Donor-Strand Exchange in Chaperone-Assisted Pilus Assembly Proceeds through a Concerted β Strand Displacement Mechanism

Han Remaut, Rebecca J. Rose, Thomas J. Hannan, Scott J. Hultgren, Sheena E. Radford, Alison E. Ashcroft, and Gabriel Waksman

1 Institute of Structural Molecular Biology at UCL/Birkbeck
2 School of Crystallography
Malet Street
London WC1E 7HX
United Kingdom
3 Astbury Centre for Structural Molecular Biology
Garstang/Astbury Building
University of Leeds
Leeds, LS2 9JT
United Kingdom
4 Department of Molecular Microbiology
Washington University Medical School
660 South Euclid Avenue
St. Louis, Missouri 63105
5 Department of Biochemistry and Molecular Biology
University College London
Gower Street
London WC1E 6BT
United Kingdom

Summary

Gram-negative pathogens commonly use the chaperone-usher pathway to assemble adhesive multisubunit fibers on their surface. In the periplasm, subunits are stabilized by a chaperone that donates a strand to complement the subunits' truncated immunoglobulin-like fold. Pilus assembly proceeds through a "donor-strand exchange" (DSE) mechanism whereby this complementary β strand is replaced by the N-terminal extension (Nte) of an incoming pilus subunit. Using X-ray crystallography and real-time electrospray ionization mass spectrometry (ESI-MS), we demonstrate that DSE requires the formation of a transient ternary complex between the chaperone-subunit complex and the Nte of the next subunit to be assembled. The process is crucially dependent on an initiation site (the P5 pocket) needed to recruit the incoming Nte. The data also suggest a capping reaction displacing DSE toward product formation. These results support a zip-in-zip-out mechanism for DSE and a catalytic role for the usher, the molecular platform at which pilis are assembled.

Introduction

A wide range of pathogenic gram-negative bacteria deploy surface-exposed adhesive multisubunit fibers that mediate attachment to biological and nonbiological surfaces (Thanassi and Hultgren, 2000). A predominant class of such organelles is assembled by the conserved chaperone-usher protein secretion system (Thanassi et al., 1998). In this secretion pathway, pilus subunits (pilins) are exported to the periplasm via the general secretory pathway, where they are captured by a cognate chaperone. The chaperone assists pilins in folding and prevents their premature polymerization by capping their interactive surfaces. The usher protein forms the outer membrane assembly platform wherein subunits are released from the chaperone and assembled into surface-exposed fibers.

The structural basis of chaperone-subunit and subunit-subunit interactions has been revealed by a number of recent crystal structures of Pap, Fim, and Caf (Choudhury et al., 1999; Sauer et al., 1999, 2002; Zavialov et al., 2003). Despite very low sequence homology, all of these pilins are composed of a similar immunoglobulin-like (Ig-like) fold that lacks the seventh, C-terminal, β strand (strand G). This missing strand leaves a deep hydrophobic groove on the pilin surface, rendering subunits unstable outside the fiber or chaperone-subunit complex. The periplasmic chaperone is composed of two Ig-like domains that meet at a right angle. As first shown by studies on the Pap and Fim systems, in the complex with the pilus subunit, strand G of the N-terminal domain of the chaperone (β strand G1) inserts a conserved motif of four alternating hydrophobic residues (termed “P1 to P4 residues”) into four binding pockets in the hydrophobic groove of the pilus subunits (named “P1 to P4 binding pockets”). The G1 strand makes main-chain/main-chain hydrogen bonding interactions with the subunit strand F and, thus, provides in trans the β strand that is lacking in the pilus subunit, so completing its Ig fold. In this complex, however, the chaperone G1 strand runs parallel to the strand F of the pilus subunit, and thus, the noncovalently assembled Ig fold that results is non-canonical. This process of chaperone action is termed “donor-strand complementation” (DSC) (Choudhury et al., 1999; Sauer et al., 1999).

Pilus assembly involves a similar principle of complementing the incomplete Ig-fold of the pilins. During pilus assembly, a 10–18 residue long Nte of an incoming pilus subunit, which is not itself involved in the Ig-like fold, inserts into the hydrophobic groove of an adjacent subunit, thereby replacing the G1 strand of the chaperone and complementing the Ig-fold of the adjacent subunit. Insertion of the Nte is antiparallel to the strand F of the complemented subunit, and thus, a canonical Ig fold is reconstituted. This process is termed DSE (Choudhury et al., 1999; Sauer et al., 1999, 2002; Zavialov et al., 2003). These Nte sequences contain a motif of alternating hydrophobic residues, termed “P2 to P5 residues” because they occupy the P2 to P5 binding pockets in the groove of the pilus subunit. DSE occurs at the outer membrane usher, releasing chaperones from the subunit-chaperone binary complexes and allowing polymerization of the subunits.

Despite the generic importance of DSE in the biogenesis of bacterial pilus assembled by the chaperone-usher pathway, the mechanism by which DSE occurs is not presently understood. In vivo, DSE occurs efficiently (on a minute timescale) at the outer membrane usher protein in the absence of an energy source. By contrast,
in vitro, DSE occurs spontaneously in a reaction that proceeds more slowly (on an hour timescale) (Jacob-Dubuisson et al., 1994; Sauer et al., 2002). Two hypothetical mechanisms for DSE have been proposed (Zavialov et al., 2003). In the simplest, the chaperone is released from the binary complex at the usher, exposing the subunit’s hydrophobic groove. This groove is then able to accommodate and bind the N-terminal extension of an incoming subunit, still in complex with the chaperone. In another hypothesis, Nte binding occurs concomitantly with the release of the chaperone, and the incoming Nte gradually displaces the chaperone G1 β strand in a zip-in-zip-out fashion.

