The role of the chaperone FimC in the formation of type 1 pili from Escherichia coli

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The role of the chaperone FimC in the formation of type 1 pili from *Escherichia coli*

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# Table of Contents

**SUMMARY** ................................................................................................................................................. 1  
**ZUSAMMENFASSUNG** ................................................................................................................................. 3  

1. **INTRODUCTION** .................................................................................................................................... 5  
   General aspects of protein folding ............................................................................................................. 5  
      *How do proteins fold?* .......................................................................................................................... 5  
   *Molecular chaperones* ............................................................................................................................. 10  
   *Folding catalysts* ....................................................................................................................................... 12  
   Fiber formation via the chaperone/usher pathway in Gram-negative bacteria ....................................... 14  
      *Overview* ........................................................................................................................................... 14  
      *Donor strand complementation* .......................................................................................................... 16  
      *The role of the usher in subunit assembly* ............................................................................................ 19  

2. **RESULTS** ............................................................................................................................................... 21  
   Chaperone-independent folding of type 1 pilus domains .......................................................................... 21  
   Pilus chaperones represent a novel type of protein folding catalyst ....................................................... 36  
   Intrinsic affinities explain the sequential order of subunits in type 1 pili and constitute the driving force for pilus assembly .......................................................................................................................... 62  

3. **ADDITIONAL ARTICLE** ......................................................................................................................... 90  
   Identification and characterization of the chaperone-subunit complex-binding domain from the type 1 pilus assembly platform FimD ...................................................................................................... 90
4. OUTLOOK .............................................................................................................. 104

5. REFERENCES ....................................................................................................... 108

CURRICULUM VITAE .................................................................................................. 116
SUMMARY

Adhesive type 1 pili from uropathogenic *Escherichia coli* strains play a crucial role during infection by mediating the attachment to and potentially invasion of the host tissue. They belong to a class of surface organelles that enable a variety of pathogenic Gram-negative bacteria to bind to host cells. These filamentous, highly oligomeric protein complexes are assembled by the chaperone/usher pathway, according to which the individual pilus subunits fold in the bacterial periplasm and form stoichiometric complexes with a specific periplasmic chaperone that is essential for pilus assembly. The chaperone subsequently delivers the subunits to an outer membrane assembly platform, termed usher, which mediates subunit assembly and translocation to the cell surface. The assembly of bacterial pili provides a model for fundamental biological problems including protein folding, secretion, and assembly into highly ordered macromolecular structures.

The focus of the thesis presented here was to elucidate the role of periplasmic pilus chaperones in the folding process of pilus subunits, and to investigate how pilus chaperones prevent premature assembly of pilus subunits. The type 1 pilus system from *E. coli* was used as a model. Type 1 pili are composed of the main structural pilus subunit FimA that assembles into a long rigid helical rod and the subunits FimF, FimG, and FimH, forming a distal tip fibrillum. Besides the pilin domain that shares high sequence homology with the other pilus subunits, FimH in addition has a lectin domain that mediates the adhesion of the pili to host glycoproteins. The chaperone and the usher required for type 1 pilus assembly are FimC and FimD, respectively.

First, folding of a subunit (the pilin domain of FimH) in the absence of the chaperone FimC was analyzed. In contradiction to a widely accepted hypothesis, the subunit was found to fold autonomously and with a cooperativity that is typical of proteins of similar size. The thermodynamic stability of the pilin domain is low (-10 kJ mol⁻¹) so that a significant fraction of the domain is unfolded even in the absence of denaturant, which may explain the high tendency of pilus subunits to aggregate in the absence of chaperone. Folding kinetics revealed that the pilin domain folds on a timescale of minutes, which is surprisingly slow for a single-domain protein. It was further found that the chaperone-subunit complex is efficiently formed only when the chaperone is present during refolding of the subunit. These results suggested
that periplasmic pilus chaperones might directly influence and probably even accelerate the folding process of pilus subunits.

To test this attractive hypothesis, folding of FimG was investigated. Unlike the pilin domain of FimH, FimG has a tryptophane residue that can be used as a sensitive probe to follow folding by fluorescence spectroscopy. Similar to the pilin domain of FimH, monomeric FimG displays a low thermodynamic stability (-12 kJ mol⁻¹) and refolds on a timescale of minutes. Surprisingly, the presence of the chaperone FimC changes the folding kinetics of FimG dramatically. It was discovered that FimC binds the non-native pilus subunit and accelerates folding by 100-fold. Moreover, the FimC-FimG complex is formed quantitatively and very rapidly 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. FimC thus represents a novel type of protein folding catalyst and simultaneously acts as a kinetic trap preventing spontaneous subunit assembly in the periplasm.

Finally, a model system was developed and used for quantitative analysis of subunit assembly, the central event in fiber formation. These experiments revealed that the intrinsic affinities of different subunits for each other correlate exactly with their position in the final pilus structure and that subunit-subunit complexes are in general thermodynamically more stable than the periplasmic chaperone-subunit complexes. Consequently, the incorporation of a subunit into the pilus is energetically favourable which explains why pilus assembly does not require cellular energy. The formation of subunit-subunit complexes in the absence of the outer membrane usher was observed to be extremely slow with apparent halftimes of several hours. Evidence was found that the outer membrane usher FimD acts as a catalyst of this reaction, ensuring that formation of subunit-subunit interactions is coupled to secretion of the assembled subunits into the extracellular space.
ZUSAMMENFASSUNG


Im ersten Teil der Arbeit wurde die Faltung einer Untereinheit (der Pilindomäne von FimH) in Abwesenheit des Faltungshelfers FimC untersucht. Im Widerspruch zu einer weitverbreiteten Hypothese faltet sich die Untereinheit eigenständig und mit einer für die Grösse dieses Proteins typischen Kooperativität. Die thermodynamische Stabilität der Pilindomäne ist gering (-10 kJ mol⁻¹), sodass auch in völliger Abwesenheit von Denaturierungsmittel ein bedeutender Anteil der Domäne ungefaltet ist. Der Faltungsprozess
der Pilindomäne erstreckt sich über einen Zeitraum von Minuten und ist damit für ein Protein mit nur einer Domäne aussergewöhnlich langsam. Weiterhin wurde entdeckt, dass sich der Komplex zwischen FimC und Pilindomäne nur dann effizient bildet, wenn FimC bereits während der Rückfaltung der Pilindomäne anwesend ist. Dies deutet darauf hin, dass periplasmatische spezifische Faltungshelfer den Faltungsprozess und womöglich sogar die Faltungsgeschwindigkeit der Untereinheiten direkt beeinflussen.


1. INTRODUCTION

General aspects of protein folding

How do proteins fold?

Protein folding is studied extensively and for diverse reasons. First, protein folding is a fascinating theoretical problem. The physical principles that direct unfolded polypeptides efficiently and on a timescale of only microseconds to minutes to their native conformation are still not understood. Second, a thorough understanding of protein folding would greatly facilitate de novo structure predictions, one of the grand challenges in the proteomics era. Third, kinetic and thermodynamic determinants of protein folding have a strong impact on the lifecycle of a given protein in vivo. This aspect receives particular attention since it became known that protein misfolding is the origin of a wide variety of severe human diseases (Dobson, 2003).

Protein folding is commonly modelled as a landscape with the free energy as a function of all possible conformations that the polypeptide can exploit. This landscape is envisaged as a funnel with a rugged interior (Fig. 1), representing the virtually infinite number of possible pathways down to the native state, with each pathway having its own distinct energetic topography (Dill and Chan, 1997). This view has emerged from theoretical studies with lattice models (Bryngelson et al., 1995) and elegantly explains why certain proteins appear to follow multiple pathways to their native conformation (Wildegger and Kiefhaber, 1997; Goldbeck et al., 1999) while other proteins fold along a single dominant pathway (Fersht, 1995). In general, four different conformational states are considered to describe the folding pathways of proteins: the denatured state, folding intermediates, the transition state and the native state.

The denatured state (D) is the starting point of protein folding and occupies the edge of the folding funnel. The individual conformations of the ensemble of denatured molecules are characterized by large entropy and low free energy. The denatured state may resemble a random coil under certain conditions (e.g. in 6 M guanidinium chloride) but simulations and experimental data indicate that denatured states have structure, especially under native conditions (Neri et al., 1992; Mayor et al., 2003; Sanchez and Kiefhaber, 2003a). These structures vary greatly for different proteins, they are extended or compact and some contain
native contacts while others comprise non-native interactions (Mok et al., 1999; Kortemme et al., 2000; Bai et al., 2001). Intrinsic propensities of certain secondary structure elements or persistent native contacts prevent D from sampling the large conformational space that a random coil occupies. Furthermore, they bias the denatured state towards native-like structures (Nauli et al., 2001).

![Figure 1. A rugged energy landscape with kinetic traps, energy barriers, and some narrow throughways to the native state. Modified from Dill and Chan, 1997.](image)

Intermediates (I) populate local free energy minima or, more figurative, basins on the free energy surface. They are separated by barriers from conformations with lower free energy. Under equilibrium conditions, most small single-domain proteins populate only the denatured and the native state. The observation of single exponential kinetic relaxation towards equilibrium can indicate that a single free energy barrier separates the two ground-states. It does however not imply that no intermediates exist on the folding pathway of such apparent two-state folders. Intermediates with high free energies are only rarely populated and thus easily escape experimental detection. This becomes evident from experiments on the closely related proteins Im7 and Im9. The folding intermediate that characterizes folding of Im7 under all conditions is not observed for Im9 at neutral pH but only at acidic pH (Gorski et al., 2001).

It is difficult to distinguish experimentally between intermediates that are obligatory precursors of the native state (on-pathway intermediates) and intermediates that cannot fold
INTRODUCTION

towards the native state but instead are in equilibrium with the denatured state (off-pathway intermediates). So far, more evidence has been found for on-pathway intermediates (Laurents et al., 1998; Bai, 1999; Bai, 2000; Capaldi et al., 2001; Sanchez and Kiefhaber, 2003a). Interestingly, high energy intermediates that are introduced into the transition state region can even accelerate protein folding (Wagner and Kiefhaber, 1999). Structures of intermediates may be largely determined by local interaction preferences, whereas in later stages of folding, non-local interactions become more important. In the case of β-lactoglobulin, a kinetic folding intermediate contains more helical structure than the native structure possibly due to the high helix propensity of the polypeptide sequence (Hamada et al., 1996; Kuroda et al., 1996; Arai et al., 1998) and also the folding intermediate of Im7 was found to have specific non-native interactions (Capaldi et al., 2002).

The transition state (TS) is the free energy barrier that separates the native state from the denatured states and folding intermediates. It can be envisaged as a saddle point in the free energy landscape that all molecules must pass if they are to fold to the native state. The ensemble of structures representing the TS may appear as a homogeneous group of related structures (Day et al., 2002) or as a heterogeneous collection of unrelated structures (Lazaridis and Karplus, 1997). When several parallel pathways lead to the native state (N), the observed transition state is the weighted average over all rate-limiting steps in all paths (Schonbrun and Dill, 2003). High resolution structures of transition states are derived from molecular dynamics simulations (Lazaridis and Karplus, 1997; Day et al., 2002) or $\phi_f$-value analysis, which compares the impact of site-directed mutations along the protein sequence on the stability of the TS and on the stability of N. Linear free energy relationships are used to relate the two effects:

$$\phi_f = \frac{\Delta \Delta G_{TS-D}}{\Delta \Delta G_{N-D}}$$  \hspace{1cm} (Eq. 1)

In order to determine $\phi_f$, the folding rate ($k_{TS-D}$) and the stability ($K_{N-D}$) of the wild-type and the mutant protein (wt and mut, respectively) are measured and used to obtain $\phi_f$ as follows (Fersht et al., 1992):

$$\phi_f = \frac{\ln(k_{TS-D}^{wt}/k_{TS-D}^{mut})}{\ln(K_{N-D}^{wt}/K_{N-D}^{mut})}$$ \hspace{1cm} (Eq. 2)
A $\phi$-value of 1 indicates that the mutation affects N and TS equally, meaning that the corresponding residue has native-like interactions in the TS, while a $\phi$-value of zero implicates that the residue has the same contacts as in D. Experiments typically yield fractional values $0<\phi<1$ among which the majority is between 0 and 0.5. This may indicate partial formation of all native contacts in a homogeneous transition state ensemble that resembles a distorted native state. Alternatively, these values may be caused by parallel folding pathways with a heterogeneous ensemble of transition states of which each has only a subset of a few native contacts.

Two types of TS are distinguished based on the distribution of $\phi$-values. In proteins with a diffuse TS, residues with $\phi$-values > 0 are spread out evenly over the entire three-dimensional structure, suggesting that the formation of a correct overall topology is the rate-limiting step in the folding process. In proteins with a polarized TS, however, large areas of the native structure have $\phi$-values very close to 0. A polarized TS indicates that formation of just a substructure is rate-limiting and that the remaining parts fold after the TS. An extensive mutational analysis of the small protein CI2 yielded a few $\phi$-values close to 1 in addition to many residues with values between 0 and 0.5. This observation led to a popular model of protein folding that was termed nucleation/condensation mechanism (Fersht, 1995).

According to this model, the rate-limiting step is a condensation of the chain (causing values between 0 and 0.5) coupled to the formation a folding nucleus (causing the values close to 1). This model was, however, challenged by the finding that the $\phi$-values close to 1 are associated with a very high experimental error and therefore in most cases not reliable (Sanchez and Kiefhaber, 2003b). This close examination of $\phi$-values revealed that proteins with a diffuse transition state (such as CI2) have extremely rarely authentic $\phi$-values above 0.5. For proteins with a polarized transition state, the upper limit is 0.8. These results imply that folding transition states in general contain no specific nucleus.

The native state is a determinant of protein folding as native-like interactions dominate the folding process. For two-state folders, the folding rates correlate strongly with the relative contact order (RCO), a measure of the average sequence separation of residues forming contacts in the native protein (Plaxco et al., 1998). Because, obviously, local contacts form faster than nonlocal ones, $\alpha$ helical proteins tend to fold faster than $\beta$ sheet proteins. Several
INTRODUCTION

lines of evidence, however, indicate that RCO per se does not determine the folding rate. First, mildly perturbing point mutations do not change RCO, but the folding rates of such variants may vary over several orders of magnitude (Itzhaki et al., 1995). Second, circular permutation of proteins dramatically alters RCO, but at least in some cases the folding rates change only modestly (Miller et al., 2002). Therefore, topology is one important kinetic determinant but it is far from being the only one.

The native state is tightly connected to the thermodynamics of protein folding. The free energy of N defines, relative to the free energy of D, the gain in energy that drives protein folding.

$$\Delta G_{\text{stab}}^0 = G_N^0 - G_D^0 = \Delta H_{\text{stab}}^0 - T \cdot \Delta S_{\text{stab}}^0$$  \hspace{1cm} (Eq. 3)

For proteins that populate only N and D under equilibrium conditions, $\Delta G_{\text{stab}}^0$ can be measured by standard methods. Both the enthalpic gain and the entropic cost have large absolute values (Jaenicke, 2000) but they almost compensate each other under physiological conditions, which is evident from the observation that the values of $\Delta G_{\text{stab}}^0$ of medium size globular proteins are in the order of only -50 kJ mol⁻¹ (Pfeil, 1998). The delicate balance of stabilizing and destabilizing interactions in proteins results in a high degree of flexibility, which has been found to be crucial for such diverse aspects as function, translocation, and degradation of proteins (Shoemaker et al., 2000; Matouschek, 2003).

Finally, it should be noted that our current knowledge about the mechanism of protein folding is largely based on experiments that observe ensembles of molecules. Protein folding, however, is an inherently heterogeneous process with a large number of microscopic pathways that lead to the native state. A thorough understanding of the principles of protein folding thus requires methods that investigate the behaviour of single molecules. One approach is fluorescence resonance energy transfer spectroscopy with single molecules (Zhuang and Rief, 2003). This technique yielded new important information on the free energy surface of protein folding (Schuler et al., 2002; Lipman et al., 2003). Another promising method are molecular dynamics (MD) simulations with atomic resolution. Such calculations depend strongly on the parameters that are used and, therefore, need experimental verification. Ideally, the same conditions should by applied in silico and in vitro. To date, the
timescales that are accessible in MD simulations are typically a few nanoseconds, while reactions that can be followed experimentally usually occur on a timescale of at least tens of nanoseconds (Mayor et al., 2003). So the gap separating theory and experiment is almost but not completely closed. Single molecule methods have in common that they potentially yield detailed information about the structures and the transient populations of I and TS, information that is essential to understand the fundamental principles of protein folding.

**Molecular chaperones**

Although protein folding is in principle a spontaneous process (Anfinsen, 1973; Dobson and Karplus, 1999) a large variety of folding helpers are required for efficient folding inside cells. They are divided into two groups according to their function. Molecular chaperones enhance the efficiency but not the rate by which certain proteins fold while folding catalysts accelerate certain rate-limiting steps of the folding process.

The principle function of molecular chaperones is to prevent misfolding and aggregation of folding proteins. Molecular chaperones in general recognize unfolded or misfolded proteins by binding to extended hydrophobic segments, which are buried upon folding and therefore only accessible in non-native protein. In contrast to small single-domain proteins, larger proteins composed of multiple domains often fold slowly and via partially folded intermediates (Jaenicke, 1999). Though compact in shape, such intermediates expose numerous hydrophobic amino acid residues and unstructured segments to the solvent for a considerable length of time and are thus prone to unspecific aggregation or, in rare cases, to ordered self-assembly, driven by interchain hydrogen bonding and hydrophobic forces (Dobson and Karplus, 1999). Such structured assemblies of misfolded protein became known as amyloid and are associated with severe human diseases (Dobson, 2003). The tendency of proteins to aggregate is greatly increased in the cell by two factors. First, while nascent chains emerge from the ribosome, they contain not all sequence information necessary for folding and therefore expose structural features of non-native protein until the entire sequence has emerged from the ribosome. Second, the cytosol is highly crowded. The cytoplasm of *E. coli* contains macromolecules at a concentration of more than 300 mg/ml (Ellis, 2001). The higher the concentration of aggregation-prone molecules is the more such non-specific interactions occur because aggregation is an intermolecular process.
INTRODUCTION

A set of chaperones in the *E. coli* cytoplasm binds to nascent chains, including trigger factor (TF), DnaK, and SecB. TF is associated with the ribosome (Deuerling et al., 1999) and binds nascent chains in a 1:1 stoichiometry. Upon release from TF, most proteins fold rapidly to their native conformation (Hesterkamp et al., 1996). In addition, TF has PPIase activity (see below), but the biological significance of this activity remains unclear (Kramer et al., 2004). Eubacterial Hsp70, termed DnaK, is not associated with the ribosome but has overlapping function with TF in binding to nascent chains and releasing them in a folding-competent conformation. The functional redundancy is evident from the observation that *E. coli* strains lacking TF or DnaK display no severe folding defects whereas strains with deficiency in both TF and DnaK are not viable (Deuerling et al., 1999; Teter et al., 1999). Binding and release of unfolded polypeptides from DnaK is ATP-dependent and requires the assistance of the Hsp40 co-chaperone DnaJ and the nucleotide exchange factor GrpE (Harrison et al., 1997; Rudiger et al., 1997; Mayer et al., 2000; Pellecchia et al., 2000; Sha et al., 2000). SecB is involved in the translocation of secretory proteins across the cytoplasmic membrane (Fekkes and Driessen, 1999). It associates with nascent chains, stabilizes their unfolded state, and targets the polypeptides to SecA, from which they are translocated, driven by the ATPase activity of SecA, through the heterotrimeric SecYEG channel (Fekkes et al., 1997; Fekkes et al., 1998; Hardy and Randall, 1991; Manting et al., 2000). *E. coli* strains lacking SecB are viable but suffer from translocation and growth defects (Shimizu et al., 1997).

The only chaperones that are essential for growth of *E. coli* under all conditions are GroEL and its co-chaperone GroES (Fayet et al., 1989). They belong to the conserved class of chaperonins which all form large cylindrical complexes of about 800 kDa enclosing a central cavity. GroEL consists of two homoheptameric rings that are stacked back-to-back. Non-native substrate that has been released from TF or the Hsp70 system is bound via hydrophobic segments to GroEL. This step is closely followed by binding of GroES, a homoheptameric ring, and translocation of the substrate into the large enclosed cavity. There, folding can occur unimpaired by interactions with other folding proteins during approximately 10 seconds. Then, GroES and the substrate are released. Non-native substrate is rapidly rebound and undergoes further folding cycles until it has reached the native conformation. The function of GroES requires large conformational movements for which the energy is provided by ATP-hydrolysis (Bukau and Horwich, 1998; Sigler et al., 1998).
INTRODUCTION

Compared to the cytoplasmic chaperones of *E. coli* that have been investigated in great detail, the periplasmic chaperones and their interplay remain poorly understood. Periplasmic chaperones work under difficult conditions. The chemical composition, ionic strength, pH and redox conditions in the periplasm may vary within a wide range. Further, ATP, which is essential for the function of many cytoplasmic chaperones, is not available. Intriguingly, many of the known periplasmic chaperones have in addition enzymatic activity. SurA, PpiD, and FkpA are peptidyl-prolyl isomerases (Tormo *et al.*, 1990; Dartigalongue and Raina, 1998; Ramm and Pluckthun, 2000; Behrens *et al.*, 2001) and DegP is a protease at elevated temperatures (Spiess *et al.*, 1999). Deletions in genes encoding periplasmic chaperones often lead to defects in the formation of outer membrane proteins (Tormo *et al.*, 1990; Chen and Henning, 1996; Rouviere and Gross, 1996). This observation suggests that an important task of periplasmic chaperones is assistance in transport and folding of outer membrane proteins. This was demonstrated directly for SkpA, which promotes the formation of soluble periplasmic intermediates of outer membrane proteins (Schafer *et al.*, 1999). The periplasm also harbours a group of highly specialized pilus chaperones that are required for the formation of a variety of fibers attached to the cell surface. These chaperones will be discussed in detail below.

**Folding catalysts**

The conformation of each peptide bond in a native protein is fixed by structural constraints. For sterical reasons, *cis* bonds are very rare in native proteins. In contrast, a given peptide bond in the ensemble of denatured protein populates statistically both the *trans* and the *cis* conformation. The ratio between *trans* and *cis* is largely determined by the combination of the two amino acids that form the peptide bond. The highest ratio of *cis* bonds in denatured protein or in peptides is found for the Xaa-Pro (prolyl) peptide bond. Depending on the identity of Xaa, 6-38 % of the prolyl peptide bonds are in *cis* (Reimer *et al.*, 1998). For nonprolyl bonds, the equilibrium fractions of bonds in *cis* are around 0.15 % (Scherer *et al.*, 1998). Prolyl peptide bonds isomerise on a timescale of 10 – 100 s (Schmid, 1992), while nonprolyl peptide bonds isomerise on a timescale of 0.5 s for *cis-trans* and 100 s for *trans-cis* (Pappenberger *et al.*, 2001). Peptide bonds with a non-native conformation do not necessarily block folding completely. It has been observed that some proteins fold to a conformation that is very similar to the native state despite non-native peptide bonds (Pappenberger *et al.*, 2001).
INTRODUCTION

2003). But frequently prolyl isomerization is rate-limiting for folding and may cause complex folding kinetics with different folding rates if folding of the fraction of denatured molecules with native-like peptide bond conformations is faster than prolyl isomerization (Schmid, 1992). Non-prolyl isomerization has so far seldom been recognized as a rate-limiting step, possibly because it occurs faster than many folding reactions (Odefey et al., 1995; Pappenberger et al., 2001).

