, the selective recognition of chaperone-subunit complexes by FimD, and its inability of binding isolated FimC, guarantees that FimC is released to the periplasm after delivery of a pilus subunit to FimD, and recycled for further steps of subunit binding and release.

Compared to previously published data on oligomeric, full-length FimD isolated from the outer *E. coli* membrane, we found that the monomeric FimD domain binds the FimC-FimH complex with about 100-fold lower affinity (\(K_D = 1.2 \ \mu M\) compared with \(K_D = 9.1 \ \text{nM}\)). A plausible explanation for this fact would be the presence of multiple FimD domains in the FimD oligomer, which may cause higher apparent affinities due to higher local concentrations of binding domains for FimC-subunit complexes compared to monomeric FimD. In addition, surface plasmon resonance generally may yield higher apparent affinities than binding experiments in solution due to local rebinding effects. Furthermore, C-terminal, periplasmically oriented regions of full-length FimD may additionally be involved in...
periplasmically oriented regions of full-length FimD may additionally be involved in recognition of FimC-subunit complexes. We however failed to express the predicted C-terminal, periplasmic domain of FimD (residues 556-833) in the periplasm. This may indicate that the alternative theoretical topology model of the assembly platforms, predicting that their entire C-terminal region is integrated into the outer membrane as β-barrel domain is correct.\textsuperscript{28}

In addition to the weaker binding of the FimC-FimH complex, there are further differences between our present binding data of FimD\textsubscript{N} to FimC-subunit complexes and surface plasmon resonance experiments obtained with the oligomer of full-length FimD.\textsuperscript{18} For example, Saulino et al.\textsuperscript{18} reported that FimC-FimF is bound with 100-fold lower affinity than FimC-FimH ($K_D = 0.9$ $\mu$M compared with $K_D = 9.1$ nM, respectively), while we only found a 3-fold lower affinity for FimC-FimF in the micromolar range (Table 1). Moreover, we could not detect binding of FimD\textsubscript{N} to FimC-FimG or FimC-FimA, while full-length FimD was reported to bind FimC-FimG and FimC-FimA with a $K_D$ of 0.7 $\mu$M and 0.2 $\mu$M, respectively.\textsuperscript{18} One possible explanation for this difference may again be the monomeric state of FimD\textsubscript{N}, in conjunction with a lower intrinsic affinity of FimD for the FimC-FimA and FimC-FimG complexes. In addition, the C-terminal FimD segment 140-833 that includes the transmembrane domain of the assembly platform may contain additional regions involved in recognition and discrimination of chaperone-subunit complexes. Finally, a very attractive explanation for the failure of FimD\textsubscript{N} to bind the FimC-FimA and FimC-FimG complexes compared to FimD would be that an intact FimD oligomer that is pre-loaded with at least one pilus subunit in its central pore (e.g. FimH) is supposed to have much higher affinity for a chaperone-subunit complex than "unloaded" FimD, because a pilus subunit bound to the central pore presents an additional binding site for the exposed donor strand of an incoming subunit bound to FimC and one N-terminal domain of FimD. Our finding that FimD\textsubscript{N} recognizes the complexes FimC-FimF and FimC-FimF\textsubscript{t} equally well indeed indicates that the donor strands of pilus subunits bound to FimC are not involved in binding to FimD\textsubscript{N}. Therefore, the donor strands of pilus subunits are likely to be solvent-exposed and capable of binding to the incomplete immunoglobulin-like fold of a subunit in the translocation pore. Such a model predicts that FimC-subunit complexes with very low or even lacking affinity for FimD\textsubscript{N}, such as the FimC-FimA complex, require pre-loaded FimD for incorporation of the incoming subunit into the pilus. This would guarantee that the pilus rod, which is exclusively composed of FimA subunits, cannot be formed prior to assembly of the tip fibrillum.
As expected from the highest affinity of FimD_N for the FimC-FimH complex, overexpression of FimD_N in the periplasm significantly reduced the incorporation of FimH at the tip of type 1 pili. However, the number of pili per cell and the average pilus length were not affected when FimH was partially missing. In contrast to previous reports, this result clearly indicates that initiation of type 1 pilus assembly is independent of FimH. Although we could not detect binding of FimD_N to the FimC-FimG complex in gel filtration experiments, we observed decreased incorporation of FimG into the tip fibrillum when FimD_N was overexpressed in the periplasm of E. coli wild type cells (Figure 6C). In addition, we also observed a lowered content of FimG in pili isolated from a fimH deletion strain. These data indicate that the presence of FimH at the assembly platform favors incorporation of FimG into pili, and are consistent with previous experiments that suggested that FimG is the subunit next to FimH within the tip fibrillum.

In summary, we have identified a self-folding, periplasmically oriented domain of a pilus assembly platform in which critical properties of intact assembly platforms are retained, i.e., exclusive recognition of chaperone-subunit complexes and highest affinity towards the chaperone-adhesin complex. The identification of FimD_N, which is monomeric and soluble in the absence of detergents, should greatly facilitate future attempts to characterize critical assembly intermediates of type 1 pili at a structural level.
Materials and methods

Bacterial strains and expression plasmids

For periplasmic protein expression, the protease-deficient *E. coli* strain HM125 was used. The *E. coli* K12 wild type strain W3110 was used in the yeast agglutination assay. The *fimH* deletion strain W3110Δ*fimH* was a gift of P. Sebbel and constructed from W3110 by allelic exchange according to Hamilton.

To test the expression of the predicted N-terminal, periplasmic domain of FimD, three constructs with different C-termini were created. The corresponding genes encoding the natural FimD signal sequence and segments of mature FimD were amplified by the polymerase chain reaction (PCR) from the genome of *E. coli* W3110. Oligonucleotide 5'-GGG ATC TCG CAT ATG TCA TAT CTG AAT TTA AG-3' was used as N-terminal primer for all constructs (Ndel site underlined). The following oligonucleotides were used as C-terminal primers: 5'-GCT GGA TCC TCA ACC ACG AGC GCG ATT AC-3' for amplification of segment 1-126 of mature FimD; 5'-GCT GGA TCC TCA GTC CCA TAA CTC AGG-3' for amplification of segment 1-134 of mature FimD; and 5'-GCA GGA TCC TTA CGC ATT ACC GGG GTC CCA TAA CTC-3' for amplification of segment 1-139 of mature FimD (BamHI site underlined). The PCR products were cloned via Ndel and BamHI into pDsbA3. The resulting expression plasmids allow periplasmic expression of the respective genes under control of the trc promoter. The expression plasmid encoding residues 1-139 of mature FimD was termed pFimDN and used for large-scale protein purification. For the expression tests of the predicted C-terminal, periplasmic domain of FimD, four constructs with different N-termini were created. The following oligonucleotides were used to amplify the corresponding segments of mature FimD from the genome of *E. coli* W3110 by PCR. As N-terminal primers: 5'-CCT ACT CAG GCT AGC GCG GGACGG GAT CAG ATG TTA G-3' for amplification of segment 544-833 of mature FimD; 5'-CCT ACT CAG GCT AGC GCG TCT TTC AGC CAC TGG TTG-3' for amplification of segment 556-833 of mature FimD; 5'-CAT ACT GAC GCT AGC GCG GGG GGA GGC GAT GGAAAT AGC-3' for amplification of segment 612-833 of mature FimD; and 5'-GGT TCT CAGGCT AGC GCG GGAGTTAGTGGTGGGGTAC-3' for amplification of segment 651-833 of mature FimD (Ndel sites underlined). Oligonucleotide 5'-GGC GGATCCTTAACGACATTCAGCTG-3' was used as C-terminal primer for all constructs (BamHI site underlined). The amplified fragments were cloned via Ndel and BamHI into pDsbA3 directly after the DsbA signal sequence.
For periplasmic coexpression of either FimGₜ or FimFₜ with FimC, the fimH gene on pFimH-FimC³⁵ was replaced with either the fimGₜ or fimFₜ gene via the XbaI and HindIII restriction sites. The fimGₜ and fimFₜ genes were amplified by PCR from the genome of W3110 using the following oligonucleotides: 5'-GGGACGCATCTAGAGGAGGGATGATTGTAATGAAAAAAGATTTGGCTGGCGCC-3' and 5'-GCACTACCTAATCCACAGCTAAGCTTATGGCCATCCGGACCGGACC-3' for amplification of fimGₜ; 5'-GGGACGCATCTAGAGGAGGGATGATTGTAATGAAAAAAGATTTGGCTGGCGCCGC-3' and 5'-CCTTCATCTAATCCACAGCTAAGCTTATGGCCATCCGGACCGGACC-3' for amplification of fimFₜ (XbaI and HindIII sites underlined). In order to facilitate purification of the FimC-subunit complexes, a His₆-tag was introduced at the C-terminus of coexpressed FimC through site-directed mutagenesis (QuikChange™, Stratagene). The resulting coexpression plasmids were termed pFimGₜ-FimCₜ₈ and pFimFₜ-FimCₜₘ, respectively. In all constructs, the sequences of genes amplified by PCR or modified by site-directed mutagenesis were verified by dideoxy sequencing.

Expression and purification of FimDₙ and FimC-subunit complexes

E. coli HM125³⁰ was transformed with either pFimDₙ, pFimGₜ-FimCₜₘ, pFimFₜ-FimCₜₘ. Cells were grown at 30 °C in 2×YT medium containing ampicillin (100 μg/ml) until an optical density at 600 nm (OD₆₀₀ₓ) of 0.7 was reached. Protein expression was induced with 1 mM IPTG. After further growth for 4 h, cells were harvested by centrifugation, suspended in 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mg/ml polymyxin B sulfate (10 ml per l of culture), and stirred for 2 h at 4 °C. After centrifugation, the supernatants (periplasmic extracts) were collected for further purification.

For purification of FimDₙ, the periplasmic extract was dialyzed against 20 mM Bis-Tris/HCl pH 6.2, and applied to a QA52 cellulose column (Whatman) equilibrated with the same buffer. FimDₙ was eluted with a linear gradient from 0 to 400 mM NaCl. Fractions containing FimDₙ were pooled, and ammonium sulfate was added to a final concentration of 900 mM. This solution was loaded onto a Phenyl Sepharose HP column (Pharmacia). FimDₙ was eluted with a linear gradient from 900 mM to 200 mM ammonium sulfate in 20 mM Bis-Tris/HCl pH 6.2. Fractions containing FimDₙ were pooled, dialyzed against 20 mM Bis-Tris/HCl pH 6.2 and loaded onto a HiPrep 16/10 Q column (Pharmacia). Pure FimDₙ was eluted with a linear gradient from 0 to 400 mM NaCl. Correct N-terminal processing of
eluted with a linear gradient from 0 to 400 mM NaCl. Correct N-terminal processing of FimDN was verified by Edman sequencing and MALDI-TOF mass spectrometry (calculated mass: 15179 Da; measured mass: 15182 Da).

For purification of the complexes FimGt–FimCHis and FimFt–FimCHis, the periplasmic extracts were dialyzed against 50 mM sodium phosphate pH 8.0, 300 mM NaCl, and applied to a Ni-NTA agarose column (Qiagen) equilibrated with the same buffer. The column was washed with the above buffer containing 20 mM imidazole/HCl. The subunit-FimCHis complexes were eluted with a linear gradient from 20 to 250 mM imidazole/HCl in 50 mM sodium phosphate pH 8.0, 300 mM NaCl. Fractions containing the complex were combined, dialyzed against 10 mM MOPS/NaOH pH 8.0, and applied to a Resource S column (Pharmacia) to remove excess FimCHis. The 1:1 complexes were eluted with a linear gradient from 0 to 200 mM NaCl. The complex between FimC with either the pilin domain of FimH (FimHC–FimC) or full-length FimH (FimH–FimC) was prepared as described previously. The identity of all purified proteins was confirmed by Edman sequencing and MALDI-TOF mass spectrometry. The yields of purified FimDN and subunit-FimC complexes were all in the range of 0.8-2.8 mg per liter of bacterial culture.

FimA was purified from intact type 1 pili expressed by the E. coli strain AAEC189 that had been transformed with the plasmid pSH2 (containing the complete fim gene cluster). AAEC189/pSH2 was grown at 37 °C in LB medium containing chloramphenicol (100 µg/ml). Cells were harvested by centrifugation and resuspended in 20 mM sodium phosphate pH 7.4, 150 mM NaCl. Pili were sheared off mechanically using a Diax 600 homogenizer (Heidolph). The debris was removed by centrifugation, and MgCl₂ was added to the supernatant to a final concentration of 0.1 M. After incubation on ice for 1 h, Mg²⁺-precipitated type 1 pili were obtained by centrifugation, and resuspended in 50 mM sodium phosphate pH 7.4. The pilus preparation was then incubated in saturated GdmCl for 3 days to dissociate and unfold the pilus subunits. Refolding of FimA was induced by diluting the GdmCl-saturated solution 1:15 with 20 mM sodium phosphate pH 7.4, 150 mM NaCl containing FimC (37.5 µM) at 3-fold molar excess over FimA (final concentration 12.5 µM). The 1:1 complex between FimA and FimC was separated from excess FimC by preparative gel filtration on a Superdex™ 26/60 column (Pharmacia), equilibrated with 20 mM sodium phosphate pH 7.4, 150 mM NaCl. The identity of the purified FimC-FimA complex (0.3 mg FimC-FimA complex per liter of bacterial culture and OD₆₀₀) was confirmed by SDS-PAGE and MALDI-TOF mass spectrometry (calculated mass of FimA: 15827 Da; measured mass: 15833 Da).
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Protein concentrations
Protein concentrations were measured by their absorbance at 280 nm\(^{37}\). Molar extinction coefficients of 10912 M\(^{-1}\) cm\(^{-1}\), 31750 M\(^{-1}\) cm\(^{-1}\), 55980 M\(^{-1}\) cm\(^{-1}\), 36210 M\(^{-1}\) cm\(^{-1}\), 32370 M\(^{-1}\) cm\(^{-1}\), 26680 M\(^{-1}\) cm\(^{-1}\), 7740 M\(^{-1}\) cm\(^{-1}\), 2680 M\(^{-1}\) cm\(^{-1}\), and 24000 M\(^{-1}\) cm\(^{-1}\), were used for FimDN, FimHc-FimC, FimH-FimC, FimGt-FimC\(_{His}\), FimFt-FimC\(_{His}\), FimA-FimC, FimHc, FimA, and FimC, respectively.

Analytical ultracentrifugation
Sedimentation equilibrium measurements were performed with a Beckman Optima XL-A analytical ultracentrifuge at 20000 rpm and 20 °C in an An60Ti rotor using double sector cells. Analysis was carried out at a protein concentration of 20 μM in 20 mM Tris/HCl pH 8.0. The data were analyzed as described previously\(^{38}\).

CD and NMR spectroscopy
Far-UV CD spectra were recorded in 20 mM HEPES/NaOH pH 7.4, 100 mM NaCl at 25 °C on a JASCO J-710 spectropolarimeter at protein concentrations of 50 μM in a 0.02 cm quartz cuvette. Ten spectra were accumulated, corrected for the buffer, and the CD signal was converted to molar mean residue ellipticity\(^{39}\). One-dimensional \(^1\)H-NMR spectrum of FimDN was recorded on a 600 MHz spectrometer (Bruker DRX 600) at 25 °C. The NMR sample (90 % H\(_2\)O/ 10% D\(_2\)O) contained FimDN at a concentration of 0.7 mM in 20 mM sodium phosphate pH 7.4, 115 mM NaCl.