In this study, we determine the mechanism of DSE with a real-time investigation of the exchange reaction involved in the Salmonella enterica Saf pilus system by using X-ray crystallography, site-directed mutagenesis, and ESI-MS. The Saf system constitutes one of four operons (lim, bcf, saf, and sth) that are present in all S. enterica subspecies I strains, which account for over 99% of clinical cases in mammals and birds (Townsend et al., 2001). The saf operon consists of only four genes, safA, B, C, and D, encoding the major pilus subunit, the chaperone, the usher, and a putative adhesin/invasin, respectively. Here, we show that DSE in the absence of the usher proceeds through formation of a transient ternary complex between the chaperone-subunit complex and the Nte of the subunit next in assembly. Ternary complex formation initiates DSE and is dependent on the P5 (F17) residue in the Nte binding to the P5 pocket of the subunit previously assembled. Our results show that this interaction critically determines the rate of strand exchange. We also provide evidence that suggests a capping mechanism that concludes DSE, whereby residue F3 (termed P*) inserts into the P* pocket of the subunit previously assembled and drives DSE toward product formation. Overall, these data suggest a concerted β strand displacement mechanism for DSE that, we hypothesize, also takes place at the usher but is accelerated by increased local concentration and proper positioning and orientation of the various partners in the DSE reaction.

Results and Discussion

Structures of the SafB-SafANtd2 and SafANtd2-Ante Complexes

Like most chaperone-subunit complexes, SafB-SafA is prone to spontaneous Nte-mediated subunit polymerization. Therefore, in order to crystallize the complex, N-terminally deleted mutants of the SafA pilus subunit were created. In the first variant constructed, termed SafANtd1, the seven-residue sequence from F3 to Q9 was deleted. This variant was found to be significantly impaired in polymerization, forming small molecular weight oligomers at neutral pH and a dimeric species at pH 8.5. The dimeric species was purified and crystallized, and its structure was elucidated by using single wavelength anomalous dispersion (SAD) phasing on the selenomethionine-labeled SafANtd1 dimer (see Table 1 and Figure S1 available in the Supplemental Data with this article online). The structure consisted of a head-to-tail “swapped” dimer in which residues 10–19 of one molecule were found bound to the groove of the adjacent subunit in the dimer. The data suggest that residues 10–19 are part of the Nte involved in DSE. To test this hypothesis, a second N-terminal deletion of SafA was created, termed SafANtd2, in which residues 9–21, comprising the complete Nte and the adjacent three-residue loop, were deleted. The SafB-SafANtd2 chaper-one-subunit complex was found to be stable, and no SafA polymers were observed. The SafB-SafANtd2 complex crystalized readily, and its structure was solved by molecular replacement using the structure of the Stae chaperone (Knight et al., 2002) as search model. In parallel, and to generate a view of SafA after DSE, the SafB-SafANtd2 complex was purified and SafB competitively removed by the addition of an excess of a 19-mer peptide encompassing the entire Nte (residues G1–S19, termed ANtd2). The resulting product, the SafANtd2-Ante complex, was also crystallized, and its structure was solved by molecular replacement using the SafANtd2 structure as search model (Figure 1).

Despite the complete lack of sequence homology with other chaperone-usher pilus subunits, the structures of SafA showed that this protein folds into an Ig-like fold with a missing C-terminal β strand (strand G), the hallmark of chaperone-pilin complexes (Sauer et al., 2002) (Figures 1A and 1B). In the SafANtd2-Ante structure (Figure 1A), the Nte peptide forms two β strands, separated by a short helical turn, that each align antiparallel with the subunit F strand and, as anticipated from the structures of other pilus assemblies (Sauer et al., 2002; Zavialov et al., 2003), thus serve as a complement for the missing strand G and complete the Ig fold of SafA. In addition to forming 13 main-chain hydrogen bonds, the Nte strand interacts with the pilus subunit through a motif of four alternating hydrophobic side chains. Two hydrophobic residues, F17 and I15, insert deeply into the hydrophobic core. These residues are termed the “P5 and P4 residues,” respectively, according to the nomenclature used in the Pap system (Sauer et al., 2002), and bind to the P5 and P4 binding pockets in the groove of SafANtd2, respectively. V13 (the P3 residue) and the aliphatic part of K11 (the P2 residue) only partially interact with the subunit core due to a twist in the longer Nte strand. Finally, near its N terminus, residue F3 of the Nte (termed the P* residue) is deeply buried into a large hydrophobic pocket (termed the P* pocket) formed by strands β1 and β2 of SafA on one side and strands C and F on the other (Figures 1A and 1D).

The structure of the SafB-SafANtd2 complex (Figure 1B) revealed that the chaperone SafB folds into two Ig-like domains that link together to form an L shaped molecule, typical of that of other periplasmic pilin chaperones (Holmgren and Branden, 1989; Pellecchia et al., 1998; Choudhury et al., 1999; Sauer et al., 1999; Knight et al., 2002; Zavialov et al., 2003). As seen in the Caf1M-Caf1 chaperone-subunit interaction (Zavialov et al., 2003), the N-terminal β strand of SafB (strand A1, Figure 1B) aligns with the A strand of the SafA subunit. The G1 strand of SafB complements the C-terminally truncated Ig-like fold of SafA in a classical DSC interaction. A motif of five alternating hydrophobic residues in the G1 strand (A114, L116, L118, L120, and I122) is positioned such that these residues interact with the hydrophobic P5–P1 pockets of the subunit’s groove (Figure 1B). Interestingly, two crystal forms of
the SafB-SafANtd2 complex were observed that differ in the extent of ordering in residue A114 of the G strand. In type I crystals, A114 is ordered and is inserted into the P5 pocket of the SafA subunit (Figure 1B, left). In type II crystals, by contrast, this residue is disordered and does not insert into the P5 pocket (Figure 1B, right). As a result, the loops and secondary structure elements in the SafA subunit that form this P5 pocket are also disordered and are not observed in the electron density. In both crystal forms, this region of the structure is not directly involved in crystal packing, but differences in crystallization conditions and crystal packing presumably explain why the two forms were captured in their respective crystals. These two structures suggest that there is an equilibrium between two states of the SafB-SafANtd2 complex as a result of weak binding of the chaperone G1 donor strand at the P5 site of the Nte binding groove, a conclusion borne out by the results of the experiments described below. Intriguingly, the dynamics of the binding of the chaperone G1 strand at the distal end of the chaperone-subunit interaction may point to a possible initiation point for G1 strand displacement during DSE (see below). In contrast, the P4–P1 pockets are completely obscured from solvent by the chaperone G1 strand (Figures 1B and 1C). Finally, the P* pocket in the SafB-SafANtd2 is also inaccessible and is obstructed by both Y142 in the SafA F strand and W103 in the b1-b2 loop. Upon DSE, this region undergoes conformational changes resulting in Y142 and W103 moving out to form the pocket and enabling F3 accommodation only after the breakdown of the SafB-SafA Ntd2 chaperone-subunit complex has occurred (Figure 1D).