A class of enzymes, peptidyl cis/trans isomerases, catalyses peptide bond isomerization. Numerous members of this class accelerate isomerization of prolyl bonds and hence are known as peptidyl prolyl cis/trans isomerases (PPIases; EC 5.2.1.8; Schmid, 2001). In E. coli, PPIases are found both in the cytoplasm (e.g. trigger factor) and the periplasm (FkpA, PpiA, PpiD, SurA). Only recently it was discovered that also nonprolyl peptide bond isomerization is catalysed by certain enzymes when DnaK of E. coli was identified as a peptidyl nonprolyl cis/trans isomerase (Schiene-Fischer et al., 2002).

Another potentially rate-limiting step in protein folding is the formation of correct disulfide bonds. Many secretory proteins, membrane proteins and proteins located in the periplasm of Gram-negative bacteria contain disulfide bonds. Such bonds are often crucial for stability and function. Disulfide bonds are formed by oxidation of two cysteine residues, usually in compartments with conditions favouring a spontaneous reaction, which, however, is slow. The oxidizing compartment of E. coli is the periplasm. It is equipped with dithiol/disulfide oxidoreductases that promote rapid formation of disulfide bonds and the isomerization of incorrect disulfide bonds (Collet and Bardwell, 2002; Hiniker and Bardwell, 2003). The main oxidant is DsbA, a soluble and very efficient enzyme of the thioredoxin family. The catalytic dithiol/disulfide pair of DsbA is highly oxidizing. Even at acidic pH, i.e. under conditions where spontaneous oxidation is extremely slow, DsbA rapidly introduces disulfide bonds into substrate proteins, due to the low pKₐ of one of the active site cysteines (Wunderlich et al., 1993b; Nelson and Creighton, 1994). The high efficiency of DsbA in oxidising newly translocated polypeptides is further enhanced by the ability of DsbA to bind unfolded proteins (Frech et al., 1996b). DsbA may be so efficient that it introduces disulfide bonds while the substrates are still in the process of translocation (Sone et al., 1997; Hiniker and Bardwell, 2004). Rapid introduction of disulfide bonds is advantageous because folding of the polypeptide chain may bury the cysteines and thus render them inaccessible to oxidation by
INTRODUCTION

DsbA (Frech et al., 1996a). Upon oxidation of a substrate protein, DsbA itself gets reduced. It is reoxidized by the integral inner membrane protein DsbB (Bardwell et al., 1993; Dailey and Berg, 1993; Missiakas et al., 1993; Grauschopf et al., 2003). From DsbB, the electrons are transferred to components of the respiratory chain (Bader et al., 1999; Kobayashi and Ito, 1999). DsbA introduces disulfide bonds very rapidly and randomly, which may lead to the formation of incorrect bonds (Wunderlich et al., 1993a). It is the task of periplasmic DsbC to isomerize such bonds into the correct pattern (Zapun et al., 1995). In order to do so, the two active site cysteines need to be kept reduced. This is achieved by the inner membrane protein DsbD, that in turn is reduced by cytoplasmic thioredoxin (Rietsch et al., 1997). The oxidizing DsbA-DsbB and the reducing DsbC-DsbD pathway are kept apart by high kinetic barriers that prevent a fast flow of electrons between the two pathways (Rozhkova et al., 2004).

Fiber formation via the chaperone/usher pathway in Gram-negative bacteria

Overview

Pathogenic Gram-negative bacteria express adhesive fibrous surface organelles that mediate binding to the sites of infection. A large number of such organelles are assembled by the chaperone/usher pathway (Sauer et al., 2000). The final fibers consist exclusively of structural subunits. These enter the periplasm through the SecYEG translocon. 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. This disulfide bond possibly enhances the stability of the final fiber structure but seems dispensable for the assembly process because E. coli strains lacking DsbA retain the ability to produce fibers (Hultgren et al., 1996). Once in the periplasm the subunits form heterodimers with dedicated periplasmic chaperones. The periplasmic chaperones assist subunit folding (Barnhart et al., 2000), prevent non-productive aggregation of the subunits (Jones et al., 1997) and also avert their spontaneous premature assembly (Soto et al., 1998). The chaperone-subunit complexes dissociate at the outer membrane where the subunits are incorporated into fibers that are secreted through oligomeric usher pores (Fig. 2). Organelles assembled by the chaperone-usher pathway are divided into pilus and nonpilus fibers (Hung et al., 1996). Their subunits have the same fold and the same principles underlie their interactions with the chaperones and with each other. Nonpilus fibers are composed of only one or two types of subunits. The best
stressed member of this class is the capsular F1 antigen from the plague pathogen *Yersinia pestis* (Zavialov et al., 2002; Zavialov et al., 2003). In contrast to nonpilus fibers, pili have a complex architecture. They are composite structures made of a rigid helical rod and a flexible

**Figure 2.** Schematic presentation of the current view of type 1 pilus assembly by the chaperone/usher pathway. As the structural pilus subunits FimA, FimF, FimG, and FimH enter the periplasm, their signal sequence is cleaved off and a disulfide bridge is introduced. The subunits then form heterodimers with a specialized pilus chaperone, FimC. These complexes bind to the outer membrane usher FimD and subsequently dissociate. While the chaperone is released back to the bulk medium of the periplasm, the subunit is incorporated into the growing pilus, which is translocated through FimD to the cell surface.
thin tip fibrillum joined to the distal end of the rod. P pili and type 1 pili from enteropathogenic *E. coli* are two prominent members of this class. The rod of type 1 pili consists of 500-3000 FimA subunits, has a diameter of 6.9 nm and is up to 2 µm long. The tip fibrillum is approximately 15 nm long and formed by the adaptor subunits FimF and FimG and the adhesive subunit FimH at the distal end of the pilus (Fig. 2; Hahn *et al*., 2002). The role of another potential subunit, FimI, remains unclear (Saulino *et al*., 2000; Valenski *et al*., 2003).

Strains of uropathogenic *E. coli* are the cause of the vast majority of urinary tract infections (Hooton and Stamm, 1997). They express a number of virulence factors that enable them to colonize the urinary tract and to persist in the face of robust host defences. The interplay between uropathogenic bacteria and the defense mechanisms in the bladder is reviewed thoroughly in Mulvey *et al.* (2000). Importantly, initial pathogenesis critically relies on the ability of the bacteria to attach to host tissue through specific adhesins. Among uropathogenic *E. coli*, type 1 pili are the most widely distributed adhesive organelles (Langermann *et al*., 1997). At their tip, type 1 pili display the subunit FimH, which consists of two domains (Fig. 3A). The C-terminal pilin domain is responsible for incorporation of FimH into the pilus, while the N-terminal lectin domain mediates mannose-sensitive binding to the glycoproteins uroplakin Ia and Ib, abundant integral membrane proteins of the bladder epithelium (Wu *et al*., 1996). Moreover, FimH triggers the internalization of bacteria into bladder cells (Martinez *et al*., 2000). Within the host cell, bacteria are protected from a variety of host defense mechanisms and also antibiotics. Internalized bacteria not only persist but in some cases even replicate (Mulvey *et al*., 2001) and thus constitute a reservoir of pathogenic bacteria, which may cause recurrent infections seen in a large portion of women with urinary tract infections (Schilling *et al*., 2002).

**Donor strand complementation**

Crystal structures of chaperone-subunit complexes revealed that the subunits have an atypical immunoglobulin (Ig)-like fold that lacks the (C-terminal) G strand, creating a large hydrophobic groove on the subunit surface. In the chaperone-subunit complex, this groove is occupied by an extrinsic β strand, provided by the chaperone (Choudhury *et al*., 1999; Sauer *et al*., 1999; Zavialov *et al*., 2003). This remarkably tight interaction is referred to as “donor strand complementation” and the extrinsic β strand is called “donor strand”. The
complemented structure of the subunit, however, has an atypical Ig-like fold because the donated strand runs parallel to the F strand of the subunit rather than antiparallel (Fig. 3B). Zavialov et al. (2003) recently showed that donor strand complementation also governs subunit-subunit interactions.

Figure 3. The Ig-like fold of subunits is complemented by extrinsic donor strands. (A) FimH, the adhesive subunit of type 1 pili, has a N-terminal lectin domain (FimH_L) and a C-terminal pilin domain (FimH_P) that is homologous to the other structural pilus subunits. FimH forms a periplasmic complex with the chaperone FimC and interacts with FimG after incorporation into the pilus (see also Fig. 2). (B) The crystal structure of the FimC-FimH complex revealed that FimH_P has an incomplete Ig-like fold unless the lacking (G-) strand is provided by another protein. The donor strand of FimC inserts parallel to the F-strand of the subunit creating an atypical Ig-like fold. Strong evidence suggests that the FimG donor strand inserts antiparallel to the F-strand of FimH_P, complementing the Ig-like fold in a canonical manner.
The role of the chaperone donor strand is taken over by the previously disordered N-terminus of a second subunit, suggesting that in the fiber, the hydrophobic groove of each subunit is occupied by the N-terminus of the adjacent subunit. In contrast to the chaperone donor strand, the strand provided by another subunit inserts parallel to the F-strand of the acceptor subunit (Fig. 3B), creating a canonical Ig-like fold as it had been predicted (Choudhury et al., 1999; Sauer et al., 1999). In the same study, Zavaliov et al. compared the structure of a subunit in the chaperone-bound state with the structure of the same subunit in the subunit-bound state. They found that the two β sheets of the Ig-like fold have low shape correlation statistics (Lawrence and Colman, 1993) in the chaperone-bound subunit, reflecting a loosely packed hydrophobic core. Meanwhile, the subunit in complex with another subunit has a tightly packed hydrophobic core as is typically observed for β barrels consisting of two β sheets. As subunits are incorporated into the fiber, they dissociate from the chaperone and instead bind to another subunit. This process involves apparently a collapse of the hydrophobic core of the subunit, which could result in a gain in free folding energy. Zavaliov et al. propose that this potential difference in free energy might constitute the driving force for fiber assembly. According to this model, the periplasmic pilus chaperones trap a high energy folding intermediate of the subunits and preserve folding energy that is released upon incorporation of the subunit into the fiber.

While recent structural data has greatly enhanced our knowledge of chaperone-subunit and subunit-subunit interactions, two important questions concerning the dynamics of such interactions remain open. (i) The role of pilus chaperones in the folding process of subunits is not clear. While some evidence suggested, that chaperones bind to unfolded subunits and subsequently assist folding (Barnhart et al., 2000), other experiments argued for a model according to which the subunits fold autonomously prior to binding to the chaperone (Pellecchia et al., 1999; Hermanns et al., 2000). (ii) It is unknown whether chaperone-subunit interactions are indeed thermodynamically less stable than subunit-subunit interactions and if this proves true, how periplasmic pilus chaperones prevent the spontaneous formation of the more favourable subunit-subunit interactions leading to premature fiber assembly.
The role of the usher in subunit assembly

The formation of each type of fiber by the chaperone/usher pathway involves two dedicated proteins, a periplasmic chaperone (FimC in Fig. 2) and an assembly platform, termed usher, in the outer membrane (FimD in Fig. 2). Both components are essential for the assembly of fibers (Norgren et al., 1987; Klemm and Christiansen, 1990). In the absence of usher, chaperone-subunit complexes accumulate in the periplasm but no fibers are assembled (Zavialov et al., 2002). PapC and FimD, the ushers required for the formation of P pili and type 1 pili, respectively, have been investigated by electron microscopy. The ushers comprise six or twelve subunits that form a ring-shaped structure with an outer diameter of 15 nm and a pore that is 2 nm wide (Thanassi et al., 1998; Saulino et al., 2000). Such a pore cannot accommodate pilus rods, which are about 7 nm wide (Bullitt and Makowski, 1995; Hahn et al., 2002). It has therefore been proposed that assembled subunits leave the periplasm as a linear array of subunits and that formation of the helical rod occurs on the bacterial surface (Thanassi et al., 1998). This model is supported by the recent observation, that subunits in principle interact head to tail with each other (Sauer et al., 2002; Zavialov et al., 2003). Hence it is conceivable that subunits are translocated in an extended linear conformation, even those that finally form the pilus rod.

For both FimD and PapC it has been found that they bind the periplasmic chaperone-subunit complexes with affinities in the nanomolar to micromolar range. In both systems, the affinities of the different complexes for the ushers vary within a wide range and, strikingly, the chaperone-subunit complexes containing the adhesin (FimH and PapG, respectively) have the highest affinity for their cognate usher. Because it is assumed that the adhesins are the first subunits to become incorporated into the pilus, this observation has led to the hypothesis that the differential affinities of the various chaperone-subunit complexes for the usher determine the sequential order of the subunits within the pilus (Dodson et al., 1993; Saulino et al., 1998).

Topology studies on another usher, FaeD, suggested a central transmembrane domain flanked by N- and C-terminal periplasmic domains (Harms et al., 1999). It appears that the N-terminal domain is responsible for binding of chaperone-subunit complexes (cf. chapter 3) while the C-terminal domain is required for subunit assembly (Thanassi et al., 2002). Despite the central
role of the ushers in fiber formation, the mechanism by which they carry out their assembly function is not understood. An elementary assembly reaction consists of the dissociation of a chaperone-subunit complex and the subsequent formation of a subunit-subunit complex. The spontaneous reaction is presumably very slow, ensuring that premature fiber formation in the periplasm is suppressed efficiently. It is however not clear whether the dissociation or the association is rate-limiting. This is an intriguing question because the usher presumably catalyses fiber formation by accelerating the rate-limiting step of the assembly reaction.
2. RESULTS

Chaperone-independent folding of type 1 pilus domains


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Chaperone-independent Folding of Type 1 Pilus Domains

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An elementary step in the assembly of adhesive type 1 pili of Escherichia coli is the folding of structural pilus subunits in the periplasm. The previously determined X-ray structure of the complex between the type 1 pilus adhesin FimH and the periplasmic pilus assembly chaperone FimC has shown that FimH consists of a N-terminal lectin domain and a C-terminal pilin domain, and that FimC exclusively interacts with the pilin domain. The pilin domain fold, which is common to all pilus subunits, is characterized by an incomplete β-sheet that is completed by a donor strand from FimC in the FimC–FimH complex. This led to unsuccessful attempts to refold isolated, urea-denatured FimH in vitro had suggested that folding of pilin domains strictly depends on sequence information provided by FimC. We have now analyzed in detail the folding of FimH and its two isolated domains in vitro. We find that not only the lectin domain, but also the pilin domain can fold autonomously and independently of FimC. However, the thermodynamic stability of the pilin domain is very low (8.0–10.0 kJ mol⁻¹) so that a significant fraction of the domain is unfolded even in the absence of denaturant. This explains the high tendency of structural pilus subunits to aggregate non-specifically in the absence of stoichiometric amounts of FimC. Thus, pilus chaperones prevent non-specific aggregation of pilus subunits by native state stabilization after subunit folding.

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Keywords: type 1 pilus; protein folding; pilus chaperones; pilus assembly; protein stability

Introduction

Type 1 pili from Escherichia coli are filamentous, proteinaceous surface organelles attached to the outer bacterial membrane. They enable uropathogenic E. coli strains to bind to mannose units of uroplakin Ia, a surface glycoprotein of urinary epithelial cells, and are important for efficient colonization of the urinary tract.⁵⁻⁹ Moreover, type 1 pili have also been shown to mediate the internalization of pathogenic E. coli strains into eukaryotic host cells where they are protected from rapid clearance and a variety of host defence mechanisms.⁶⁻⁷ Type 1 pili consist of up to 3000 protein subunits that assemble into filaments with a diameter of about 7 nm and a typical length of 1–2 μm.⁶ Each pilus consists of a right-handed helical rod that is composed of FimA subunits. In addition, a thin, linear tip fibrillum is linked to the distal end of the rod. The fibrillum is formed by the two adaptor proteins FimE and FimG, and the mannose-binding adhesin FimH.⁷⁻⁸ In addition to the structural pilus subunits FimA, FimE, FimG, and FimH, two more proteins encoded by the type 1 pilus gene cluster are essential for type 1 pilus biogenesis: The assembly platform FimD which anchors the pilus to the bacterial surface and mediates translocation of pilus subunits through the outer E. coli membrane⁵,⁶ and the chaperone FimC which forms transient, non-covalent complexes with individual pilus subunits and delivers the subunits to FimD.⁵,⁶ This assembly mechanism, termed
RESULTS

Chaperone-independent folding of subunits

Figure 1. Three-dimensional structure of the FimC–FimH complex, and production of the isolated FimH domains. (a) Ribbon diagram of the X-ray structure of the FimC–FimH complex. FimH and FimC are depicted in dark and light grey, respectively. The N-terminal lectin domain of FimH (top, residues 1–156) harbors both tryptophan residues (W103 and W146) of the adhesin. Only the C-terminal pilin domain (residues 160–279) of FimH interacts with FimC. Both domains are connected by a short linker peptide (residues 157–159) from which T158 is highlighted. (b) Ribbon representation of the pilin domain of FimH (residues 160–279, dark grey) in the FimC–FimH complex, with the G1 donor strand from FimC (residues 101–116, light grey) completing the β-fold of the pilin domain. (c) SDS-PAGE of the four purified proteins used in this study. A reducing SDS-containing gel (18% w/v polyacrylamide) stained with Coomassie blue is shown. Lane 1, molecular mass standard; lane 2, FimH; lane 3, recombinant lectin domain of FimH (residues 1–159); lane 4, recombinant pilin domain of FimH (residues 158–279); lane 5, FimC.

Chaperone-usher pathway, has been postulated for more than 20 different adhesive organelles of Gram-negative pathogens. The X-ray structure of the FimC–FimH complex has revealed that FimH consists of two independent β-sheet domains that do not interact with each other in the complex. The two FimH domains have entirely different functions. The N-terminal lectin domain is responsible for recognition of mannose units on receptor proteins. The C-terminal domain, termed pilin domain, mediates the integration of FimH into the pilus, possibly by specific interactions with FimG. Moreover, the pilin domain is the only part of FimH that interacts with FimC (Figure 1(a)). The pilin domain also shares high sequence homology with the other structural pilus subunits and thus serves as a general model for the structure and the folding of individual pilus subunits.

Previous experiments have indicated that the chaperone FimC prevents non-productive aggregation of pilus domains, and presumably also prevents premature assembly of pilus subunits in the periplasm. A further function that has been suggested for FimC is the direct assistance of folding of structural pilus subunits on the basis of the following lines of evidence. First, it was found for the related P pilus system that peptides corresponding to the conserved C-terminal segments of the structural pilus subunit PapG and PapK bind to the periplasmic chaperone PapD. Plus chaperones may thus recognize the C-terminal segment of pilus subunits prior to subunit folding, possibly indicating pilus subunit folding in the chaperone-bound state. However, the affinity of FimC towards the C-terminal peptide segment of FimH is several orders of magnitude lower than the affinity for the entire FimH. Second, the crystal structures of the FimC–FimH and the PapD–PapK complexes revealed that the immunoglobulin-like folds of both the FimH pilin domain and of PapK contain incomplete β-sheets to which a lacking β-strand is donated by the chaperone (Figure 1(b)). This mechanism is termed donor strand complementation. The absence of the chaperone would lead to a partial exposure of the hydrophobic cores of the pilin domains. FimC in these structural data it was concluded that pilin domains, in contrast to the general view of sequence-encoded tertiary structure formation, are not capable of autonomous folding due to lacking sequence information, and strictly require plus chaperones to adopt their tertiary structures. Moreover, initial in vitro experiments indicated that FimH aggregates completely upon refolding in vitro unless FimC is present. Donor strand complementation has also been proposed as a basic mechanism underlying the interactions between subunits in intact pili. According to this model, the lacking strand of pilus subunits is provided by an N-terminal extension from the neighboring pilus subunit. This is strongly supported by the finding that a FimH variant, which was extended at the C terminus by the presumed donor-strand of the subunit FimG, proved to be soluble in the absence of FimC and could be refolded in vitro.

In the present study, we have tested the hypothesis that pilin domains lack sequence information for autonomous folding in a detailed study on the
in vitro folding of FimH. The type 1 pilus adhesin FimH, which is located at the tip of the pili, was chosen as a model subunit because the typical N-terminal donor strand extension of pilin domains is absent in FimH and replaced by the lectin domain. Both FimH and its isolated pilin domain are thus not capable of self-assembly by intramolecular donor strand complementation, which eliminates donor strand-mediated, specific posttranslational modifications. FimH during refolding experiments. Like all other structural pilus subunits, the pilin domain of FimH contains a conserved disulfide bond. Here, we examined exclusively the in vitro folding of the disulfide-intact pilin domain as we focused on the question whether folding of the pilin domain requires the presence of the chaperone FimC or not. Folding in vitro, however, is most likely also limited by disulfide bond formation through DsbA.

In contrast to previous studies, we compare folding and stability of full-length FimH with the properties of its isolated pilin and lectin domains. We demonstrate that, in contrast to previously described models pilin domains can spontaneously adopt native tertiary structure in the absence of periplasmic pilus chaperones or other pilus subunits, but are only marginally stable without association partners.

Results

Expression and purification of FimH and its isolated domains

As FimH is only soluble in the E. coli periplasm as a complex with FimC, we co-expressed FimH with FimC in the E. coli periplasm with a dicistronic operon and first purified the FimC–FimH complex for the preparation of FimH. Expression of the FimC–FimH complex in the protease-deficient E. coli strain HM 125 yielded large quantities of the complex (20 mg per litre of bacterial culture). Full-length FimH was obtained after dissociation of the complex through simultaneous unfolding of FimC and the pilin domain of FimH (see below) with 4 M urea, and separation of FimH and FimC by ion exchange chromatography in the presence of 4 M urea (Figure 1(a)). Expression plasmids for the recombinant, isolated domains of FimH were on the basis of the three-dimensional structure of FimH in the FimC–FimH complex (Figure 1(a)). The N-terminal lectin domain of FimH (residues 1–156), extended by the linker peptide connecting both domains (residues 157–159), was secreted to the periplasm with the DsbA signal sequence and purified from the periplasmic fraction by conventional chromatography (Figure 1(c)). As we assumed that the isolated pilin domain, like fulllength FimH, would be insoluble in the periplasm in the absence of FimC, the pilin domain of FimH, extended at the N terminus by the last two residues from the linker (residues 158–279) was also co-expressed with FimC. The periplasmic expression yields of the FimC–pilin domain complex were similar to those obtained for the FimC–FimH complex. In addition, we could apply the purification protocol developed for FimH to purify the isolated pilin domain (Figure 1(c)). Correct N-terminal processing of the secreted proteins was verified by Edman sequencing and MALDI-TOF mass spectrometry. The overall yields of purified proteins were 10 mg, 11 mg and 5 mg per litre of bacterial culture for FimH, the lectin and the pilin domain, respectively.