GdmCl-dependent folding transitions
For equilibrium unfolding transitions of FimDN, the native protein was diluted with 20 mM HEPES/NaOH pH 7.4, 100 mM NaCl containing different concentrations of GdmCl and incubated at 25 °C for 18 h. For measuring refolding transitions, FimDN was first denatured in 5 M GdmCl, 20 mM HEPES/NaOH pH 7.4, 100 mM NaCl for 1 h at room temperature. Unfolded FimDN was then diluted with the above buffer containing different GdmCl concentrations, and incubated at 25 °C for 18 h. The final protein concentration was 5 μM in all samples. Transitions were followed by far UV-CD at 218 nm, evaluated according to the two-state model of folding using a six-parameter fit\(^{40}\), and normalized.
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Differential scanning calorimetry

Measurements were carried out on a VP-DSC micro-calorimeter (Micro-Cal Inc.) at a FimDN concentration of 40 μM in 20 mM sodium phosphate pH 7.4, 115 mM NaCl. The heating rate was 1 °C min⁻¹. Reversibility was tested by repeated heating of the same sample after rapid cooling, and proved to be about 90%. The raw data were corrected for the instrumental buffer-buffer baseline and converted to apparent molar heat capacities. The calorimetric enthalpy (ΔHcal) was obtained by integration of the heat absorption peak after subtraction of the intrinsic heat capacity. Data were fitted according to a two-state transition with ORIGIN (Micro-Cal Inc.).

Isothermal titration calorimetry

Titration experiments were performed with a MCS titration microcalorimeter (Micro-Cal Inc.) at 25 °C. Aliquots of 9 μl of a 290 μM FimDN solution in 20 mM sodium phosphate pH 7.4, 115 mM NaCl were injected into the sample cell containing either 1.36 ml of a solution of FimC-FimH, FimC-FimHc, or FimCHis-FimFt (20 μM each) in the same buffer. The duration of the injection was 11.5 s, and the delay between injections was 300 s. The raw data after integration were corrected for non-specific heat effects, normalized for the molar concentration and analyzed according to a 1:1 stoichiometry of binding with ORIGIN (Micro-Cal Inc.).

Analytical gel filtration

Analytical gel filtration experiments were performed on a Superdex™ 75 HR 10/30 column (Pharmacia) equilibrated with 20 mM sodium phosphate pH 7.4, 115 mM NaCl at a flow rate of 20 ml h⁻¹ at room temperature. All proteins were incubated in the above buffer at a final protein concentration of 20 μM for 1 h at room temperature and then applied to the column. We observed that the isolated pilin domain of FimH (FimHc) is very aggregation-prone and adsorbs non-specifically to the gel filtration column. Non-specific adsorption could be prevented when isolated FimHc was mixed with a 10-fold excess of the synthetic peptide NH3⁺-TENTLQLAIISRIKR-CO₂⁻ corresponding to the FimC donor strand prior to gel filtration. Eluted proteins were detected by their absorbance at 226 nm.
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**Yeast agglutination assay**

*E. coli* strains W3110, W3110ΔfimH and W3110 harboring pFimD_N were grown in 5 ml of M9 minimal medium containing 1 g/l ammonium sulfate, 2 g/l glucose and 100 μg/ml ampicillin in the presence or absence of 1 mM IPTG at 37 °C for 2 days without shaking. After centrifugation, bacteria were suspended in the above buffer to a final OD600 of 5.0. A 250 μl aliquot of the bacterial suspension was mixed with 50 μl of a 10 % (w/v) suspension of dry yeast in 20 mM sodium phosphate pH 7.4, 150 mM NaCl. The extent of agglutination was examined after 5 min.

**Electron microscopy**

Bacteria were grown as described for the yeast agglutination assay. Adsorption of bacteria was performed for 2 min onto glow discharged carbon films mounted on 400 mesh copper grids. They were negatively stained with 1 % uranyl acetate for 15 s. Electron micrographs were recorded on a Philips CM12 electron microscope at an acceleration voltage of 100 kV.

**Immunoblot analysis of subunit content of type 1 pili**

Bacteria were grown in a volume of 1 l as described for the yeast agglutination assay, and pili were isolated as described above. Pilus subunits were dissociated by boiling in 0.1 M HCl for 5 min prior to addition of SDS-sample buffer, separated on 18 % (w/v) polyacrylamide-SDS gels and transferred onto PVDF membranes. Membranes were incubated for 1 h with 8 % (w/v) non-fat dry milk in 20 mM Tris/HCl pH 7.4, 130 mM NaCl, and then for 1 h with either anti-FimA, -FimF, -FimG or -FimH antibodies. After extensive washing, the membranes were incubated for 1 h with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma), and stained as described.
Abbreviations used for the various constructs in this study:

FimD<sub>N</sub>, N-terminal, periplasmically oriented domain of FimD (residues 1-139 of mature FimD); FimH<sub>C</sub>, C-terminal pilin domain of FimH (residues 158-279 of FimH); FimF<sub>t</sub>, FimF truncated at its N-terminus by the supposed donor strand extension (residues 13-154 of mature FimF); FimG<sub>t</sub>, FimG truncated at its N-terminus by the supposed donor strand extension (residues 13-144 of mature FimG).
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Acknowledgements

We thank Dr René Brunisholz and Dr Thomas Denzinger for Edman sequencing and MALDI-TOF mass spectrometry, Dr Peter Tittmann for assistance with electron microscopy, Pascal Bettendorff for NMR measurement, and Andrea Fritz for helping with the preparation of FimC-subunit complexes. We are also grateful to Dr Hauke Lilie of the Martin-Luther-Universität Halle-Wittenberg, Halle, Germany, for his help in ultracentrifugation analysis.
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Figure legends

Figure 1: Predicted domain topology of FimD. (A) Schematic drawing of the predicted transmembrane topology of FimD based on a model for FaeD\(^9\). The individual domains are depicted as boxes, and the corresponding amino acid residues are indicated according to the sequence alignment of FimD with FaeD. (B) Alignment of the predicted N-terminal domain of FaeD with the N-terminal domain of FimD (natural signal sequences are shown in italics). Amino acid residues are numbered according to the mature FimD sequence. Identical residues are boxed in black, conserved residues in gray. Triangles indicate the three different C-termini of the putative N-terminal FimD domain analysed in this study. The first predicted β-strand of the transmembrane domain of FaeD is indicated by an open arrow\(^9\). Clustal W\(^{43}\) was used to align the sequences.

Figure 2: FimD\(_N\) is a structured monomer. (A) Sedimentation equilibrium analysis of FimD\(_N\) at pH 8.0 and 20 °C. Fitting of the data yielded a monodisperse system with an apparent molecular mass of 13020 ± 182 Da (solid line). The lower panel shows the residual error between the fitted and observed values. Inset: Coomassie-stained SDS gel showing the enrichment of FimD\(_N\) during purification. Lane S, molecular mass standard; lane 1, total periplasmic extract of induced cells; lane 2, pooled fractions after purification on QA52; lane 3, pooled fractions after purification on phenyl sepharose; lane 4, purified FimD\(_N\) eluted from HiPrep 16/10 Q. (B) Far-UV CD spectra of native (solid lines) and unfolded (dotted lines) FimD\(_N\) at pH 7.4 and 25 °C.

Figure 3: FimD\(_N\) is a stable, autonomous folding unit with defined tertiary structure. (A) GdmCl-dependent folding transition of FimD\(_N\) at pH 7.4 and 25 °C. Unfolding (closed circles) and refolding (open circles) were followed by far-UV CD at 218 nm. Original data were fitted according to the two state model and normalized (solid line). FimD\(_N\) shows a midpoint of transition at 2.3 ± 0.03 M GdmCl, a m-value of 14.3 ± 1.1 kJ mol\(^{-1}\) M\(^{-1}\) and a ΔG of -33.0 ± 2.4 kJ mol\(^{-1}\). (B) Temperature-dependence of the molar heat capacity of FimD\(_N\) (40 μM) at pH 7.4 followed by differential scanning calorimetry. The experimentally observed data (squares) is nearly superimposable with the fit (solid line) for the reversible denaturation of FimD\(_N\) according to the two state model, yielding a T\(_m\) of 66.8 ± 0.2 °C and ΔH\(_{cal}\) of 254.5 ± 3.0 kJ mol\(^{-1}\). The dashed line represents the repeated heating scan of the same sample after
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rapid cooling. The dotted line represents the intrinsic heat capacity change. (C) One-dimensional 600-MHz $^1$H-NMR spectrum of FimD$_N$ at pH 7.4 and 25 °C.

**Figure 4:** Analytical gel filtration experiments on a Superdex 75 HR column at pH 7.4 and 25 °C. FimD$_N$ (20 μM) was incubated with equimolar amounts of the respective proteins for 1 h prior to loading onto the column. (A) Binding of FimD$_N$ to the FimC-FimH$_C$ complex. Solid line, FimD$_N$ incubated together with FimC-FimH$_C$; dashed line, FimC-FimH$_C$; dotted line, FimD$_N$.Inset: SDS-PAGE analysis of FimD$_N$ (lane 1), FimC-FimH$_C$ (lane 2) and the peak fraction (10.5 ml elution volume) collected from the run with the FimD$_N$-FimC-FimH$_C$ ternary complex. (B) FimD$_N$ neither binds the isolated FimC, nor the isolated FimH$_C$. Solid lines, FimD$_N$ incubated together with FimC and FimH$_C$, respectively; dashed lines, FimC and FimH$_C$, respectively; dotted line, FimD$_N$. (C) Binding of FimD$_N$ to the FimC-FimF$_t$ complex. Solid line, FimD$_N$ incubated together with FimC-FimF$_t$; dashed line, FimC-FimF$_t$; dotted line, FimD$_N$. Identical results were obtained when full-length FimF was used instead of FimF$_t$. (D) Monomeric FimD$_N$ does not bind the FimC-FimG$_t$ complex in vitro. Solid line, FimD$_N$ incubated together with FimC-FimG$_t$; dashed line, FimC-FimG$_t$; dotted line, FimD$_N$. Identical results were obtained when full-length FimG was used instead of FimG$_t$.

**Figure 5:** Analysis of binding of FimD$_N$ to FimC-subunit complexes at pH 7.4 and 25 °C by isothermal titration calorimetry. As an example, the titration of the FimC-FimH$_C$ complex with FimD$_N$ is shown. Upper panel: The heat released upon consecutive 9 μl injections of a solution of FimD$_N$ (290 μM) into the sample cell containing the FimC-FimH$_C$ complex (20 μM). Lower panel: Integrated heats after correction for non-specific heat effects and normalization for the molar concentration. The solid line represents the best non-linear least-squares fit of the data with a 1:1 stoichiometry of binding.

**Figure 6:** Overexpression of FimD$_N$ in vivo competitively diminishes incorporation of FimH and FimG at the tip of type 1 pili. (A) Yeast agglutination assay. E. coli strains W3110, W3110ΔfimH and W3110 harboring pFimD$_N$ were mixed with yeast cells. Cells producing FimD$_N$ in the presence of 1 mM IPTG were compared with non-induced cells. The ability of the strains to agglutinate yeast cells is indicated at the bottom. (B) Electron micrographs of IPTG-induced W3110/pFimD$_N$ (a) and non-induced W3110/pFimD$_N$ (b). (C) Immunoblot analysis of the subunit composition of pilus preparations from different bacterial strains.
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Identical amounts of pilus preparations were boiled for 5 min in 0.1 M HCl to dissociate fimbrial subunits before SDS-PAGE. Blotted proteins were probed with rabbit anti-FimA antibodies (a), anti-FimF antibodies (b), anti-FimG antibodies (c), and anti-FimH antibodies (d), respectively.
**Table 1**: Thermodynamic parameters obtained from calorimetric titrations of FimD with FimC-subunit complexes at pH 7.4 and 25 °C

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<th>Chaperone-subunit complex</th>
<th>$K_D$ (10$^{-6}$ M)</th>
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<th>$\Delta G$ (kJ mol$^{-1}$)</th>
<th>$\Delta S$ (J mol$^{-1}$ K$^{-1}$)</th>
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Table 1
Figure 1

A

Extracellular space

135-555

Outer membrane

FimD

FaeD

MSYLNLRLYQRNTQCLHIRKHRLAGFFVRLVVA

MKKYVTTKSVOPVAFLTLTSLVMSAVLG

1-134

H$_3$N$^+$

CO$_2^-$

Periplasm

B

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<th>FaeD</th>
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Figure 1
Figure 2

**A**

- Graph showing absorbance at 280 nm vs. radius (cm).
- Insert with molecular weight markers (kDa) and bands labeled S, 1, 2, 3, 4.

**B**

- Graph showing ellipticity (degree cm² dmol⁻¹) vs. wavelength (nm).
- Data points indicating changes at different wavelengths.
Figure 3

(A) Fraction of native molecules vs. [GdmCl] (M)

(B) Molar heat capacity (kJ K⁻¹ mol⁻¹) vs. Temperature (°C)

(C) NMR spectrum of H₂O
Figure 4

(A) Absorbance at 226 nm

(B) Absorbance at 226 nm

(C) Absorbance at 226 nm

(D) Absorbance at 226 nm

Figure 4
Results

chaperone-subunit complex binding domain of FimD

Figure 5
Figure 6

A

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B

C

Figure 6
2.2 Structural basis of chaperone–subunit complex recognition by the type 1 pilus assembly platform FimD

Mireille Nishiyama, Reto Horst, Oliv Eidam, Torsten Herrmann, Oleksandr Ignatov, Michael Vetsch, Pascal Bettendorff, Ilian Jelesarov, Markus Grütter, Kurt Wüthrich, Rudi Glockshuber and Guido Capitani

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Abstract

Adhesive type 1 pili from uropathogenic *Escherichia coli* are filamentous protein complexes that are attached to the assembly platform FimD in the outer membrane. During pilus assembly, FimD binds complexes between the chaperone FimC and type 1 pilus subunits in the periplasm and mediates subunit translocation to the cell surface. Here we report NMR and X-ray protein structures of the N-terminal substrate recognition domain of FimD (FimD<sub>N</sub>) before and after binding of a chaperone–subunit complex. FimD<sub>N</sub> consists of a flexible N-terminal segment of 24 residues, a structured core with a novel fold, and a C-terminal hinge segment. In the ternary complex, residues 1–24 of FimD<sub>N</sub> specifically interact with both FimC and the subunit, acting as a sensor for loaded FimC molecules. Together with *in vivo* complementation studies we show how this mechanism enables recognition and discrimination of different chaperone–subunit complexes by bacterial pilus assembly platforms.
Results

Introduction

A wide variety of pathogenic bacteria possess adhesive surface organelles ("pili") that mediate binding to host tissue. These highly oligomeric, filamentous protein complexes are anchored to the outer bacterial membrane (Jones et al., 1995). Type 1 pili from uropathogenic *Escherichia coli* strains are required for bacterial attachment to mannose units of the glycoprotein receptor uroplakin Ia on the surface of urinary epithelial cells, and thus mediate the first critical step in the infection process (Mulvey et al., 1998; Zhou et al., 2001). In addition, type 1 pili are responsible for bacterial invasion and persistence in target cells (Baorto et al., 1997; Martinez et al., 2000). The quaternary structure of type 1 pili is characterized by a 6.9 nm wide pilus rod consisting of a right-handed, helical array of 500 to 3000 copies of the most abundant structural subunit, FimA, and a linear tip fibrillum composed of the adhesin FimH and several copies of the subunits FimG and FimF (Hahn et al., 2002; Jones et al., 1995) (Figure 1).