<table>
<thead>
<tr>
<th>Data Collection</th>
<th>Radiation</th>
<th>Resolution (Å)</th>
<th>Reflections Total/Unique</th>
<th>I/σ(I)</th>
<th>ComPLETENESS (%)</th>
<th>Rmerge</th>
</tr>
</thead>
<tbody>
<tr>
<td>(SafANtd1)2</td>
<td>1.5418, Raxis</td>
<td>20–1.78</td>
<td>201192/23089</td>
<td>22.8 (5.1)</td>
<td>97.3 (95.7)</td>
<td>4.8 (18.2)</td>
</tr>
<tr>
<td>(SafANtd1)2 Se peak</td>
<td>0.9793, ID14-4</td>
<td>20–2.05</td>
<td>203805/15222</td>
<td>33.2 (17.8)</td>
<td>100.0 (100.0)</td>
<td>5.3 (8.7)</td>
</tr>
<tr>
<td>SafB-SafANtd2 Type I</td>
<td>1.0726, ID23-1</td>
<td>25–2.0</td>
<td>78100/20162</td>
<td>13.3 (4.0)</td>
<td>99.9 (100.0)</td>
<td>9.5 (29.6)</td>
</tr>
<tr>
<td>SafB-SafANtd2 Type II</td>
<td>0.9792, ID23-1</td>
<td>25–1.8</td>
<td>112190/27689</td>
<td>17.5 (4.3)</td>
<td>91.5 (89.7)</td>
<td>6.6 (28.1)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Structure Determination Data set: (SafANtd1)2 Se peak</th>
<th>Resolution: 20–2.05 Å</th>
<th>Phasing power: acentric: 1.96</th>
<th>Rullen (ano): acentic: 0.68</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOM: acentric: 0.46</td>
<td>FOM: centric: 0.11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Refinement</th>
<th>(SafANtd1)2</th>
<th>SafB-SafANtd2 Type I</th>
<th>SafB-SafANtd2 Type II</th>
<th>SafANtd2-ANte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Å)</td>
<td>20–1.78</td>
<td>20–2.0</td>
<td>20–1.8</td>
<td>20–1.85</td>
</tr>
<tr>
<td>Number of reflections</td>
<td>Total 22,411</td>
<td>19,127</td>
<td>26,305</td>
<td>15,203</td>
</tr>
<tr>
<td>Work set</td>
<td>21,257</td>
<td>18,101</td>
<td>24,926</td>
<td>14,399</td>
</tr>
<tr>
<td>Test set</td>
<td>1154</td>
<td>1026</td>
<td>1379</td>
<td>804</td>
</tr>
<tr>
<td>Rwork (%)</td>
<td>18.4</td>
<td>18.1</td>
<td>19.0</td>
<td>17.1</td>
</tr>
<tr>
<td>Rfree (%)</td>
<td>21.7</td>
<td>22.9</td>
<td>22.8</td>
<td>20.0</td>
</tr>
<tr>
<td>Numbers of atoms</td>
<td>Protein 2345</td>
<td>2537</td>
<td>2390</td>
<td>1103</td>
</tr>
<tr>
<td>Water 257</td>
<td>198</td>
<td>274</td>
<td>131</td>
<td></td>
</tr>
<tr>
<td>Wilson B factor (Å²)</td>
<td>15.8</td>
<td>23.0</td>
<td>18.3</td>
<td>18.6</td>
</tr>
<tr>
<td>Average B factor (Å²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rmsd stereochemistry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bonds (Å)</td>
<td>0.010</td>
<td>0.014</td>
<td>0.016</td>
<td>0.009</td>
</tr>
<tr>
<td>Angles (Å)</td>
<td>1.641</td>
<td>1.591</td>
<td>1.533</td>
<td>1.193</td>
</tr>
<tr>
<td>Rmsd B factor (Å²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main chain 1.4</td>
<td>1.6</td>
<td>1.5</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Side chain 0.8</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

Values for high-resolution shell are in parenthesis. Rmerge = ∑j | Ij − <I> | / ∑j Ij, where Ij = observed intensity and <I> = average intensity of multiple observations of symmetry-related reflections. Rullen(ano) = ∑j | | | ΔFobs | | (obs) − ΔFcalc | | (calc) | | / | | ∑j ΔFcalc | | (calc), where Fobs| | (obs) is the structure factor amplitude difference between Bijvoet pairs and ΔFcalc = 2 F00 | | FH sin(aPH−aH), with P and H the protein and heavy atom substructure, respectively. FOM, figure of merit. Rwork = ∑j Fo | | (obs) − Fc | | (calc) / | | ∑j Fo | | (calc), where Fobs| | (obs) and Fcalc| | (calc) are the observed and calculated structure factors, respectively. Rmsd stereochemistry is the deviation from ideal values, Rmsd B factors are the deviations between bonded atoms.