In vitro folding and thermodynamic stability of FimH and its isolated domains

As a prerequisite for studying the folding of FimH and its two isolated domains in vitro, all three proteins were characterized spectroscopically in their native and guanidinium chloride (GdmCl)-denatured states, as well as under conditions (1.8 M GdmCl) where the lectin domain of full-length FimH is folded and the pilin domain is unfolded (see below). Fluorescence spectroscopy proved to be a useful and selective tool to follow folding of the lectin domain, because it contains all tryptophan residues of FimH (W103 and W146). Surprisingly, however, the fluorescence spectrum of the native lectin domain was essentially dominated by tyrosine fluorescence when the protein was excited at 280 nm (simultaneous excitation of tyrosine and tryptophan residues) (Figure 2(a)). Only after unfolding of the lectin domain in 6 M GdmCl, a typical tryptophan fluorescence spectrum with a maximum at 350 nm was observed (Figure 2(a)). This indicates almost complete quenching of tryptophan fluorescence in the native state of the lectin domain. The fluorescence spectra of intact FimH and the isolated lectin domain are similar, except that the tyrosine fluorescence component (maximum at ~310 nm) of FimH (Figure 2(e)) is higher due to the additional tyrosine residues from the pilin domain (Figure 2(a), (c) and (e)). The tyrosine fluorescence of the isolated pilin domain was comparably weak and changed only slightly upon unfolding in 1.8 M GdmCl (Figure 2(c)). Therefore, we used circular dichroism (CD) spectroscopy to follow folding of the pilin domain. When FimH or the pilin domain were purified from the corresponding FimC complexes according to the protocol described above, protein preparations in 4 M urea were obtained where the pilin domain was unfolded (data not shown). After dialysis against 0.25 M GdmCl, the isolated pilin domain adopted a conformation that displays a far-UV CD spectrum typical for a folded β-sheet protein with a minimum around 215 nm (Figure 2(d)). Full-length FimH also exhibited a minimum at 215 nm after dialysis, but displayed an additional maximum at 232 nm (Figure 2(f)). As the same maximum is observed in the spectrum of the native lectin domain (Figure 2(b)), the CD spectrum of full-length FimH appears as the sum
of the spectra of the individual domains. As expected, all three proteins showed similar, random coil-like CD spectra in the presence of 6 M GdmCl (Figure 2(b), (d) and (f)). The spectrum of full-length FimH in the presence of 1.8 M GdmCl, however, is remarkable. It resembles a spectrum of a mixture of the native lectin domain and the unfolded pilin domain in that it still shows the maximum at 232 nm, but lacks the minimum around 215 nm (Figure 2(b), (d) and (f)). Overall, the spectroscopic characterization of FimH and its isolated domains indicated that both the isolated pilin domain and the pilin domain in the context of FimH can be refolded in vitro to a native-like
RESULTS

Chaperone-independent folding of subunits

Figure 3. GdmCl-induced equilibrium transitions of (b) FimH and (a) its isolated domains at pH 7.0 and 25 °C. Unfolding (△) and refolding (▽) of the isolated lectin domain, and unfolding of the lectin domain in the context of FimH (●) was monitored by tryptophan fluorescence at 350 nm (excitation at 280 nm). Refolding of the isolated pilin domain (■) and the pilin domain in the context of FimH (●) was followed by the far-UV CD signal at 210 nm. The original data were fitted according to the two-state model and normalized (continuous lines). (c) Three-state analysis of GdmCl-dependent folding of FimH. The fractions of the native state (with both domains folded (●)), of the unfolding intermediate consisting of the native lectin and the unfolded pilin domain (●), and of the fully unfolded state (△) at GdmCl concentrations between zero and 6 M were calculated from the transitions of the domains in the context of FimH shown in (b).

Table 1. Thermodynamic stabilities of the isolated lectin and pilin domain and of the domains in the context of FimH in 10 mM Mops/NaOH (pH 7.0) at 25 °C.

<table>
<thead>
<tr>
<th></th>
<th>ΔG (kJ mol⁻¹)</th>
<th>Cooperativity (kJ mol⁻¹ M⁻¹)</th>
<th>Midpoint of transition (M GdmCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lectin domain</td>
<td>-43.0 ± 3.9</td>
<td>14.6 ± 1.3</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>Lectin domain in the context of FimH</td>
<td>-45.2 ± 4.1</td>
<td>15.1 ± 1.4</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>Pilin domain</td>
<td>-9.5 ± 0.9</td>
<td>13.8 ± 1.2</td>
<td>0.09 ± 0.06</td>
</tr>
<tr>
<td>Pilin domain in the context of FimH</td>
<td>-7.7 ± 0.9</td>
<td>8.8 ± 0.8</td>
<td>0.07 ± 0.10</td>
</tr>
<tr>
<td>Pilin domain*</td>
<td>-14.2 ± 1.8</td>
<td>12.6 ± 1.3</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

* In 20 mM Tris–HCl (pH 8.0), 20% (v/v) glycerol at 25 °C.

conformation, that the domains of FimH fold independently, and that the lectin domain is significantly more stable than the pilin domain.

We next measured GdmCl-dependent equilibrium transitions of FimH and its isolated domains under conditions where aggregation of FimH and the pilin domain during refolding proved to be minimal (pH 7.0, 25 °C, low ionic strength, protein concentrations ≤10 μM) (Figure 3 and Table 1). Folding of the isolated lectin domain and of the lectin domain in the context of FimH was followed by the decrease in tryptophan fluorescence. Folding of the isolated pilin domain and the pilin domain in the context of FimH was analyzed by the increase in the far-UV CD signal at 210 nm. Figure 3(a) shows that the isolated pilin domain exhibits a cooperative folding transition, clearly indicating autonomous folding to an intact tertiary structure. Moreover, two-state evaluation of the transition yielded a cooperativity of folding (n-value) of 13.8 (±1.2) kJ mol⁻¹ M⁻¹, which is in good agreement with the n-value predicted for a protein of 122 amino acids (13.2 kJ mol⁻¹ M⁻¹). The intrinsic stability of the pilin domain is, however, rather low (−9.5 kJ mol⁻¹; Table 1), with the consequence that a significant fraction of the protein (about 2%) is unfolded even in the absence of denaturant at pH 7.0. The stability of the pilin domain in the context of FimH proved to be identical with that of the isolated pilin domain within experimental error (Figure 3(b) and Table 1). The somewhat lower n-value of pilin domain folding in the context of FimH may be explained by binding of the unfolded pilin domain to the still folded lectin domain (see below), which would cause a decrease in the difference in accessible surface area (ΔASA) between the native and unfolded pilin domain.

The isolated lectin domain proved to be significantly more stable that the pilin domain (−43 (±3.9) kJ mol⁻¹). Its folding was also compatible with the two-state assumption (measured n-value: 14.6 (±1.3) kJ mol⁻¹ M⁻¹; predicted n-value for a 159 residue protein: 16.5 kJ mol⁻¹ M⁻¹; Table 1). The refolded lectin domain is spectroscopically identical with the native protein. We
RESULTS

Chaperone-independent folding of subunits

also tested whether the refolded lectin domain is functional. For that purpose, the refolded lectin domain was incubated with D-mannose immobilized on agarose in the presence or absence of 500 mM D-mannose. The refolded pilin domain bound quantitatively to the immobilized ligand, and binding was competitively inhibited by soluble D-mannose. The folding equilibrium of the lectin domain in the context of FimH was indistinguishable within experimental error from that of the isolated lectin domain (Figure 3(b) and Table 1). In summary, the comparison of denaturant-dependent unfolding transitions of FimH with the transitions of its isolated FimH domains demonstrated that the pilin domain is capable of folding in the absence of FimC, and that both FimH domains fold independently and do not interact with each other in full-length FimH. Folding of FimH can thus be described by the simple three-state equilibrium:

\[
K_{eq} = \frac{[N]}{[U][I]}
\]

where N is native FimH with both domains folded, I is the unfolding intermediate of FimH in which the pilin is unfolded and the lectin domain is native, and U represents the fully denatured state. Figure 3(c) shows the corresponding three-state analysis, deduced from the equilibrium transitions of the individual domains in the context of FimH (Figure 3(b)), and demonstrates that the intermediate is maximally populated (98%) at 2.1 M GdmCl.

FimC prevents non-specific aggregation of FimH

As FimH was reported to aggregate completely after dilution from 9 M to 0.45 M urea in a previous study,\textsuperscript{22} we analyzed conditions influencing non-specific aggregation of FimH during refolding in 10 mM Mops/NaOH (pH 7.0) at 25°C by varying the ionic strength in the refolding buffer. We performed this set of experiments at higher FimH concentrations (14 μM) compared to the equilibrium folding experiments (∼10 μM) to favor the second-order aggregation reaction\textsuperscript{26,27} relative to folding. Moreover, aggregation was additionally favored by using lower GdmCl concentrations (0.13 M) compared to the equilibrium transitions (at least 0.25 M). In addition, we started refolding of FimH from 2.0 M GdmCl where the lectin domain is folded and the pilin domain is unfolded (see above). This excluded contributions to aggregation from folding of the lectin domain and allowed selective observation of pilin domain-dependent aggregation. When FimH was rapidly diluted from 2.0 M to 0.13 M GdmCl in the absence of NaCl, about 50% of the protein precipitated, while the rest stayed soluble (Figure 4(a)). Increase of ionic strength in the refolding buffer strongly promoted aggregation of FimH, so that more than 90% of FimH was found in insoluble aggregates when 500 mM NaCl was included in the refolding reaction (Figure 4(a)). This indicates that non-specific hydrophobic interactions, which are favored at high ionic strength, are responsible for FimH aggregation during refolding.

![Graph](image)

Figure 4. Dependence on ionic strength and the presence of FimC of FimH aggregation during refolding at pH 7.0 and 25°C. FimH with a folded lectin domain but unfolded pilin domain in 2 M GdmCl was diluted to final concentrations of 14 μM FimH and 0.13 M GdmCl in 10 mM Mops/NaOH (pH 7.0) containing different concentrations of FimC and NaCl. Equal amounts of the soluble (s) and the insoluble (i) fraction after dilution and incubation for 30 minutes were subjected to SDS-PAGE. SDS-containing gels were stained with Coomassie blue. (a) Influence of ionic strength on aggregation of FimH during refolding in the absence of FimC or in the presence of two molar equivalents of FimC. (b) FimC (zero to two molar equivalents relative to FimH) was tested for its ability to prevent aggregation of FimH during refolding in 500 mM NaCl.
When two molar equivalents of FimC relative to FimH were included in the same set of refolding experiments, more than 90% of the FimH stayed soluble after refolding, even at the highest ionic strength investigated (500 mM NaCl). Protein concentrations were 14 μM and 28 μM for FimH and FimC, respectively, allowing formation of the FimC–FimH complex, as the complex has a Kₜ value in the sub-micromolar range. To establish the stoichiometry of the interaction between FimC and FimH during FimH refolding, we varied the concentration of FimC relative to FimH under conditions where FimH aggregation in the absence of FimC was maximal (500 mM NaCl). Figure 4(b) shows that maximal inhibition of aggregation was achieved at a 1:1 ratio between FimC and FimH, where 90% of FimH stayed soluble. Only a small further increase in soluble FimH was observed when FimC was present at a twofold molar excess over FimH. In contrast, only about 50% of FimH stayed soluble when 0.5 molar equivalents of FimC relative to FimH were present (Figure 4(b)). This demonstrates that FimC functions stoichiometrically as inhibitor of FimH aggregation by formation of soluble 1:1 complexes with FimH.

The pilin domain binds to FimC after refolding in the absence of FimC

In a previous study it was reported that no FimC–FimH complex is formed if FimC is added to FimH after refolding from 9 M to 0.45 M urea. This had led to the hypothesis that pilin domains cannot be refolded in vitro to a conformation that is competent of chaperone binding. As this contradicted our finding that the pilin domain of FimH adopts a defined tertiary structure, we examined in detail the ability of the pilin domain to bind FimC after refolding in the absence of the chaperone. The following precautions were taken in these experiments. (i) Instead of FimH, we used the isolated pilin domain, since it shows better solubility properties than FimH at low denaturant concentrations. (ii) As the equilibrium unfolding experiments (Figure 3(a)) had revealed that the pilin domain is partially denatured even in the absence of denaturant at pH 7.0, we searched for alternative buffer conditions where the pilin domain is more stable than in 10 mM Mops/NaOH, pH 7.0. We found that the pilin domain was significantly stabilized in 20 mM Tris/HCl (pH 8.0) containing 20% (v/v) glycerol (Figure 5(a)). The free energy of folding increased from −9.5 ± 0.9 kJ mol⁻¹ to −14.2 ± 1.8 kJ mol⁻¹ under these conditions (Table 1). Moreover, we observed a decreased aggregation tendency during refolding of the pilin domain at high protein concentrations in the presence of glycerol (data not shown). Similar effects of glycerol have been described for other proteins. We therefore used 20 mM Tris–HCl (pH 8.0), 20% (v/v) glycerol as a buffer system for our further experiments. (iii) We established the kinetics of pilin domain folding to make sure that refolding was completed prior to the addition of FimC in our binding experiments. Refolding was followed by the increase in the far-UV CD signal at 210 nm after manual dilution of the pilin domain from 2.5 M to 0.25 M GdmCl. Figure 5(b) shows that pilin domain folding is comparably slow and can be described by a single-exponential reaction with a half-time of 105 seconds (apparent rate constant of folding: 0.0086 s⁻¹). The observed signal amplitude of 3360 deg cm⁻² dmol⁻¹ agrees well with the value of 3400 deg cm⁻² dmol⁻¹ expected from the equilibrium transition. The kinetic parameters were independent of protein concentration between 2.5 μM and 10 μM. Since peptidyl-prolyl trans/cis isomerization could account for the slow folding of the pilin domain, it was tested whether the periplasmic peptidyl-prolyl cis/trans isomerase (PPlase) from E. coli accelerates folding of the pilin domain. Therefore, the pilin domain (10 μM) was also refolded in the presence of 1 μM PPlase. Although the catalytic activity of PPlase is not affected under the applied refolding conditions (residual GdmCl concentration of 0.25 M), the folding rate of the pilin domain did not change in the presence of the enzyme (data not shown; see also Discussion).

To test the pilin protein for its ability to bind FimC after refolding, the pilin domain was renatured under the same conditions as in the above refolding kinetics (dilution from 2.5 M to 0.25 M GdmCl in 20% (v/v) glycerol (pH 8.0)) and incubated for ten minutes to allow complete refolding (cf. arrow in Figure 5(b)). Then one molar equivalent of FimC was added, and formation of the FimC–pilin domain complex was investigated with analytical gel filtration (Figure 5(c)). In a control experiment, we found that the refolded pilin domain alone irreversibly adsorbs to the column under these conditions and is thus not eluted from the column. The elution profile (Figure 5(c)) yielded two overlapping peaks, corresponding to the FimC–pilin domain complex (retention volume: 11.1 ± 0.2 ml) and monomeric FimC (retention volume: 11.9 ± 0.2 ml). A quantitative evaluation of the elution profile revealed that 62 ± 5% of the FimC molecules were eluted in complex with the pilin domain, demonstrating that the pilin domain can adopt a biologically active tertiary structure after refolding in vitro in the absence of FimC. In a control experiment, FimC was already included at the onset of the refolding reaction, and the reaction products were again separated by gel filtration (Figure 5(d)). Analysis of the relative peak areas revealed that 86 ± 5% of the FimC molecules were found in the complex (Figure 5(d)), showing that the chaperone–pilin domain complex is formed more efficiently when FimC is already present during pilin domain folding.
RESULTS

Chaperone-independent folding of subunits

Figure 5. Refolding of the isolated pilin domain of FimH in the presence of 20% (v/v) glycerol and subsequent binding to FimC. All experiments were performed at pH 8.0 and 25 °C. Refolding (a) and (b) was recorded by following the increase in ellipticity at 210 nm. (a) The refolding transition of the pilin domain at pH 8.0 was fitted according to the two-state model (continuous line) and normalized. (b) Refolding kinetics of the pilin domain after dilution from 2.5 to 0.25 M GdmCl in 20 mM Tris–HCl (pH 8.0), 20% (v/v) glycerol (final protein concentration: 6 μM). The increase in ellipticity at 210 nm was fitted with a single-exponential function (continuous line). The residuals are shown in the inset. (c) and (d) Analysis of binding of the pilin domain to FimC by analytical gel filtration on a Sephadex 75 HR column. The retention volumes of FimC and of the FimC–pilin domain complex are indicated. The free pilin domain irreversibly adsors to the column and is therefore not detected in the elution profile. The individual peaks areas of FimC and the heterodimer (dotted lines) were derived after deconvolution of the elution profile (continuous line) with the program PeakFit. (c) The pilin domain (5 μM) was refolded by dilution from 2.5 M to 0.25 M GdmCl for ten minutes, allowing essentially complete refolding (indicated by the arrow in (b)). At this time point, one molar equivalent of FimC was added. After additional 20 minutes of incubation, the sample was applied to the gel filtration column. (d) As a control, an identical experiment as in (c) was performed except that one molar equivalent of FimC was already present at the onset of pilin domain refolding.

Discussion

Folding of the structural type 1 pilus subunits in the periplasm and the formation of stoichiometric complexes between pilus subunits and specific periplasmic chaperones are key events in type 1 pilus biogenesis. Previous data on the folding of the model subunit FimH and its interactions with FimC had led to the hypothesis that bacterial pilus subunits lack sequence information to fold autonomously into an assembly-competent active tertiary structure.1420,22 This hypothesis was deduced from the three-dimensional structures of the chaperone–pilus subunit complexes PapD–PapK and FimC–FimH, which had shown that both PapK and the pilin domain of FimH are lacking a
RESULTS

Chaperone-independent folding of subunits

β-strand for completion of their β-fold, and that the lacking strand is donated to the pilin domains by the corresponding chaperone. Moreover, unsuccessful attempts to refold FimH from 9 M urea in the absence of FimC had led to irreversible aggregation of FimH, which supported the view of the requirement of interactions with a chaperone for folding of the FimH pilin domain.

In the present study, we tested this hypothesis that challenges the dogma of sequence-encoded tertiary structure formation by comparing the in vitro folding of FimH and its two isolated domains. Although there is now increasing evidence for coupling of folding and binding for numerous proteins that are unstructured as isolated polypeptides, we could show that no extrinsic information is required for folding of the pilin domain of FimH. We obtained the first information on the intrinsic thermodynamic stability of a pilin domain. Our conclusions are on the basis of the following observations. First, both the isolated pilin domain as well as the pilin domain in the context of FimH showed sigmoidal, cooperative, and two-state compatible folding transitions in the presence of GdmCl, which is a hallmark of tertiary structure formation. Second, the observed m-values of folding of the pilin domain were exactly within the expected range of a 122 residue protein folding according to the two-state model. Third, the isolated pilin domain showed typical β-sheet far-UV CD spectra, as expected from its structure in the FimC–FimH complex. Firth, the pilin domain, refolded in the absence of FimC, proved to be capable of binding to FimC. Nevertheless, the intrinsic thermodynamic stability of the pilin domain, both in its isolated form and in the context of FimH, proved to be very limited (8–10 k mol⁻¹) and revealed that a small, but significant fraction of the pilin domain is unfolded at pH 7.0 in the absence of denaturant. Irreversible aggregation of unfolded pilin domains, pulling unfolded, but refolding-competent polypeptides from the folding equilibrium, is thus a very likely explanation for the high tendency of FimH and the pilin domain to aggregate non-specifically in the absence of FimC, both in vivo and in vitro. The low intrinsic stability of the pilin domain also indicates that intermolecular interactions between neighboring pilus subunits contribute strongly to the enormous overall stability of type 1 pili, which, for example, cannot be dissociated by 7.5 M GdmCl. These interactions are possibly mediated by N-terminal donor strands from neighboring subunits and additional surface contacts and might be the driving force for pilus assembly, which is ATP-independent.

The fact that stoichiometric amounts of FimC prevent non-specific aggregation of FimH and the FimH pilin domain can be well explained by the strong binding of FimC to the native subunits, which simultaneously decreases the fraction of unfolded, aggregation-prone subunits that are present in the subunit’s folding equilibrium. We thus propose from our data that prevention of non-specific aggregation of pilus subunits in the periplasm by binding and native state stabilization is another function of FimC, in addition to its task of preventing specific, premature subunit assembly and delivering pilus subunits to the assembly platform FimD.

Schembri et al. have previously purified the isolated lectin domain of FimH and demonstrated that it binds α-d-mannose. Here we show that, in contrast to the isolated pilin domain, the lectin domain displays high intrinsic thermodynamic stability (−43.0(±3.9) k mol⁻¹). This finding is in accordance with the donor-strand complementation model for the intersubunit contacts in type 1 pili and the X-ray structure of FimH, which predicts that the lectin domain, which is located at the tip of the pili, functions as an isolated domain and does not form contacts with other pilus subunits. Thus, in contrast to the pilin domains, which mutually stabilize each other in the intact pili, the lectin domain must be intrinsically stable to cope with destabilizing environmental conditions which may be present in e.g. the urinary tract.

There are several possible reasons why others had previously failed to refold chemically denatured FimH in vitro. We found that the refolding conditions were very critical for successful reconstitution of FimH and the FimH pilin domain, as both proteins are rather aggregation-prone. Specifically, we had to use low protein concentrations, low ionic strength, and rapid dilution into the refolding buffer to suppress formation of non-specific aggregates during reconstitution. As we could demonstrate that the pilin domain of FimH folds cooperatively in the absence of FimC, we believe that non-specific aggregation was misinterpreted as folding incompetence in a previous study. Moreover, autonomous folding and a certain intrinsic stability of isolated pilin domains may also be a prerequisite for pilus assembly, because monomeric pilin domains are likely to occur transiently after dissociation of chaperone–pilin domain complexes prior to incorporation of pilin domains into the pilus.

Despite the fact that we could refold the pilin domain of FimH completely in vitro, we observed only partial (62(±5)%)) reconstitution of the FimC–pilin domain complex when FimC was added to refolded FimH in stoichiometric amounts and complex formation was analyzed by gel filtration (Figure 5(c)). As we also observed incomplete, albeit more efficient (86(±5)%)) reconstitution of the complex when FimC was present in stoichiometric amounts during pilin domain refolding (Figure 5(d)), non-specific adsorption of the pilin domain to the gel filtration column (see above) may have occurred in this experiment. This may have been caused by partial dissociation of the FimC–pilin domain complex during the chromatography, and/or non-quantitative complex formation at the beginning of the chromatography due to residual GdmCl concentrations.
from the refolding experiment. Despite these technical restrictions, the analysis of the reconstitution of the FimC–pilin domain complex by gel filtration clearly showed that the pilin domain is capable of binding to FimC after refolding, and that FimC facilitates complex formation when already present during refolding.

For the related P pilus system it has been reported that pilus assembly in the living E. coli cell occurs very rapidly and is presumably completed in less than five minutes. If one assumes similar rates of pilus assembly for type 1 pili, the observed slow folding of the FimH pilin domain (105 seconds half-time; Figure 5b) suggests that pilus domain folding may be catalyzed in vivo. Formation of the conserved disulfide bond present in all type 1 pilus subunits, which appears to precede formation of chaperone-subunit complexes in vitro, has been shown to be catalyzed by the dithiol oxidase DsbA. A possible reason for the observed, slow folding of the disulfide-intact pilin domain of FimH is peptidyl-prolyl cis/trans isomerization, as this reaction is expected to occur on a similar timescale. The X-ray structure analysis of the FimC–FimH complex revealed that the Leu172-Pro173 peptid bond of the FimH pilin domain might be in the cis conformation. Our kinetic data are consistent with Pro173 in cis in the folded pilin domain, as we observed the full CD signal amplitude from the equilibrium transition, in contrast to a calculated fraction of about 40% fast folding molecules for the trans conformer. Overall, the slow folding reaction of the pilin domain raises the question of whether pilin domain folding is catalyzed in vivo. It has been suggested that the P-pilus chaperone PapD interacts with folding pilin domains in vivo by recognizing the conserved C-terminal segments of P pilus subunits and possibly another conserved N-terminal region in the subunits. The improved reconstitution of the FimC–pilin domain complex when FimC was present during refolding of the pilin domain is another hint that periplasmic pilus chaperones might directly influence the folding of pilus subunits. Nevertheless, it has not been established so far whether FimC binds to unfolded type 1 pilus subunits and/or folding intermediates of subunits. The in vitro refolding conditions for the isolated pilin domain of FimH developed here will allow us to address this intriguing question.