Biogenesis of type 1 pili is governed by the chaperone-usher pathway (Sauer et al., 2004; Thanassi and Hultgren, 2000). The assembly machinery is composed of two specialized classes of proteins: a periplasmic chaperone and an outer membrane assembly platform, which is also referred to as the usher. The periplasmic type 1 pilus chaperone FimC forms stoichiometric complexes with pilus subunits, catalyzes their folding, and transports them to the assembly platform FimD in the outer membrane (Jones et al., 1993; Vetsch et al., 2004) (Figure 1). The X-ray structure of the FimC–FimH complex (Choudhury et al., 1999), as well as the structures of the related chaperone–subunit complexes PapD–PapK (Sauer et al., 1999), PapD–PapE (Sauer et al., 2002) and Caf1–Caf1M (Zavialov et al., 2003) have shown that pilus subunits have an incomplete immunoglobulin-like fold that lacks the seventh, C-terminal β-strand (referred to hereafter as "pilin fold"). In chaperone–subunit complexes, the missing β-strand is provided by a polypeptide segment of the chaperone, the "donor strand", which is inserted parallel to the sixth strand of the subunit (Choudhury et al., 1999; Sauer et al., 1999; Sauer et al., 2002; Zavialov et al., 2003). In the assembled pilus, an N-terminal extension of about 15 residues, preceding the pilin fold, acts as the donor strand and complements the pilin fold of the adjacent pilus subunit (Sauer et al., 2002; Zavialov et al., 2003). In this way, each subunit provides its own donor strand to the preceding subunit and accepts a donor strand from the following subunit. In contrast to the chaperone–subunit complexes, the orientation of the inserted donor strand in the pilus is antiparallel to the sixth β-strand of the preceding
Results

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subunit. Structure comparison of a chaperone-bound subunit and the same subunit in complex with another subunit indicates that a conformational transition of the pilin fold occurs upon exchange of the donor strand during subunit assembly (Sauer et al., 2002; Zavialov et al., 2003). It has been proposed that this conformational change is the driving force for pilus assembly (Sauer et al., 2002; Zavialov et al., 2003). This hypothesis is further supported by the observations that pilus assembly is independent of ATP and of an electrochemical gradient (Jacob Dubuisson et al., 1994), and that subunit-subunit complexes are thermodynamically more stable than chaperone-subunit complexes (Vetsch et al., 2004).

The type 1 pilus assembly platform FimD is a multifunctional outer membrane protein of 833 residues (Klemm and Christiansen, 1990), which not only anchors the pilus to the cell surface, but simultaneously recognizes FimC-subunit complexes in the periplasm and mediates translocation of folded subunits through the outer membrane (Saulino et al., 2000; Saulino et al., 1998). In spite of the fundamental role of assembly platforms in pilus biogenesis, no structural information on the atomic level is available to date. Based on electron microscopy data on its P pilus homologue PapC, FimD is supposed to form a pore of about 2 nm diameter into the outer membrane (Saulino et al., 2000; Li et al., 2004). This pore size would be wide enough for translocation of individual folded subunits from the periplasm to the cell surface, but not for translocation of the helical pilus rod, which appears to attain its final quaternary structure only on the cell surface (Bullitt and Makowski, 1995).

In a previous study, we showed that FimD possesses an N-terminal periplasmic domain, FimD_N, comprising residues 1–139. FimD_N is soluble in the absence of detergents, folds autonomously, and specifically binds FimC-subunit complexes with micromolar affinities although FimC or pilus subunits alone are not recognized (Nishiyama et al., 2003). In accordance with these data, the recognition site of chaperone–subunit complexes in PapC was also localized to the N-terminal 124 residues (Ng et al., 2004). Nevertheless, it has been discussed controversially whether the N-terminal chaperone–subunit binding region of pilus assembly platforms is an independent periplasmic domain (Harms et al., 1999, Nishiyama et al., 2003), or belongs to the porin-like β-barrel transmembrane domain of FimD (Henderson et al., 2004; Ng et al., 2004).

Here we report nuclear magnetic resonance (NMR) and X-ray protein structures that provide snapshots of the initial step of pilus formation at the site of the assembly platform, i.e. the chaperone–subunit recognition domain of an assembly platform before and after binding of a chaperone–subunit complex. The NMR structure of isolated FimD_N reveals that this domain consists of a flexible, N-terminal “arm” (residues 1–24), a structured “core” (residues 25–125)
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with a novel polypeptide fold, and a potential hinge segment (residues 126–135) that connects the structured core to the transmembrane region of FimD. The most remarkable feature of FimD is its flexible N-terminal arm, which adopts a defined conformation only upon binding to the complex between FimC and the pilin domain of FimH (FimHp, residues 158–279 of FimH), as revealed by the 1.8 Å crystal structure of the ternary FimD-FimC-FimHp complex. The structural data, in conjunction with biochemical experiments and *in vivo* complementation studies, suggest a mechanism in which the assembly platform utilizes its flexible N-terminal segment 1–24 to accomplish specific recognition of different chaperone–subunit complexes.
Results

Results and Discussion

The NMR solution structure of free FimD<sub>N</sub> reveals a previously unknown fold with mobile chain ends

Initial NMR experiments with FimD<sub>N</sub>(1-139) (residues 1-139 of FimD) showed that this construct is susceptible to N-terminal degradation when incubated for several days at 25 °C and a concentration of 1 mM, most likely due to minute protease contaminations. Edman sequencing and mass spectrometry revealed non-specific N-terminal degradation of FimD<sub>N</sub>(1-139) with cleavage after residues Leu9, Ala10, Gln13 and Ser20 (data not shown). Moreover, measurement of [15N,1H]-NOEs showed that the segment 1–24 of the polypeptide chain is flexibly disordered (Supplementary Figure S1). We then incubated the ternary complex formed by FimD<sub>N</sub>(1-139), FimC and the pilin domain of FimH (FimH<sub>p</sub>) under identical conditions. In the complex, we observed specific and quantitative cleavage of FimD<sub>N</sub>(1-139) at a single site close to the C-terminus (Ala125), but no N-terminal degradation was observed. Comparison of the thermal stabilities of FimD<sub>N</sub>(1-139) and its truncated variants FimD<sub>N</sub>(25-139) and FimD<sub>N</sub>(1-125) at pH 7.4, which were monitored by the far-UV circular dichroism signal at 218 nm, showed identical transition midpoints (T<sub>m</sub>) of 67.6 ± 0.5 °C for all the constructs (Supplementary Table S1). Combined with the aforementioned NMR data, the thermal denaturation data show that the segment 25–125 of FimD<sub>N</sub>(1-139) adopts a stable tertiary structure, independent of whether or not the terminal chain segments are present.

Based on these data, we decided to perform a NMR structure determination of the N- and C-terminally truncated protein fragment FimD<sub>N</sub>(25–125) (Figure 2, A and B). The scaffold of the tertiary structure is formed by a three-stranded, antiparallel β-sheet (β<sub>1</sub> to β<sub>3</sub>) consisting of residues 31–39, 42–53 and 60–62, and a two-stranded, antiparallel β-sheet (β<sub>4</sub> and β<sub>5</sub>) comprising residues 101–105 and 110–114, respectively. The two β-sheets are connected by a peptide segment comprising a single-turn 3<sub>10</sub>-helix (residues 76–78), and the α-helices α<sub>1</sub> (residues 66–72) and α<sub>2</sub> (residues 93–96). The invariant cysteine pair (Cys63 and Cys90; cf. Figure 4) forms a disulfide bond stabilizing this peptide segment. A second 3<sub>10</sub>-helix (residues 117–119) is located close to the C-terminus. The helices α<sub>1</sub> and α<sub>2</sub> are packed tightly against the β-sheets, with Met72 of α<sub>1</sub> in direct contact with Met44 and Leu39 of β<sub>2</sub> and Leu113 of β<sub>5</sub>, and Leu93 of α<sub>2</sub> in contact with Ala102 of β<sub>4</sub> and Leu113 of β<sub>5</sub>. The helices α<sub>1</sub> and α<sub>2</sub> pack at an angle of 50° to each other, with pronounced hydrophobic interactions between Leu69 and
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Leu93. A comparison of the structure of FimDN(25–125) with the structures deposited in the Protein Data Bank (PDB) (Berman et al., 2000) using the DALI server (Holm and Sander, 1998) identified no structural homologues. The two structurally most closely related proteins, PDB entries 1SFO and 1T0Y, exhibited Z-scores of 2.1 and 2.0, respectively, with r.m.s.d. values for the Cα atoms of 3.8 and 3.6 Å over 51 and 58 aligned residues, respectively. This shows that FimDN(25–125) represents a previously unknown polypeptide fold. In addition, the NMR data confirm that FimDN(25–125) forms a self-folding periplasmic domain that precedes the transmembrane domain of FimD, and they are in clear-cut contrast with models predicting that the N-terminal region of the assembly platform belongs to the β-barrel transmembrane domain (Ng et al., 2004; Henderson et al., 2004).

In order to study the role of the segment 126–139, which is supposed to connect FimDN(25–125) to the transmembrane domain of FimD (according to a topology prediction program by Martelli et al. (2002), residue 138 is the first residue of a transmembrane β-barrel of FimD), we further solved the NMR structure of FimDN(25–139) (Figure 2C). Except for the additional C-terminal residues, the structure of FimDN(25–139) is in very close agreement with that of FimDN(25–125), with a r.m.s.d. of 1.0 Å for the Cα atoms of the residues 30–120. Interestingly, the segment 126–135 is not disordered in FimDN(25–139), even though it does not adopt a regular secondary structure (Figure 2C). Although the residues 136–139 show negative [15N,1H]-NOE values indicative of high-frequency internal motions, those of the residues 121–135 are positive, suggesting rotational tumbling with an effective rotational correlation time similar to that for overall tumbling of the globular domain (Supplementary Figure S1). In addition, we identified a network of long-range NOEs connecting side chain protons of Trp133 with Val49, Leu64, Thr65, Glu68 and Met72 of the globular domain. These NOEs define a unique position of the aromatic ring of Trp133 in a binding pocket on the surface of the folded domain FimDN(25–125) (Figure 2D). We interpret these observations in terms of a rapid, intramolecular association/dissociation equilibrium between the domain FimDN(25–125) and the segment 126–135. The fact that FimDN(25–139) and FimDN(25–125) have identical Tm-values indicates that the C-terminal segment 126–135 dissociates in a spectroscopically silent fashion from the folded core of FimDN, presumably at a temperature below the observed Tm-value (Supplementary Table S1). As will be discussed below, there are indications that the intramolecular association/dissociation equilibrium between the domain FimDN(25–125) and the polypeptide segment 126–135 might be related
to a spatial rearrangement of residues 1-125 relative to the transmembrane domain of FimD when chaperone–subunit complexes are bound.

X-ray structure determination of the FimD\(_N(1-125)\)-FimC-FimH\(_P\) ternary complex

The search for optimal crystallization conditions of a ternary complex between FimD\(_N\), FimC, and a bound pilus subunit led us to use protein constructs without disordered segments that might impair crystallization. We therefore investigated the requirement of the flexible segment 1\--24 and the C-terminal region 126–139 of FimD for the recognition of FimC–subunit complexes. In addition, we used the C-terminal pilin domain of FimH (FimH\(_P\), residues 158–279 of FimH) instead of full-length FimH, because the structure of the FimC–FimH complex (Choudhury et al., 1999) had revealed that FimC interacts exclusively with FimH\(_P\). Moreover, the interaction between FimC and FimH\(_P\) through donor strand complementation is representative for all FimC–pilus subunit complexes (Choudhury et al., 1999), and the lectin domain is not required for recognition of the FimC–FimH complex by FimD\(_N(1-139)\) (Nishiyama et al., 2003). Taking these facts into account, we tested the ability of the truncated FimD\(_N\) variants FimD\(_N(12-139)\), FimD\(_N(25-139)\) and FimD\(_N(1-125)\) to bind the FimC–FimH\(_P\) complex in vitro. Analytical gel filtration revealed that residues 1–24 are strictly required for the formation of the FimD\(_N\)-FimC-FimH\(_P\) ternary complex (Figure 5B), although they are disordered in the NMR structure of isolated FimD\(_N(1-139)\) (Supplementary Figure S1). The requirement of segment 1–24 was confirmed by the observation that deletion of residues 1–12 or residues 1–24 in full-length FimD completely abolished the ability of plasmid-encoded FimD to restore type 1 pilus formation in an E. coli fimD deletion strain (W3110\(\Delta fimD\)) (Figure 5A). In contrast, residues 126–139 in FimD\(_N\) are not needed for the formation of the ternary complex in vitro, since the variant FimD\(_N(1-125)\) exhibits the same affinity towards the FimC–FimH\(_P\) complex as full-length FimD\(_N(1-139)\) (Supplementary Table S2).

Based on these results, we crystallized the ternary complex between FimD\(_N(1-125)\), FimC, and FimH\(_P\), and obtained two different crystal forms, A and B, with space groups P\(_{6}\)\(_3\) and P\(_2\)\(_1\)\(_2\)\(_1\), respectively. The structure of the ternary complex was solved with data collected from a single crystal of form B at 1.8 Å resolution through molecular replacement based on the structure of the FimC–FimH complex (Choudhury et al., 1999). Structure refinement resulted in R-factor and free R-factor values of 0.19 and 0.22, respectively (Table 2). The final model encompasses residues 1–205 of FimC, residues 158–279 of FimH\(_P\), and residues 1–9 and 19–125 of FimD\(_N(1-125)\) (Figure 3). Residues 10–18 of FimD\(_N(1-125)\) were not
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inclusion of the model due to missing electron density. The lack of electron density in this region was confirmed by computation of a simulated-annealing omit map. As the FimD segment 10–18 is also disordered in the electron density map obtained from crystal form A, the lack of density in this region appears to be intrinsic to the FimD-FimC-FimHp complex. The nature of the structural disorder was further investigated by measurements of heteronuclear $^{15}$N,$^1$H-NOEs for FimD in the ternary complex (Figure 2F), which showed that the effective rotational correlation time of the residues 10–18 is significantly shorter than that for the structured parts of the protein. Interestingly, the residues Asn5 and Arg7 have positive $^{15}$N,$^1$H-NOE values, which is an indication that these residues get immobilized upon complex formation.

Both the N-terminal arm 1–24 and the structured core 25–125 of FimD contribute to recognition of FimC–subunit complexes

The crystal structure of the FimD-FimC-FimHp complex reveals a unique mechanism for recognition of the chaperone–subunit complex by the bacterial pilus assembly platform. FimC interacts via its N-terminal domain (residues 1–116) with both the pilin domain of FimH, through donor strand complementation, and with the globular core of FimD (residues 25–125). The FimD core thereby forms no direct contacts with the pilin domain (Figure 3A). Comparison of the NMR structure of isolated FimD(25–125) and the crystal structure of FimD(1–125) in the ternary complex reveals that the core of FimD does not undergo significant conformational changes upon binding to the FimC–FimHp complex (r.m.s.d. = 1.2 Å for the Cα atoms of residues 28–121) (Supplementary Figure S2A). Moreover, the structures of FimC and FimHp in the ternary complex are closely similar to those in the previously published FimC–FimH binary complex, with some local differences (see Supplementary Material). Importantly, the residues 1–24 of the N-terminal “arm”, which are completely unstructured in free FimD, become ordered upon complex formation and specifically interact with both FimC and the bound pilin domain (Figure 3A). The interactions formed by the N-terminal FimD arm comprise 60% of the total interface area of 1260 Å² between FimD(1–125) and the FimC–FimHp complex. The other 40% of the contact area are contributed by the folded core FimD(25–125), which exhibits a complementary surface to FimC. The total interface area of FimD(1–125) in the ternary complex is in good agreement with the average value of 1210 Å² that was calculated for a set of protein–protein complexes with dissociation constants in the micromolar range (Nooren and Thornton, 2003) ($K_D = 1.2$
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µM for the interaction between FimD_N and the FimC–FimHp complex (Nishiyama et al., 2003)).