### Biochemical Characterization of DSE In Vitro

Two mechanisms have been proposed for DSE (Zavialov et al., 2003). In one mechanism, the binding of the chaperone-subunit complex to the usher destabilizes the chaperone-subunit interaction and leads to complete chaperone dissociation before DSE occurs. In the other mechanism, the Nte of one subunit progressively zips...
into the groove of another subunit while the G1 strand of the chaperone bound in that groove zips out.

The zip-in-zip-out model has two important implications, which we set out to test. First, during the exchange process, a ternary complex between the chaperone subunit and the Nte of the next subunit coming into assembly is predicted to form. Second, for the subunit's Nte to displace the chaperone G1 strand, an entry (or initiation) point is required. There are two classes of chaperone-usher pathways, FGL and FGS, characterized by either a long (FGL) or short (FGS) F1G1 loop in the chaperone (Hung et al., 1996). In available chaperone-subunit structures of FGS chaperone-usher systems (PapD-PapK, PapD-PapE Ntd2, and FimC-FimH [Choudhury et al., 1999; Sauer et al., 1999, 2002] and also PapD-PapA [D. Verger and G.W., unpublished data]), the P5 pocket is the only pocket not occupied by side chains of the chaperone G1 strand (Figure 1C).

In order to determine the mechanism of DSE and to test the implications of the zippering model outlined above, DSE experiments on the SafB-SafANtd2 complex were set up in vitro with the wild-type (wt) ANte 19-mer peptide together with a series of four ANte peptide variants containing a single alanine substitution at the P5 residue (F17), P4 (I15), P3 (V13), or P* (F3) position (the ANte peptide with an alanine substitution at P2 was not...
soluble). In a first set of experiments, purified SafB-SafANtd2 complex was incubated in vitro with increasing concentrations of each peptide (0, 1, 2, 5, 10, and 20 ANte:SafB-SafANtd2 molar ratios) at pH 8.0. After a 24 hr incubation at ambient temperature, the extent of the DSE reaction termed "DSE efficiency" (as indicated by the decrease of the SafB-SafANtd2 complex and the release of SafB) was monitored by native PAGE. The resulting data (Figure 2A) showed that for wt ANte, an equimolar amount of peptide drives the DSE reaction to near completion. By contrast, for the F17A ANte peptide, the bulk of the SafB-SafANtd2 complex remains intact after 24 hr, even in the presence of a 20-fold molar excess of the wt ANte peptide. The ANte peptides containing the I15A and V13A substitutions required from 10- to 20-fold and 5-fold molar excess, respectively, to drive the DSE reaction to completion. Finally, for the F3A ANte mutant peptide, DSE is significantly perturbed, with significant SafB-SafANtd2 remaining intact after 24 hr incubation even in the presence of a 20-fold molar excess of peptide. Thus, the five ANte peptides can be ranked according to their DSE efficiency in the following order: wt ANte > V13A > I15A > F3A >> F17A. The results described above indicate that the P5 residue is the most important site in the Nte and point to the importance of the P5 pocket in DSE.

To verify that the observed differences in DSE efficiency do not result from aberrant binding of the mutant peptides out of register in the SafA binding groove, the complexes of SafANtd2 bound to each of the ANte peptide variants were purified and crystallized and their structures determined (see Table S1 and Figure S2). The results confirmed that all peptides bind with the same register as wt ANte and the SafA structure within the various complexes remains essentially unchanged. The conformational properties of the unbound state of each peptide were also analyzed by using far UV CD and shown to be identical and lacking in significant regular secondary structure (data not shown).

The results described above indicate that the P5 residue is the most important site in the Nte and point to the importance of the P5 pocket in DSE. To test this hypothesis further, we next examined the role of residue 114 of SafB in the SafB-SafANtd2 complex (Figure 1B) in the DSE reaction. Based on the observation of conformational dynamics of A114 at the P5 pocket (see above), we speculated that transient local dissociation at this site in the complex might expose the P5 pocket to the incoming ANte peptide, suggesting that conformational dynamics at this site may be crucial for the initiation of DSE. Mutating A114 to a more bulky hydrophobic residue, therefore, may increase the stability of the chaperone-subunit complex at this site, thus decreasing the ability of the complex to undergo DSE. To investigate this, residue 114 in SafB was substituted with valine (SafBA114V) or phenylalanine (SafBA114F) and DSE was measured in vitro as described above using the wt F17A mutant peptide is significantly more impaired in its ability to promote DSE, consistent with the P5 residue playing a critical role in DSE.

To verify that the observed differences in DSE efficiency do not result from aberrant binding of the mutant peptides out of register in the SafA binding groove, the complexes of SafANtd2 bound to each of the ANte peptide variants were purified and crystallized and their structures determined (see Table S1 and Figure S2). The results confirmed that all peptides bind with the same register as wt ANte and the SafA structure within the various complexes remains essentially unchanged. The conformational properties of the unbound state of each peptide were also analyzed by using far UV CD and shown to be identical and lacking in significant regular secondary structure (data not shown).

The results described above indicate that the P5 residue is the most important site in the Nte and point to the importance of the P5 pocket in DSE. To test this hypothesis further, we next examined the role of residue 114 of SafB in the SafB-SafANtd2 complex (Figure 1B) in the DSE reaction. Based on the observation of conformational dynamics of A114 at the P5 pocket (see above), we speculated that transient local dissociation at this site in the complex might expose the P5 pocket to the incoming ANte peptide, suggesting that conformational dynamics at this site may be crucial for the initiation of DSE. Mutating A114 to a more bulky hydrophobic residue, therefore, may increase the stability of the chaperone-subunit complex at this site, thus decreasing the ability of the complex to undergo DSE. To investigate this, residue 114 in SafB was substituted with valine (SafBA114V) or phenylalanine (SafBA114F) and DSE was measured in vitro as described above using the wt
ANte peptide. In both cases, by using an equimolar concentration of peptide and a 24 hr incubation time, significantly less product was formed than observed for the wt complex, with the SafB (A114V)-SafANtd2 and SafB(A114F)-SafANtd2 complexes requiring at least a 10-fold or 20-fold excess of wt ANte peptide for completion of the reaction, respectively (Figure 2B). Stabilizing the interaction between SafB and the P5 pocket of SafA thus results in less efficient DSE reactions, confirming that occupation of the P5 pocket by the incoming Nte plays an important role in the DSE process.