Materials and Methods

Construction of expression plasmids

Standard molecular cloning techniques were performed as described. The plasmid pFimH–FimC allows co-expression of FimH and FimC from a dicistronic operon under control of the trc promoter. The plasmid pCT-FimH–FimC for periplasmic co-expression of the isolated FimH pilin domain with FimC was obtained from pFimH–FimC by deletion of the genetic sequence encoding the N-terminal 157 amino acid residues of mature FimH (lectin domain) through site-directed mutagenesis with the mutagenesis primer 5’–CAT CGC AGC CGC CAG ATG ACC AGG CAT TTA CCG–3’

Expression and purification of FimC, FimH, the isolated FimH domains

QA52, CM52, and SE52 cellulose were purchased from Whatman (Maidstone, UK), Q-Sepharose™ Fast Flow was from Amersham Pharmacia Biotech (Uppsala, Sweden). All chromatographic steps were carried out at room temperature.

Expression of FimC and FimH was carried out in E. coli HB101 harboring pFimH–FimC. The cells were grown at 30 °C in LB medium containing ampicillin (100 µg ml⁻¹). At an absorbance density of 600 nm (A600) of 1.5, isopropyl-β-d-thiogalactoside (IPTG) was added to a final concentration of 1 mM. After further growth for 12 hours, the bacteria were harvested by centrifugation. The pellet was suspended in cold 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mg ml⁻¹ polymyxin B sulfate (10 ml per litre of culture) and stirred at 4 °C for two hours. After centrifugation, the supernatant (periplasmic extract) was dialyzed against 20 mM Tris–HCl (pH 8.0) and applied to a QA52 column equilibrated with the same buffer. The flow-through containing the FimH–FimC complex was dialyzed against 20 mM Mops/NaOH (pH 7.0) and loaded onto a CM52 column equilibrated with 20 mM Mops/NaOH (pH 7.0). The complex was eluted with a linear gradient from 0 to 400 mM NaCl. Fractions containing the complex were pooled and dialyzed against 10 mM Mops/NaOH (pH 7.0). At this stage, the protein preparation could be stored at 4 °C for up to three months. In order to obtain pure FimH, aliquots from the above solution of the FimC–FimH complex were mixed with 9 M urea to a final concentration of 4 M urea. This solution was applied to a CM52 column in 4 M urea, 10 mM Mops/NaOH (pH 6.6), and pure FimH was collected in the flow-through. The mass of FimH was verified by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS): the measured mass (29,078 Da) was in agreement with the calculated value (29,065 Da). The overall yield was 10 mg pure FimH per litre of bacterial culture. FimH was always prepared freshly from the FimC–FimH complex for folding studies in vitro, as

RESULTS

Chaperone-independent folding of subunits
isolated FimH aggregated even when stored in 4 M urea, 10 mM Mops/NaOH (pH 6.6) for more than two weeks. FimC was either purified as described, or obtained after elution from the above CM32 column with a linear gradient from 0 mM to 400 mM NaCl in 4 M urea, 10 mM Mops/NaOH (pH 6.6). Fractions containing unfolded FimC were pooled and FimC was refolded by dialysis against distilled water. The protein was stored at −20 °C.

For production of the isolated pilin domain of FimH, *E. coli* HM 125 transformed with pCT-FimHM–FimC was grown at 30 °C in 2×YT medium containing ampicillin (100 μg mL−1) for 16 h. After induction with 1 mM IPTG at an A600 of 1.0, the cells were grown further for 12 h. Periplasmic extracts were then prepared as described above, and the pilin domain was purified to homogeneity with exactly the same protocol as described for the full-length FimH. The identity of the isolated pilin domain was confirmed by amino-terminal sequencing and MALDI-MS (calculated mass: 12,267 Da; measured: 12,262 Da). Typical yields were around 5.0 mg pilin domain per litre of bacterial culture. Like full-length FimH, the pilin domain was stored at 4 °C as a complex with FimC.

For expression of the isolated lecin domain, *E. coli* HM 125 transformed with pNT-FimH was grown in 2×YT medium containing ampicillin (100 μg mL−1) to an A600 of 1.0, and expression was induced with 1 mM IPTG. After further growth for 12 h, cells were subject to periplasmic extraction (see above). The extract was acidified by addition of 0.11 volumes of 1 M acetic acid/NaOH (pH 4.5), dialyzed against 10 mM acetic acid/NaOH (pH 4.5) and then loaded onto a SE52 column equilibrated with the same buffer. The flow-through was collected and its pH was adjusted to 8.0 by addition of 1 M Tris–HCl (pH 8.2). This solution was then applied to a Q-Sepharose column equilibrated with 40 mM Tris–HCl (pH 8.0). The flow-through contained the pure lecin domain that was dialyzed against water and stored at −20 °C. The identity of the purified protein was confirmed by amino-terminal sequencing and MALDI-MS (calculated mass: 16,964 Da; measured: 16,964 Da). This method yielded around 11 mg pure protein per litre of bacterial culture. Periplasmic PPlase from *E. coli* was expressed and purified as described. 33

Protein concentrations

Protein concentrations were measured by absorption at 280 nm according to Gill & von Hippel. 34 Molar extinction coefficients (ε280) of 24,000 M−1 cm−1, 31,750 M−1 cm−1, 31,900 M−1 cm−1, 24,665 M−1 cm−1, 7740 M−1 cm−1, and 6146 M−1 cm−1 were used for native FimC, the native FimC–pilin domain complex, unfolded FimH, the native lecin, the unfolded pilin domain of FimH, and *E. coli* PPlase, respectively.

CD and fluorescence spectroscopy

CD measurements were performed with a Jasco J-715 spectropolarimeter at 25 °C in 0.1 cm quartz cuvettes. Spectra were measured at protein concentrations of 10 μM in 20 mM Tris–HCl (pH 8.0) containing different concentrations of GdmCl. The far-UV range between 200 and 260 nm was measured at a wavelength of 210–200 nm, depending on GdmCl concentration) was scanned at 20 nm min−1 with a 2 nm band width and a response of 0.5 second. Ten spectra were accumulated, corrected for the corresponding buffer, and the CD signal was converted to residue ellipticity ([θ]residue/deg cm2 dmol−1). 35 CD signals at a constant wavelength of 210 nm were recorded for two minutes with 2 nm band width and averaged. Buffer conditions and protein concentrations are indicated in the legends of Figures 2, 3, and 5. Refolding of the pilin domain was monitored for 25–60 minutes by the increase in the CD signal at 210 nm, using a resolution of 0.5 second and a response of two seconds.

Fluorescence measurements were performed at 25 °C on an Hitachi F-4500 spectrofluorimeter in 1.0 cm (emission path) × 0.2 cm (excitation path) quartz cuvettes. The excitation wavelength was 280 nm in all measurements (2.5 nm or 5 nm band width). Emission spectra were recorded between 290 nm and 450 nm (5 nm band width) at a scan speed of 60 nm min−1 or 240 nm min−1 with a response of two or 0.5 seconds, respectively. Protein concentrations for recording spectra were 5 μM in 20 mM Tris–HCl (pH 8.0) containing different concentrations of GdmCl, and all spectra were corrected for the buffer. GdmCl-dependent equilibrium transitions of the lecin domain were followed at a protein concentration of 1 μM in 10 mM Mops/NaOH (pH 7.0) by the change in fluorescence at 350 nm. The fluorescence signal was recorded with a band-width of 5 nm for 30 seconds and averaged.

GdmCl-dependent folding transitions

Denaturant-induced equilibrium transitions were measured at 25 °C after incubation of proteins for 18 hours in 10 mM Mops/NaOH (pH 7.0) containing different GdmCl concentrations. Equilibrium transitions of the isolated lecin domain and of the lecin domain in the context of full-length FimH were followed by the change in tryptophan fluorescence at 350 nm. In order to prepare the lecin domain in the context of FimH for unfolding studies, FimH was first dialyzed against 1.2 M GdmCl in 10 mM Mops/NaOH (pH 7.0), where the pilin domain is unfolded and the lecin domain is native. Prior to unfolding or refolding of the isolated lecin domain, the protein was first dialyzed against 10 mM Mops/NaOH (pH 7.0) containing either zero or 6 M GdmCl, respectively. To induce unfolding or refolding, aliquots of the above solutions were diluted 1:10 with 10 mM Mops/NaOH (pH 7.0) containing different concentrations of GdmCl. The final protein concentration was 1 μM in all samples. The far-UV CD signal at 210 nm was used to monitor refolding of the isolated pilin domain and of the pilin domain in the context of FimH. For refolding transitions of the isolated pilin domain, the protein was dialyzed against 2.5 M GdmCl, 10 mM Mops/NaOH (pH 7.0) or against 2.5 M GdmCl, 20% (v/v) glycerol, 20 mM Tris–HCl (pH 8.0). These solutions were then diluted to a protein concentration of 10 μM with 10 mM Mops/NaOH (pH 7.0) or with 20% (v/v) glycerol, 20 mM Tris–HCl (pH 8.0), respectively, containing different GdmCl concentrations. To record unfolding of the lecin domain in the context FimH, FimH was dialyzed against 2.4 M GdmCl, 10 mM Mops/NaOH (pH 7.0). Under these conditions, the pilin domain is unfolded, while the lecin domain is native. Aliquots of the FimH solution (70 μM) were then diluted 1:10 with 10 mM Mops/NaOH (pH 7.0) containing different concentrations of GdmCl. All transitions were evaluated according to a two-state model using the linear extrapolation method and
normalized. The corresponding six-parameter fit was applied to all original data except for the transitions of the pilin domain at pH 7.0, where no pre-transition baseline was obtained due to low protein stability. In this case, a five-parameter fit was used, assuming a slope of zero for the pre-transition baseline.

For the three-state analysis of the GdmCl-dependent folding of FimH, the fractions of the native protein (N), the unfolding intermediate (I), and the fully unfolded protein (U) were calculated from the GdmCl dependence of the equilibrium constants of each transition in the context of the full-length FimH according to equations (1) and (2), where $\Delta G_{UI}, \Delta G_{NI}, \Delta G_{NN}$ correspond to the free energies of folding, and $n_{UI}$ and $n_{NN}$ are the cooperativities of folding of the lectin and the pilin domain, respectively:

$$k_{UI} = \exp[-(\Delta G_{UI} + n_{UI}(\text{GdmCl})/RT)]$$

$$k_{NN} = \exp[-(\Delta G_{NN} + n_{NN}(\text{GdmCl})/RT)]$$

**Analysis of aggregation of FimH during refolding of the pilin domain**

For analysis of FimH precipitation during refolding of its pilin domain, a concentrated solution of FimH (210 μM) in 2 M GdmCl, 10 mM Mops/NaOH (pH 7.0) was prepared. The solution was diluted 1:15 to a final volume of 300 μl with 10 mM Mops/NaOH (pH 7.0) containing different concentrations of FimC and NaCl (final FimH concentration: 14 μM). In one set of experiments, the concentration of FimC was varied from zero to two molar equivalents (0–28 μM) at a constant NaCl concentration of 500 mM. In another set of experiments, the FimC concentration was kept constant at 28 μM and the NaCl concentration was varied between 0 mM to 500 mM. After rapid mixing, all samples were incubated at 25 °C for 20 minutes and then centrifuged at maximal speed for ten minutes in a tabletop centrifuge. The supernatants (300 μl) were collected, and the pellets were washed with 10 mM Mops/NaOH (pH 7.0), 200 mM NaCl and suspended in 300 μl of 10 mM Mops/NaOH (pH 7.0), 4 M urea. Aliquots of 20 μl from the soluble and the insoluble fractions were then applied to a SDS-containing gel (15% w/v acrylamide), and protein bands were stained with Coomassie blue.

**Refolding kinetics of the pilin domain**

Refolding kinetics of the isolated pilin domain were measured at pH 8.0 and 25 °C by following the increase in ellipticity at 210 nm. The unfolded pilin domain was first dialyzed against 2.5 M GdmCl, 20 mM Tris–HCl (pH 8.0). The protein concentration was adjusted to 100 μM, 50 μM, or 25 μM by dilution with the same buffer. To induce refolding, aliquots of these solutions were mixed manually with nine volumes of 20 mM Tris–HCl (pH 8.0) and immediately transferred to a 0.1 cm cuvette in the CD sample chamber. The same experiments were also performed with 20% (v/v) glycerol included in all buffers. To test the influence of periplasmic E. coli PPlase on the folding of the pilin domain, the pilin domain (100 μM in 2.5 M GdmCl, 20 mM Tris–HCl (pH 8.0)) was diluted with nine volumes of 1.1 μM PPlase in 20 mM Tris–HCl (pH 8.0). Final concentrations were 10 μM pilin domain, 1 μM PPlase and 0.25 M GdmCl. All refolding kinetics could be well fitted with a single exponential function (Figure 5(b)). However, due to the comparably low signal-to-noise ratio in the kinetic experiments, we cannot completely exclude bi- or multi-exponential kinetics.

**Analysis of binding of the pilin domain to FimC by analytical gel filtration**

Binding of the pilin domain of FimH to FimC after refolding was investigated in 20 mM Tris–HCl (pH 8.0), 20% (v/v) glycerol. In order to induce refolding, a stock solution (50 μM) of the pilin domain in 2.5 M GdmCl, 20 mM Tris–HCl (pH 8.0), 20% (v/v) glycerol was diluted 1:10 with 20 mM Tris–HCl (pH 8.0), 20% (v/v) glycerol. After ten minutes of incubation at 25 °C, one molar equivalent of FimC was added to the refolded pilin domain. The sample was then incubated for another 20 minutes. Final protein concentrations were 4.9 μM for both proteins in 0.25 M GdmCl. Alternatively, aliquots of the stock solution of the pilin domain were diluted 1:10 with buffer containing one molar equivalent of FimC, so that FimC was already present at the onset of refolding. These samples were also incubated for 30 minutes. All mixtures were then directly analyzed by gel filtration on a Superdex™ 75 HR 10/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mM sodium phosphate (pH 7.4), 115 mM NaCl, at a flow rate of 20 μl/hour (volume of applied samples: 100 μl). Eluted proteins were detected by their absorbance at 226 nm. The elution profiles were evaluated with the program PeakFit® (SBI) using the Exponentially Modified Gaussian (EMG) model. In the experiments shown in Figure 5(c) and (d), two overlapping peaks with the same asymmetry were identified, corresponding to the FimC–pilin domain complex and monomeric FimC. On the basis of the individual peak areas obtained after deconvolution of the elution profiles and the extinction coefficients at 226 nm of FimC (211,800 M⁻¹ cm⁻¹) and the FimC–pilin domain complex (303,500 M⁻¹ cm⁻¹), the fractions of monomeric FimC and the FimC–pilin domain complex were calculated. The extinction coefficients at 226 nm for FimC and the FimC–pilin domain complex were determined by measuring the absorbance at 226 nm of corresponding protein samples at known protein concentrations. The isolated pilin domain adsorbs non-specifically to the column under the chosen conditions (possibly due to complete removal of residual denaturant at the beginning of the chromatography) and is not observed in the eluate.

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**References**


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Pilus chaperones represent a novel type of protein folding catalyst

\section*{RESULTS \hspace{0.5cm} Pilus chaperones accelerate subunit folding}

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Adhesive type 1 pili from uropathogenic \textit{Escherichia coli} strains play a crucial role during infection by mediating the attachment to and potentially invasion of the host tissue. These filamentous, highly oligomeric protein complexes are assembled by the “chaperone-usher” pathway\textsuperscript{1}, according to which the individual pilus subunits fold in the bacterial periplasm and form stoichiometric complexes with a periplasmic chaperone that is essential for pilus assembly\textsuperscript{2-4}. The chaperone subsequently delivers the subunits to an assembly platform in the outer membrane, which mediates subunit assembly and translocation to the cell surface\textsuperscript{5-8}. Here we show that the periplasmic type 1 pilus chaperone FimC binds non-native pilus subunits and accelerates folding of the subunit FimG by 100-fold. Moreover, we find that the FimC·FimG complex is formed quantitatively and very rapidly 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. FimC thus represents a novel type of protein folding catalyst and simultaneously acts as a kinetic trap preventing spontaneous subunit assembly in the periplasm.

Type 1 pili from \textit{E. coli} are composed of the main structural pilus subunit FimA forming the pilus rod, and subunits FimF, FimG and the adhesin FimH, which form the distal tip fibrillum\textsuperscript{9} (Fig. 1a). The X-ray structures of chaperone-subunit and subunit-subunit complexes from different adhesive pilus systems have shown that pilus subunits have an immunoglobulin-like fold lacking a $\beta$-strand\textsuperscript{10-13}. In chaperone-subunit complexes this incomplete fold of the pilus subunit is completed by a polypeptide segment of the chaperone,
RESULTS  

Pilus chaperones accelerate subunit folding

...termed donor strand\textsuperscript{10-13}, while in the assembled pilus an N-terminal extension of the adjacent pilus subunit acts as the donor strand\textsuperscript{12-14} (Fig. 1a). Based on the above complementation principles, we designed an experiment that mimics pilus subunit folding in the periplasm. To test whether a folding subunit preferentially binds to the chaperone or another folded, but not yet incorporated subunit, the chemically denatured subunit FimG was refolded in the presence of both FimC and FimF. In order to inhibit self-polymerization of FimG and FimF, we used a FimG variant (FimG\textsubscript{t}) lacking the 14 amino-terminal donor strand residues, and a FimF variant (FimF\textsubscript{F}) elongated at its C-terminus by a FimF donor strand that allows intramolecular donor strand complementation (cf. Supplementary Fig. S1). The only complexes that can form in this system are the heterodimers FimC·FimG\textsubscript{t} and FimF\textsubscript{F}·FimG\textsubscript{t} (Fig. 1d). We refolded FimG\textsubscript{t} (6.8 µM) in the presence of equimolar concentrations of FimC and FimF\textsubscript{F}, separated the products after different reaction times with gel filtration, and analysed eluted fractions by SDS-PAGE. Figs. 1b, c, d show that the FimC·FimG\textsubscript{t} complex was formed quantitatively and very rapidly within the dead time of the experiment. However, FimG\textsubscript{t} then slowly dissociated from FimC and formed a 1:1 complex with FimF\textsubscript{F} with an apparent half time of 6.6 h (Figs. 1b, c, d). After 72 hours, 96±4 % of refolded FimG\textsubscript{t} is in complex with FimF\textsubscript{F}. Together, these data demonstrate that the FimF\textsubscript{F}·FimG\textsubscript{t} complex is thermodynamically more stable than the FimC·FimG\textsubscript{t} complex, and that FimC acts as a kinetic trap for folding pilus subunits preventing rapid formation of subunit-subunit complexes. As a control, we demonstrated that FimG\textsubscript{t} alone can be refolded quantitatively, and that the native protein exhibits a low thermodynamic stability in the absence of a donor strand (\(\Delta G_{\text{folding}} = -13 \text{ kJ mol}^{-1}\), Supplementary Fig. S2a, b).

To investigate the kinetics of folding of FimG\textsubscript{t} we employed stopped-flow tryptophan fluorescence. FimG\textsubscript{t} was refolded by 1:11 dilution of the denaturant guanidinium chloride (GdmCl) from 2.75 to 0.25 M. Fig. 2a shows that spontaneous folding of FimG\textsubscript{t} is a slow process that can be described by the sum of two exponential functions (rate constants of 0.14 s\textsuperscript{-1} and 0.031 s\textsuperscript{-1}, cf. Table 1). In addition, we observed a rapid fluorescence increase in the dead time of stopped-flow mixing (15±2 % of total amplitude, Supplementary Fig. S3), indicating rapid formation of at least one folding intermediate. As the \(\beta\)-fold of FimG is complemented either by the FimC donor strand in the FimC·FimG complex or by the N-terminal donor strand of the adjacent subunit FimF in the intact pilus (Fig. 1a), we next...
RESULTS

Pilus chaperones accelerate subunit folding

refolded FimGₜ in the presence of a 40-fold excess of the corresponding donor strand peptides (DS₉FimC and DS₉FimF, respectively). Figs. 2b and c show that the donor strand peptides do not significantly accelerate FimGₜ folding. Rapid formation of the intermediate in the dead time of the stopped-flow experiment was retained, and only the slower of the two exponential phases was slightly faster (Table 1). As we see a significant decrease in the fluorescence signal of the FimGₜ-peptide complexes after 100 s of refolding relative to that of free FimGₜ (Fig. 2a, b, c), we can exclude that lack of acceleration stems from failure of the peptides to bind. The change in fluorescence was used to determine the affinity of FimGₜ for DS₉FimC by fluorescence titration. The obtained data are consistent with a 1:1 stoichiometry of binding, with an apparent Kₐ of 6.6 µM for the DS₉FimC·FimGₜ complex (Fig. 2e). We next measured the folding kinetics of FimGₜ in the presence of a five-fold molar excess of the self-complemented acceptor subunit FimFF. Similar to the donor strand peptides, the intact donor subunit FimFF has no influence on the folding kinetics of FimGₜ, except for a slight acceleration of the second slow refolding phase (Fig. 2d, Table 1).

To investigate if the refolding reaction of FimGₜ might be influenced by additional interactions with FimC other than those with the FimC donor strand, we refolded FimGₜ in the presence of the complete chaperone FimC. To avoid strong fluorescence contributions from FimC, we used a tryptophan-free FimC variant (FimCₙₚ) that is fully functional and can complement FimC deficiency in vivo (Supplementary Fig. S4). Strikingly, FimGₜ folding in the presence of full-length chaperone is changed dramatically. Four exponential functions with rate constants of 38 s⁻¹, 1.9 s⁻¹, 0.24 s⁻¹, and 0.026 s⁻¹ were required to fit the fluorescence trace (Fig. 3a), which apparently lacked the burst phase observed in spontaneous FimGₜ folding. The phase with highest fluorescence amplitude was also the fastest of the three rates (38 s⁻¹), and the trace suggests accumulation of a hyperfluorescent folding intermediate (Fig. 3a).

Since two of the kinetic phases of FimGₜ folding in the presence of FimCₙₚ were significantly faster than the two observable phases in spontaneous folding (Table 1), FimCₙₚ likely accelerated formation of native FimGₜ. To substantiate that formation of native FimGₜ is accelerated in the presence of FimC, we performed interrupted refolding experiments¹⁵, which exclusively detect the formation of native FimGₜ during the refolding reaction. These experiments make use of the fact that, in a mixture of native molecules and folding
intermediates, native molecules unfold with a characteristic rate constant at a given denaturant concentration, while any folding intermediate unfolds significantly faster\(^\text{15}\). The amplitude of the slowest unfolding phase is thus directly proportional to the concentration of native molecules after the corresponding time of refolding. To compare the folding reaction in presence of donor strand peptide with the folding reaction in presence of full-length chaperone, FimG\(_t\) was allowed to refold in the presence of either a 40-fold excess of DS\(_{\text{FimC}}\) or a fivefold excess of FimC\(_{\text{YY}}\). After different times of refolding, the reactions were again transferred to unfolding conditions and the amplitudes of the unfolding reactions were measured. Remarkably, in the presence of donor strand peptide, formation of native FimG\(_t\) can be described by a single first-order rate constant of 0.0041 s\(^{-1}\)(Fig. 3b), which is one order of magnitude slower than the slowest rate detectable in the fluorescence trace (Fig. 2b, Table 1). Therefore, a spectroscopically silent, rate-limiting reaction leads from an unstable DS\(_{\text{FimC}}\cdot\text{FimG}_t\) intermediate to the native complex.