The N-terminal arm 1–24 of FimD_N thus serves as a sensor that selectively detects loaded FimC molecules. Because the arm is the only FimD_N region that forms contacts with the chaperone-bound subunit, it may be exclusively responsible for the discrimination of the different FimC–subunit complexes by the assembly platform (Saulino et al., 1998). FimD binds to different FimC–subunit complexes with different affinities, which is a key element for correct initiation of pilus assembly and for the correct ordering of the subunit incorporation into the pilus (Saulino et al., 1998; Nishiyama et al., 2003). The X-ray structure of the FimD_N(1–125)–FimC–FimHp complex thus predicts that the common element of the interactions of FimD_N with the four different FimC–subunit complexes (Figure 1) is the contact area between the N-terminal FimC domain and the structured domain 25–125 (Figure 3D). This contact area alone is, however, neither sufficient for binding of FimC–subunit complexes to FimD_N (cf. Figure 5), nor for stable binding of the free chaperone to the assembly platform (Saulino et al., 1998; Nishiyama et al., 2003). The fact that FimC alone is not bound by FimD ensures that FimC is released to the periplasm for another reaction cycle as soon as the bound subunit dissociates from the ternary complex and is delivered to the translocation pore.

Conserved hydrophobic interactions dominate the recognition of the FimC–FimHp complex by FimD_N(1–125)

A striking feature of the N-terminal FimD_N arm is the crowding of the three aromatic residues Phe4, Phe8 and Phe22 in the sequence, which make hydrophobic contacts with the FimC–FimHp complex. As shown in Figures 3B and 3C, Phe8 protrudes deeply into the hydrophobic core of the FimC–FimHp interface and interacts with residues Ile90 and Gln104 of FimC. Specific contacts from FimD_N to FimHp are formed to the FimHp residues Gln269 and Ile271 (Figure 3B). The interaction between the globular domain FimD_N(25–125) and the N-terminal FimC domain is stabilized by hydrophobic interactions and hydrogen bonds, as well as by a salt bridge between Asp36 of FimD_N and Arg66 of FimC (Figure 3D). Sequence alignment shows that the corresponding salt bridge is also present in the PapD–PapC contact area (Arg68 in PapD and Asp35 in PapC), which provides a rationale for the finding that the Arg68 in PapD is required for P pilus biogenesis in vivo (Hung et al., 1999) (see also Figure 4). In addition to specific side chain contacts, there are also main-chain hydrogen bonds between FimC and the FimD_N core (Figure 3D). Furthermore, a hydrophobic cluster is formed...
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by Leu28 and Tyr33 of FimD_N and Pro52 of FimC, and Gly107 of FimD_N makes hydrophobic contact with Asn63 of FimC. Overall, multiple interactions in the protein–protein interface thus define the specificity of the FimC–FimD contact, making FimC and FimD a functional chaperone/assembly platform pair (Jones et al, 1993).

The biological significance of the recognition of FimC–subunit complexes by FimD_N

In order to test the biological significance of the molecular interactions between FimD_N and the chaperone–subunit complex observed in the X-ray structure of the FimD_N–FimC–FimHp complex, we performed functional tests after replacing individual amino acids in FimD (Phe4Ala, Phe8Ala, Phe22Ala, Tyr33Ala, Asp36Ala, Gln109Ala) or FimC (Leu32Gly, Leu32Glu, Ile90Arg) that form specific contacts in the interface between FimD_N and the FimC–FimHp complex (cf. Figure 3). The FimD mutations were first introduced into full-length FimD, and the mutant proteins were tested for their ability to complement FimD deficiency in an E. coli fimD deletion strain through agglutination assays with yeast cells (Mirelman et al, 1980). We then introduced the same mutations into FimD_N(1–139), expressed and purified the mutant proteins, and tested them for their ability to form ternary complexes with the FimC–FimHp complex in vitro. Similarly, the FimC variants Leu32Gly, Leu32Glu, Ile90Arg were analyzed for FimC complementation in the fimC deletion strain W3110ΔfimC, and the corresponding purified variants were tested for formation of a ternary complex with wild-type FimD_N(1–139) and FimHp. The results from these experiments are summarized in Figure 5, which shows that all the mutations leading to the loss of formation of the ternary complex in vitro also lead to the complete or partial loss of the formation of functional type 1 pili in vivo (cf. also Supplementary Figure S3A).

The FimC variants Leu32Glu and Ile90Arg completely lost biological activity and no longer formed ternary complexes in vitro (Figure 5), showing that the conserved residues Leu32 and Ile90 are required for recognition of chaperone–subunit complexes by FimD_N. Only the FimC variant Leu32Gly was active, which provides a rationale for the fact that residue 32 is either Leu or Gly in the entire chaperone family (cf. Figure 4). All FimC variants retained the ability to form the binary complex with FimHp (Figure 5B), which was expected given the fact that the FimC residue exchanges are located opposite to the subunit-binding site (Figure 3A). In FimD variants, all amino acid replacements and truncations in the arm 1–24 completely abolished FimD function, although the variant proteins were expressed at the same level as wild-type FimD (Supplementary Figure S3B). These data explain the finding of Ng et al (2004) that alanine substitution of Phe3 in the N-terminal segment of the P pilus assembly.
platform PapC, and deletion of residues 3–12 in FimD or residues 2–11 in PapC abolish pilus biogenesis (cf. also Figure 4). In contrast, the FimD replacements Tyr33Ala, Asp36Ala, and Gln109Ala in the contact area of FimD_N(25–125) did not disrupt FimD function in vivo. However, isothermal titration calorimetry showed that the affinity of the corresponding FimD_N(1–139) variants for the FimC–FimH_p complex was lowered ($K_D = 7.1 \mu M$ and $4.2 \mu M$ for the variants Asp36Ala and Gln109Ala, respectively, as compared to $1.2 \mu M$ for the wild-type protein; Supplementary Table S2). Overall, the FimD mutagenesis experiments indicate that the contacts formed by the N-terminal FimD arm of residues 1–24 make the dominant energetic contributions to the recognition of chaperone–subunit complexes, and are thus crucial for the function of assembly platforms. In particular, the hydrophobic interactions formed by the aromatic residues Phe4 and Phe22 in the N-terminal arm of FimD are indispensable for ternary complex formation. Intriguingly, the positions 4 and 22 of homologous assembly platforms are strongly conserved (Figure 4).

**TROSY-NMR chemical shift mapping indicates a movement of the C-terminal hinge segment of FimD_N upon binding of the FimC–FimH_p complex**

To further analyze the conformational changes associated with the formation of the ternary FimD_N–FimC–FimH_p complex, we mapped chemical shift variations between isolated FimD_N(1–139) and FimD_N(1–139) in the ternary complex with $^{15N,1H}$-TROSY-NMR, using $^{15N,2H}$-labeled FimD_N(1–139) and unlabeled FimC–FimH_p complex. Figure 2E shows that only eight residues in FimD_N(1–139) have $[^{15N,1H}]$-chemical shift variations, $\Delta \delta_{NH}$, larger than 0.075, namely Asn5, Arg7, Asp36, Arg47, Arg125, Glu131, Trp133, and Asp134. Large chemical shift changes for Asn5, Arg7, and Asp36 can readily be rationalized by the X-ray structure of the ternary FimD_N(1–125)–FimC–FimH_p complex, since these residues form specific contacts either with FimC or the FimH pilin domain. The extensive chemical shift changes observed for Arg125, Glu131, Trp133 and Asp134 suggest a movement of the C-terminal hinge segment upon binding of the FimC–FimH_p complex. When we modeled the C-terminal hinge segment 126–139 of the NMR structure of FimD_N(25–139) into the X-ray structure of the ternary complex, we observed steric clashes between the side chain of Asn138 in FimD and the surface of FimC for all calculated NMR conformers (Supplementary Figure S2B). Particularly significant is the large chemical shift change of Arg47. In the NMR structure of FimD_N(25–125) (shown in red in Supplementary Figure S2C) and in the X-ray structure of the ternary complex (shown in green in Figure S2C), the side chain of Arg47
adopts a bent conformation. In the X-ray structure and in some of the NMR conformers of FimD\textsubscript{N}(25–125) the guanidinium group acts as a hydrogen bond donor to the main chain carbonyl oxygen of Asp48. This conformation of Arg47 is not observed in the NMR structure of FimD\textsubscript{N}(25–139) (shown in blue in Figure S2C), since it would lead to clashes with the residues Pro130, Trp133 and Pro135 of the C-terminal hinge segment. Instead, the side chain of Arg47 protrudes into the bulk solvent (Supplementary Figure S2C). These observations suggest that upon binding of the FimC–FimHp complex, the side chain of Arg47 is set free to move, and most likely assumes again the hydrogen-bonded conformation observed in the absence of the C-terminal hinge segment (i.e., in the X-ray structure, and in the NMR structure of FimD\textsubscript{N}(25–125)). Most importantly, the above-mentioned model considerations reveal that FimC and the bound subunit would collide with the hydrophobic part of the outer membrane, if one assumes that the first β-strand of the transmembrane β-barrel of FimD starts with residue 138, as indicated by the topology prediction program described by Martelli \textit{et al} (2002). Taken together with the observation that segment 126–139 of FimD\textsubscript{N}(1–139) was proteolytically degraded only when complexed with FimC–FimHp, this observation provides convincing evidence for a model in which a partial or complete displacement of the C-terminal hinge segment of FimD\textsubscript{N} relative to the folded core (residues 1–125) occurs upon formation of the ternary complex. FimD\textsubscript{N}(1–139) would thus exist in an "open" conformation capable of binding chaperone-subunit complexes, and a "closed" conformation in which Trp133 is bound to its pocket on the surface of the FimD\textsubscript{N} core 25–125. A movement of FimD\textsubscript{N}(1–139) could also be related to the delivery of pilus subunits to the translocation pore and to the release of the free chaperone to the periplasm for the next assembly cycle.

\textbf{Concluding remarks}

The \textit{E. coli} type 1 pilus system serves as a prototype for adhesive surface organelles produced by a large number of pathogenic bacteria. The presently described structural studies on FimD\textsubscript{N}, with and without bound chaperone-subunit complex, shed light on the molecular basis of the initial interaction of the assembly platform with the chaperone–subunit complex. To further elucidate the cascade of reaction steps following this initial binding event, additional biochemical and structural information on the full-length outer membrane assembly platform will be required. Understanding the molecular details of the mode of action of assembly platforms can be expected to provide a basis for the development of novel antimicrobial agents that would block the assembly of the virulence determinants and hence their adhesion to host tissue.
Materials and methods

Plasmids
Mutations corresponding to amino acid replacements were introduced into the following plasmids, using the QuikChange site-directed mutagenesis kit (Stratagene). For FimD variants, plasmid pfimD_his was used, which contains the entire fimD gene with a C-terminal hexahistidine tag. This plasmid was generated by cloning the genetic fimD sequence into pBAD30 (Guzman et al, 1995) via the KpnI and HindIII restriction sites. The plasmid pfimC-T7term served as template for mutagenesis of fimC. It contains the fimC gene under the control of the T7 promoter. Amino acid replacements for in vitro mutagenesis experiments were introduced into the following plasmids: pfimD_N (Nishiyama et al, 2003), encoding residues 1–139 of mature FimD, was used as the template for N-terminal FimD constructs, and for the construction of the truncated FimD_N variants FimD_N(25–139), FimD_N(1–125) and FimD_N(25–125). pCT-FimH-FimC (Vetsch et al, 2002) was used for mutagenesis of the fimC gene. The nucleotide sequences of all the plasmids used in this study are available upon request.

NMR sample preparation and data collection
^{13}C,^{15}N-labelled FimD_N constructs were obtained by growing E. coli strain HM125 carrying the appropriate plasmids in minimal medium containing[^13]C_6]-\beta-glucose and ^{15}NH_4Cl as the sole carbon and nitrogen sources, respectively. Uniformly deuterated and ^{15}N-labelled FimD_N(1–139) was obtained by growing cells in minimal medium containing 99% \textit{H}_2O, [^{12}C_6]-\beta-glucose and ^{15}NH_4Cl. All NMR measurements were performed at 20 °C and pH 7.0, either on a Bruker DRX 500 spectrometer equipped with a cryogenic probe head, or on Bruker DRX 750 and 900 spectrometers.

For the backbone resonance assignment, the following experiments were recorded: 3D HNCA, 3D HNCACB, 3D CBCA(CO)NH, 3D HNCO, 3D HC(C)H-TOCSY and 3D HC(C)H-COSY (Wider, 1998). Distance constraints were obtained from three NOE experiments with mixing times of 50 ms, i.e., 3D ^{15}N-resolved [^1H,^1H]-NOESY, and two 3D ^{13}C-resolved [^1H,^1H]-NOESY spectra with the ^{13}C carrier frequency in the aliphatic or aromatic regions, respectively. The data sets used for obtaining the sequence-specific resonance assignments were interactively peak picked using the programs XEASY (Bartels, 1995) and CARA (R. Keller et al, unpublished).
The assignments of the cross-peaks in the 2D \[^{15}N,^1H\]-TROSY spectra (Pervushin et al., 1998) of FimD\(_N\)(1–139) in the ternary complex with FimC and FimHp were obtained in two steps: First, the backbone \(^{15}N,^1H\) chemical shift assignments from free FimD\(_N\) were mapped onto the 2D \[^{15}N,^1H\]-TROSY spectrum of FimD\(_N\)(1–139) in the complex. Second, the resulting tentative assignments were confirmed by sequential \(^1H^N,^1H^N\) connectivities obtained from a 3D \(^{15}N\)-resolved \[^{15}N,^1H\]-NOESY spectrum.

Measurements of heteronuclear NOEs were performed at 20 °C on a Bruker DRX 750 spectrometer. The NOE spectra and the reference spectra were integrated using XEASY.

**NMR Structure Calculation**

The NOESY spectra were automatically analyzed with the new in-house software packages ATNOS (Herrmann et al., 2002b) for automated peak picking and NOE identification in 2D homonuclear and 3D heteronuclear-resolved NOESY spectra, and CANDID (Herrmann et al., 2002a) for automated NOE assignment of NOESY cross peaks. The program DYANA (Guntert et al., 1997) was used to perform simulated annealing in torsion angle space. The input for ANTOS/CANDID/DYANA consisted of the chemical shifts obtained from the sequence-specific resonance assignment, and of the three aforementioned NOESY spectra. The standard protocol with seven cycles of peak picking, NOE assignment and 3D structure calculation was applied (Herrmann et al., 2002a, b). During the first six cycles of computation, ambiguous constraints (Nilges, 1997) were used. At the outset of the spectral analysis, highly permissive criteria were used to identify a comprehensive set of peaks in the NOESY spectra, and only the knowledge of the covalent polypeptide structure and the chemical shifts were exploited to guide NOE cross peak identification and NOE assignment. In the second and subsequent cycles, the intermediate protein three-dimensional structures served as an additional guide for the interpretation of the NOESY data. The output of ANTOS/CANDID/DYANA consisted of assigned NOE peak lists for each input spectrum, and a final set of meaningful upper limit distance constraints that constituted the input for the DYANA 3D structure calculation algorithm. For each cycle of 3D structure calculation, torsion angle constraints for the backbone dihedral angles derived from \(\text{Ca}^\alpha\) chemical shifts (Spera and Bax, 1991) were added to the CANDID output. For the final structure calculation in cycle 7, only those distance constraints were retained that could be unambiguously assigned based on the protein three-dimensional structure from cycle 6. The 20 conformers with the lowest residual DYANA target function values obtained from cycle 7 were energy-refined in a water shell with the program OPALp (Koradi et al., 2000; Luginbuhl et al., 1996),
using the AMBER force field. The program MOLMOL (Koradi et al, 1996) was used to
analyze the protein structure and to prepare the figures of the NMR structures. The atomic
coordinates of 20 energy-minimized DYANA conformers each of FimD_N(25–125) and
FimD_N(25–139) have been deposited in the Protein Data Bank, with entry codes 1ZDX and
1ZDV, respectively.