Kinetics of DSE Measured in Real Time by ESI-MS

To investigate the kinetics of the DSE process in more detail, the SafB-SafA\(_{\text{Ntd2}}\) complex was incubated with the peptides described above and the rate of DSE measured directly and in real time with ESI-MS under nondenaturing conditions (Loo, 1997; Ashcroft, 2005). This technique is ideal for monitoring reactions in solution in real time due to its ability to resolve, identify, and quantify individual components within a complex mixture (van Duijn et al., 2005; Videler et al., 2005). First, the ability to retain a stable complex of the SafB-SafA\(_{\text{Ntd2}}\) complex
within the mass spectrometer in 10 mM aqueous ammonium acetate at pH 5.5 was assessed (Figure 3A). The resulting spectrum showed predominantly a species of measured mass 37,119.7 Da, consistent with that expected of the intact complex (theoretical mass 37,123.1 Da). The purified SafB-SafA\textsubscript{Ntd2} complex was next incubated with an equimolar ratio of the wt A\textsubscript{Nte} peptide (measured mass 2068.4 Da; theoretical mass 2068.3 Da), and the decline of the complex and concurrent appearance of SafA\textsubscript{Ntd2}-A\textsubscript{Nte} (measured mass 15,191.8 Da; theoretical mass 15,193.0 Da) and SafB (measured mass 23,996.9 Da; theoretical mass 23,998.4 Da), the products of DSE, were monitored (Figures 3B–3D). Remarkably, a species corresponding to a ternary complex of SafB-SafA\textsubscript{Ntd2} bound to the A\textsubscript{Nte} peptide (involving the 11+, 12+, and 13+ charge states in the region m/z 3000–3600, measured mass 39,199.9 Da; theoretical mass 39,191.4 Da) was detected clearly in these reactions immediately upon addition of the peptide (Figure 3B), its concentration decreasing with time, concomitantly with the appearance of the reaction products (Figures 3B–3D). The data thus exclude the hypothesis that DSE occurs via transient dissociation of the pilin and chaperone but instead provide strong evidence in support of a zip-in-zip-out mechanism. Consistent with the results shown in Figure 2A, 24 hr after the addition of the peptide, the reaction had reached completion, with negligible evidence for either SafB-SafA\textsubscript{Ntd2} or ternary complex remaining; instead, the two products SafA\textsubscript{Ntd2}-A\textsubscript{Nte} and SafB were the only species observed (Figure 3D).

The experiment was repeated with the A\textsubscript{Nte} peptide derivatives with alanine residues substituted in the P\textsuperscript{*} (F3A), P3 (V13A), P4 (I15A), or P5 (F17A) position, as well as a control reaction in the absence of any peptide. As with the reaction with the wt A\textsubscript{Nte} peptide, the masses of all species observed were within 0.02% of their theoretical value. Representative data for the F17A peptide are shown in Figures 3E–3H. The resulting spectra showed that this peptide is highly inefficient in facilitating DSE, consistent with the results presented in Figure 2A. Indeed, for this peptide, relatively low concentrations of ternary complex were formed and DSE proceeded extremely inefficiently, with ~50% of the starting material remaining after incubation with peptide for 24 hr (Figure 3H). To provide detailed insights into the ability of each peptide sequence to facilitate the DSE reaction, the m/z ions for each species were summed, integrated, and normalized to the total ion count for all species (see the Supplemental Data for details). In this manner, the rate of DSE for each peptide could be directly compared (Figure 4). Monitoring the decline of the SafB-SafA\textsubscript{Ntd2} complex showed that the reaction with the wt A\textsubscript{Nte} peptide proceeds rapidly, reaching completion within 3 hr (Figure 4A). The V13A and F3A peptides followed similar kinetics, whereas the reactions with the peptides F17A and I15A resulted in a slower decline of the initial SafB-SafA\textsubscript{Ntd2} complex, such that significant concentration of the starting material persisted for over 24 hr. The SafB-SafA\textsubscript{Ntd2} complex showed no decrease in intensity in the absence of peptide (Figure 4A).

The appearance of the ternary reaction intermediate, as well as the reaction products SafA\textsubscript{Ntd2}-A\textsubscript{Nte} and SafB, could also be measured and quantified with accuracy within the same experiment (see the Supplemental Data for details) (Figures 4B–4D). All peptides bound rapidly (although to varying extents) to SafB-SafA\textsubscript{Ntd2} to form a ternary complex in the 2 min dead time of these experiments, demonstrating the generic importance of ternary complex formation in DSE and showing that peptide binding in all cases occurs more rapidly than product release. The rate of production of the SafA\textsubscript{Ntd2}-A\textsubscript{Nte} complex and free SafB mirror the decline of the ternary complex for each peptide. The reaction proceeds most rapidly in the presence of the wt A\textsubscript{Nte}, F3A, and V13A peptides, whereas the kinetics of DSE using the F17A peptide are clearly slower, and those initiated by the I15A peptide are of an intermediate rate. The rates of substrate decrease and product increase are complementary and follow the order wt ≥ F3A ≥ V13A > I15A >> F17A. The dramatic difference in the
kinetic signature of DSE induced by the F17A ANte peptide explains why a significant amount of substrate is still observed after a 24 hr incubation of SafB-SafANtd2 with even a 20-fold molar equivalent of F17A ANte peptide as observed by using native PAGE (Figure 2A).