The kinetics of formation of native FimG\(_t\) in the presence of FimC\(_{\text{YY}}\) are strikingly different: the majority of molecules (64±5 \%) reach the native state with a rate constant of 0.49±0.08 s\(^{-1}\), which is more than 100-fold faster than in presence of the DS\(_{\text{FimC}}\) peptide. The remaining fraction (36±5 \%) folds 20-fold slower (0.025±0.009 s\(^{-1}\)), but still 6-fold faster than in the presence of DS\(_{\text{FimC}}\) (Fig. 3b, Table 1). The rate of the slower folding reaction coincides with typical values of prolyl cis-trans isomerization\(^\text{25}\), and the fraction of 36±5 \% slow folding species is very similar to the calculated value of 28 \% FimG\(_t\) molecules with at least one cis prolyl peptide bond\(^\text{16}\). Thus, assuming that both prolyl peptide bonds in native FimG\(_t\) are trans, the slower folding fraction may be caused by cis-trans isomerization of prolyl peptide bonds in FimG\(_t\).

The time constants and the relative sizes of the amplitudes in the interrupted folding experiment are very similar to the values observed for the two slowest phases in fluorescence-detected folding (Fig. 3a and Table 1). The two slowest phases can thus be assigned to formation of native FimG\(_t\), whereas the two fastest phases must be caused by the transient formation of FimG\(_t\) folding intermediates. The fast accumulation of a hyperfluorescent intermediate that is only observed in the presence of the chaperone (cf. Fig. 2a, b, 3a) is unambiguous proof of binding of the chaperone FimC to an early folding intermediate or the completely unfolded substrate. From this observation and the experiment described in Figs.
1b, c, and d, we conclude that unfolded subunits (or early folding intermediates) have a higher affinity for the chaperone than for other pilus subunits. Once they are folded, however, their affinity for the chaperone is lower than for other pilus subunits. This mechanism ensures that the chaperone captures folding subunits to prevent premature pilus assembly while energy is preserved to promote the subsequent incorporation of subunits into the pilus.

The striking acceleration of folding to native FimGt by more than 100-fold from 0.0042 s\(^{-1}\) to 0.49 s\(^{-1}\) has important implications for the kinetics of pilus assembly \textit{in vivo}. Previous studies on the rate of SEC-dependent protein translocation into the \textit{E. coli} periplasm\(^{17}\) and the rate of pilus assembly\(^{18}\), which in the case of type 1 pili consists of 500-3000 pilus subunits\(^{9}\), indicate that both translocation of an individual subunit into the periplasm and its incorporation into the growing pilus occurs on the timescale of a few seconds. In contrast, uncatalyzed folding of pilus subunits takes minutes (Fig. 3b, \(^{19}\)). The large acceleration of subunit folding by FimC is thus critical for efficient pilus biogenesis, since it overcomes the kinetic bottleneck of the assembly process.

It is remarkable that FimC accelerates folding of substrate molecules in view of the fact that classical molecular chaperones enhance the efficiency but not the rate by which their substrates fold\(^{20}\). Nevertheless, FimC is a typical chaperone in that it forms complexes with non-assembled substrates and also inhibits non-specific aggregation of subunits\(^{19,21}\). The acceleration of pilus subunit folding by FimC described here requires defined contacts between the folding polypeptide and the tertiary structure of the chaperone, and is reminiscent of proteins that are synthesized as precursors with pro regions that are transiently necessary for folding\(^{22}\). A well characterized example is alpha-lytic protease, in which the pro region dramatically accelerates folding to the native protease\(^{23}\). While pro regions act as intramolecular folding catalysts, periplasmic pilus chaperones function as intermolecular catalysts that are released through delivery of native subunits to the growing pilus for the next round of catalysis. The common feature of pro regions and periplasmic chaperones as folding catalysts may be that they both transiently become part of the tertiary structure of their substrate. So far, natural, intermolecular protein folding catalysis has only been known for peptide bond cis-trans isomerization and disulfide bond formation, two prominent, rate-limiting steps in protein folding\(^{24-26}\). In this context FimC represents a novel type of protein folding catalyst.
folding catalyst that either accelerates certain rate-limiting steps along the folding pathway of pilus subunits or even changes the folding mechanism of its substrates entirely.

Methods

Plasmids. For cytoplasmic expression of C-terminally (His)$_6$-tagged FimC, the genetic sequence of mature FimC was amplified by the polymerase chain reaction (PCR) from pfimC$^{27}$ and introduced into pET-11a (Novagen). The resulting plasmid pfimC-his-cyt was used to create the (His)$_6$-tagged FimC variant FimC$_{YY}$ (QuickChange, Stratagene). For cytoplasmic expression of FimFF, the gene encoding mature fimF was amplified by PCR from genomic DNA of *E. coli* W3110, using a 3’ primer that added a short linker (DNKQ) to the FimF C-terminus, followed by the FimF donor strand (ADSTITIRGYVRDN). The PCR product was ligated into pET-11a yielding pfimFF-cyt. All PCR primers used are given in Table S1 (Supplementary Information).

Proteins and Peptides. FimFF, and (His)$_6$-tagged FimC and FimC$_{YY}$ were expressed as soluble proteins in the cytoplasm of *E. coli* Tuner (DE3) (Novagen) carrying the plasmids pfimFF-cyt, pfimChis-cyt, and pfimCW36YW84Yhis-cyt, respectively. FimFF was purified by anion exchange and hydrophobic interaction chromatography. The single disulfide bond in FimF$_F$ was formed during purification by air oxidation, which was verified by Ellman’s assay after unfolding of FimF$_F$ in 6.0 M GdmCl. (His)$_6$-tagged FimC and FimC$_{YY}$ were purified by immobilized metal affinity and cation exchange chromatography. In order to obtain FimG$_t$, the FimC·FimG$_t$ complex was first expressed and purified as described$^{28}$. (His)$_6$-tagged FimC was then removed by immobilized metal affinity chromatography after dissociation of the complex under denaturing conditions (2.75 M GdmCl, pH 8.0). The HPLC-purified peptides DS$_{FimC}$ and DS$_{FimF}$ were obtained from Jerini and >95 % pure. Protein concentrations were determined via the extinction coefficients at 280 nm (FimC: 22900 M$^{-1}$cm$^{-1}$; FimC$_{YY}$: 14300 M$^{-1}$cm$^{-1}$; FimF$_F$: 10900 M$^{-1}$cm$^{-1}$; FimG$_t$: 12200 M$^{-1}$cm$^{-1}$). Peptide concentrations were
measured via the extinction coefficients at 205 nm (DS_{FimC}: 47400 M^{-1}cm^{-1}; DS_{FimF}: 49700 M^{-1}cm^{-1}).

**Kinetics of FimC binding and subunit assembly.** Unfolded FimGt in 20 mM Tris/HCl, 2.75 M GdmCl, pH 8.0 was diluted 11-fold into 20 mM Tris/HCl, pH 8.0 containing FimC and FimF at 25 °C. The final concentration of all three proteins was 6.8 µM. At different times after mixing, samples were removed and subjected to analytical gel filtration at 4 °C on a Superdex 75 HR 10/30 column (Amersham Biotech). The protein composition of the eluted fractions (500 µl each) was quantified by densitometric analysis of the Coomassie-blue stained SDS-gels.

**Stopped-flow fluorescence.** Reactions were measured in an SX-18MV instrument (Applied Photophysics) at 25 °C.

FimGt refolding kinetics were followed in 20 mM Tris/HCl, 0.25 M GdmCl, pH 8.0. Unfolded FimGt in 2.75 M GdmCl was diluted 11-fold either with 20 mM Tris/HCl or buffer containing FimC_{YY}, FimF, DS_{FimC}, or DS_{FimF}. The final FimGt concentration was 1.0 µM in all experiments. FimC_{YY} and FimF were used at 5-fold, and the DS_{FimC} and DS_{FimF} peptides at 40-fold excess over FimGt, establishing pseudo-first-order conditions for binding to FimGt. Excitation and emission wavelengths were 280 and 330 nm, respectively. Each measurement was repeated at least 10 times, data were averaged, and corrected for the buffer and a slight signal decrease over time caused by photobleaching.

To determine the apparent affinity of FimGt for the DS_{FimC} peptide, FimGt (1 µM) was refolded at different DS_{FimC} concentrations. The dependence of the folding amplitude on DS_{FimC} concentration was used to determine the apparent dissociation constant.

For interrupted refolding experiments in the presence of FimC_{YY}, unfolded FimGt (in 2.75 M GdmCl) was diluted 11-fold with 20 mM Tris/HCl, pH 8.0 containing FimC_{YY} to final protein concentrations of 1 µM FimGt and 5 µM FimC_{YY}. After various times of refolding (t_i),
the GdmCl concentration was again rapidly increased from 0.25 to 2.7 M, and the unfolding kinetics were monitored at 330 nm (excitation at 295 nm; dead time of sequential mixing $\approx 40$ ms). The amplitudes corresponding to unfolding of native $\text{FimC-FimG}_t$ were normalized against the unfolding amplitudes of $\text{FimG}_t$ that had been completely refolded in the presence of $\text{FimC}_{YY}$. This yielded the fraction of native $\text{FimG}_t$ at $t_i$. Interrupted folding of 1 $\mu$M $\text{FimG}_t$ in the presence of 40 $\mu$M $\text{DS}_{\text{FimC}}$ was measured in the same manner, except that unfolding of the native $\text{DS}_{\text{FimC}}$-$\text{FimG}_t$ complex was induced by 4.1 M GdmCl, 50 mM glycine/HCl, pH 1.8, and that additional data points at 1800 and 3600 s were obtained by a combination of manual mixing (initiation of refolding) and stopped-flow mixing (detection of unfolding).

References


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Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to R.G. (e-mail: rudi@mol.biol.ethz.ch).
Table 1  **Reciprocal time constants and amplitudes of FimG₈ folding**

<table>
<thead>
<tr>
<th>Protein or peptide in the refolding buffer</th>
<th>$1/\tau_1$ (s⁻¹)</th>
<th>$A_1$ (%)</th>
<th>$1/\tau_2$ (s⁻¹)</th>
<th>$A_2$ (%)</th>
<th>$1/\tau_3$ (s⁻¹)</th>
<th>$A_3$ (%)</th>
<th>$1/\tau_4$ (s⁻¹)</th>
<th>$A_4$ (%)</th>
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</thead>
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<tr>
<td>a</td>
<td>0.14±0.01</td>
<td>38±2</td>
<td>0.031 ±0.001</td>
<td>62±2</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>aDSFimC</td>
<td>0.15±0.01</td>
<td>42±3</td>
<td>0.038 ±0.002</td>
<td>58±3</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>aDSFimF</td>
<td>0.14±0.02</td>
<td>54±8</td>
<td>0.041 ±0.006</td>
<td>46±8</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>aFimFF</td>
<td>0.13±0.03</td>
<td>35±9</td>
<td>0.034 ±0.004</td>
<td>65±8</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>aFimCYY</td>
<td>38±3</td>
<td>91±3</td>
<td>1.9±0.5</td>
<td>32±5</td>
<td>0.24±0.15</td>
<td>-17±8</td>
<td>0.026 ±0.003</td>
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<td>bDSFimC</td>
<td>0.0042</td>
<td>100</td>
<td>-</td>
<td>-</td>
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<tr>
<td>bFimCYY</td>
<td>0.49±0.08</td>
<td>64±5</td>
<td>0.025 ±0.009</td>
<td>36±5</td>
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</table>

a  Fluorescence-detected folding kinetics  
b  Interrupted refolding experiments, monitoring formation of native FimG₈
RESULTS  

Pilus chaperones accelerate subunit folding

Figure 1  FimC forms transient complexes with folding pilus subunits and acts as kinetic assembly trap.  

a, Schematic representation of donor strand complementation in chaperone-subunit complexes and in the quaternary structure of type 1 pili.  

b, and c, Refolding of chemically denatured FimGt (6.8 µM) in the presence of equimolar concentrations of FimC and the self-complemented donor subunit FimFF at pH 8.0 and 25 °C. After different times of refolding products were separated by gel filtration, and proteins in eluted fractions were analysed quantitatively by SDS-PAGE.  

d, Reaction scheme and apparent time constants of transient formation (cf. Fig. 3b) and disappearance of the FimC·FimGt complex.

Figure 2  Folding kinetics of FimGt (1 µM) at pH 8.0 and 25°C, detected by tryptophan fluorescence.  

a, spontaneous refolding.  

b, refolding in the presence of 40 µM DSFimC peptide (15-mer, sequence: TENTLQLAIISRIKR).  

c, refolding in the presence of 40 µM DSFimF (15-mer, sequence: ADSTITIRGYVRDN).  

d, refolding of FimGt in the presence of the self-complemented donor subunit FimFF (5 µM). Arrows indicate the expected initial fluorescence of FimGt (cf. Supplementary Fig. S3). All fluorescence traces in a-d are characterized by a rapid burst phase, followed by a slow fluorescence increase that can be fitted with a sum of two exponentials (solid lines).  

e, DSFimC binds specifically to FimGt during FimGt folding. FimGt was refolded in the presence of different concentrations of DSFimC, and the folding amplitude was plotted against the DSFimC concentration. The data yielded a dissociation constant of 6.6 µM and a 1:1 stoichiometry of binding (solid line).

Figure 3  FimC accelerates folding of FimGt by more than 100-fold.  

a, Fluorescence kinetics at pH 8.0 and 25 °C of FimGt refolding (1 µM) in the presence of 5 µM FimCYY. Data from 30 independent experiments were averaged. A sum of four exponentials was required to fit the data (solid line). Arrows indicate the expected initial fluorescence (1; cf. Supplementary Fig. S3), and the initial fluorescence from
the fit (2), respectively. The dashed line shows the fit to the fluorescence trace of FimGt folding in the presence of DS\textsubscript{FimC} (data from Fig. 2b). b, Formation of native FimGt during refolding in the presence of FimC\textsubscript{YY} (●), or in the presence of the DS\textsubscript{FimC} peptide (▲). Data are consistent with a sum of two exponentials for folding in the presence of FimC\textsubscript{YY}, and with a single exponential in the case of folding in the presence of DS\textsubscript{FimC} (solid lines).
RESULTS

Pilus chaperones accelerate subunit folding

Figure 1

(a) chaperone-pilin complex

(intact pilus)

FimH
FimG
FimG
FimF
FimA
FimA

(FimA subunits)

(b) 0.1 h

FimC
FmF/F
FimG

4 h

FimC
FimF/F
FimG

24 h

FimC
FimF/F
FimG

(c) Fraction of FimC or FmF/F in complex with FimG.

Elution volume (mL)

(d) unfolded FimG

FimF/F

τ = 2.0 s

FimF/F

τ = 6.6 h

FimG

FimC

FimC

FimC

Figure 1
RESULTS

Pilus chaperones accelerate subunit folding

Figure 2
RESULTS

Pilus chaperones accelerate subunit folding

Figure 3

(a) 

(b) 

Figure 3
### Supplementary Information Table S1. Primer sequences.

<table>
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<tr>
<th>Cloning step</th>
<th>Primer 1</th>
<th>Primer 2</th>
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<td>Amplification of a gene encoding FimC with a carboxy-terminal His&lt;sub&gt;6&lt;/sub&gt;-tag and without the natural signal sequence</td>
<td>5'-GG AGTGGCCTTA GGTGCG-3'</td>
<td>5'-GAC GCA TGG CGC GGA TCC TTA GTG GTG GTG GTG ATG ATG TTC CAT TAC GCC CGT C-3'</td>
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<td>Exchange of the codon encoding W36 of FimC by a codon encoding Y</td>
<td>5'-GTA CCT ATT TAA TTC AAT CGT ACG TGG AAA ATG CCG ATG GTG TAA AGG-3'</td>
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<td>Exchange of the codon encoding W84 of FimC by a codon encoding Y</td>
<td>5'-GGA CCG GGA AAG TTT ATT CTA TAT GAA CGT TAA AGC GAT TCC G-3'</td>
<td>5'-CGG AAT CGC TTT AAC GTT CAT ATA GAA TAA ACT TTC CCG GTC C-3'</td>
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<tr>
<td>Amplification of FimF&lt;sub&gt;F&lt;/sub&gt; without the natural signal sequence</td>
<td>5'-GGC CAG GCC TAC GCG TCA TAT GGC GGA TAG CAC GAT TAC TAT C-3'</td>
<td>5'-CGG GTC GCG ATG CGC ATG GAT CCT TAG TTA TCT CTG ACA TAA CCG CGA ATA GTA ATC GTG CTA TCG GCC TGT TTG TTA TCC TGA TAT TCA AGA GTG AAG G-3'</td>
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**Supplementary Figure S1** Overview over type1 pilus subunits and donor strand peptides used in this study. The variant FimG\textsubscript{t} lacks the 14 amino-terminal residues of mature FimG, which are predicted to serve as donor strand for a neighbouring subunit. The construct FimF\textsubscript{F} consists of FimF (with the natural, amino-terminal donor strand) extended at the carboxy-terminus with the tetrapeptide linker DNKQ and another FimF donor strand that intramolecularly complements the \(\beta\)-fold of the protein. The peptides corresponding to the donor strands of FimC (DS\textsubscript{FimC}) and FimF (DS\textsubscript{FimF}) have an artificial arginine residue at their carboxy-terminus (in italics) that was added to increase the solubility of the peptides.

**Supplementary Figure S2** Spectroscopic and thermodynamic properties of FimG\textsubscript{t}. 

**a**, Fluorescence spectra of 2 \(\mu\)M FimG\textsubscript{t} in 0.25 M GdmCl (---) and 2.75 M GdmCl (——) at pH 8.0 (excitation at 280 nm). **b**, The change in fluorescence intensity at 330 nm was used to record a GdmCl-dependent equilibrium folding transition of FimG\textsubscript{t}. FimG\textsubscript{t} in 20 mM Tris/HCl pH 8.0 containing 0.25 or 4.0 M GdmCl was diluted with 20 mM Tris/HCl, pH 8.0 containing different GdmCl concentrations (final FimG\textsubscript{t} concentration: 1.0 \(\mu\)M), incubated at 25 \(^\circ\)C for 48 hours, and the fluorescence was measured in a HITACHI F-4500 fluorescence spectrometer at 330 nm (10 nm band width; excitation at 280 nm, 5 nm band width). The data for unfolding (●) and refolding (○) were evaluated according to the two-state model and normalized. Due to low protein stability, no pre-transition baseline could be obtained, which was therefore assumed to have a slope of zero. The calculated free energy of folding is \(-12.6\pm1.5\) kJ mol\(^{-1}\), the cooperativity is \(17.2\pm1.8\) kJ mol\(^{-1}\) M\(^{-1}\).

**Supplementary Figure S3** Detection of the burst phase in folding of FimG\textsubscript{t} in the absence of the chaperone. A solution of unfolded FimG\textsubscript{t} (11 \(\mu\)M) in 20 mM Tris/HCl pH 8.0, 2.75 M GdmCl was diluted 1:11 by stopped-flow mixing with 20 mM Tris/HCl containing different concentrations of GdmCl. The initial fluorescence signal after
mixing (●) was plotted against the final GdmCl concentration (bottom x-axis). Linear extrapolation of the data points to 0.25 M GdmCl (dashed line) revealed an initial burst phase in the refolding kinetics at 0.25 M GdmCl (x-axis on top) that corresponds to 15±2 % of the entire refolding amplitude.

**Supplementary Figure S4** Comparison of spectroscopic, thermodynamic and functional properties of FimC and the tryptophan-less variant FimC<sub>YY</sub>.  

**a**, Fluorescence spectra of FimG<sub>i</sub> (▲), FimC (●), and the tryptophan free variant FimC<sub>YY</sub> (■) in 0.25 M GdmCl at pH 8.0 and 25 °C upon excitation at 280 nm. All protein concentrations were 2 µM. The tryptophan fluorescence of FimC is approximately four times more intense than that of FimG<sub>i</sub>, while FimC<sub>YY</sub> only displays tyrosine fluorescence. Because folding of FimG<sub>i</sub> is ideally followed at 330 nm (see Fig. S2a), experiments in the presence of wild type FimC would suffer from a strong background signal caused by FimC. When FimC<sub>YY</sub> is used instead of FimC, the background signal is comparably small.  

**b**, Far-UV CD spectra of FimC (dots) and FimC<sub>YY</sub> (squares) at 25 °C, pH 8.0 in the absence of denaturant (black) or in the presence of 6 M GdmCl (grey).  

**c**, The far-UV CD signal at 218 nm was used to monitor GdmCl-dependent unfolding (●) and refolding (◦) of FimC or unfolding (■) and refolding (□) of FimC<sub>YY</sub>. The data were analysed based on the two-state model and normalized. The free energies of folding are -32.4±1.5 kJ mol<sup>-1</sup> and -23.5±1.4 kJ mol<sup>-1</sup>, the cooperativities 37.5±1.7 kJ mol<sup>-1</sup> M<sup>-1</sup> and 36.4±2.2 kJ mol<sup>-1</sup> M<sup>-1</sup> for FimC and FimC<sub>YY</sub>, respectively. The data demonstrate that FimC<sub>YY</sub> is less stable than FimC but sufficiently stable to be fully native under our standard conditions for FimG<sub>i</sub> refolding (0.25 M GdmCl).  