Crystallization and X-ray structure determination of the ternary complex
FimD_N(1–125) and the FimC–FimH_p complex were expressed and purified as described
(Nishiyama et al, 2003). The ternary complex was obtained by mixing equimolar amounts of
FimD_N(1–125) and FimC–FimH_p, and subsequent purification on a Superdex 75 26/60 size-
exclusion column (Amersham Biosciences) equilibrated in 20 mM sodium phosphate
(pH 7.4), 115 mM NaCl. The homogenous complex was dialyzed against 10 mM Tris/HCl
(pH 8.0) and concentrated. Using the sitting drop vapor diffusion method, we obtained two
different crystal forms. Crystals with space group P6_3 (form A) with 3 complexes per
asymmetric unit were obtained using a reservoir solution containing 0.02 M TAPS/NaOH
(pH 9.2) and 18% PEG 5000 MME. Crystals with space group P2_1_2_1 (form B) with one
complex per asymmetric unit were obtained using a reservoir solution containing 0.1 M
MES/NaOH (pH 6.5) and 15% PEG 6000.

Diffraction data were collected using synchrotron radiation at the Swiss Light Source and the
structure of the ternary complex was solved by molecular replacement with AMoRe (Navaza,
1994) and refined with CNS (Brunger et al, 1998). The data collection and refinement
statistics are given in Table 2. Details of data collection, structure solution and refinement are
given in Supplementary Material. The atomic coordinates and structure factors have been
deposited in the Protein Data Bank with entry code 1ZE3.

Characterization of FimC and FimD variants
All variants of FimD_N were expressed in E. coli strain HM125 and purified as described
previously for wild type FimD_N (Nishiyama et al, 2003). Periplasmic expression and
purification of complexes between FimC variants and FimH_p was carried out as described
(Vetsch et al, 2002).

Yeast agglutination assays were performed as previously described (Nishiyama et al, 2003)
with E. coli strains W3110ΔfimC and W3110ΔfimD transformed with plasmids encoding the
respective wild-type proteins or variants. W3110ΔfimC and W3110ΔfimD were constructed
from *E. coli* K12 wild-type strain W3110 by allelic exchange (Hamilton, 1989) and the Red disruption system (Datsenko and Wanner, 2000), respectively.

Analytical gel-filtration experiments were carried out at pH 7.4 and 25 °C with initial protein concentrations of 60 μM as described (Nishiyama *et al*, 2003).

Protein concentrations were determined via the specific protein absorbance at 280 nm.
Acknowledgements

We thank the staff of the beamline X06SA at the Swiss Light Source (Villigen, Switzerland), especially Takashi Tomizaki, for support in X-ray data collection. We are also grateful to Beat Blattmann (University of Zurich) for help in protein crystallization, René Brunisholz (Protein Service Laboratory, ETHZ) for Edman sequencing and Peter Tittmann for assistance with electron microscopy. M.N. thanks Kaspar Hollenstein for his valuable comments on the manuscript. This project was funded by the Schweizerische Nationalfonds, the ETH Zurich and the University of Zurich within the framework of the NCCR Structural Biology program.
Results

Structural basis of chaperone-subunit complex recognition by FimD

References


Results

Structural basis of chaperone-subunit complex recognition by FimD


### Results

**Structural basis of chaperone-subunit complex recognition by FimD**

Table 1: Input for the structure calculation and characterization of the energy-minimize NMR structures of FimD$_N$ (25–139) and FimD$_N$ (25–125)

<table>
<thead>
<tr>
<th>Quantity</th>
<th>FimD$_N$(25–139)</th>
<th>FimD$_N$(25–125)</th>
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<tr>
<td>NOE upper distance limits</td>
<td>2928</td>
<td>2953</td>
</tr>
<tr>
<td>Dihedral angle constraints</td>
<td>94</td>
<td>94</td>
</tr>
<tr>
<td>Residual target function, Å$^2$</td>
<td>1.57 ± 0.33</td>
<td>1.20 ± 0.43</td>
</tr>
<tr>
<td>Residual NOE violations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number ≥0.1Å</td>
<td>31 ± 6 (24–45)</td>
<td>22 ± 5 (5–29)</td>
</tr>
<tr>
<td>Maximum, Å</td>
<td>0.14 ± 0.01 (0.12–0.15)</td>
<td>0.14 ± 0.01 (0.12–0.17)</td>
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<td>Residual dihedral angle violations</td>
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<tr>
<td>Number ≥2.5 deg.</td>
<td>0 ± 1 (0–2)</td>
<td>1 ± 1 (0–3)</td>
</tr>
<tr>
<td>Maximum, deg.</td>
<td>1.82 ± 1.12 (0.35–4.54)</td>
<td>2.89 ± 1.32 (1.57–7.44)</td>
</tr>
<tr>
<td>Amber energies, kcal/mol</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>-4323.89 ± 76.56</td>
<td>-4129.35 ± 55.45</td>
</tr>
<tr>
<td>Van der Waals</td>
<td>-327.64 ± 13.24</td>
<td>-292.04 ± 16.14</td>
</tr>
<tr>
<td>Electrostatic</td>
<td>-4948.89 ± 74.37</td>
<td>-4673.08 ± 52.16</td>
</tr>
<tr>
<td>r.m.s.d. from ideal geometry</td>
<td></td>
<td></td>
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<tr>
<td>Bond lengths, Å</td>
<td>0.0078 ± 0.0001</td>
<td>0.0079 ± 0.0002</td>
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<tr>
<td>Bond angles, deg.</td>
<td>2.035 ± 0.044</td>
<td>2.022 ± 0.067</td>
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<td>r.m.s.d. to the mean coordinates, Å$^b$</td>
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<tr>
<td>bb (35–120)</td>
<td>0.43 ± 0.06 (0.33–0.54)</td>
<td>0.40 ± 0.06 (0.28–0.54)</td>
</tr>
<tr>
<td>ha (35–120)</td>
<td>0.74 ± 0.07 (0.62–0.95)</td>
<td>0.74 ± 0.06 (0.67–0.90)</td>
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<td>Ramachandran plot statistics $^c$</td>
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<tr>
<td>Most favored regions (%)</td>
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<td>71</td>
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<td>Additional allowed regions (%)</td>
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<td>26</td>
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<tr>
<td>Generously allowed regions (%)</td>
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<td>Disallowed regions (%)</td>
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<td>1 ± 1 (0–3)</td>
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<td>Van der Waals</td>
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<td>Ramachandran plot statistics $^c$</td>
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<tr>
<td>Disallowed regions (%)</td>
<td>1</td>
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a. Except for the three top entries, the average value for the 20 energy-minimized conformers with the lowest residual DYANA target function values and the standard deviation among them are given. For the residual violations and the r.m.s.d. values, the range from the minimum to the maximum value is given in parentheses.

b. bb indicates the backbone atoms N, C$^\alpha$, C$^\beta$; ha stands for “all heavy atoms”. The numbers in parentheses indicate the residues for which the r.m.s.d. was calculated.

c. As determined by PROCHECK (Laskowski et al., 1993).

---

Table1
Table 2: Summary of crystallographic data collection and refinement statistics

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<td>Average B-factor (Å²)</td>
<td>18.8</td>
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*last shell: 1.91–1.84 Å
Results  Structural basis of chaperone-subunit complex recognition by FimD

Figure legends

**Figure 1**: Schematic model of type 1 pilus assembly by the chaperone-usher pathway. The periplasmic chaperone FimC forms stoichiometric complexes with the newly translocated pilus subunits (FimA, FimG, FimF, FimH). In these complexes, FimC donates its G1 donor strand to the individual subunits, thereby completing the immunoglobulin-like fold of the subunits. FimC-subunit complexes diffuse to the assembly platform (usher) FimD, which specifically recognizes FimC-subunit complexes via its periplasmic, N-terminal segment of residues 1-139. Subsequently, FimC is released to the periplasm, and the subunit is delivered to the translocation pore of FimD, where it is supposed to interact with the previously incorporated subunit via donor strand exchange. The pilus rod, composed of FimA subunits, assembles into its helical quaternary structure on the cell surface. IM, inner membrane; OM, outer membrane.

**Figure 2**: NMR studies on FimDN. (A) Polypeptide backbone of FimDN(25-125) represented by a bundle of 20 energy-minimized DYANA conformers. Selected positions along the polypeptide chain are identified with sequence positions. (B) Ribbon drawing of one of the twenty energy-minimized conformers. β1-β5 and α1-α2 indicate five β-strands and two α-helices, respectively. The disulfide bridge Cys63-Cys90 is drawn in yellow. The chain ends are identified by the letters N and C. (C) NMR structure of FimDN(25-139) represented by a bundle of 20 energy-minimized DYANA conformers showing only the polypeptide backbone. The chain ends are identified by the letters N and C. The C-terminal residues 125-139 are shown in magenta. (D) Close-up view of the surface of one of the twenty energy-minimized conformers of FimDN(25-139). Relative to (C), the structure has been rotated by approximately 90° about a vertical axis. The backbone of the C-terminal stretch 125-139 is drawn in magenta, and the side chain of Trp133 is indicated in red. Those side chains, which show long-range NOE connectivities with Trp133 are drawn in bronze. In total, 14 long-range upper-distance limits between Trp133 and the rest of the protein (shown in cyan) define the position of the aromatic ring of Trp133. (E) Chemical shift variations of FimDN upon binding to FimC-FimHp. \( \Delta \delta_{Av} \) is the weighted average of the \(^{15}\text{N}\) and \(^{1}\text{H}\) chemical shifts, \( \Delta \delta_{Av} = \sqrt{0.5(\Delta \delta_H^2 + 0.2\Delta \delta_N^2)} \) (Pellecchia et al, 1999). (F) Heteronuclear \([{^{15}\text{N}},^{1}\text{H}]-\text{NOE}\) measurements of FimDN(1-139) in the FimDN-FimC-FimHp ternary complex. Values

77
between 0.5 and 1 indicate well-structured parts of the protein, values <0.5 manifest increased flexibility.

**Figure 3:** X-ray structure of the ternary FimD\(_N\)(1–125)–FimC–FimH\(_P\) complex. (A) Ribbon diagram of the ternary complex, with FimD\(_N\)(1–125) depicted in green, FimC in cyan and the pilin domain FimH\(_P\) in yellow. The G\(_1\) donor strand of FimC is colored in blue. A black dashed line indicates residues 10–18 of FimD\(_N\), for which no electron density was observed. The N- and C-termini of FimD\(_N\) are labeled in green. (B) Close-up view of the hydrophobic contacts between Phe8 of the N-terminal FimD\(_N\) tail (green) and residues from FimC (cyan) and FimH\(_P\) (yellow). The final 2mFo-DFc electron density map is contoured at 1σ level. (C) Stereo representation of the tail interface. Residues from FimD\(_N\), in stick model, are shown in green. The molecular surfaces of FimC (slate-grey) and FimH\(_P\) (light yellow) are shown in semitransparent mode. Residues contributing to the FimC and FimH\(_P\) surfaces and interacting with FimD\(_N\) are shown in more intense color: cyan for FimC and yellow for FimH\(_P\) residues, respectively. Residues from the G\(_1\) donor strand of FimC contributing to the molecular surface appear in blue. (D) Stereo representation of the interface between FimC and the folded FimD\(_N\) core 25–125. Some hydrogen bonds between FimC and the FimD\(_N\) core are depicted as thin dashed lines. Color-coding is as in (A). The figure was prepared with Pymol (www.pymol.org).

**Figure 4:** Multiple sequence alignments of N-terminal domains of assembly platforms (A) and periplasmic chaperones (B). Sequences are identified by their SWISS-PROT IDs. Residue numbering refers to mature FimD (A) and FimC (B). Identical residues are boxed in red, conserved ones are highlighted in yellow. Secondary structure elements derived from the X-ray structure of the ternary complex are shown in green (FimD\(_N\)(1–125)) and cyan (FimC). Residues of FimD\(_N\)(1–125) interacting (5.0 Å distance cutoff) with FimC and FimH\(_P\) are indicated with cyan and yellow triangles, respectively. Residues interacting with both FimC and FimH\(_P\) are indicated with black triangles. FimC residues involved in contacts (5.0 Å distance cutoff) with FimD\(_N\)(1–125) are indicated with green triangles. The alignment was generated using CLUSTAL W (Thompson *et al*, 1994) and displayed with ALSCRIPT (Barton, 1993).

**Figure 5:** Analysis of amino acid replacements and deletions in FimD, FimD\(_N\)(1–139), and replacements in FimC with respect to type 1 pilus biogenesis *in vivo* and formation of ternary
FimD<sub>N</sub>(1–139)–FimC–FimH<sub>p</sub> complexes in vitro. (A) Yeast agglutination assays, probing the formation of functional type 1 pili through agglutination with yeast cells. The <i>E. coli</i> strains W3110ΔfimD and W3110ΔfimC were transformed with expression plasmids carrying the indicated FimD and FimC variants, respectively. Agglutination intensities are indicated as (−) no agglutination, (±) weak or (+) strong. The ability of FimD<sub>N</sub>(1–139) and FimC variants to form the ternary complex, as well as the ability of FimC variants to bind FimH<sub>p</sub> in vitro are indicated as “yes” (+) or “no” (−). n.d., not determined. (B) Analytical gel filtration at pH 7.4 and 25 °C, probing the effect of mutations in FimD<sub>N</sub>(1–139) or FimC on the formation of the FimD<sub>N</sub>(1–139)–FimC–FimH<sub>p</sub> complex.
Figure 1

Results

Structural basis of chaperone-subunit complex recognition by FimD

Figure 1
Figure 2

A

B

C

D

E

F
Figure 3

A

B

C

D

Figure 3
Results

Structural basis of chaperone-subunit complex recognition by FimD

Figure 4

A

B

Figure 4
Figure 5

A

FimD variants

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FimC variants

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B

FimD$_N$ variants

FimC variants

Figure 5
Results and Discussion

Conformational changes in the chaperone–subunit complex upon binding to FimD_N

The structures of FimC and FimH_P in the ternary complex are overall closely similar to those in the previously published binary complex (PDB entries 1QUN and 1KIU) (Choudhury et al., 1999; Hung et al., 2002). The r.m.s.d. value is 1.3 Å for the 318 common Ca atoms (chains A and B from PDB entry 1QUN were used for the comparison). In a region where electron density was unambiguous in our structure of the ternary complex, we observed a register shift involving 5 residues in the A strand of FimH_P (residues 169–173), which are in direct contact with the donor strand G_1 of FimC. Using the structure factors available for the PDB entry 1KIU, we recalculated an electron density map for the ternary complex after simulated annealing at 5000 K and B-factor refinement. The revised density and a bioinformatic analysis (M. Sippl, personal communication) lead to a model that, in contrast to 1QUN and 1KIU, does not exhibit a register shift with respect to the ternary complex. After taking this into account, significant conformational changes can still be observed near the binding site of the donor strand. Specifically, the FimD_N arm of residues 1–24 pushes Met93 of FimC towards FimH upon binding, in order to accommodate Tyr3 of FimD_N. This leads to a twisting of the segment of residues 92–100 of FimC, which precedes the donor strand, and the flexible loop 213–217 of FimH_P is pushed away. The rotation of FimC Met93 also causes additional rearrangements in FimH_P. These include that through a contact with the C^β atom of FimH_P Thr169, the FimC Met93 side chain forces the residues 165–170 of FimH_P to adopt a different conformation. As a consequence, the side chain of FimH_P Val168, which is solvent-exposed in the binary complex, is pushed into a hydrophobic groove formed by Tyr31, Pro91, Leu105, Ile107 of FimC and Ala165 of FimH_P (Supplementary Figure S4).