Because substitution of F17 with Ala in the ANte peptide results in the most dramatic reduction in the rate of DSE, we next asked whether the nature of the side chain inserting in the P5 pocket affects the kinetics of DSE. Two further ANte peptide variants were thus synthesized in which F17 was mutated to Ile or to Val and the kinetics of DSE measured by using ESI-MS. These peptides showed DSE kinetics intermediate in rate between those observed with the wt and F17A ANte peptides, with F17I displaying more rapid kinetics than F17V (Figure 5A). Thus, the nature of the side chain inserting in the P5 pocket plays an important role in determining the rate of DSE.

In the chaperone-subunit complex, the subunit’s P5 pocket is only partially occupied by residue A114 of the chaperone. Accessibility of the P5 pocket is thus dependent on the dynamic motions affecting the F1G1 loop where residue A114 lies. We have shown above that decreasing the accessibility of the P5 pocket by mutating A114 to a bulkier residue, either F or V, results in decreased product formation (Figure 2B). We next asked whether decreasing P5 pocket accessibility also affects the kinetics of product formation. ESI-MS was thus used to monitor the kinetics of DSE of SafB_{A114V}–SafANtd2 and SafB_{A114F}–SafANtd2 when challenged with wt ANte. Both the A114F mutation (Figures 3I–3L) and the A114V mutation (Figure S3) have a dramatic effect on the DSE reaction, reducing the amount of ternary complex formed and decreasing substantially the rate of product formation. A comparison of the rate of product formation for the three complexes is presented in Figure 5B and shows that wt SafB–SafANtd2 displays the fastest kinetic behavior, SafB_{A114V}–SafANtd2 reacts with intermediate kinetics, and SafB_{A114F}–SafANtd2 reacts the most slowly. Together, these results demonstrate that kinetics of DSE are significantly reduced when the P5 pocket is made less accessible by the insertion of a bulky residue in the P5 pocket, consistent with the P5 pocket acting as the entry point mediating ternary complex formation.

Somewhat surprisingly, although the reactions with the wt, V13A, and F3A ANte peptides proceed with similar rates (Figure 4A), the reaction with the F3A ANte peptide resulted in the SafB–SafANtd2 complex remaining at a constant ~30% of its original concentration after reaching equilibrium. Also, Figure 2A indicates that at equimolar concentrations of F3A ANte peptide and SafB–SafANtd2 complexes, ~80% of the original complex is left intact after 24 hr. These results suggest that (1) DSE induced by the F3A ANte peptide proceeds to an equilibrium between the various reaction species, and (2) this equilibrium appears to be pH dependent, as more DSE substrate (SafB–SafANtd2) is left unreacted under native PAGE condition (pH 8.0) than under ESI-MS conditions (pH 5.5). To test that indeed a “reverse” DSE reaction occurs with the F3A ANte peptide and that the resulting equilibrium is pH dependent, an equimolar amount of SafB (25 μM) was added to purified SafANtd2 subunit in complex with wt or F3A ANte peptide, and formation of the SafB–SafANtd2 complex was monitored by using native PAGE. These reactions were carried out at both pH 8.0 and pH 5.5. As shown in Figure 2C, the reverse DSE reaction with F3A ANte results in a significant concentration of DSE substrate being formed, whereas the DSE reaction with wt ANte did not. Furthermore, the DSE equilibrium was shown to be pH dependent, with increased SafB–SafANtd2 reforming at pH 8.0 compared with pH 5.5. Thus, destabilization of the interaction at the P* pocket by substituting the P* residue with an Ala results in the DSE reaction being displaced toward DSE substrate (SafB–SafANtd2) formation. Moreover, our structural data show that, in the SafB–SafANtd2 complex, the P* pocket is not formed and is not available for binding until the SafB–SafANtd2 complex has dissociated. Binding of the P* residue requires a conformational change in the subunit’s C terminus, away from the conformation it adopts in the chaperone-subunit complex where it is in a firm electrostatic interaction with the conserved arginine/lysine pair at the chaperone’s interdomain cleft (Arg8/Lys112 in the Pap nomenclature). Together, these results suggest that the role of the P* residue is to act as a capping device, locking the Nte’s N terminus and the subunit’s C terminus in place, thereby ending the DSE reaction and displacing the reaction toward product (SafANtd2–ANte) formation.

DSE with Full-Length SafA Subunits The biochemical and biophysical experiments described above strongly suggest that DSE is initiated by ternary complex formation at the P5 pocket and may end by the capping of the P* pocket. To determine whether this proposed mechanism also occurs in the full-length proteins, the F17A, I15A, and V13A mutations were introduced individually into the full-length SafA protein and the various resulting SafB–SafA complexes were purified and analyzed after 1 and 24 hr by using...
native PAGE (Figure 6). Native PAGE monitors both chaperone release from the SafB-SafA complex, indicative of the extent of DSE, and formation of SafA polymers as indicated by a smear of higher molecular weight products (Figure 6). In all four cases, the extent of chaperone release mirrors the extent of SafA polymer formation and is in agreement with the order of DSE efficiencies observed with the Nte peptides, with the observed order of chaperone displacement $\text{wt} \gg V13A > I15A \gg F17A$. Therefore, these experiments carried out with the full-length protein yield the same conclusions drawn from the experiments carried out with the peptide variants, validating the use of A$_{\text{Nte}}$ peptides to model this reaction.

**Molecular Mechanism of DSE In Vivo**

The results presented here demonstrate that DSE occurs via the formation of a transient ternary chaperone-subunit-subunit complex, consistent with the zip-in-zip-out mechanism originally proposed by Zavialov et al. (2003). We show, in addition, by using X-ray crystallography and real-time ESI-MS experiments, that DSE critically involves an interaction between the P5 pocket of the subunit already assembled and the P5 residue of the Nte of the incoming subunit and also that DSE ends with the insertion of the P* residue of the Nte of the incoming subunit in the P* pocket of the subunit already assembled (Figure 7A).