**d**, The pilin domain of FimH (FimH<sub>P</sub>, Ref.19) was coexpressed with either FimC or FimC<sub>YY</sub> in the periplasm of *E. coli*. Periplasmic extracts of bacteria at different times after induction of protein expression with IPTG (indicated above the Coomassie-stained gel) were prepared and subjected to SDS-PAGE. As overexpressed pilus subunits only accumulate in
the soluble, periplasmic fraction when functional FimC is coexpressed, the results demonstrate that FimC\textsubscript{YY} retains the ability to bind pilus subunits and inhibit subunit misfolding and aggregation \textit{in vivo}.
Supplementary Figure S1
RESULTS

Pilus chaperones accelerate subunit folding

Supplementary Figure S2
Supplementary Figure S3
RESULTS

Pilus chaperones accelerate subunit folding

Supplementary Figure S4

Graphs and images showing fluorescence, UV absorbance, and gel electrophoresis data.
Intrinsic affinities explain the sequential order of subunits in type 1 pili and constitute the driving force for pilus assembly

T. Spirig, M. Vetsch, C. Puorger & R. Glockshuber
Abstract

Type 1 pili of uropathogenic *Escherichia coli* belong to a class of filamentous surface organelles expressed by various pathogenic bacteria. These appendages consist exclusively of protein subunits and they are associated with the capability of the bacteria to attach to the sites of infection. The assembly of bacterial pili by a dedicated periplasmic pilus chaperone and a specialized outer membrane usher is one of the most thoroughly studied examples of macromolar assembly. Extensive studies *in vivo* and *in vitro* investigated the interactions between periplasmic pilus chaperones and subunits or the binding of such chaperone-subunit complexes to the outer membrane usher. In contrast, little is known about the incorporation of subunits into the growing pilus, i.e. the establishment of subunit-subunit contacts. Here we present a model system derived from the type 1 pilus assembly-machinery that we used for a quantitative analysis of this central event of pilus assembly. Our results show that the intrinsic affinities of different subunits for each other correlate with their position in the final pilus structure. We further found that subunit-subunit complexes are thermodynamically more stable than the periplasmic chaperone-subunit complexes. Consequently, the incorporation of a subunit into the pilus is energetically favourable which in turn explains why pilus assembly does not require cellular energy. The formation of subunit-subunit complexes in the absence of the outer membrane usher was extremely slow with apparent halftimes of several hours. We provide evidence that the outer membrane usher acts as the catalysts of this reaction, ensuring that the formation of subunit-subunit interactions is coupled to secretion of the assembled subunits into the extracellular space.
Introduction

Type 1 pili of uropathogenic *Escherichia coli* belong to a class of filamentous surface organelles expressed by various bacteria (Sauer *et al.*, 2000). These proteinaceous appendages are usually associated with the capability of the bacteria to attach to certain eucaryotic cell types. Type 1 pili are critical in the ability of uropathogenic *E. coli* to cause bladder infections (Langermann *et al.*, 1997). They mediate binding of the bacteria to mannose residues of uroplakin Ia, a glycoprotein on the luminal surface of bladder epithelium (Zhou *et al.*, 2001). Type 1 pili consist of a long stiff helical rod and a short flexible tip fibrillum at the distal end. They are composed of the four structural pilus subunits FimA, FimF, FimG, and FimH. FimA forms the pilus rod that is a 6.9 nm wide tube and has a length of up to 2 μm. The tip fibrillum is composed by FimF, FimG and FimH. FimH differs from the other subunits in important aspects. While all other subunits fold into one domain, FimH contains an additional domain. This domain is responsible for the adhesive properties of type 1 pili as it binds to mannose and hence it is called lectin domain (Choudhury *et al.*, 1999). Further, FimH caps the pilus interacting with a single subunit. All other subunits, in contrast, bind to two other subunits (Fig. 1A). The role of another potential subunit, FimI, remains unclear. While it was widely believed that FimI is not necessary for the expression of functional pili (e.g. Saulino *et al.*, 2000), new experiments argue for the opposite (Valenski *et al.*, 2003). Type 1 pili are assembled by the chaperone/usher pathway that is required for the formation of over 30 adhesive surface organelles in a wide range of Gram-negative bacteria (Sauer *et al.*, 2000).

During pilus biogenesis, the subunits are secreted to the periplasm via the Sec-apparatus where they form heterodimers with dedicated periplasmic chaperones. The periplasmic chaperones assist subunit folding (Barnhart *et al.*, 2000), prevent non-productive aggregation of the subunits (Jones *et al.*, 1997) and also inhibit their spontaneous premature assembly (Soto *et al.*, 1998). The chaperone-subunit complexes dissociate at the outer membrane where the subunits are incorporated into growing fibers that are secreted through oligomeric usher pores. The chaperone and the usher of the type 1 pilus system are FimC and FimD, respectively (Fig. 1A).

Crystal structures of chaperone-subunits complexes revealed that the subunits have an atypical immunoglobulin-like fold that lacks the final C-terminal strand, creating a large hydrophobic groove on the subunit surface. In the chaperone-subunits complex, this groove is occupied by
an extrinsic $\beta$ strand, provided by the chaperone (Choudhury et al., 1999; Sauer et al., 1999; Zavialov et al., 2003). This remarkably tight interaction is called “donor strand complementation” and the extrinsic $\beta$ strand is referred to as “donor strand”. Zavialov et al. (2003) recently showed that donor strand complementation also governs subunit-subunit interactions. The role of the chaperone donor strand is taken over by the previously disordered N-terminus of a second subunit, suggesting that in the fiber, the hydrophobic groove of each subunit is occupied by the N-terminus of the adjacent subunit. In the same study, Zavialov et al. compared the structure of a subunit in the chaperone-bound state with the structure of the same subunit in the subunit-bound state. They found that the two $\beta$ sheets of the immunoglobulin-like fold have low shape correlation statistics (Lawrence and Colman, 1993) in the chaperone-bound subunit, reflecting a loosely packed hydrophobic core. Meanwhile, the subunit in complex with another subunit has a tightly packed hydrophobic core as is typically observed for $\beta$ barrels consisting of two $\beta$ sheets. As subunits are incorporated into the fiber, they dissociate from the chaperone and instead bind to another subunit. This process involves apparently a collapse of the hydrophobic core of the subunit, which could result in a gain in free folding energy. Zavialov et al. propose that this difference in free energy might constitute the driving force for fiber assembly. According to this model, the periplasmic pilus chaperones trap a high energy folding intermediate of the subunits and preserve folding energy that is released upon incorporation of the subunit into the fiber.

Recent in situ immuno-electron microscopy experiments revealed that the sequential orientation of subunits in type 1 pili is FimA-FimF-FimG-FimH (Hahn et al., 2002). Several factors could contribute to this order: the relative abundance of the different chaperone-subunit complexes in the periplasm, the different affinities of chaperone-subunit complexes for the usher FimD, and the intrinsic affinities of the subunits for each other. It is known that among all chaperone-subunit complexes, the FimC-FimH has the highest affinity for the usher FimD (Saulino et al., 1998) and also for the isolated N-terminal domain of FimD constituting the domain of FimD that binds chaperone-subunit complexes (Nishiyama et al., 2003). This high affinity ensures that the FimH repertoire of the periplasm is exploited efficiently and that most if not all pili contain a FimH subunit at their tip, which is the only possible position for FimH in the pilus because FimH lacks a N-terminal donor strand (Fig.1A).
In contrast to the well characterized interplay between chaperone-subunit complexes and the usher, very little is known about the thermodynamics and kinetics that govern the dissociation of the chaperone from a subunit and the subsequent formation of a new subunit-subunit interaction, despite the fact that the establishment of subunit-subunit contacts is the central event of pilus formation. In order to shed light on these crucial determinants of pilus assembly, we investigated the replacement of the chaperone FimC by other subunits from chaperone-subunit complexes. We tested the hypothesis that subunit-subunit complexes may be thermodynamically more stable than chaperone-subunit complexes. Moreover, we compared the affinity of different subunits for each other to address the question how the intrinsic affinities correlate with the order of the subunits in the final pilus structure. And finally we performed initial experiments to investigate the role of the outer membrane usher FimD on the replacement of the chaperone FimC by other subunits from chaperone-subunit complexes.
Results

Prevention of subunit self-polymerization by protein design. The structural subunits of type 1 pilus possess the intrinsic ability to polymerize (Fig. 1A). This property leads to complex mixtures of various oligomers as soon as two different subunits are mixed, making an exact analysis impossible. Because we attempted to investigate the formation of subunit-subunit complexes quantitatively, we decided to inhibit self-polymerization by two different strategies: (i) We constructed truncated variants of FimF and FimG (FimF<sub>t</sub> and FimG<sub>t</sub>), which are depleted of their N-terminal donor strand and are thus no longer able to complement any other subunit (Fig. 1B). Like FimH that naturally lacks a donor strand, FimF<sub>t</sub> and FimG<sub>t</sub> can only accept donor strands of either the chaperone FimC or other subunits. Therefore, we refer to FimF<sub>t</sub>, FimG<sub>t</sub>, and FimH as acceptors. (ii) In order to obtain subunits that are restricted to donor function, we fused 14 amino acids representing the donor strand of FimF to the C-termini of FimF and FimG. This additional donor strand is expected to fold into the groove that is occupied by the chaperone in the chaperone-subunit complex. These self-complemented variants, called FimFF<sub>F</sub> and FimGF<sub>F</sub> (Fig. 1B) are unable to accept a donor strand from any other protein, but they still can complement the fold of other subunits and are thus referred to as donors.

The donors FimFF<sub>F</sub> and FimGF<sub>F</sub> were expressed in the cytoplasm of E. coli and purified to homogeneity. Far-UV circular dichroism (CD) spectra of both proteins under native conditions show a minimum at 217 nm, a typical property of proteins with high β sheet content and consistent with the expected immunoglobulin-like fold (Fig. 2A). In addition a maximum at 230 nm is observed. In the presence of 6 M guanidinium chloride (GdmCl), both proteins appear completely unfolded. FimFF<sub>F</sub> and FimGF<sub>F</sub> contain both one tryptophan residue each dominating their fluorescence spectra (Fig. 2B). Upon unfolding in 6 M GdmCl, a blue shift from 340 to 350 nm and a decrease in fluorescence intensity is observed. The CD and fluorescence spectra indicate that both FimFF<sub>F</sub> and FimGF<sub>F</sub> have clearly defined secondary and tertiary structures. Wild-type subunits have an incomplete immunoglobulin fold and a small free energy of folding (Vetsch et al., 2002). In FimFF<sub>F</sub> and FimGF<sub>F</sub>, however, the fold is complemented intramolecularly, which could lead to a significant stabilization of the native state. To test this hypothesis, we recorded GdmCl-induced folding transitions by following changes in intrinsic tryptophan fluorescence. For both proteins a hysteresis separates the data
RESULTS  Intrinsic affinities of pilus subunits

Intrinsic affinities of pilus subunits for unfolding and refolding. Not even after six days of incubation at 25 °C equilibrium is reached (Fig 2C). As a quantitative analysis of the data would require an equilibrium between unfolding and refolding reaction, only a qualitative description is possible based on our data. We estimate that the midpoints of transition are close to 2 and 3 M GdmCl for FimF₆ and FimG₆, respectively, once the unfolding and refolding reactions have reached equilibrium. So both proteins appear several times more stable than their wild-type counterparts that have transition midpoints below 0.8 M GdmCl (M. V. unpublished data).

Formation of subunit-subunit complexes. The crucial step in pilus assembly is the dissociation of a chaperone-subunit complex and the formation of a new subunit-subunit contact (Fig. 3A). A comprehensive series of experiments was carried out that mimic this key event in pilus formation. Chaperone-bound acceptors (FimC·FimF₆, FimC·FimG₆, FimC·FimH) were incubated with equimolar amounts of donors (FimF₆, FimG₆) in all six possible combinations. We expected that some acceptors might dissociate from the chaperone and instead bind to the donors as shown schematically in Fig. 3B. The mixtures were incubated during 4 to 100 hours to allow relaxation towards the binding equilibria and then applied to gel filtration. Due to their size, complexes elute at a lower volume than their individual components. This is illustrated in Fig. 3C where the elution profiles of all educts and additionally of FimC, one of the potential products, are shown. The FimC-subunit complexes all elute at a lower volume (peak maxima between 10.5 and 11.0 ml) than monomeric FimC (peak maximum at 11.7 ml). The eluate of the gel filtration column was collected in fractions of 500 µl and their protein content was analyzed by SDS-PAGE (Fig. 4). We quantified densitometrically what fraction of acceptor was in the newly formed donor-acceptor complex and what fraction had remained in the chaperone-acceptor complex. A summary of the data is shown in Fig. 5 where the fractions of donors that have replaced the chaperone FimC from the chaperone-acceptor complexes are shown. It is evident that in most cases the donors (FimF₆ and FimG₆) indeed replace the chaperone FimC leading to the formation of various subunit-subunit complexes. Only FimG₆ did not replace FimC from the FimC·FimF₆ complex (Fig. 5A, triangles). All acceptors seem to have a preference for one of the two donors. FimF₆ forms only a complex with FimF₆, FimG₆ preferentially binds to FimF₆, while FimH associates more readily with FimG₆. Strikingly, all observed reactions are very slow. Single exponential fits of the data
RESULTS

Intrinsic affinities of pilus subunits reveal apparent time constants between 20 h (FimC·FimGt + FimF) and 77 h (FimC·FimFt + FimF).

Influence of outer membrane on the formation of subunit-subunit complexes. The slow formation of subunit-subunit interactions that we observed in vitro is in sharp contrast with pilus assembly in vivo that takes place on a timescale of minutes (Striker et al., 1994) arguing for a catalysis of subunit-subunit association in vivo. In order to search for the potential catalyst we prepared outer membrane from E. coli expressing high amounts of type 1 pili. To confirm the identity of the outer membrane preparation, it was analysed by SDS-PAGE. The two major bands had apparent masses of approximately 38 and 40 kDa. Protein sequencing revealed that they represented OmpA and OmpC, respectively. In addition the strongest band in the range of 50-200 kDa was sequenced and identified as FimD. The N-terminus of FimD was Asp-Lys-Tyr-Phe-Asn-Pro, supporting the result of (Nishiyama et al., 2003) who described that FimD possesses an unusual long signal sequence of 45 amino acids. The outer membrane was incubated with 20 μM FimC·FimGt and 20 μM FimF. After 3 hours of incubation at 25 °C the sample was mixed with SDS buffer and subjected to SDS PAGE without prior heating (Fig. 6, lane 6). While subunit-subunit complexes in general remain intact when incubated at 25 °C in SDS, they dissociate upon incubation at 95 °C (e.g. (Saulino et al., 2000)). Polyacrylamide gels with samples that have not been boiled thus permit a direct analysis of subunit-subunit complex formation. A comparison of the reaction of FimC·FimGt and FimF in the absence and the presence of outer membrane (Fig. 6, lane 5) reveals that the outer membrane containing FimD promoted an accelerated formation of FimF·FimGt. A control with purified FimF·FimGt shows that this complex is resistant towards dissociation in 2 %(w/v) SDS at 25 °C as anticipated.
Discussion

Many pathogenic bacteria express adhesive surface organelles that mediate targeting to the sites of infection. Over 30 of these fibers are formed via the conserved chaperone-usher pathway (Sauer et al., 2000). These organelles consist exclusively of protein-subunits. The key event in fiber assembly is the dissociation of these subunits from the periplasmic pilus chaperone and their incorporation into the growing pilus at the outer membrane. We developed an in vitro system that allows the controlled formation of subunit-subunit complexes and thus established the basis for a quantitative thermodynamic and kinetic description of the process of subunit oligomerization.

As model proteins we choose the subunits FimF, FimG, and FimH from the tip fibrillum of type 1 pili of E. coli. Both FimF and FimG have a donor strand and, in addition, a groove that can accommodate the donor strand of another protein. This explains their ability to self-polymerize which leads to heterogeneous populations of oligomers whenever FimF or FimG are involved. In order to prevent self-polymerization, we modified FimF and FimG such that they can only act as acceptors (FimF′, FimG′, Fig. 1B) or only as donors (FimF and FimG, Fig. 1B). The donors possess an additional C-terminal donor strand that is expected to complement the immunoglobulin-like fold intramolecularly and thus to increase the stability of the otherwise incomplete fold. This is indeed the case as shown by denaturant dependent folding transitions (Fig. 2C). The hysteresis between the unfolding and the refolding reaction hints at a high kinetic barrier between the native and the unfolded state of self-complemented subunits. Donor strands thus not only increase the thermodynamic stability of the immunoglobulin-like fold, but also the kinetic stability against unfolding. This observation is of important biological relevance because unfolding of a single subunit would lead to a break of the pilus and as a consequence to a loss of function of the entire pilus because the adhesive subunit FimH at the tip of the pilus and the bacterial cell were separated. Kinetic stability as a mechanism for longevity has also been observed for extracellular proteases that, like pili, experience a harsh environment (e.g. (Cunningham et al., 1999). Furthermore, FimF and FimG, in contrast to wild-type FimF and FimG, do not form any homooligomers (Fig. 3C), confirming the prediction that the additional donor strand indeed folds into the hydrophobic groove that within the oligomeric pilus is occupied with the donor strand of the adjacent subunit.
RESULTS Intrinsic affinities of pilus subunits

In an extensive series of experiments, we tested whether a certain donor (Fim$_F$, Fim$_G$) can replace the chaperone(FimC) from different chaperone-acceptor complexes (FimC·Fim$_F$, FimC·Fim$_G$, FimC·FimH). For 5 out of the 6 tested combinations, this was indeed the case, indicating that, in general, chaperone-subunit complexes are thermodynamically less stable than subunit-subunit complexes (Fig. 5A, B, C). This result substantiates the hypothesis of Zavialov and colleagues (2003) who proposed that the replacement of the chaperone from a chaperone-subunit complex by another subunit might be thermodynamically favourable and that this gain in free energy might be the driving force for pilus assembly.

FimH is located exclusively at the tip of type 1 pili as shown by in situ immuno-electron microscopy experiments (Hahn et al., 2002). The role of FimH as a cap of the oligomeric pilus structure is also supported by the observation that FimH lacks the N-terminal donor strand that all other subunits possess. We found that FimH binds preferentially to FimG (Fig. 5C), while FimG prefers FimF as donor (Fig. 5B), and FimF accepts only FimF as donor (Fig. 5A). The same order (FimH-FimG-FimF) has been described for pili assembled in vivo (Hahn et al., 2002). Thus the intrinsic affinities of the subunits for each other not only constitute the driving force for their assembly but in addition also determine their sequential order in the final pilus structure. Our data further shows that the subunits in general have overlapping stereospecificity. FimH for instance forms a stable complex not only with Fim$_G$ but also Fim$_F$ (Fig. 5C). This allows an alternative order and explains why bacteria with a lesion in the fim$_F$ or the fim$_G$ gene still assemble adhesive pili (Russell and Orndorff, 1992).

Interestingly, the Fim$_G$·Fim$_F$ complex is only formed in very low amounts, indicating that the FimC·Fim$_F$ complex is much more stable (Fig. 5A). So once FimF is part of the growing pilus, no FimG will be incorporated anymore. Because neither Fim$_F$ nor Fim$_G$ is able to efficiently replace the chaperone from the chaperone-Fim$_F$ complex (Fig. 5A), it is likely that FimA is the only subunit that acts as a high affinity donor towards FimF. These observations argue for a role of FimF as an adapter subunit linking the flexible tip fibrillum to the rigid helical pilus rod that is exclusively composed of FimA.

Although subunit-subunit complexes are more stable than chaperone-subunit complexes, they form very slowly. Under our conditions (20 µM of each protein, 25 °C, pH 8), none of the reactions seems to have reached equilibrium even after 100 hours of incubation. It is likely that the reactions are faster at e.g. 37 °C but in any case, a large kinetic barrier apparently
RESULTS

Intrinsic affinities of pilus subunits

prevents the spontaneous establishment of subunit-subunit complexes. Such a barrier constitutes an elegant mechanism that inhibits premature unproductive pilus assembly in the bacterial periplasm. Productive assembly, however, must be catalyzed as entire pili consisting of several hundred of subunits assemble within minutes (Striker et al., 1994). The most obvious candidates for catalysts are the outer membrane ushers, which bind the chaperone-subunit complexes, transport the subunits through the membrane and anchor the pili to the cell surface. To test this tempting idea, we analyzed the reaction leading from chaperone-bound FimGt to FimFt-bound FimGt in the presence of outer membrane containing the outer membrane usher FimD. Under our experimental conditions the formation of the subunit-subunit complex (FimFt·FimGt) was indeed accelerated in the presence of FimD (Fig. 6). Although we cannot rule out the possibility that not FimD but a different protein or even a non-protein component of the outer membrane preparation caused the observed acceleration, our finding supports the hypothesis that FimD has catalytic activity promoting the establishment of subunit-subunit interactions. It, however, contradicts earlier results suggesting that assembly of subunits requires an entirely intact outer membrane (Jacob-Dubuisson et al., 1994). But even in the presence of FimD, the reaction appears slow and is not completed within 3 hours of incubation. A reason for the apparently slow kinetics even in the presence of the usher FimD could be that FimD suffers from potent product inhibition. Possibly the newly formed FimFt·FimGt complex has a higher affinity for the usher than the FimC·FimGt complex and thus blocks or at least slows down further catalysis because the usher is only present in catalytic amounts relative to FimC·FimGt and FimFt. Our experiment is the first step towards an understanding of the role of the outer membrane usher in the establishment of subunit-subunit interactions and further experiments addressing this question will yield important insights into the key event of pilus assembly. The data on the uncatalyzed formation of subunit-subunit contacts that we provide here is the basis for such work.
Materials and Methods

Construction of expression plasmids. pfimH-fimChis: To facilitate purification a sequence coding for a hexahistidine tag was added to the 3’ end of fimC in the plasmid pFimH-FimC (Pellecchia et al., 1999) by site directed mutagenesis (QuickChange, Stratagene, CA, USA).

pfimFt-fimChis and pfimGt-fimChis: The genes encoding FimFt and FimGt were constructed by PCR from genomic DNA of E. coli W3110 as template with a 5’ primer that replaced the first 35 codons of fimF or fimG with the DsbA signal sequence. With these PCR products, the gene encoding the C-terminal domain of FimH in plasmid pCT-FimH-FimC (Vetsch et al., 2002) was substituted via Xba I and Hind III, leading to plasmids pfimFt-fimC and pfimGt-fimC. Subsequently, a sequence coding for a hexahistidine tag was fused to the 3’ end of fimC by site directed mutagenesis (QuickChange). The plasmid pfimFt-fimChis allows coexpression of FimFt and FimC from a dicistronic operon under control of the trc promoter. FimFt is a truncated variant of FimF, lacking the 14 N-terminal residues, which correspond to the putative donor strand of FimF. The DsbA signal sequence is used to direct FimFt to the periplasm where it can bind to coexpressed FimC. FimGt is expressed from the plasmid pfimGt-fimChis analogously to FimFt.

pfimFF-cyt and pfimGF-cyt: PCR was used to generate a DNA fragment encoding fimFF (or fimGF), from genomic DNA of E. coli W3110 as template, with a 5’ primer that led to the removal of the signal sequence of fimF (or fimG) and a 3’ primer that joined a short linker and the donor strand sequence of fimF to the 3’ end of the fimF (or fimG) gene. The PCR product was ligated into the Nde I and BamH I restriction sites of pET-11a. The resulting plasmids pfimFF-cyt and pfimGF-cyt allow expression of self-complemented variants of FimF and FimG, respectively, in the E. coli cytoplasm.

Expression and purification of FimC·FimFt, FimC·FimGt, FimC·FimH, FimFF, and FimGF. Ni-NTA Superflow was obtained from Quiagen (Hilden, Germany), Phenyl Sepharose™ High Performance, Mono Q, Resource S, and Superdex 75 columns were from Amersham Biosciences (Buckinghamshire, UK). QA52 was purchased from Whatman (Maidstone, UK). All chromatographic steps were carried out at 4 °C.
RESULTS

Intrinsic affinities of pilus subunits

The chaperone-subunit complexes FimC·FimFt, FimC·FimGt, and FimC·FimH were obtained by coexpressing the subunits FimFt, FimGt, or FimH with FimC in E. coli HM 125 (Meerman and Georgiou, 1994) harbouring the plasmids pfimFt-fimChis, pfimGt-fimChis, or pfimH-fimChis, respectively. The cells were grown at 30 °C in 2YT medium containing 100 µg ml⁻¹ ampicillin. When an optical density at 600 nm (OD₆₀₀) of 0.7 was reached, IPTG was added to a final concentration of 1 mM. After further growth for 4 hours, the bacteria were harvested by centrifugation. The pellet was suspended in cold 50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, 1 mg/ml polymyxin B sulfate, pH 7.5 and stirred at 4 °C for two hours. After centrifugation, the supernatant was dialyzed against 50 mM NaPi, 300 mM NaCl, pH 8.0 and applied to a Ni-NTA superf flows column. Chaperone-subunit complexes were eluted with a gradient from 20 to 300 mM imidazole, dialyzed against 10 mM Mops/NaOH, pH 6.8 and loaded onto a Resource S column. Pure complexes eluted in a gradient from 0 to 200 mM NaCl. The protein was dialyzed against 20 mM Tris/HCl, pH 8.0 and stored at −80 °C. The yields per liter bacterial culture were 8, 2, and 20 mg for FimC·FimFt, FimC·FimGt, and FimC·FimH, respectively.