Materials and methods

X-ray structure determination of the FimD_N(1–125)–FimC–FimH_P ternary complex

Crystals were cryo-protected either by stepwise addition of 2-methyl-2,4-pentanediol to a final concentration of 20% (form A), or of ethylene glycol to 20% (form B). Data were collected at 100 K using synchrotron radiation (beamline X06SA at the Swiss Light Source) and processed with DENZO (Otwinowski and Minor, 1996) and with programs of the CCP4
suite (Collaborative Computational Project, 1994). The structure of the ternary complex was solved by molecular replacement with AMoRe (Navaza, 1994), using the data from crystal form A. The search model was a truncated version of the binary FimC-FimH complex [(Choudhury et al., 1999), PDB entry 1QUN] in which the FimH lectin domain had been deleted. Additional electron density for FimD_N(1–125) appeared already with the initial molecular replacement phases. Several cycles of model building with O (Jones et al., 1991) and structure refinement with CNS (Brunger et al., 1998) yielded a nearly complete model of FimD_N(1–125) and improved density for the other two proteins. The model of the ternary complex obtained in this way was used for straightforward molecular replacement with AMoRe against the 1.84 Å orthorhombic data. Refinement was carried out with CNS, using a bulk solvent correction and applying no sigma cut-off. Water molecules were added at positions where clear electron density was seen in Fo-Fc maps and where the environment allowed reasonable hydrogen bonding to the protein or to other water molecules. Structure validation was performed with the programs PROCHECK (Laskowski et al., 1993) and WHAT_CHECK (Hooft et al., 1997).

Characterization of FimC and FimD variants

To analyze the expression of FimD variants in the outer membrane, W3110ΔfimD encoding wild-type fimD or fimD variants were grown in M9 minimal medium without shaking. After two days, cells were harvested, resuspended in 20 mM Tris/HCl (pH 8.0), and lysed by sonication. After unbroken cells were removed by centrifugation, Sarkosyl (N-lauroylsarcosine sodium salt) was added to the supernatant to a final concentration of 0.5%, and the mixture was rocked for 20 min at 25 °C to selectively solubilize the inner membrane (Nikaido, 1994). The outer membrane was then pelleted by centrifugation and resuspended in 20 mM sodium phosphate (pH 7.4), 115 mM NaCl. Immunoblot analysis was performed with anti-PentaHis antibodies (Qiagen), and an alkaline phophatase-conjugated secondary antibody (Pierce).

To analyze the expression of FimC variants in the periplasm, W3110ΔfimC harboring plasmid-encoded wild-type fimC or fimC variants were grown for two days in M9 minimal medium without shaking. Periplasmic extracts and immunoblot analysis with anti-FimC antibodies were performed as described previously (Nishiyama et al., 2003).

Isothermal titration calorimetry was carried out as described previously (Nishiyama et al., 2003). Thermal unfolding of truncated FimD_N variants was measured by far-UV CD at 218...
nm with a protein concentration of 10 μM in 20 mM sodium phosphate (pH 7.4), 115 mM NaCl. The experimental data were analyzed according to the two-state model of folding, using a six-parameter fit (Pace et al, 1998). Electron micrographs of *E. coli* fimD deletion strains complemented with plasmids encoding different FimD variants were recorded as described previously (Nishiyama et al, 2003).
Results

Structural basis of chaperone-subunit complex recognition by FimD

References


### Results

*Structural basis of chaperone-subunit complex recognition by FimD*

**Table S1:** Thermal unfolding of truncated FimD<sub>N</sub> variants at pH 7.4.

<table>
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**Table S2:** Thermodynamic parameters obtained from calorimetric titrations of various FimD<sub>N</sub> variants with the FimC–FimH<sub>p</sub> complex at pH 7.4 and 25 °C. The n-value denotes the binding stoichiometry calculated from the calorimetric data.

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<th>ΔG (kJ mol&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>ΔS (J mol&lt;sup&gt;-1&lt;/sup&gt; K&lt;sup&gt;-1&lt;/sup&gt;)</th>
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Table S1, S2
**Figure legends**

**Figure S1**: Heteronuclear $[^{15}N, ^1H]$-NOEs of free FimD$_N$(1–139). Values between 0.5 and 1 indicate well-structured parts of the protein, values < 0.5 manifest increased flexibility.

**Figure S2**: (A) C$^\alpha$-Superposition of the FimD$_N$(25–139) NMR solution structure on the crystal structure of the ternary complex. Both models are shown in C$^\alpha$-trace representation. The NMR model is depicted in red, and FimD$_N$ bound to FimC and FimH in the crystal structure is depicted in green. Residues 126–139 of FimD$_N$ (present only in the NMR structure) are depicted in blue. The NMR conformer with the smallest r.m.s.d. to the mean coordinates of the bundle of 20 DYANA conformers was chosen for the superposition. A r.m.s.d. of 1.19 Å was calculated for the C$^\alpha$-atoms of the 94 aligned residues 28–121 of FimD. The right panel shows the same superposition as in the left panel, rotated by ~90° around the vertical axis. (B) Close-up view of the C-terminal hinge segment of FimD$_N$. (C) Stereo close-up view of the FimD$_N$ region around Arg47. The NMR solution structures of FimD$_N$(25–139) (blue) and FimD$_N$(25–125) (red) are shown in backbone mode and superimposed on the crystallographic ternary complex (green). Arg47, Asp48 and residues 130, 133 and 135 of FimD$_N$ are shown in stick mode. Residues from the C-terminus of FimD$_N$ that would clash with Arg47 in the conformation observed in the X-ray structure or in the NMR structure of FimD$_N$(25–125) are labeled.

**Figure S3**: (A) Electron micrographic studies on *E. coli* strains W3110ΔfimD complemented with plasmids encoding FimD variants FimD$^{p4A}$, FimD$^{p8A}$ and wild-type FimD. The arrows show type 1 pili. (B) Expression of FimD variants in the outer membrane (OM). OMs were isolated from strain W3110ΔfimD cells used to express the indicated FimD variants. Samples were incubated for 5 min at 95 °C in SDS sample buffer prior to electrophoresis. FimD was detected by immunoblotting with the anti-Penta-His antibodies (Qiagen). (C) Expression of FimC variants in the periplasm. Periplasmic extracts were prepared from strain W3110ΔfimC cells used to express the indicated FimC variants. FimC was detected with the anti-FimC antibodies.

**Figure S4**: Stereo view of the conformational changes taking place in FimH$_p$ and FimC upon binding of FimD$_N$ to the binary complex. The structure of the ternary complex (FimD$_N$ in...
green, FimC in cyan and FimHp in yellow) is superimposed onto that of the binary complex (both FimC and FimH in magenta; see Results and Discussion for details). Black arrows indicate the displacements caused by the binding of FimD\textsubscript{N} (especially Tyr3): FimC Met93, FimHp Thr169 and Val168 are displaced and Val168 is pushed into a hydrophobic groove formed by Tyr31, Pro91, Leu105 and Ile107 of FimC and by Ala165 of FimHp.
Results  Structural basis of chaperone-subunit complex recognition by FimD

Figure S1

![Graph showing NOE interactions with residue numbers]

Figure S1
Figure S2

A

B

C

Figure S2
Results  Structural basis of chaperone-subunit complex recognition by FimD

Figure S3

A

F4A  F8A  WT

1.0 μm  1.0 μm  1.0 μm

B

FimD →

F4A  F8A  F22A  Y33A  D38A  Q109A  Δ1-11  Δ1-24  WT  Vector

C

FimC →

L32G  L32E  I90R  WT  Vector

Figure S3
Figure S4
2.3 Preliminary studies on the assembly platform FimD

Mireille Nishiyama and Rudi Glockhuber
Introduction

Assembly of type 1 pili is governed by the chaperone/usher pathway that requires two specialized proteins: the periplasmic chaperone FimC and the outer membrane assembly platform, also termed usher, FimD (reviewed in Hung and Hultgren, 1998; Sauer et al., 2000; Thanassi et al., 1998a). FimC forms stoichiometric complexes with subunits and catalyzes their folding by donating a β-strand to complete the Ig-like fold of subunits (Jones et al., 1993; Vetsch et al., 2004). This interaction is termed donor strand complementation and also governs subunit-subunit interactions in the mature pili (Choudhury et al., 1999; Sauer et al., 2002; Zavialov et al., 2003). At FimD, it is likely that the donated β-strand of FimC is exchanged for the N-terminal extension of an incoming subunit. Dissociation of FimC therefore promotes type 1 pilus assembly. To date, only limited analysis has been carried out to elucidate the assembly process taking place at the outer membrane. The only reasonably characterized property of the assembly platform is the recognition and binding of chaperone-subunit complexes (Chapter 2.1; Saulino et al., 1998). The elucidation of why and how chaperone dissociation takes place only at the site of the outer membrane is currently one of the biggest challenges in the field of pilus assembly.

In the foregoing chapters the N-terminal periplasmic domain of FimD (FimD_N) that comprises 139 residues was characterized in detail. FimD_N is an autonomous folding unit that selectively binds FimC-subunit complexes. This is achieved by the flexible “arm” that adopts a defined conformation only upon binding of the FimC-subunit complexes (chapter 2.2). Since FimD_N did not accelerate the donor strand exchange reaction (data not shown), this activity must reside in a different part of FimD. Our attempt to produce the predicted C-terminal domain of FimD in the periplasm was not successful. It is possible that this domain is stabilized by a segment of the transmembrane domain and therefore is not stable if expressed alone, or does not exist.

Besides the solved structure of FimD_N no structural information at atomic level is available for bacterial assembly platforms. Moreover, topology prediction for known assembly platforms is still ambiguous (Harms et al., 1999; Henderson et al., 2004; Valent et al., 1995). However, taking our data on FimD_N into account (chapter 2.1; chapter 2.2), the topology model that predicts 22 transmembrane β-strands and two, N- and C-terminally located,
periplasmic domains for an assembly platform seems to be more conceivable at present (Harms et al., 1999). Regarding the oligomeric state of assembly platforms, there are studies with controversial results. Both PapC and FimD have been shown to assemble into ring-shaped oligomers composed of 6-12 subunits with a central pore (Saulino et al., 2000; Thanassi et al., 1998b). However, examination of FaeD did not show any evidence for such oligomerization (Harms et al., 1999). In contrast, a recent study revealed that PapC forms a dimer with a twin-pore configuration (Li et al., 2004). Assembly platforms are expected to function in the same manner and consequently exist in the same oligomeric state. The accurate determination of the oligomeric state therefore is of fundamental importance for understanding the physiological mode of action of the assembly platform in pilus assembly.

Towards the goal to elucidate the entire pilus assembly process, data on the molecular mechanism of full-length FimD is required. Therefore, initial work on FimD has been taken up. In the presented study an appropriate expression system was established that allows over-production of FimD in *E. coli*. Furthermore, preliminary studies on the full-length FimD had been performed.
Results and Discussion

Expression of fimD impairs cell growth

To facilitate protein purification, a sequence encoding a hexahistidine (His\textsubscript{6})-tag was attached to the 3' end of the fimD gene by PCR. This engineered gene fragment was first cloned into the pBR322-derived plasmid pDsba3 under control of the lac promoter/operator (Hennecke et al., 1999). Sequencing results, however, revealed that in all tested clones, one or two nucleotide deletions were introduced into the fimD gene. When these deletions were corrected by site-directed mutagenesis, new deletions were generated in other positions of the gene, preferentially in the segment coding for the natural signal sequence. Such spontaneous deletions have been reported for some recombinant genes whose products are toxic for cells (Dolja et al., 1993; Hartung et al., 2001). If it is assumed that basal expression of fimD caused by transcription from the leaky lac promoter impairs cell growth and thus leads to selection of mutants with incorrect fimD sequences, vector systems with tightly controlled promoters should avert the toxic effects associated with fimD over-expression. To test this hypothesis, the engineered fimD gene fragment was subcloned into a pBAD vector (pBAD30) containing the tightly controlled arabinose-inducible \( P_{\text{BAD}} \) promoter (Guzman et al., 1995). This vector system has been employed successfully for expression of genes coding for toxic proteins, both soluble and membrane-inserted (Auer et al., 2001, Li et al. 2001; Beaber et al., 2004). The fact that all sequenced clones of the resulting expression plasmid (pBAD30\_fimD\textsubscript{His}) indeed contained the correct fimD fusion product, strongly suggests that basal fimD expression from the leaky lac promoter is detrimental to cell growth. To test whether His\textsubscript{6}-tagged FimD is functional in vivo, the ability of pBAD30\_fimD\textsubscript{His} to complement FimD deficiency in an \textit{E.coli} fimD deletion strain was tested through agglutination assays with yeast cells (Mirelman et al., 1980). As shown in Figure 1, the missing agglutination of the fimD-deficient strain can be restored almost to the wild-type level by pBAD30\_fimD\textsubscript{His} indicating that the His\textsubscript{6}-tag does not interfere with the functionality of FimD. Since the assays were carried out in the absence of arabinose, it appears that a few copies of FimD, synthesized even without induction, are sufficient to complement the fimD-deficiency. When glucose was added to the growth medium, bacteria failed to assemble functional pili (data not shown). This indicates that the expression of fimD is completely suppressed by catabolic repression. The presence of arabinose in the growth medium led to a very low cell density, but cells were able to assemble
Results

Preliminary studies on FimD

functional pili. This observation further supports the hypothesis that over-production of FimD impairs cell growth (see below).

**Tagged FimD can be over-produced at high levels**

FimD over-production was tested in *E. coli* strains HM125 and MC1061 harboring the plasmid pBAD30_fimD<sub>His</sub>. The strain MC1061 is araD-deficient and therefore not able to utilize arabinose as a carbon source, while the strain HM125 is deficient in major proteases, but can metabolize arabinose. When uninduced, both strains show a similar growth profile and reach a maximal optical density at 600 nm (OD<sub>600</sub>) of about 4.0 (Figure 2). Once protein production was induced by arabinose, strain MC1061 stopped dividing immediately after addition of the inducer, whereas HM125 cells kept growing until an OD<sub>600</sub> of around 3.0 before they started to lyse (Figure 2). SDS-PAGE analysis revealed that *E. coli* HM125 expressed fimD at a level approximately twice as high as MC1061 (data not shown). The toxicity of over-expressed fimD might reside in the fact that the gene product perforates the outer membrane. In wild-type *E. coli* harboring the chromosomal fim gene cluster, FimD is produced only as part of the entire gene cluster together with the chaperone FimC and the pilus subunits (Brinton, 1965), and the pores are likely to be occupied by pili. To “plug” the pores, fimD could be coexpressed with a plasmid encoding fimC and fimH. The L-shaped adhesin FimH is thought to bind to FimD such that the lectin domain is inserted into the pore of FimD, while the pilin domain bound to FimC interacts with the N-terminal periplasmic domain of FimD (chapter 2.1; Barnhart et al., 2003). However, a more likely explanation for the toxicity of over-expressed fimD is the perturbation of the membrane physiology in the host organism. This is supposed to be the reason for the toxicity of many other over-produced membrane proteins that are not pore-forming. Highest yield of FimD was obtained when protein production was induced in HM125 with 0.1 % arabinose for one hour. Under these conditions, the expression level of FimD is as high as the major outer membrane proteins OmpA and OmpC (Figure 3).