In vivo, chaperone-subunit complexes are assembled into fibers at the outer membrane usher. Complexes of the usher with various intermediates in type I and P pilus assembly invariably show the presence of the chaperone attached to the growing fiber (Saulino et al., 1998, 2000; Ng et al., 2004), suggesting that the chaperone of the last incorporated subunit does not dissociate.

---

**Figure 6. In Vitro DSE of Full-Length SafB-SafA Complexes**

Native PAGE showing release of SafB, dissociation of the SafB-SafA complex, and polymerization of wt and mutants (SafB-SafA$_{\text{F17A}}$, SafB-SafA$_{\text{I15A}}$ and SafB-SafA$_{\text{V13A}}$) subunit-chaperone complexes after 1 hr (after elution of the SafB-safA complexes off the Phenyl-Sepharose column [time 0], there is a 1 hr dead time for buffer exchange and concentration before the samples can be examined by native PAGE) and 24 hr incubations. In all experiments, the full-length SafA protein was used. SafA polymers migrate as a smear of higher molecular weight species that just enter the gels. Unspecific release of SafB was monitored with the nonpolymerizing SafB-SafA$_{\text{Ntd2}}$ complex (C) as a control. For the F17A mutant, a second species with electrophoretic mobility close to the SafB-SafA complex was observed (indicated with an asterisk [*]). The identity of this species is unclear.

**Figure 7. Model for the Mechanism of DSE In Vitro and In Vivo**

(A) Schematic representation of DSE in vitro. Chaperone and subunit are labeled (i) and (ii), respectively. In the chaperone, strands G$_1$ and F$_1$ are represented as solid black lines. In the subunit, strand F, which directly interacts with the G$_1$ donor strand, is depicted in blue. An incoming Nte (depicted in red) forms a ternary complex with the chaperone-subunit complex at the P5 pocket (indicated by a thicker line). DSE then proceeds and terminates by dissociation of the chaperone-subunit complex and insertion of the P* residue in the P* pocket.

(B) Schematic representation of a single incorporation cycle at the usher (see text). Chaperone and usher are colored gray and light blue, respectively. For clarity, subunits are differentiated by color (yellow, red, green, orange, and blue), with the last incorporated subunit in orange and the incoming subunit in blue. The N-terminal and C-terminal domains of the usher are indicated.
from the base of the fiber. These observations also favor a mechanism in which DSE occurs concomitantly with the release of the chaperone rather than a stepwise model in which DSE requires the release of the chaperone before the Nte of an incoming chaperone-subunit can bind in the groove of the subunit at the base of the growing fiber. We propose, therefore, that the mechanisms of DSE both in vitro, as demonstrated here, and in vivo at the usher occur in a similar manner. In the absence of the usher, DSE is typically a slow process occurring over several hours (see for example, Figure 4). By contrast, in vivo formation of pili at the usher occurs in minutes (Jacob-Dubuisson et al., 1994), a feat that may be accomplished by the usher binding and orientating incoming chaperone-subunit complexes in order to create a proximity effect, thus accelerating the DSE reaction. Related studies suggest the presence of two distinct chaperone-subunit binding sites at the usher. For example, copurification studies with the FimD usher have demonstrated that chaperone-subunit complexes can form stable interactions with a C-terminal 40 kDa fragment of the usher (Saulino et al., 1998). In addition, a periplasmic N-terminal domain in the usher has been identified that binds the chaperone-subunit complexes and retains the differential affinity toward specific chaperone-subunit complexes seen in the intact usher (Thanassi et al., 2002; Nishiyama et al., 2003, 2005; Ng et al., 2004). These results have led to the hypothesis that the usher exposes two chaperone-subunit binding platforms. At the first site, the last incorporated chaperone-subunit complex at the base of the growing fiber is accommodated; the second interaction site would then bind incoming chaperone-subunit complexes (Ng et al., 2004).

A schematic diagram for the hypothetical mechanism of DSE at the usher is shown in Figure 7. This scheme incorporates the zip-in-zip-out model for DSE suggested here (Figure 7A), together with current knowledge of the role of the usher in this reaction (Figure 7B). At the start of a new cycle of subunit incorporation, the growing fiber, with the chaperone of the most recently added subunit at the base of the nascent pilus, is accommodated in the C-terminal binding site of the usher. Next (step 1), the usher N-terminal domain solicits a chaperone-subunit complex from the periplasmic pool and orients it (step 2) so that the incoming subunit’s Nte is ideally positioned to initiate the DSE reaction with the subunit at the fiber base (a potential model of this is shown in Figure S4). The DSE reaction could then proceed as demonstrated here in vitro, with the Nte initiating assembly by interaction with the P5 pocket, proximal to the incoming complex, and continuing in a zip-in-zip-out process to displace the chaperone from the base of the fiber (step 3). The usher could potentially further catalyze this process by interacting with the chaperone G1 strand to destabilize the donor-strand complementation interaction and facilitate the release of the donor strand. Upon completion of the DSE reaction, the chaperone of the now penultimate subunit is displaced from the base of the fiber and is released into the periplasm (step 4). This liberates the C-terminal chaperone-subunit recognition site that now becomes available to bind the newly incorporated chaperone subunit still associated with the N-terminal domain. A difference in affinity for chaperone-subunit complexes could then ensure that the newly incorporated chaperone subunit is shifted from the N-terminal to the C-terminal recognition site (step 5), liberating the former for a new round of the cycle (step 6). While further structural, thermodynamic, and kinetic data are required to confirm the model presented, the mechanism proposed is able to combine elegantly all of the current biological and biochemical data on pilus assembly with the insights into the mechanism of DSE in vitro elucidated here.