For expression of FimFF and FimGF, E. coli Tuner (DE3) cells transformed with pfimFF-cyt or fimGF-cyt were grown at 37 °C (FimFF) or 30 °C (FimGF) in LB medium containing 100 µg ml⁻¹ ampicillin. When an OD₆₀₀ of 0.8 was reached, IPTG was added to a final concentration of 1 mM and the cells were grown for another 5 hours. The cells were harvested by centrifugation and lysed by three passages trough a EmulsiFlex C5 Homogenizer. After removal of insoluble material by centrifugation, the cell extracts were dialyzed against 20 mM Tris/HCl, 0.1 mM EDTA, pH 8.0. The solution containing FimFF was applied to a QA52 column and protein was eluted in a linear gradient from 0 to 400 mM NaCl. Ammonium sulfate was added to the fractions containing FimFF to a final concentration of 1.4 M. This solution was loaded onto a Phenyl Sepharose column equilibrated in 20 mM Bis-Tris/HCl, 1 mM EDTA, 1.4 M (NH₄)₂SO₄, pH 6.5. FimFF was eluted in a gradient from 1.4 M to 0 M (NH₄)₂SO₄, dialyzed against 10 mM Tris/HCl, pH 8.0 and applied to a Mono Q column. Pure FimFF was eluted with a gradient from 0 to 120 mM NaCl, dialyzed against 5 mM Tris/HCl, pH 8.0 and stored at −80 °C. Using the Ellman assay (Ellman, 1959), we found that after purification, the two cysteines of FimFF had formed a disulfide bridge in 97 % of the molecules. The mass of isolated FimFF measured by MALDI-TOF (18218 Da) is in good
agreement with the calculated value (18217 Da). Per 1 litre of bacterial culture, 6 mg FimFF were obtained. The purification protocol of FimGF was identical to the protocol for FimFF with the exception that 1.2 M (NH₄)₂SO₄ (instead of 1.4 M) was used for hydrophobic interaction chromatography. The two cysteines of FimGF had formed a disulfide bridge in 90 % of the molecules. The identity of the purified protein was confirmed by N-terminal sequencing and MALDI-MS (calculated mass: 16904 Da; measured mass: 16905 Da). The overall yield was 16 mg FimGF per litre of bacterial culture.

To obtain FimFF·FimGt complex, FimC·FimGt was incubated with a threefold molar excess of FimFF in 20 mM Tris/HCl, pH 8.0 during 24 h at 25 °C. Under these conditions, 50% of FimC is replaced from FimGt by FimFF. FimFF·FimGt was purified by Ni-NTA as described above and gel filtration with a Superdex 75 column equilibrated in PBS.

**Preparation of E. coli outer membrane.** *E. coli* AAEC 189 transformed with pSH2 (Orndorff and Falkow, 1984) were grown at 37 °C for 14 h with shaking (160 rpm) to an OD₆₀₀ of 2.4. The bacterial cells were harvested by centrifugation (3000 g, 12 min, 4 °C), washed once with 20 mM Tris/HCl, 1 mM EDTA, pH 8.0, resuspended in the same buffer and disrupted as described above. Cellular debris was removed by centrifugation (12000 g, 10 min, 4 °C). The supernatant containing the total membrane fraction was sedimented by an additional centrifugation step (180000 g, 1 h, 4 °C). The pellet was rinsed with 20 mM Tris/HCl, 1 mM EDTA, pH 8.0 and resuspended by 20 strokes with a dounce homogenizer (Fischer, Schwerte, Germany) in 20 mM Tris/HCl, 1 mM EDTA, 1 % (w/v) Sarkosyl, pH 8.0 and rocking for 1 h at 4°C in order to solubilize the inner membrane. The suspension was subsequently subjected to centrifugation (100000 g, 1 h, 4 °C) and washed once in 20 mM Tris/HCl, 1 mM EDTA, pH 8.0. The resulting pellet contained essentially only outer membrane components and was resuspended in 20 mM Tris/HCl, 1 mM EDTA, pH 8.0 as described above. The yield for FimD was estimated from Coomassie-stained SDS polyacrylamide gels to be approximately 60 µg per litre of bacterial culture. Samples of the outer membrane preparation were stored at −80°C.

**Protein concentrations.** Protein concentrations were measured by absorbance at 280 nm as described (Gill and von Hippel, 1989). Molar extinction coefficients of 32370 M⁻¹ cm⁻¹,
RESULTS  
Intrinsic affinities of pilus subunits

36210 M$^{-1}$ cm$^{-1}$, 56100 M$^{-1}$ cm$^{-1}$, 10806 M$^{-1}$ cm$^{-1}$, and 12550 M$^{-1}$ cm$^{-1}$ were used for FimC·FimF, FimC·FimG, FimC·FimH, FimF, and FimG, respectively.

Fluorescence and circular dichroism spectra. GdmCl (AA-grade) was obtained from NIGU (Waldkrainburg, Germany). All spectra were measured at 25 °C and corrected for the buffer, 20 mM Tris/HCl pH 8.0 containing different concentrations of GdmCl. Fluorescence spectra were recorded in a HITACHI F-4500 fluorescence spectrometer in 1.0 cm (emission path) x 0.2 cm (excitation path) quartz cuvettes. The excitation wavelength was 280 nm (5 nm band width). Emission spectra were recorded between 290 and 450 nm (10 nm band width) at a scan speed of 60 nm/min with 0.5 s response. Far-UV circular dichroism spectra were collected with a Jasco J-715 spectropolarimeter in 0.1 cm quartz cuvettes at a scan speed of 50 nm/min with 2 s response. 10 spectra were averaged and the signal was converted to mean residue ellipticity [$\Theta$]$_{MRW}$ according to (Schmid, 1989).

GdmCl-dependent folding transitions. To induce unfolding or refolding, protein either in 20 mM Tris/HCl, pH 8.0 or in 6 M GdmCl, 20 mM Tris/HCl, pH 8.0 were diluted 1:45 with 20 mM Tris/HCl, pH 8.0, containing different concentrations of GdmCl. The final protein concentration was 1.6 µM in all samples. After incubation at 25 °C for 20 hours or 6 days, transitions were recorded in a HITACHI F-4500 fluorescence spectrometer. Folding transitions were followed by exciting the samples at 280 nm and recording the fluorescence emission at 338 or 327 nm for FimF or FimG, respectively. The experimental data were analyzed based on the two-state model as described (Santoro and Bolen, 1988).

Analysis of subunit-subunit complex formation by gel filtration and SDS-PAGE. Different combinations of acceptors in complex with FimC (FimC·FimF, FimC·FimG, FimC·FimH) and donor components (FimF, FimG) were mixed at concentrations of 20 µM each. The samples were incubated in 20 mM Tris/HCl pH 8.0 at 25 °C for different time spans before being applied to a Superdex 75 HR 10/30 gel filtration column (Amersham Biotech, Buckinghamshire, UK). The column was equilibrated in PBS at a flow rate of 20 ml/h. Sample volumes of 200 µl were injected, the fraction volume was 500 µl. The protein composition of the collected fractions was analyzed by SDS-PAGE and further evaluated densitometrically to calculate the ratio of monomeric donor and donor in complex with acceptor. In a set of control experiments, donors alone (FimF, FimG or FimC) or
acceptors in complex with FimC alone (FimC·FimF, FimC·FimGt, or FimC·FimH) were also subjected to analytical gel filtration. Single elution peaks were observed for these preparations, confirming their oligomeric homogeneity.

**Analysis of the influence of outer membrane on subunit-subunit complex formation.** To follow subunit-subunit complex formation in the presence of outer membrane, FimC·FimGt complex and FimF and outer membrane containing FimD were mixed in 20 mM Tris/HCl, pH 8.0. In a control experiment, no outer membrane was added. The final concentration of FimC, FimGt, and FimF was 20 µM each. After 3 hours of incubation at 25 °C, the samples were mixed with sample buffer containing no reducing agent at 25 °C and applied to SDS PAGE in a cooled unit. The SDS concentration was 2 % (w/v) in the sample buffer and in the gel.
Acknowledgements

We thank Ulla Grauschopf for helpful advice, and René Brunisholz for protein sequencing.
References


RESULTS

Intrinsic affinities of pilus subunits


RESULTS

Intrinsic affinities of pilus subunits

Legends to figures

**Figure 1.** (A) Current model of type 1 pilus assembly in *E. coli*. The subunits FimA, FimF, FimG, and FimH are translocated by the Sec-machinery to the periplasm where DsbA introduces the conserved disulfide bond. The periplasmic chaperone FimC then binds the subunits and accelerates their folding. The chaperone-subunit complexes dissociate at the outer membrane where they are incorporated into the growing pilus, which is secreted through the outer membrane usher FimD. The final pilus structure consists of a rigid helical rod formed by FimA and a flexible tip fibrillum composed of FimF, FimG, and FimH. (B) Schematic presentation and primary sequences of the subunits and the chaperone used in this study. The subunits FimF and FimG were modified in two different ways in order to prevent self-oligomerization: On the one hand, their 14 N-terminal amino acids, corresponding to their donor strands, were truncated, yielding FimFt and FimGt. While these variants are unable to complement other subunits, they retain the ability to accept donor strands from the chaperone or other subunits. We refer to them as acceptors. On the other hand, FimF and FimG were self-complemented by adding the donor strand of FimF to their C-termini, yielding FimFF and FimGF. These variants can only act as donors. FimH is located at the tip of type 1 pili and lacks a donor strand. FimH is thus naturally only an acceptor. Instead of wild-type FimC, we used a variant with a C-terminal hexahistidine-tag to simplify purification. We refer to this variant as FimC throughout this study.

**Figure 2.** Spectroscopic and thermodynamic properties of the donor-subunits FimFF and FimGF at 25 °C and pH 8.0. (A) Far-UV CD spectra of 10 µM FimFF and FimGF in the absence of denaturant (solid line) or in the presence of 6 M GdmCl (dashed line). (B) Fluorescence spectra of 3 µM FimFF and FimGF in the absence of denaturant (solid line) or in the presence of 6 M GdmCl (dashed line). The excitation wavelength was 280 nm. (C) Denaturant-dependent unfolding (closed symbols) and refolding (open symbols) transitions at 25 °C and pH 8.0. Transitions of 1.6 µM protein were recorded after 20 h (circles) and 6 d (triangles) of incubation. The excitation wavelength was 280 nm, emission was measured at 338 (FimFF) or 327 nm (FimGF). The original data were normalized. The solid lines are only meant to guide the eye of the reader.

**Figure 3.** Schematic presentation of the elementary reaction in pilus assembly and a model system for a quantitative *in vitro* analysis of this reaction. (A) Incorporation of subunits into
the growing pilus is the key event of pilus formation and proceeds in the cell as follows: Subunit n that was last incorporated into the pilus remains bound to the chaperone. After binding of another chaperone-subunit complex to the usher, the chaperone bound to subunit n is replaced by subunit n+1. (B) In order to investigate the formation of new subunit-subunit contacts in detail, an experimental setup was designed that mimicks this key event of pilus assembly. Complexes between the chaperone FimC and subunits that are restricted to acceptor function (FimC·FimFt, FimC·FimGt, and FimC·FimH) were incubated with donor subunits (FimFF and FimGF). If the affinity of a certain acceptor subunit is higher for a donor subunit than for the chaperone, the schematically shown reaction takes place and subunit-subunit complexes form. (C) Retention volumes of different proteins and protein complexes subjected to gel filtration. The solid lines are the profiles of the chaperone-acceptor complexes (FimC·FimFt, FimC·FimGt, and FimC·FimH) and of the donors (FimFF and FimGF) that were the educts of the reactions described in Fig. 4. The dashed line shows the profile of monomeric FimC.

**Figure 4.** Time-resolved analysis of the replacement of the chaperone FimC from chaperone-acceptor complexes at 25 °C and pH 8.0. The chaperone-acceptor complexes FimC·FimFt, FimC·FimGt, and FimC·FimH were incubated together with the donors FimFF and FimGF in all 6 possible combinations. The final concentrations of all proteins were 20 µM. The reaction mixture was applied to gel filtration after 4, 24, and 100 hours. The eluate was fractionated and the protein composition of the individual fractions was evaluated by SDS-PAGE. The Coomassie-stained SDS polyacrylamide gels are shown. On top of each series of gels, the elution volume (10-15 ml) of the corresponding fractions is indicated.

**Figure 5.** Formation of subunit-subunit complexes as a function of time. The gels shown in Fig. 4 were densitometrically evaluated and it was calculated which fraction of the acceptors FimFt (A), FimGt (B), or FimH (C) was in complex with the donor FimFF (circles) or FimGF (triangles) and which fraction remained bound to the chaperone FimC. The estimated error in the calculation is ±10 % for the data points at 4 and 24 h and 20 % for the data points at 100 h, where in many cases degradation was observed (cf. Fig. 4). The solid lines represent single exponential fits of the data.
**Figure 6.** Influence of an outer membrane preparation on the formation of the FimF-FimG complex. Coomassie blue-stained SDS polyacrylamide gel (17% (w/v)) with samples that were not boiled before electrophoresis. Lane 1: FimC-FimG; lane 2: FimF; lane 3: outer membrane preparation; lane 4: FimF-FimG; lane 5: FimC-FimG was incubated together with FimF at 25 °C for 3 hours; lane 6: same as in lane 5 but additionally outer membrane preparation was included. The arrowheads indicate the positions of marker proteins.
RESULTS

Intrinsic affinities of pilus subunits

Figure 1

A

B

FimF_t

FimF_F

FimG_t

FimG_F

FimH

FimC

Figure 1
RESULTS

Intrinsic affinities of pilus subunits

Figure 2
RESULTS
Intrinsic affinities of pilus subunits

Figure 3

A

B

C

Absorbance at 226 nm

Retention volume (ml)

Figure 3
RESULTS

Intrinsic affinities of pilus subunits

Figure 4
RESULTS

Intrinsic affinities of pilus subunits

Figure 5

A

Fraction of FimF in complex with FimF or FimG

Time (h)

B

Fraction of FimG in complex with FimF or FimG

Time (h)

C

Fraction of FimH in complex with FimF or FimG

Time (h)

Figure 5
RESULTS

Intrinsic affinities of pilus subunits

Figure 6

97 kDa
66 kDa
45 kDa
31 kDa
22 kDa
14 kDa

1  2  3  4  5  6

FimD
FimF_F·FimG_{lt}
FimC
FimF_F
FimG_{lt}

Figure 6
3. ADDITIONAL ARTICLE

Identification and characterization of the chaperone-subunit complex-binding domain from the type 1 pilus assembly platform FimD


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

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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 (FimD⁶) comprising the N-terminal 139 residues of FimD. Purified FimD⁶ is a monomeric, soluble protein that specifically recognizes complexes between FimC and individual type 1 pilus subunits, but does not bind the isolated chaperone, or isolated subunits. In addition, FimD⁶ 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 FimD⁶ 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 FimD⁶ and its ternary complexes with FimC and individual pilus subunits opens the avenue to structural characterization of critical type 1 pilus assembly intermediates.

Keywords: assembly platform; chaperone/usher pathway; Escherichia coli; FimD; type 1 pili

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 bind specifically 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 pili consists of a right-handed helical rod that is composed of the main structural subunit FimA, and a short tip fibril joined to the rod. The tip fibrillum is believed to be a linear array of three 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 single-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 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 non-specific 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. FimD consists of 833 residues and shares 20–30% sequence identity with the known bacterial pilus assembly platforms. Data on the related P pilus assembly platform PapC indicate that assembly platforms are ring-shaped oligomers of six to 12 subunits forming a central pore in the outer membrane. 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. Moreover, FimD anchors the pilus to the outer E. coli membrane, and specifically recognizes FimC-subunit complexes with different affinities. 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. 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 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, 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 1(A)). 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.

Figure 1 Predicted domain topology of FimD. (A) A drawing of the predicted transmembrane topology of FimD based on a model for FaeD. 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. CLUSTAL W was used to align the sequences.
that interacts exclusively with chaperone-subunit complexes in vitro. Furthermore, we show that overexpression of soluble FimD\textsubscript{N} in the periplasm of E. coli wild-type cells competitively diminishes incorporation of FimH at the tip of type I 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 1(A) 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{20} 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. On the basis of 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 dshB 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 residue 126, 134 or 139 of the mature FimD protein (Figure 1(B)), were obtained in large quantities as soluble proteins in the periplasm. To ensure that no functionally important residue of the N-terminal FimD domain is missing, we focussed on the longest, expressed construct (C terminus at residue 139) and termed this fragment FimD\textsubscript{N}. FimD\textsubscript{N} 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 A\textsubscript{oo} (Figure 2(A), inset). Edman sequencing and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry showed that FimD\textsubscript{N} has an unusually long signal sequence of 45 residues. Cleavage of the signal sequence after residue 45, however, is in agreement with the consensus sequence of E. coli signal peptidase.\textsuperscript{21} On the basis of this result, we

**Figure 2.** FimD\textsubscript{N} is a structured monomer. (A) Sedimentation equilibrium analysis of FimD\textsubscript{N} at pH 8.0 and 20 °C. Fitting of the data yielded a monodisperse system with an apparent molecular mass of 13,020(±182) Da (continuous line). The lower panel shows the residual error between the fitted and observed values. Inset: Coomassie-stained SDS/polyacrylamide gel showing the enrichment of FimD\textsubscript{N} during purification. Lane S, molecular mass standard; lane 1, total periplasmic extract of induced cells; lane 2, pooled fractions after chromatography on QAS2; lane 3, pooled fractions after chromatography on phenyl Sepharose; lane 4, purified FimD\textsubscript{N} eluted from HiPrep 16/10 Source 30Q. (B) Far-UV CD spectra of native (continuous lines) and unfolded (dotted lines) FimD\textsubscript{N} at pH 7.4 and 25 °C.
numbered the residues of FimD according to the sequence of the mature FimDₙ (Figure 1(A) and (B)).

**FimDₙ is a monomeric, autonomous folding unit**

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

We next investigated the oligomerization state of purified FimDₙ by analytical ultracentrifugation. Equilibrium sedimentation analysis at pH 8.0 and 20 °C (Figure 2(A)) yielded a monodisperse species with a molecular mass of 13,020(±182) Da, which is in good agreement with the calculated mass of 13,179 Da for the mature FimDₙ monomer. This suggests that the N-terminal domain of FimD alone is not sufficient to trigger association of

![Graph A](image1)

![Graph B](image2)

**Figure 3.** FimDₙ is a stable, autonomous folding unit with defined tertiary structure. (A) GdmCl-dependent folding transition of FimDₙ at pH 7.4 and 25 °C. Unfolding (filled circles) and refolding (open circles) were followed by far-UV CD at 218 nm. Original data were fit according to the two-state model and normalized (continuous line). FimDₙ shows a midpoint of transition at 2.3(±0.03) M GdmCl, an m-value of 14.3(±1.1) kJ mol⁻¹ M⁻¹ and a ΔG value of −33.0(±2.4) kJ mol⁻¹. (B) Temperature-dependence of the molar heat capacity of FimDₙ (40 μM) at pH 7.4 followed by differential scanning calorimetry. The experimentally observed data (squares) are nearly superimposable with the fit (continuous line) for the reversible denaturation of FimDₙ according to the two-state model, yielding a T_m of 66.8(±0.2) °C and ΔH_m of 254.5(±3.0) kJ mol⁻¹. The broken line represents the repeated heating scan of the same sample after rapid cooling. The dotted line represents the intrinsic heat capacity change. (C) One-dimensional 600 MHz ¹H NMR spectrum of FimDₙ 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. FimD₆ (20 μM) was incubated with equimolar amounts of the respective proteins for one hour prior to loading onto the column. (A) Binding of FimD₆ to the FimC–FimH₆ complex. Continuous line, FimD₆ incubated together with FimC–FimH₆; broken line, FimC–FimH₆; dotted line, FimD₆. Inset: SDS-PAGE analysis of FimD₆ (lane 1), FimC–FimH₆ (lane 2) and the peak fraction (10.5 ml elution volume) collected from the run with the FimD₆–FimC–FimH₆ ternary complex. (B) FimD₆ does not bind the isolated FimC or the isolated FimH₆. Continuous lines, FimD₆ incubated together with FimC and FimH₆, respectively; broken lines, FimC and FimH₆, respectively; dotted line, FimD₆. (C) Binding of FimD₆ to the FimC–FimF₆ complex. Continuous line, FimD₆ incubated together with FimC–FimF₆; broken line, FimC–FimF₆; dotted line, FimD₆. Identical results were obtained when full-length FimF was used instead of FimF₆. (D) Monomeric FimD₆ does not bind the FimC–FimG₆ complex in vitro. Continuous line, FimD₆ incubated together with FimC–FimG₆; broken line, FimC–FimG₆; dotted line, FimD₆. Identical results were obtained when full-length FimG was used instead of FimG₆.

FimD subunits to ring-shaped oligomers of full-length FimD in the outer membrane.

To test whether purified FimD₆ represents a cooperative folding unit with intact tertiary structure, denaturant- and heat-induced unfolding transitions were recorded (Figure 3(A) and (B)). Unfolding and refolding of FimD₆ in GdmCl, followed by far-UV CD at 218 nm, yielded cooperative, fully reversible transitions (Figure 3(A)). 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 (n-value) of 14.3(±1.1) kJ mol⁻¹ M⁻¹ is in good agreement with the n-value expected for a 139 residue protein (13.9 kJ mol⁻¹ M⁻¹).22 Thermal unfolding of FimD₆ was followed by differential scanning calorimetry and proved to be reversible, as two successive scans of the same sample were almost superimposable (Figure 3(B)). 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 (ΔH_m) of 254.5 ± 3.0 kJ mol⁻¹.
ADDITIONAL ARTICLE

Chaperone-subunit Complex Binding Domain of FimD

(Figure 3(B)). The van’t Hoff enthalpy ($\Delta H_{\text{vH}}$) 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 $\Delta H_{\text{vH}} / \Delta H_{\text{cal}}$ of 0.96 is in good agreement with a two-state thermal unfolding process of FimD$_H$.$^{23}$ Overall, these data clearly demonstrate that FimD$_H$ 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$_H$ (Figure 3(C)).

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.$^{29}$ In addition to full-length FimH, we used its isolated pilin domain, FimH$_C$, because FimH$_C$ is the only part of FimH that interacts with FimC, has the same overall fold as all other type 1 pilus subunits,$^{27}$ and a lower tendency than the full-length protein to aggregate non-specifically.$^{34}$ 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 4(A) 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 4(B)). 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 4(A), 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). Titrations data revealed a 1:1 stoichiometry of binding and a dissociation constant ($K_d$) of 1.2 μM (Figure 5; Table 1). Moreover, the thermodynamic parameters show that binding of FimD$_N$ to the FimC–FimH$_C$ complex is enthalpy-driven ($\Delta H = -50.2(\pm 11.1)$ kJ mol$^{-1}$) and entropically unfavorable ($\Delta S = -57.0$ J K$^{-1}$ mol$^{-1}$) (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.

![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 continuous line represents the best non-linear, least-squares fit of the data with a 1:1 stoichiometry of binding.