**FimD can be obtained in high purity but presumably in an instable, aggregation prone conformation**

Outer membrane was prepared by selectively solubilizing proteins of the inner membrane with the anionic detergent N-lauroylsarcosine (Sarkosyl). Sarkosyl, with one negative charge
per hydrocarbon chain, has a similar charge density as LPS, and is supposed to mimic LPS in the outer membrane (Nikaido, 1994). Figure 3 shows the enriched outer membrane containing FimD and the two major outer membrane proteins OmpA and OmpC after treatment of total membranes with Sarkosyl. Initial solubilization experiments were performed with some widely used detergents in concentrations of 1–3 % (w/v). Detergent concentrations in the examined range had no significant influence on the solubilization efficiency (data not shown). While 75 % of OmpA and OmpC were solubilized by the mild nonionic detergent n-dodecyl-ß-D-maltoside (DDM) or the zwitterionic detergent Zwittergent 3-14, only 10 % of FimD_His was extracted from the membrane by these detergents. Other tested detergents, except for lithium dodecyl sulfate (LDS), showed similar solubilization efficiencies for FimD_His as judged from SDS-PAGE (Figure 4). The strongly ionic detergent LDS extracted > 90 % of proteins from the outer membrane including the FimD_His protein. Despite its denaturing character, there are cases where LDS was successfully used for solubilization and purification without loss of protein activity (Sugawara and Nikaido, 1992). However, care should be taken when using this detergent for membrane protein preparation. Using the milder detergents DDM or Zwittergent 3-14, the yield of solubilized FimD_His was around 0.4 mg per gram of outer membrane, i.e. per 1/2 l of bacterial culture. Prolonged incubation times or different membrane to detergent ratios did not result in a higher yield of detergent soluble FimD_His (data not shown). Solubilized FimD_His was purified by immobilized metal affinity chromatography either in DDM or Zwittergent 3-14 resulting in a protein preparation of about 95 % purity (Figure 5A).

The far-UV CD spectrum of FimD_His after purification indicates a high content of β-strand conformation (Figure 5B), suggesting that FimD has a β-barrel structure, as expected for an outer membrane protein (Schulz, 2000). Gel filtration analysis of purified FimD_His in either DDM or Zwittergent 3-14 showed broad elution profiles indicative of protein aggregation (data not shown). A binding experiment further revealed that only a small fraction of FimD_His was able to bind the FimC–FimH complex (Figure 5C). At the same time, it was noticed that the detergent interferes with protein–protein interactions, as the binding of FimD_His to FimC–FimH is stronger in DDM than in Zwittergent 3-14 (Figure 5C). A recently published study on PapC, the FimD homologue in the P pilus system, reports that the C-terminal His6-tag might induce aggregation of the tagged PapC (Li et al., 2004). The hypothesis, that purified FimD_His is not in its native conformation is supported by the finding, that it did not
show heat-modifiable mobility in SDS-PAGE typical for β-barrel proteins (Figure 5A) (Kent and Wisnieski, 1983; Schnaitman, 1973), which is also reported for FimD and PapC (Thanassi et al., 1998b). This so-called heat modifiability, i.e. the correctly folded monomer migrates faster than the heat-denatured form in SDS-PAGE, arises from the extreme stability of β-barrel proteins that retain their native fold even in the presence of SDS when unheated. This property is used as a rapid means for screening protein conformation, for example, in mutational studies (Puntervoll et al., 2002; Voulhoux et al., 2003). Altogether, these observations suggest that the FimD protein prepared in this study might be in an instable, aggregation prone conformation.
Results

Preliminary studies on FimD

Outlook

In the presented study, initial results on the assembly platform FimD were obtained. As noticed, there is yet room for improvement concerning its preparation and analysis. Firstly, the yield of detergent soluble protein has to be increased. Although a suitable expression system was found that allows FimD production in high quantities, only a small fraction of protein could be extracted from the membrane. Based on several observations that outer membrane proteins are often difficult to solubilize, most purification protocols involve selective membrane extractions in which contaminating outer membrane proteins are pre-extracted before solubilization of the target protein (Locher and Rosenbusch, 1997; Puntervoll et al., 2002). The fact that the tested detergents extracted other proteins more efficiently than FimD indicates that they would be good candidates for pre-extraction. Subsequently, FimD could be extracted with a detergent that not only efficiently solubilizes FimD, but at the same time keeps it in its stable conformation. The importance of the choice of the right detergent on the stability and functionality of membrane proteins was reported in a number of studies (Auer et al., 2001; Engel et al., 2002; Li et al., 2001). In case of human liver monoamine oxidase B, the protein was not stable enough to retain its native conformation during the extraction from Pichia pastoris membranes. Therefore, the membrane was first digested with phospholipase A2 so that the protein could be mildly solubilized using 0.5 % Triton X-100 (Newton-Vinson et al., 2000). Whether such treatment can be applied to FimD extraction remains to be tested. The yield of detergent soluble FimD might be further increased when extraction is carried out under harsher conditions e.g. at higher temperatures. This was successfully used to solubilize TolC or FhuA from the E. coli outer membrane (Koronakis et al., 1997; Locher and Rosenbusch, 1997).

The second point to be considered is that the purified FimD must retain its stability and functionality in the chosen detergent system. Since solubilization of proteins from membranes requires delipidation, removal of the surrounding lipids leads in some cases to a loss of protein activity. This feature is pronounced for proteins that contain strong lipid-binding sites (Ferguson et al., 2000). For example, the enzymatic activity of the outer membrane protease OmpT is dependent on the presence of lipopolysaccharide (LPS) (Kramer et al., 2000). Regarding assembly platforms, LPS is reported to influence the conformation of PapC (Thanassi et al., 1998b). Whether LPS or lipids play an important role for stability or/and functionality of FimD remains to be elucidated. Besides, the presence of an affinity tag might
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also influence protein stability. A recently published study on PapC reports that the C-terminal His<sub>6</sub>-tag might induce aggregation of the tagged PapC (Li et al., 2004). Since the N-terminus of FimD is flexible and located in the periplasm (chapter 2.2), an N-terminal tag seems to be preferred to a C-terminal tag whose location is unknown so far. However, the fact that the N-terminus of FimD is essential for the interaction with FimC–subunit complexes, an affinity tag at this site would interfere with the functionality of FimD. To circumvent these problems, a protease cleavage site close to the affinity tag should be considered that allows the study of the tag-free protein.

Lastly, in order to assess the functionality of the purified FimD in different detergents, an appropriate assay has to be established. The fact that assembly platforms are neither enzymes catalyzing chemical reactions, nor transporters makes it difficult to carry out quantitative measurements. Apart from this, the only well-characterized property of assembly platforms is that they bind different chaperone–subunit complexes with different affinities (chapter 2.1; Saulino et al., 1998). Other potential functions, e.g. catalyzing dissociation of the chaperone from the chaperone–subunit complexes, or accelerating polymerization of pilus subunits, could not be verified so far. Whether cofactors or an energy source are required for the functionality of assembly platforms remains to be elucidated as well.
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Materials and Methods

Bacterial strains and expression plasmids
For protein expression, the protease-deficient E. coli strain HM125 (Meerman and Georgiou, 1994) or MC1061 that is araD-deficient was used (Casadaban and Cohen, 1980). Yeast agglutination assays were performed with W3110ΔfimD, which was prepared from strain W3110 using the Red disruption system (Datsenko and Wanner, 2000) by C. Puorger and D. Caminada.

To attach a hexahistidine (His₆)-tag coding sequence to the 3' end of fimD, the fimD gene including the segment coding for its natural signal sequence was amplified from the genome of E. coli W3110 by PCR. For cloning into pDsbA3 oligonucleotides 5'-GGG ATC TCG CAT ATG TCA TAT CTG AAT TTA AG-3' and 5'-GCA AGATCT TTAGTGATGGTG ATG GTG GTG ACG ACA TTC TGC TGA TAG C-3' (NdeI and BglII sites underlined) were used. For cloning into pBAD30 oligonucleotides 5'-CCG GGT ACC AGGAGGAAT TCA CCA TGT CAT ATC TGA ATT TAA GAC-3' and 5'-CCC AAG CTT AGT GAT GGT GAT GGT GGT GAC GAC ATT CAG CTG ATA GC-3' (KpnI and HindIII sites underlined) were used. Here, the ribosomal binding site (AGG AGG) is encoded by the forward primer and is located immediately after the KpnI restriction site. The PCR products were then cloned into the appropriate vectors using the above mentioned restriction sites. The resulting expression plasmids were termed pDsbA3_fimD_His and pBAD30_fimD_His. Sequences of the engineered fimD gene was verified by dideoxy sequencing.

Yeast agglutination assay
Yeast agglutination assay was performed as described in chapter 2.1 with E.coli strain W3110ΔfimD.

Over-production of FimD_His
E.coli strains HM125 and MC1061 were transformed with the plasmid pBAD30_fimD_His. Cells were grown at 30 °C in 2xYT medium containing ampicillin (100 μg/ml) to an optical density at 600 nm (OD₆₀₀) of 0.7. Protein production was induced with 0.01–0.1 % (w/v) L-arabinose. Highest yield of FimD_His was obtained when protein production was induced in

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HM125 with 0.1 % (w/v) L-arabinose for one hour. The identity of the protein was confirmed by the N-terminal sequencing and immunoblot with a Penta-His antibody (Qiagen).

Preparation of the outer membrane

Cells were harvested, washed and resuspended in 20 mM Tris/HCl (pH 8.0) containing DNaseI (final concentration 100 µg/ml) and MgCl₂ (final concentration 2 mM). Cells were broken by two passages through an EmulsiFlex C5 Homogenizer (Avestin Inc., Ottawa, Canada). Unbroken cells were removed by low speed centrifugation (5,000 x g, 10 min, 4 °C). Sarkosyl (N-Lauroylsarcosine sodium salt, Fluka) was added to the supernatant to a final concentration of 1 % (w/v), and the mixture was incubated for 30 min at 25 °C to selectively solubilize the inner membrane. The outer membrane was pelleted by ultracentrifugation (100,000 x g, 60 min, 4 °C).

Extraction of FimD<sub>His</sub> from the outer membrane

The outer membrane was resuspended in 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA and solubilized in different detergents at final concentrations of 1-3 % (w/v) for one hour at either 4 °C or 25 °C, or over night at 4 °C (25 mg membrane/ml buffer). Tested detergents were lithium dodecyl sulfate (LDS; Fluka), n-dodecyl-β-D-maltoside (DDM; Calbiochem), CHAPS (Calbiochem), Zwittergent 3-14 (Calbiochem), Elugent (Calbiochem), N,N-dimethyldodecylamine-N-oxide (LDAO; Fluka), and n-Octyl-β-D-glucopyranoside (OG; Calbiochem). After ultracentrifugation (100,000 x g, 60 min, 4 °C), the supernatant and the insoluble material were analyzed by SDS-PAGE.

Purification of FimD<sub>His</sub> by immobilized metal affinity chromatography

For FimD<sub>His</sub> purification the outer membrane was solubilized by rocking for one hour at 4 °C with 1 % (w/v) of DDM in 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA (25 mg membrane/ml buffer). Alternatively, 1 % (w/v) of Zwittergent 3-14 was used. After spinning out insoluble material (100,000 x g, 60 min, 4 °C), the outer membrane extract was diluted with 20 mM Tris/HCl (pH 8.0), 150 mM NaCl to lower the detergent concentration to 0.1 % (w/v). Subsequently, the supernatant was applied to a Ni-NTA Superflow Column (Qiagen) equilibrated with 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.1 % (w/v) detergent. The column was washed with the above buffer containing 20 mM of imidazole before FimD<sub>His</sub> was eluted with a linear gradient from 20 mM to 250 mM imidazole. Fractions containing
**Results Preliminary studies on FimD**

FimD\textsubscript{His} were pooled, and concentrated with an Amicon Ultra-15 unit (100,000 MWCO) (Millipore). To remove imidazole, the protein preparation was dialyzed against 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.1 % (w/v) detergent (DDM or Zwittergent 3-14).

**Protein concentrations**

Protein concentrations were determined by measuring absorbance at 280 nm using the molar extinction coefficient of 131,810 M\(^{-1}\)cm\(^{-1}\). The calculation was done under the assumption that FimD\textsubscript{His} is monomeric.

**CD spectroscopy**

Far-UV CD spectra were recorded in 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.1 % (w/v) detergent (DDM or Zwittergent 3-14) at 25 °C on a JASCO J-710 spectropolarimeter at a protein concentration of 0.5 μM in a 0.1 cm quartz cuvette. 10 spectra were averaged and the signal was converted to mean residue ellipticity [\(\Theta\)]\textsubscript{MRW} according to Schmid (Schmid, 1989).

**SDS-PAGE**

Electrophoresis was carried out at room temperature, using 10 % separating and 4 % stacking gels containing 0.4 % (w/v) SDS (Laemmli, 1970). Samples were loaded on the gel either directly or after 5 min incubation at 100 °C. Loading buffer contained 100 mM DTT and 2 % (w/v) SDS.

**Analytical gel filtration**

Analytical gel filtration was performed at 4 °C on a Superose 6 HR 10/30 (Amersham Biosciences) equilibrated with 20 mM Tris/HCl (pH 8.0), 300 mM NaCl, 0.1 % (w/v) detergent (DDM or Zwittergent 3-14). Protein concentrations were 10 μM. Detection wavelength was 226 nm. The following proteins were used as molecular weight markers: Thyroglobulin (670 kDa), Gamma globulin (158 kDa), Ovalbumin (44 kDa), and Myoglobin (17 kDa).

**Binding experiment**

The FimC–FimH complex was mixed with FimD\textsubscript{His} (5 μM) either in an equimolar ratio or in 5-fold excess in 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.1 % (w/v) detergent (DDM or Zwittergent 3-14). After incubation for 30 min at 25 °C, Ni-NTA slurry was added and rocked.
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for 15 min at 25 °C prior to centrifugation. The supernatant was removed and proteins were precipitated with organic solvent (Wessel and Flugge, 1984). Pellets were extensively washed and subsequently rocked in the above mentioned buffer containing 300 mM of imidazole for 15 min at 25 °C. After centrifugation, the supernatant was removed and proteins were precipitated as described. Samples were boiled for 5 min and subjected to SDS-PAGE.
Acknowledgements

We thank Linda Thöny-Meyer for helpful discussions and providing the plasmid pBAD30 and the *E.coli* strain MC1061, and Chasper Puorger and Daniel Caminada for preparing the *E.coli* W3110Δ*fimD* strain.
Results

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Results

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Figure legend

Figure 1: Yeast agglutination assay. This assay is based on the specific recognition of mannose units on the cell wall of yeast by *E. coli* cells expressing intact type 1 pili leading to agglutination of yeast cells (Mirelman et al., 1980). *E. coli* strain W3110ΔfimD was complemented with either the plasmid pBAD30_fimD_{His} or the vector pBAD30 alone. Agglutination properties of the wild-type strain W3110 and W3110ΔfimD are also shown.

Figure 2: Growth curves of *E. coli* HM125 (a) and MC1061 (b) harboring the expression plasmid pBAD30_fimD_{His}. FimD production was induced at an OD_{600} of 0.7 with different arabinose concentrations. (●) 0 %, (○) 0.01 %, (♦) 0.05 %, (○) 0.1 % (w/v) arabinose.

Figure 3: SDS-PAGE analysis of the membrane preparation showing the enrichment of FimD in the outer membrane. Lane M, molecular mass standard; lane 1, total membrane fraction after cell lysis; lane 2, supernatant after solubilizing the inner membrane with 1 % (w/v) of Sarkosyl; lane 3, enriched outer membrane. Samples were boiled for 5 min prior to application onto the gel.

Figure 4: SDS-PAGE analysis of the detergent soluble and insoluble proteins after incubation of the outer membrane in 20 mM Tris/HCl (pH 8.0), 150 mM NaCl containing 1 % (w/v) of detergent at 25 °C for one hour. Lane M, molecular weight standard. “S” indicates the soluble fraction, and “I” insoluble material after centrifugation.

