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The Solution Structure of a Domain from the *Neisseria meningitidis* Lipoprotein PilP Reveals a New β-Sandwich Fold

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Type IV pili are long, thin fibres, which extend from the surface of the bacterial pathogen *Neisseria meningitidis*; they play a key role in adhesion and colonisation of host cells. PilP is a lipoprotein, suggested to be involved in the assembly and stabilization of an outer membrane protein, PilQ, which is required for pilus formation. Here we describe the expression of a recombinant fragment of PilP, spanning residues 20 to 181, and determination of the solution structure of a folded domain, spanning residues 85 to 163, by NMR. The N-terminal third of the protein, from residues 20 to 84, is apparently unfolded. Protease digestion yielded a 113 residue fragment that contained the folded domain. The domain adopts a simple β-sandwich type fold, consisting of a three-stranded β-sheet packed against a four-stranded β-sheet. There is also a short segment of 3_{10} helix at the N-terminal part of the folded domain. We were unable to identify any other proteins that are closely related in structure to the PilP domain, although the fold appears to be distantly related to the lipocalin family. Over 40 homologues of PilP have been identified in Gram-negative bacteria and the majority of conserved residues lie within the folded domain. The fourth β-strand and adjacent loop regions contain a high proportion of conserved residues, including three glycine residues, which seem to play a role in linking the two β-sheets. The two β-sheets pack together to form a crevice, lined with conserved hydrophobic residues: we suggest that this feature could act as a binding site for a small ligand. The results show that PilP and its homologues have a conserved, folded domain at the C-terminal end of the protein that may be involved in mediating binding to hydrophobic ligands.

Keywords: lipoprotein; secretin; pilus biogenesis; *Neisseria meningitidis*; NMR

Introduction

The bacterium *Neisseria meningitidis* causes meningitis and septicaemia, and is a significiant public health problem in developed and developing countries. Type IV pili play a pivotal role in the pathogenesis of this bacterium: they are responsible for adhesion to host cell surface receptors and therefore play a crucial role in colonisation and infection. Type IV pili consist of long (>1–5 μm), thin (60–70 Å), mechanically strong polymeric fibres, which extend from the surfaces of many pathogenic Gram-negative bacteria, including meningococci.[1–3] They contain tip adhesins, which mediate cellular attachment to epithelial tissue receptors[3] and therefore have a crucial role in the colonisation of meningococci.[4] They have also been shown to be involved in several other bacterial processes,

Abbreviations used: $PilP^{(20-181)}$, recombinant fragment of PilP containing residues 20–181; $PilP^{(69-181)}$, C-terminal fragment of PilP (residues 69–181); NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; HSQC, heteronuclear single quantum coherence.

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including bacterial auto-agglutination,[5] variation of target tissue specificity[6] and natural competence for transformation of DNA.[7]

At least 15 separate proteins are known to be required for pilus formation in *N. meningitidis*.[8] One of these is PilQ, an outer membrane protein and member of the GSP secretin superfamily, members of which translocate a variety of macromolecules across the outer membrane.[9–11] Expression of PilQ is mandatory for pilus formation[12] and the assembled protein is believed to form a portal or gateway to permit passage of the pilus fibre across the outer membrane. Members of the secretin superfamily share significant homology within their C-terminal sequences; notable members include PulD from *Klebsiella oxytoca*, which is a component of the type II secretion system,[13] and secretins from type III secretion systems, YscC from *Yersinia enterocolitica*[14] and MxiD from *Shigella flexneri*.[15] Secretins are expressed in the cytoplasm, exported across the inner membrane and a signal sequence cleaved from the N-terminus; assembly of PilQ in the outer membrane is promoted by Omp85[16] and PilW.[8] Electron microscopy studies have shown that the secretins form large multimeric structures; for example, meningococcal PilQ has a subunit molecular mass of 80 kDa[11] and assembles into a large "lantern-like" structure with *C*12 quasi-symmetry.[17] Similar studies on other secretins have shown *C*12[13] and *C*14 symmetry.[18] The process of secretin oligomerization within the periplasm is dependent on auxiliary lipoproteins that are associated with each secretin.[19] These proteins are generally small lipoproteins that are also exported with a signal sequence, which is subsequently cleaved. A conserved cysteine residue at the N terminus is esterified by the addition of a fatty acid, which anchors the protein to the membrane.[20] The cognate lipoprotein for *Neisseria*l PilQ is PilP, a protein of about 18 kDa predicted molecular mass, after cleavage of its signal sequence. A PilP mutant of *Neisseria gonorrohoeae* is non-piliated and shows reduced levels of PilQ multimer formation.[21] PilP is therefore presumed to be involved in the assembly or stability of the PilQ secretin: without complete PilQ assembly, pili are not correctly formed.