<table>
<thead>
<tr>
<th>Chaperone-subunit complex</th>
<th>$K_d$ (10$^{-6}$ M)</th>
<th>$\Delta H$ (kJ mol$^{-1}$)</th>
<th>$\Delta G$ (kJ mol$^{-1}$)</th>
<th>$\Delta S$ (J mol$^{-1}$ K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FimC–FimH$_C$</td>
<td>1.23 ± 0.07</td>
<td>-50.2 ± 1.2</td>
<td>-33.7 ± 0.1</td>
<td>-57.0</td>
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<tr>
<td>FimC–FimH</td>
<td>0.96 ± 0.04</td>
<td>-52.0 ± 1.0</td>
<td>-34.3 ± 0.1</td>
<td>-59.4</td>
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<tr>
<td>FimC–Fim$_N$</td>
<td>3.35 ± 0.23</td>
<td>-50.8 ± 1.0</td>
<td>-31.3 ± 0.2</td>
<td>-65.4</td>
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with complexes between FimC and the subunit FimF, FimG or FimA. Besides full-length FimF and FimG, we used the variants FimF<sub>i</sub> and FimG<sub>i</sub>, 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. 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<sub>i</sub> in the ternary complex. In gel-filtration experiments and subsequent calorimetric titrations, we observed selective recognition of the FimC–FimF<sub>i</sub> complex by FimD<sub>i</sub> with slightly weaker binding (K<sub>D</sub> = 3.4 μM) compared to FimC–FimH<sub>i</sub>, but otherwise very similar, enthalpy-driven binding characteristics (Figure 4(C); Table 1). In contrast to the FimC–FimH and FimC–FimF<sub>i</sub> complexes, we could not detect binding of FimD<sub>i</sub> to the FimC–FimG<sub>i</sub> or the FimC–FimA complex in gel-filtration experiments that were performed under identical conditions (see Figure 4(D) for the experiment with FimC–FimG<sub>i</sub>). Thus, a potential affinity of FimD<sub>i</sub> for the FimC–FimG<sub>i</sub> or FimC–FimA complexes, must be at least tenfold to 100-fold lower than that observed for the FimC–FimH and FimC–FimF<sub>i</sub> complexes.

Expression of FimD<sub>i</sub> competitively diminishes incorporation of FimH at the tip of type 1 pili

As FimD<sub>i</sub> formed soluble, ternary complexes with FimC and the adhesin FimH, we reasoned that overexpression of soluble FimD<sub>i</sub> 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<sub>i</sub> expression plasmid for their ability to agglutinate with yeast cells when grown in the presence and in the 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 6(A) shows that agglutination of E. coli W3110 with yeast was diminished significantly when FimD<sub>i</sub> expression was induced by IPTG, while a wild-type-like agglutination phenotype

Figure 6. Overexpression of FimD<sub>i</sub> 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<sub>i</sub> were mixed with yeast cells. Cells producing FimD<sub>i</sub> 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<sub>i</sub> (a), and non-induced W3110/pFimD<sub>i</sub> (b). (C) Immunoblot analysis of the subunit composition of pilus preparations from different bacterial strains. Identical amounts of pilus preparations were boiled for five minutes 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.
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 6(A)). 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 FimDα had no influence on the phenotype of pilated W3110 cells with regard to the overall number of pili per cell and the average length distribution of type 1 pili (Figure 6(B)). This suggests that overexpression of FimDα 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 FimDα on the relative distributions of the four 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/ pFimDα cells were purified by precipitation with magnesium ions26 and analyzed for their content of individual subunits by immunoblotting after separation of the subunits by SDS-PAGE. Figure 6(C) shows that incorporation of FimH into type 1 pili is indeed decreased about threefold upon overexpression of FimDα in the periplasm, while FimDα had no significant effect on the incorporation or relative distribution of FimF and FimA. However, even though we could not detect binding of FimDα to FimC−FimG complexes in vitro, we found that the amount of FimG was decreased in pili isolated from bacteria overexpressing FimDα (Figure 6(C)), indicating that FimH favors incorporation of FimG into pili. This is supported by the reduced relative amount of FimG in pili isolated from the fimH deletion strain W3110ΔfimH (Figure 6(C)).

**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,21 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.22 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.21,22 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 7 pilus assembly platform PapC, which indicated that N-terminal regions of PapC are required for the recognition of chaperone-subunit complexes.27 Moreover, the selective recognition of chaperone-subunit complexes by FimDα, 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 FimDα domain binds the FimC−FimH complex with about 100-fold lower affinity (K_d = 1.2 μM compared with K_d = 9.1 nM).28 A plausible explanation for this fact would be the presence of multiple FimDα 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 FimDα. In addition, surface plasmon resonance generally may yield higher apparent affinities than binding experiments in solution due to local rebinding effects.29 Furthermore, C-terminal, periplasmically oriented regions of full-length FimD may additionally be involved in recognition of FimC-subunit complexes. However, we 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.29

In addition to the weaker binding of the FimC−FimH complex, there are further differences between our present binding data of FimDα to FimC-subunit complexes and surface plasmon resonance experiments obtained with the oligomer of full-length FimD.21 For example, Saulino et al. reported that FimC−FimF is bound with 100-fold lower affinity than FimC−FimH (K_d = 0.9 μM compared with K_d = 9.1 nM, respectively), while we found only a threefold lower affinity for FimC−FimF in the micromolar range (Table 1). Moreover, we could not detect binding of FimDα 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 µM and 0.2 µM, respectively. One possible explanation for this difference may again be the monomeric state of FimD$_{\alpha}$ 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$_{\alpha}$ 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 core (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$_{\alpha}$ recognizes the complexes FimC–FimF and FimC–FimG equally well indeed indicates that the donor strands of pilus subunits bound to FimC are not involved in binding to FimD$_{\alpha}$. 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$_{\alpha}$ 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 composed exclusively of FimA subunits, cannot be formed prior to assembly of the tip fibrillum.

As expected from the highest affinity of FimD$_{\alpha}$ for the FimC–FimH complex, overexpression of FimD$_{\alpha}$ in the periplasm reduced the incorporation of FimH at the tip of type 1 pili significantly. However, the number of pilus per cell and the average pilus length were not affected when FimH was partially missing. In contrast to previous reports, this result indicates clearly that initiation of type 1 pilus assembly is independent of FimH. Although we could not detect binding of FimD$_{\alpha}$ to the FimC–FimG complex in gel-filtration experiments, we observed decreased incorporation of FimG into the tip fibrillum when FimD$_{\alpha}$ was overexpressed in the periplasm of E. coli wild-type cells (Figure 6C). In addition, we 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.$^{1,2,3}$

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$_{\alpha}$, 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 HMI125 was used.$^{24}$ The *E. coli* K12 wild-type strain W3110$^{25}$ was used in the yeast agglutination assay. The fimH deletion strain W3110ΔfimH was a gift from P. Sebbel and constructed from W3110 by allelic exchange according to Hamilton.$^{26}$

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'-G GGG ATC TCG CATATG TCA TAT CGC AAT TTA AGC-3' was used as N-terminal primer for all constructs (NdeI site underlined). The following oligonucleotides were used as C-terminal primers: 5'-GGT CCA TCA ACC ACG ACG CCC ATT AC-3' for amplification of segment 1–126 of mature FimD; 5'-GGT GGAATTC TCA GTC CCA TAA CTC AGG-3' for amplification of segment 1–134 of mature FimD; and 5'-GGT CGATC TTA CCG ATT AAT 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 NdeI and BamHI into pDH6A,$^{23}$ The resulting expression plasmids allow periplasmic expression of the respective genes under control of the tac promoter. The expression plasmid encoding residues 1–139 of mature FimD was termed pFimD, 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'-GGT CCA TCA ACC ACG ACG CCC ATT AC-3' for amplification of segment 1–126 of mature FimD; 5'-GGT CCA TCA ACC ACG ACG CCC ATT AC-3' for amplification of segment 1–134 of mature FimD; and 5'-GGT CCA TCA ACC ACG ACG CCC ATT AC-3' for amplification of segment 1–139 of mature FimD (BamHI site underlined). Oligonucleotide 5'-GGT CCA TCA ACC ACG ACG CCC ATT AC-3' was used as C-terminal primer for all constructs (BamHI site underlined). The amplified fragments were cloned via NdeI and BamHI into pDH6A directly after the DhaA signal sequence.

For periplasmic coexpression of either FimG or FimF, with FimC, the fimH gene on pFimH–FimC$^{35}$ was replaced with either the fimG or fimF gene via the XhoI and HindIII restriction sites. The fimG and fimF genes
were amplified by PCR from the genome of W3110 using the following oligonucleotides: 5'-GG CAC GCA TCTAGA GGA GGG ATG ATT GTA ATG AAA AAG ATT TCG GGT GGC C-3' and 5'-GCA TCA CGT TCA ATA CCT ACA GCT AAC TCT AGT GCC ATG TCA CGG ACC GGA CC-3' for amplification of fimC; 5'-GG GAC GCA TCTAGA GGA GGG ATG ATT GTA ATG AAA AAG ATT TCG CTC CGC TTC GGT GTA ATG AAA AAG ATT TCG GGT GGC C-3' and 5'-GCA TCA CGT TCA ATA CCT ACA GCT AAC TCT AGT GCC ATG TCA CGG ACC GGA CC-3' for amplification of fimI, (XbaI and HindIII) sites underlined. In order to facilitate purification of the FimC-subunit complexes, a His-tag was introduced at the C terminus of coexpressed FimC through site-directed mutagenesis (QuickChange™, Stratagene). The resulting coexpression plasmids were termed pFimC-His-FimC-ini and pFimF-FimC-ini, respectively. In all constructions, the sequences of genes amplified by PCR or modified by site-directed mutagenesis were verified byideoxy sequencing.

Expression and purification of FimDn, and FimC-subunit complexes

E. coli HM1259 was transformed with either pFimDn, pFimC-ini-FimC-ini, or pFimF-FimC-ini. Cells were grown at 30°C in 2xYT medium containing ampicillin (100 µg/ml) until an absorbance at 600 nm (A600) of 0.7 was reached. Protein expression was induced with 1 mM IPTG. After further growth for four hours, cells were harvested by centrifugation, suspended in 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mg/ml of polymyxin B salt (10 µl/ml of culture), and stirred for two hours 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-Cl (pH 6.2), and applied to a QAE52 cellulose column (Whatman) equilibrated with the same buffer. FimDn was eluted with a linear gradient from 0–400 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–200 mM ammonium sulfate in 20 mM Bis-Tris-Cl (pH 6.2). Fractions containing FimDn were pooled, dialyzed against 20 mM Bis-Tris-Cl (pH 6.2) and loaded onto a HiPrep 16/10 Source 300 column (Pharmacia). Pure FimDn was eluted with a linear gradient from 0–400 mM NaCl. Correct N-terminal processing of FimDn was verified by Edman sequencing and MALDI-TOF mass spectrometry (calculated mass 15,179 Da; measured mass 15,182 Da).

For purification of the complexes FimC-ini-FimC-ini, and FimC-FimC-ini, the periplasmic extracts were dialyzed against 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, and applied to a Ni-NTA agarose column (Qagen) equilibrated with the same buffer. The column was washed with the above buffer containing 20 mM imidazole–HCl. The subunit–FimC-ini complexes were eluted with a linear gradient from 20 mM–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 5 column (Pharmacia) to remove excess FimC-ini. The 1:1 complexes were eluted with a linear gradient from 0 mM to 200 mM NaCl. The complex between FimC with either the pilin domain of FimH (FimHn–FimC) or full-length FimH (FimH–FimC) was prepared as described.23 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/liter of bacterial culture and A600.

FimA was purified from intact type 1 pili expressed by the E. coli strain AAEC189 which had been transformed with the plasmid pSH27 containing the complete fim gene cluster. AAEC189/pSH2 was grown at 37°C in LB medium containing chloramphenicol (100 µg/ml).

Cells were harvested 48 hours after inoculation in 20 mM sodium phosphate (pH 7.4), 150 mM NaCl. Pili were sheared off mechanically using a Dixa 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 one hour, 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 three days to disassociate 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 threefold 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 A600) was confirmed by SDS-PAGE and MALDI-TOF mass spectrometry (calculated mass of FimA 15,827 Da; measured mass 15,833 Da).

Protein concentrations

Protein concentrations were determined by measuring their absorbance at 280 nm.30 Molar extinction coefficients of 10,912; 31,750; 55,980; 36,210; 32,370; 26,680; 7740; 2680; and 2400 M-1 cm-1, were used for FimDn, FimHn–FimC, FimH–FimC, FimCn–FimCn, FimF–FimFm, FimA–FimC, FimHn, FimA, and FimC, respectively.

Analytical ultracentrifugation

Sedimentation equilibrium measurements were performed with a Beckman Optima XL-A analytical ultracentrifuge at 20,000 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.30

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 0.2 cm quartz cuvette. Ten spectra were accumulated, corrected for the buffer, and the CD signal was converted to molar mean residue ellipticity.40 One-dimensional 1H NMR spectrum of FimDn was recorded on a 600 MHz spectrometer (Bruker DRX 600) at 25°C.
ADDITIONAL ARTICLE

Chaperone-subunit Complex Binding Domain of FimD

The NMR sample (90% $^1$H$_2$O, 10% $^2$H$_2$O) contained FimD$_x$ 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 FimD$_x$, 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 hours. For measuring refolding transitions, FimD$_x$ was first denatured in 5 M GdmCl, 20 mM Hepes–NaOH (pH 7.4), 100 mM NaCl for one hour at room temperature. Unfolded FimD$_x$ was then diluted with the above buffer containing different concentrations of GdmCl, and incubated at 25 °C for 18 hours. 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 with a six-parameter fit, and normalized.

Differential scanning calorimetry

Measurements were carried out on a VP-DSC microcalorimeter (Micro-Cal Inc.) at a FimD$_x$ 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 ($\Delta H_m$) was obtained by integration of the heat absorption peaks after subtraction of the intrinsic heat capacity. Data were fitted according to a two-state transition with ORIGIN (Micro-Cal Inc.).

Isothermal titration calorimetry

Titrations experiments were performed with a MCS titration microcalorimeter (Micro-Cal Inc.) at 25 °C. Aliquots of 9 μl of a 290 μM FimD$_x$ 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–FimH$_x$, or FimC$_x$–FimF (20 μM each) in the same buffer. The duration of the injection was 11.5 seconds, and the delay between injections was 300 seconds. 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$^{	ext{TM}}$ 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 one hour at room temperature and then applied to the column. We observed that the isolated pilin domain of FimH (FimH$_x$) is very aggregation-prone and adsorbs nonspecifically to the gel-filtration column. Non-specific adsorption could be prevented when isolated FimH$_x$ was mixed with a tenfold excess of the synthetic peptide NH$_2$-TENTLQLAHSIRIKR-CO$_2$$_-$ corresponding to the FimC donor strand prior to gel-filtration. Eluted proteins were detected by their absorbance at 226 nm.

Yeast agglutination assay

E. coli strains W3110, W3110ΔfimH and W3110 harboring pFimD$_x$ were grown in 5 ml of M9 minimal medium containing 1 g/l of ammonium sulfate, 2 g/l of glucose and 100 μg/ml of ampicillin in the presence or absence of 1 mM IPTG at 37 °C for two days without shaking. After centrifugation, bacteria were suspended in the above buffer to a final A$_{600}$ 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 five minutes.

Electron microscopy

Bacteria were grown as described for the yeast agglutination assay. Adsorption of bacteria was performed for two minutes onto glow-discharged carbon films mounted on 400-mesh copper grids. They were negatively stained with 1% (w/v) uranyl acetate for 15 seconds. 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 11 as described for the yeast agglutination assay, and pili were isolated as described above. Pili subunits were dissociated by boiling in 0.1 M HCl for five minutes prior to addition of SDS-sample buffer, separated on 18% (w/v) SDS/polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were incubated for one hour with 8% (w/v) non-fat dry milk in 20 mM Tris–HCl (pH 7.4), 130 mM NaCl, and then for one hour with either anti-FimA, anti-FimE, anti-FimG or anti-FimH antibodies. After extensive washing, the membranes were incubated for one hour with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma), and stained as described.

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4. OUTLOOK

The focus of my work was the investigation of the role of the periplasmic pilus chaperone FimC in subunit folding and the assembly of subunits. The results of my experiments shed light on previously obscure events in pilus formation and in addition provide the basis for a more detailed analysis of pilus assembly.

Subunit folding

The ability of pilus chaperones to accelerate subunit folding is highly fascinating. The experiments that led to this conclusion were technically demanding for three reasons: First, structural pilus subunits are always prone to aggregation, especially in the absence of a donor strand. Second, the subunit on which I concentrated my efforts, FimGt, displays a comparably small fluorescence change upon folding. All experiments, therefore, suffer from a low signal/noise ratio. Third, it appears that FimGt binds only weakly to FimC and even less strongly to FimCYY. Hence, it was necessary to always work with excess concentrations of chaperone to ensure that FimGt was saturated with chaperone. Some of these limitations could be overcome by using FimFt (cf. the third section of the result part) as a model protein instead of FimGt. The fluorescence of FimFt increases during folding and even much more upon binding to FimC. Moreover, FimFt binds tightly to both FimC and FimCYY which allows e.g. for a variation of the FimC concentration during refolding of FimFt. Spontaneous refolding of FimFt is very slow and therefore unsuitable for stopped-flow measurements while FimC-assisted folding is fast (unpublished results). An analysis of FimFt folding would thus require a combination of stopped-flow and manual mixing experiments. Despite this disadvantage, I suggest that FimFt is used rather than FimGt in future experiments that investigate FimC-assisted subunit folding. Studies on the chaperone-assisted folding of FimFt could address the following topics: Does the chaperone bind to denatured subunits or to folding intermediates? Does FimFt folding also involve the fast accumulation of an intermediate as it has been seen for FimGt? How fast is the potential intermediate formed and how fast does it disappear? How does folding of a reduced subunit compare to folding of an oxidized disulfide-bonded subunit? Is the formation of native FimFt monophasic, or is it biphasic as for FimGt? Is a potential second phase reduced in the presence of PPlase? Are certain residues in FimC or in FimFt critical for formation of the complex or for folding of FimFt? Residues critical for
binding might also be identified by sampling peptide libraries of FimF, FimH (the only subunit with known structure) and FimA (the major subunit deserves, in principle, major attention) with FimC and FimC variants as described for classical chaperones (Rudiger et al., 1997). This alternative approach could reveal fundamental insights into the principles of chaperone-subunit complex formation.

Spontaneously refolding subunits are prone to aggregation, which can be suppressed efficiently by donor strand peptides that, importantly, seem to only subtly influence the folding kinetics of subunits. However, since the affinity of donor strand peptides for folding subunits is relatively low (in the range of a few μM), large quantities of peptide are required to achieve conditions under which the folding subunits are saturated with peptide. To circumvent this disadvantage, one might use subunits with an artificial intramolecular donor strand that prevents aggregation. Initial measurements have been performed with FimG\textsubscript{tF}, which was constructed, expressed and purified by Thomas Spirig and Andrea Fritz (unpublished results). FimG\textsubscript{tF} corresponds to mature FimG depleted of the natural N-terminal donor strand and extended at the C-terminus by the linker DNKQ and the donor strand of FimF (14 amino acid residues). Surprisingly, the folding kinetics of FimG\textsubscript{tF} do not resemble the folding kinetics of FimG\textsubscript{t} in the presence of soluble FimF donor strand peptide, but instead remind strongly of FimC\textsubscript{YY}-assisted FimG\textsubscript{t} folding. It thus appears that the artificial intramolecular donor strand of FimG\textsubscript{tF} works as a mini-chaperone. Elongation of subunits at the C-terminus as in FimG\textsubscript{tF} abolishes binding to FimC (Peter Sebbel, unpublished results).

Hence variants of FimF\textsubscript{t} and FimG\textsubscript{t} with the donor strand of FimC at their N-terminus should be constructed. Such variants would be extremely valuable for investigations on the folding mechanism of pilus subunits if they bound to the chaperone when refolded in the presence of chaperone and self-complement when no chaperone is available during refolding. It can however not be excluded that intramolecular donor strands, due to their high effective concentration, always competitively inhibit the chaperone from binding.

A surprising result that emerged from the interrupted refolding experiments with FimG\textsubscript{t} is that FimG\textsubscript{t} is kinetically much more stable in complex with the FimC donor strand peptide than in complex with the entire chaperone FimC. It is tempting to speculate that this discrepancy is due to the orientation of the donor strand. While the donor strand is parallel to the F strand of FimG\textsubscript{t} in the chaperone-subunit complex, it is possible that the peptide inserts in the reverse
orientation, creating a canonical, and maybe therefore much more stable, Ig-like fold. Possibly, the context of the entire chaperone forces the donor strand to complement the fold of the subunit in an energetically unfavourable orientation. This might constitute the mechanism that preserves folding energy that is released only when the subunit is incorporated into the pilus. To test this attractive hypothesis, one might determine the NMR structure of any complex between a subunit and the FimC donor strand peptide.

Concerning subunit folding, I finally would like to present a working hypothesis that is based on my own work, preliminary experiments of Chasper Puorger and on the results of Zavialov et al. (2003). Spontaneous subunit folding may first lead to a folding intermediate that strongly resembles the chaperone-bound conformation of a subunit, characterized by a loosely packed hydrophobic core and a relative low kinetic and thermodynamic stability. This conformation possibly converts slowly to a more rigid structure that is similar to the subunit-bound form of the subunit and unable to bind the chaperone or another subunit. In this context, it might be rewarding to perform interrupted folding experiments with FimGt using the unfolding conditions that C. P. has established to distinguish between the formation of a kinetically unstable intermediate and the more stable “native” state during FimGt folding.

**Assembly of subunits**

Spontaneous assembly of subunit is surprisingly slow and with all likelihood catalysed by the usher FimD. The assembly reaction comprises two steps: dissociation of the chaperone-subunit complex and association of two subunits. It is not clear whether the dissociation or the association is rate-limiting. This is an intriguing question because the usher presumably catalyses fiber formation by accelerating the rate-limiting step of the assembly reaction. A simple experiment could shed light on these determinants of subunit assembly. By varying the concentrations of all components in an experiment where the replacement of the chaperone by a subunit from a chaperone-subunit complex is followed, it should be possible to detect whether the (concentration-independent) dissociation or the (concentration-dependent) association is rate-limiting. In order to obtain exact data, it might be necessary to improve the analytical methods. One possibility is that analytical ion-exchange is used instead of the combination of gel filtration and SDS-PAGE in order to quantify the changes in concentration of the different products and educts in the replacement reaction. Further,
proteolysis as it was often observed after long incubation periods (e.g. 100 hours) hampers an accurate interpretation of the data. One possibility to reduce the impact of proteolysis is an acceleration of the reaction. First experiments indicate that this can be achieved with low denaturant concentrations as for example 0.25 M GdmCl.

So far, our investigations on subunit assembly concentrated on the minor subunits FimF, FimG, and FimH. These proteins are presumably not fundamentally different from the major subunit FimA and should therefore be suitable models to investigate pilus assembly. But for a safer comparison with e.g. \textit{in vivo} assembly rates, experiments should be extended to FimA, which is assumed to constitute > 99 \% of the pilus. Finally, the data on spontaneous pilus assembly that are presented in this thesis need to be complemented with experiments on FimD catalysed pilus formation. Preparation of active FimD appears to be the greatest challenge in the context of such attempts.
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5. REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


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