Figure 5: Preliminary data on the purified FimD_{His} protein. (A) SDS-PAGE analysis of the purified FimD_{His} at 25 °C. Lane M, molecular mass standard. Samples were loaded on the gel either without heating (−) or after 5 min incubation at 100 °C (+). (B) Far-UV CD spectrum of FimD_{His} (0.5 μM) in 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.1 % (w/v) Zwittergent 3-14 at 25 °C. The spectrum recorded with DDM-purified FimD_{His} in 0.1 % (w/v) DDM was superimposable to that presented here. (C) SDS gels of the binding experiment, in which the ability of FimD_{His} to bind FimC-FimH was tested (c.f. Materials and Methods). The FimC-FimH complex was mixed with FimD_{His} either in an equimolar ratio or in 5-fold excess in 0.1 % (w/v) DDM (left gel) and Zwittergent 3-14 (right gel). When there is binding, the FimC-FimH complex is eluted together with FimD_{His} from the Ni-NTA beads. S, supernatant after spinning down the resin; Ni, fraction eluted from Ni-NTA resins by 300 mM imidazole.
To test whether FimC–FimH binds unspecifically to Ni-NTA beads, the complex was incubated with the resin in the absence of FimD_{His} (lanes marked with *).
Results

Preliminary studies on FimD

Figure 1

Yeast agglutination

+   -   +   -

W3110ΔfimD/ pBAD30        W3110ΔfimD/ pBAD30
pEIMD             pBAD30
W3110          W3110ΔfimD

Figure 1
Results

Figure 2

(a)  

Post-induction time (h)

OD<sub>600</sub>

(b)  

Post-induction time (h)

OD<sub>600</sub>
Figure 3

Results

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Figure 3

M 1 2 3

FimD (91.4 kDa)

OmpC (40.4 kDa)

OmpA (37.2 kDa)
Figure 4

Results

Preliminary studies on FimD

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Figure 4

[Diagram showing gel electrophoresis results with markers for kDa and bands labeled FimD, OmpC, and OmpA.]
Figure 5

A

B

C

Figure 5
3. DISCUSSION

The aim of the thesis presented here was the closer investigation of the *E.coli* type 1 pilus assembly platform FimD. Initial work was focussed on an N-terminal, periplasmic domain of FimD (FimDN) that was found to be involved in binding of FimC-subunit complexes. For further elucidation of the interaction, structures of FimDN at atomic level were determined both before and after binding of a FimC-subunit complex. The structures shed light on the mechanism, by which the assembly platform specifically recognizes chaperone-subunit complexes. Lastly, work on the full-length assembly platform had been taken up. The obtained results serve as a good starting point for subsequent studies on FimD.

The 15.2 kDa FimDN domain comprising the first 139 amino acid residues turned out to be a self-folding unit with a high thermodynamic stability ($\Delta G = -33.0$ kJ mol$^{-1}$) that retains the ability of FimD to selectively bind FimC-subunit complexes. Its affinities towards these binary complexes, however, differ from those reported for the full-length assembly platform (Saulino et al., 1998). For example, full-length FimD binds FimC–FimH with 100-fold higher affinity than FimDN ($K_D = 9.1$ nM and $K_D = 0.9$ µM, respectively). On the contrary, both full-length FimD and FimDN bind FimC–FimF complexes with nearly the same affinity ($K_D = 0.9$ µM and $K_D = 3.4$ µM, respectively). These observations in conjunction with the fact that FimD, in contrast to FimDN, binds the isolated lectin domain (Barnhart et al., 2003) suggest that another part of FimD possibly contributes to the binding of the lectin domain explaining the much higher affinity of full-length FimD towards the FimC–FimH complex. Since both domains of FimH do not interact with each other, it is likely that the lectin domain is inserted into the pore of FimD, allowing simultaneous binding of the pilin domain to FimDN. In this way, the interactive groove of FimH occupied by FimC is exposed to the periplasm. The observation that chaperone-adhesin complexes are bound with the highest affinities to the cognate assembly platforms and that the adhesin is found at the tip of the pilus led to the hypothesis that the relative affinities of the assembly platform for the various chaperone-subunit complexes determine the order of the subunits within the pilus. At least, this mechanism would ensure that a substantial fraction of pili bear an adhesin at their tip. The above-mentioned experiments of Saulino et al., in which the affinities of full-length assembly platforms towards their cognate chaperone-subunit complexes were determined by surface plasmon resonance, however, contain some ambiguities. Firstly, one of the authors concluded
Discussion

that PapC used in that study is highly prone to aggregation (Li et al., 2004). This indicates that the protein used in the study might be partly aggregated and accordingly, the measured affinities might not reflect that of native proteins. As discussed below, the observation that His-tagged FimD is prone to aggregation was made in this thesis as well. Secondly, it is known that surface plasmon resonance may yield higher apparent affinities than binding experiments in solution due to local rebinding effects (Nieba et al., 1996). Moreover, the fact that the protein is bound to the chip surface may lead to partial denaturation of protein or shielding interactive surfaces. Taking these facts into account, the present binding data on full-length assembly platforms should be interpreted with care.

The fact that FimDₙ binds FimC–FimF and FimC–FimFₜ (FimFₜ: FimF truncated at its N-terminus by the predicted donor strand extension) equally well indicates that the donor strand of the pilus subunit is likely to be solvent-exposed and capable of binding to the incomplete Ig-like fold of a subunit in the translocation pore. In this way, the C-terminal groove of the pilus subunit would present an additional binding site for the next subunit in the assembly line. The intrinsic affinities of different subunits for each other thereby are expected to be an important factor that ensures the correct order of subunit incorporation into the pilus. Such a model explains why chaperone-subunit complexes with very low affinities to FimDₙ are still incorporated into the pilus, provided that FimD is pre-loaded with an appropriate subunit. This might be true for FimC–FimG and FimC–FimA complexes whose affinities towards FimDₙ seem to be at least 10- to 100-fold lower than that observed for the FimC–FimH and FimC–FimF complexes. As far as FimC–FimA complexes are concerned, this would guarantee that the pilus rod, which is composed exclusively of FimA subunits, cannot be formed prior to assembly of the tip fibrillum.

The structure determination of FimDₙ, both in isolated form and in complex with FimC–FimHₚ, contributed substantially to our understanding on the mode of action of FimD in the recognition of chaperone-subunit complexes. The crystal structure of the ternary FimDₙ–FimC–FimHₚ complex revealed that the key element of FimD for recognition of chaperone-subunit complexes is the “arm” (residues 1–24), which was completely unstructured in isolated FimDₙ as uncovered by the NMR structure. This N-terminal extension gets ordered upon complex formation and forms a protrusion that specifically interacts with both the chaperone FimC and the bound pilin domain FimHₚ. The arm is indeed the only region in FimDₙ that forms contacts with the chaperone-bound subunit, and is thus
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exclusively responsible for the selective binding of the different chaperone-subunit complexes. The fact that the arm interacts with both the chaperone and the subunit allows assigning another important function to the arm. It acts as cargo sensor, in that it enables FimD to discriminate between chaperone molecules loaded with pilus subunits and free chaperones. This in turn prevents that the assembly platform is occupied by an unloaded chaperone that would stall pilus assembly. The fact that the assembly platform does not bind the isolated chaperone has been confirmed by both in vitro and in vivo experiments (Dodson et al., 1993; Saulino et al., 1998).

The X-ray structure of the ternary complex further revealed that the common element of the interactions of FimD\textsubscript{N} with the four different FimC-subunit complexes is the contact between the N-terminal FimC domain and the structured "core" of FimD\textsubscript{N} (residues 25–125). Whereas a single point mutation in the arm was sufficient to abolish binding of FimD\textsubscript{N} to FimC-subunit complexes completely, point mutations in the core did not lead to complete destruction of interaction between FimD\textsubscript{N} and FimC-subunit complexes, but only to reduced affinities. The rather rugged interface between the FimD\textsubscript{N} core and the N-terminal FimC domain might ensure that the assembly platform recognizes only the chaperone (complexed by a subunit) belonging to the same fimbrial system. Considering that several pilus systems may coexist simultaneously in a single bacterium, this would guarantee the specificity of the assembly machinery.

An intriguing hint at the function of the C-terminal hinge segment (residues 126–139) of FimD\textsubscript{N} was obtained by the NMR structure determination of FimD\textsubscript{N} in conjunction with the TROSY experiment, in which chemical shift variations between isolated FimD\textsubscript{N} and FimD\textsubscript{N} in complex with FimC-FimH\textsubscript{p} were mapped. According to the NMR structure, the segment 125-139 adopts no regular secondary structure, but is not completely disordered. In addition, the invariant tryptophan residue 133 is positioned in a binding pocket on the surface of the FimD\textsubscript{N} core (residues 25–125). Furthermore, comparison of the thermal stability of FimD\textsubscript{N} with that of the C-terminal truncated variant (residues 1–125) revealed identical transition midpoints of 67.6 ± 0.5 °C suggesting that the C-terminal segment does not tightly interact with the core of FimD\textsubscript{N}. Even though the C-terminal segment is not directly involved in binding of the FimC-subunit complexes, a number of amino acid residues (Arg125, Glu131, Trp133, and Asp134) in this segment showed strong chemical shift changes in the TROSY experiment suggesting a movement of this segment upon binding of the FimC-FimH\textsubscript{p}.
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complex. These results altogether indicate an intramolecular association/dissociation equilibrium between the FimDN core and the segment 126–139 that is potentially related to a movement of residues 1–125 relative to the transmembrane domain of FimD upon binding of FimC–subunit complexes to FimD. The observation that segment 126–139 of FimDN was proteolytically degraded only when FimDN was complexed with FimC–FimHp further supports the hypothesis that the C-terminal segment 126–139 is displaced from the core of FimDN upon binding of a FimC–subunit complex resulting in a flexible C-terminal segment. FimDN(1–139) would thus exist in an “open” conformation cable of binding chaperone-subunit complexes, and a “closed” conformation in which Trp133 is bound to its pocket on the surface of the FimDN core 25–125. A movement of FimDN(1–139) could also be related to the delivery of pilus subunits to the translocation pore and to the release of the free chaperone to the periplasm for the next assembly cycle. This hypothesis needs to be verified by further experimental evidence.

The study on FimDN together with the structural information on isolated FimDN and the ternary FimDN–FimC–FimHp complex shed light on the initial interaction of FimD with chaperone-subunit complexes. Towards the goal to elucidate the entire pilus assembly process, data on the mode of action of full-length FimD is required. Therefore, initial work on FimD had been taken up. Given the observation that over-production of FimD impairs cell growth, an expression system with a tightly regulated promoter was required for the expression of the fimD gene. Using the pBAD vector system with an arabinose-inducible promoter (Guzman et al., 1995), FimD can be produced at high levels. Purified FimD, however, is likely to be in an instable, non-native conformation. Therefore, appropriate conditions for obtaining active FimD have to be found. To achieve this, the following points should be considered. Firstly, a suitable detergent has to be found in which FimD retains its stability and activity. Secondly, the influence of an affinity tag on protein stability (in this study, a His6-tag was attached to the C-terminus of FimD) should be investigated. A recently published study on PapC reports that the C-terminal His6-tag might induce aggregation of the tagged PapC (Li et al., 2004). Thirdly, the oligomeric state of FimD should be determined in a reliable way as the oligomeric states of different assembly platforms are currently discussed controversially (Thanassi et al., 1998b, Harms et al., 1999, Li et al., 2004). Since assembly platforms from different pilus systems are thought to function in the same manner, they are expected to exist in the same oligomeric state. The exact determination of the oligomeric state of the assembly platform therefore is of great importance for understanding their
physiological mode of action in pilus assembly. Fourthly, an appropriate activity assay has to be established that allows to assess the functionality of the FimD protein in detergent solution.

To date, studies on assembly platforms have been mainly focused on their properties to bind chaperone-subunit complexes. The molecular basis of the processes that follow this initial binding event is largely unknown. It is accepted that the next reaction step comprises the dissociation of the chaperone from the chaperone-subunit complex, which allows subunit-subunit interaction to occur. As spontaneous assembly of subunits is very slow, the donor strand exchange reaction is expected to have a high activation energy. Presumably, the assembly platform has a mechanism that lowers this energy barrier and therefore works as a catalyst in the donor strand exchange reaction. In future work, this hypothesis needs to be verified by experimental evidence. The fact that the presence of FimD\textsubscript{N} did not accelerate the donor strand exchange reaction under the tested conditions (data not shown) implies that the catalysis might be carried out by a segment of the assembly platform apart from the N-terminal domain. A potential candidate would be a putative C-terminal, periplasmic domain (residues 556-833) predicted based on the topology model of FaeD in conjunction with its sequence alignment with FimD (Harms et al., 1999). Another topology prediction program (Martelli et al., 2002) reveals that this C-terminal domain may start at residue 659 in FimD. Currently, it is controversially discussed if the C-terminal segment of the assembly platform forms an independent periplasmic domain, or if it is part of the transmembrane domain. Our attempts to express this predicted C-terminal domain (constructs that start at residue 544, 556, 611, 617, or 651 of the mature FimD protein) in the periplasm of the \textit{E.coli} strain HM125 were not successful (data not shown). It is possible that this domain is stabilized by another segment of FimD \textit{in vivo} and therefore not stable if expressed alone. Another candidate that could serve as catalytic site for the donor strand exchange reaction would be a loop that protrudes into the periplasmic space. Interestingly, an unusual long periplasmic loop of 109 residues is predicted to be located between \(\beta\)-sheets 4 and 5 using the topology prediction program described by Martelli et al. (2002).

Donor strand exchange comprises two steps, namely dissociation of the chaperone-subunit complex and association of two pilus subunits. Of these two, the rate-limiting step is most likely accelerated by the assembly platform. It could catalyze the dissociation of the chaperone from the chaperone-subunit complex by donating a segment that would serve as a transient donor strand for the subunit. Such an assembly intermediate is expected to be
thermodynamically more stable than an isolated subunit, which would be formed in an uncatalyzed reaction. Trapping such intermediates would be a challenging task, as they probably exhibit short half-lives. Provided that the assembly platform accelerates the second step in the donor strand exchange, i.e. the association of two subunits, this could be achieved by an oligomeric state of the assembly platform. Since the association reaction is concentration-dependent, an oligomeric assembly platform that increases the local concentration of chaperone-subunit complexes would provide an ideal subunit-subunit interaction site. Exact determination of the oligomeric state of FimD is therefore important for better understanding of the mode of action of the assembly platform in this assembly process. In addition, subunit-subunit interactions could be accelerated, if rebinding of the chaperone to the uncapped subunit could be prevented by the assembly platform, e.g. by steric shielding, once the chaperone has dissociated. The small and flexible solvent-exposed donor strand of the subsequent subunit might still be able to bind to the shielded subunit in the translocation pore. The fact that the formation of chaperone-subunit complexes is much faster than that of subunit-subunit complexes (Vetsch et al., 2004) in conjunction with the observation that the polymerization of FimA does not occur rapidly in vitro despite the high dissociation rate constant of FimC-FimA complexes (A. Ignatov and M. Vetsch, unpublished results) suggests that the rate-limiting step in the donor strand exchange is more likely the association of two subunits rather than the dissociation of the chaperone from the subunit. Whether FimD accelerates these reactions is one of the questions that need to be addressed in future work.

The assembly platforms are likely to be the site of pilus subunit translocation across the outer membrane. How does the pore look and how are the subunits translocated through it to reach the cell surface? Structural studies on FimD will help answering these questions. Information on the role of FimD in the donor strand exchange reaction together with structural data will provide fundamental insight into the assembly process of type 1 pili that serve as a prototype of highly oligomeric, adhesive bacterial surface organelles.
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CURRICULUM VITAE

Name: Mireille Nishiyama

Date and place of birth: July 13th 1975 in Odawara, Japan

Nationalities: Swiss and Japanese

Education:

1982 – 1988 Primary school, Minamiashigara-shi, Japan

1988 – 1992 Junior high school, Minamiashigara-shi, Japan

1992 – 1994 High school, Atsugi-Kotogakko, Atsugi-shi, Japan

1994 – 1995 Preparatory Course for Swiss Universities, Fribourg, Switzerland with subsequent entrance examination at the ETH Zurich, Switzerland

1995 – 2000 Studies in Pharmaceutical Sciences at the ETH Zurich, Switzerland

2001 – 2005 PhD studies at the Institute for Molecular Biology and Biophysics, ETH Zurich, Switzerland
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