β Strand Insertions/Displacements in Other Biological Processes

The impact of this work may extend beyond the study of the mechanism of pilus biogenesis by the chaperone-usher pathway to other biological processes involving β strand displacement and/or insertion. For example, serine proteases are regulated by formation of dead-end complexes with serpins, a process that involves insertion of the inhibitory loop into the serpin’s β sheet structure (Ye and Goldsmith, 2001). Formation of amyloid fibrils could also be mediated by a strand swap zipper mechanism similar to that demonstrated here for DSE. Here, polymerization involves the assembly of protein subunits or peptides into fibrils extending to micrometers in length, the molecular basis of which is the sequential insertion/juxtaposition of a β strand into a central β sheet of subunits already assembled into a fibril. This possibility is particularly appealing for amyloid fibrils formed from β sheet containing proteins, such as β2-microglobulin (β2-m) or antibody light chains, which also have a native Ig fold, as well as other β sheet containing proteins associated with protein polymerization disorders (Chow et al., 2004). For β2-m, for example, we have recently shown that extension of amyloid fibrils at neutral pH involves assembly of native-like subunits in a mechanism that proceeds most rapidly in variants in which the N-terminal strand is destabilized and could thereby be available to zip into the growing fibril (Jahn et al., 2006).

The mechanism determined here for DSE in pilus assembly proposes that an initiation point is essential for the zipper mechanism to start: in the case of pilus biogenesis, this is provided by an unoccupied or transiently occupied P5 pocket. The identification of initiation sites in other systems will not only present strong evidence for a generic role of this mechanism in other protein assembly mechanisms but also provide a handle for biomedical intervention in the wealth of disorders involving bacterial invasion, protein polymerization, or amyloid formation.
subunit that do not form part of the pilin Ig domain (sequence GSFPLNSEQQKDVFYVSSMGWEL for the wt ANte peptide [underlined residues indicate the positions 3, 13, 15, and 17, which were alternatively mutated to Ala in the F3A, V13A, I15A, and F17A ANte peptide variants; purchased from Severn Biotech, UK, and QCB, USA]). Peptide stocks at 5 mM were dialyzed against 5 mM ammonium acetate pH 5.5. In vitro DSE experiments were performed by incubating 40 μM SafB-SafANtd2 complex with a 0-, 1-, 2-, 5-, 10-, or 20-fold molar excess of the peptide in 50 mM Tris-HCl pH 8.0 at room temperature. For the reverse DSE reaction, 25 μM of purified SafANtd2-ANte complex containing wt or F3A ANte peptide was incubated with a 0- or 1-fold molar equivalent of purified SafB in 50 mM ammonium acetate pH 5.5 or Tris-HCl pH 8.0 at room temperature. Completion of the forward and reverse DSE reactions was monitored after 24 hr on 8% native Tris-Glycine polyacrylamide gels stained with SimplyBlue stain (Invitrogen). In the full-length protein DSE experiments, wt and mutant SafB-SafA complexes (SafB-SafA114F, SafA-SafA114M) were allowed to polymerize during a 24 hr incubation at room temperature in 50 mM Tris-HCl pH 8.0 at a 50 μM protein concentration. SafB release was monitored on 8% native Tris-Glycine polyacrylamide gels as above. Unspecific release of SafB was monitored by using the nonpolymerizing SafB-SafANtd2 complex as a control.

ESI-MS

Equimolar quantities of SafB-SafANtd2 and wt ANte peptide (final concentrations = 50 μM) were incubated at 25°C in 10 mM ammonium acetate pH 5.5. The reactions were sampled from an initial time point of 1–2 min to a maximum period of 92 hr. Similar experiments were performed with the F3A, V13A, I15A, and F17A complexes at various times. For details of the parameters used, and also the tentative noncovalent interactions when the complexes were transferred into the gas phase. For our experiments, each purified complex was incubated in water bath for 24 hr on 8% native Tris-Glycine polyacrylamide gels stained with SimplyBlue stain (Invitrogen). One aliquot of reaction mixture and subsequent analysis by nano-electrospray ionization mass spectrometry (nanoESI-MS). Each individual aliquot was placed in a gold-plated borosilicate capillary that was then positioned in the nano-ESI source of a Q-Tof I mass spectrometer (Waters Corp./Micromass UK Ltd, Manchester, UK) for analysis. The mass spectrometer parameters were optimized so as to maintain noncovalent interactions when the complexes were transferred into the gas phase. For details of the parameters used, and also the data processing and normalization procedures employed, see the Supplemental Data.

The thermal stability of different SafANtd2-ANte complexes was assessed by incubating each purified complex in a water bath for 15 min at different temperatures (over the range 25°C–80°C) before ESI-MS analysis. This allowed the relative amount of complex remaining intact at each temperature to be measured.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, four figures, and one table and can be found with this article online at http://www.molecule.org/cgi/content/full/22/6/831/DC1/.

Acknowledgments

We thank staff at the ID14-4, ID23-1, ID29, and ID23-2 beam lines at the European Synchrotron Radiation Facility for access and help. This work was funded by Medical Research Council (MRC) grant G0203052 to P.A.-D. The Q-ToF was purchased with funds from HEFCE, the University of Leeds, and Waters/Micromass UK Ltd. P.J.R. is a Biotechnology and Biological Sciences Research Council (BBSRC)/Co-operative Awards in Science and Engineering (CASE) student; S.E.R. is a BBSRC Professorial Fellow.

Received: January 5, 2006
Revised: April 19, 2006
Accepted: May 30, 2006
Published: June 22, 2006

References


Accession Numbers

Coordinates for all the structures described above have been submitted to the PDB (entry codes 2co3, 2co6, 2co7, 2co4, 2co1, 2cny, 2cnz, and 2co2 for the Sâ£B-SÅ£AÅœ11 dimer, Sâ£B-SÅ£AÅœ2 crystal form I, Sâ£B-SÅ£AÅœ2 crystal form II, SÅ£AÅœ2 bound to wild-type, F17Å£, I15Å£, V13Å£, and F3Å£ Åœ14 peptide variants, respectively).