To date there is only one report of the determination of the 3D structure of an auxiliary/pilot protein: MxiM from *S. flexneri.*[22] MxiM promotes the assembly of the secretin MxiD, a component of type III secretion system needle complex.[23,24] The crystal structure of MxiM shows a ten-stranded discontinuous or "cracked" barrel structure, with an α-helix interrupting the regular β-barrel. The protein forms a linear hydrophobic groove, capable of binding lipid ligands.[22] Binding of a peptide fragment, derived from the MxiD secretin, was shown to compete with lipid binding to MxiM: Lario *et al.* proposed that this feature could permit release of MxiD upon interaction of MxiM with outer membrane phospholipid.[22] Although there are some functional similarities between MxiM from *S. flexneri* and neisserial PilP, there is no significant sequence homology between the two proteins. In order to investigate the structural basis for PilP function and to compare its structure with MxiM, we have determined the structure of a folded fragment of PilP by NMR. The results show that meningococcal PilP adopts a three plus four stranded β-sandwich fold that is different from MxiM, although both proteins appear to have a hydrophobic pocket that could act as a binding site.

Results and Discussion

Identification of the folded domain of PilP

Previous work by Koomey and co-workers on PilP from *N. gonorrhoeae* has shown that the protein is covalently attached to a lipid moiety.[21] A Prosite scan of the PilP leader sequence region between Leu6 and Cys16 predicted that this is a prokaryotic lipid attachment site (PS00013), indicating that 15 amino acid residues of the leader sequence are removed by a specific lipoprotein signal peptidase (signal peptidase II) and then a lipid is attached to Cys16 at the N terminus.[25] Our initial expression construct therefore removed residues 1–19 from the coding sequence, including the Cys residue at position 16, which forms a thioester bond to the fatty acid in the mature lipoprotein, to give a recombinant protein ($PilP^{(20\text{-}181)}$) that contained 162 residues from PilP. The 2D NMR ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) spectrum of this recombinant protein, analysed at pH 6.1, shows evidence for a folded domain, as well as a significant proportion of flexible residues indicated by the sharp, poorly dispersed signals (Figure 1(a)). The poorly dispersed signals mainly disappear in the spectrum of the same sample at high pH (8.5) due to increased rates of amide proton exchange (Figure 1(a)), significantly simplifying the spectrum and suggesting that the corresponding regions are indeed flexible and exposed to the solvent. The flexible residues corroborate with the high degree of disorder predicted for this region (data not shown). The signals that disappear at higher pH were shown, after examination of the final structure, to coincide with exposed loop regions (Figure 2). Interestingly, the majority of the chemical shifts from residues in the folded domain were invariant between pH 6.1 and 8.5 (Figure 1(a)), suggesting that the structure of the folded domain does not change, and the bulk of the residues in the folded domain are well protected from exchange with the solvent. Conveniently, this property allowed the assignment of NMR signals at high pH for long, consecutive stretches of the sequence without the interference of strong signals from the flexible regions. However, missing data for the loop regions at high pH (Figure 2) made structure calculation impractical, so the process of signal assignment was repeated at lower pH, where the bulk of assignments for the residues within the folded domain were preserved.