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PEPTIDES INHIBIT COMPLEXATION OF THE BACTERIAL CHAPERONE PAPD AND REVEAL POTENTIAL TO BLOCK ASSEMBLY OF VIRULENCE ASSOCIATED PILI

Katarina Flemmer[a], Zheng Xu[b], Jerome S. Pinkner[b], Scott J. Hultgren[b] and Jan Kihlberg[a]*

[a]*Organic Chemistry 2, Chemical Center, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden, and* [b]*Department of Molecular Microbiology, Box 8230, Washington University School of Medicine, St Louis, MO 63110-1093, USA.*

Abstract: Peptides as small as octamers were found to inhibit protein-protein complex formation between the PapD chaperone and the pilus adhesin PapG. The intermolecular hydrogen bonding pattern in the crystalline complex of PapD with a peptide from PapG was revealed to be important also for complex formation in solution. The role of the peptide side chains was indicated to be slightly different as compared to in the crystal.

Introduction

Attachment of bacteria to host cells is a key event in the development of infections since it allows the pathogens to avoid elimination by flows of cleansing body fluids. Most gram-negative pathogens produce long hairlike extracellular protein appendages called pili which mediate specific attachment to glycolipids and glycoproteins located in cell membranes.[1,2] Uropathogenic *E. coli* assemble virulence associated P pili which present the adhesin PapG for attachment to the disaccharide α-D-galactopyranosyl-(1-4)-β-D-galactopyranose [Galα(1-4)Gal] present in glycolipids on kidney epithelium cells.[3,4] P pili are supramolecular protein structures which consist of six different types of subunits assembled into the pilus under the influence of the molecular chaperone PapD.[1,2] The PapD chaperone forms complexes with the pilus subunits in the bacterial periplasm thereby capping interactive surfaces on the subunits, preventing non-productive subunit aggregation, and inducing proper folding of the subunits.

Over 20 periplasmic chaperones which assemble pili in pathogenic bacteria, such as *E. coli*, *K. pneumoniae*, and *H. influenzae*, have been shown to have significant homology to PapD[5], suggesting that they have structures similar to that of PapD[6]. A few conserved residues in the chaperone family, such as the invariant Arg^8 and Lys^{112} located in the cleft between the two domains of PapD, have no apparent structural function[5] and were shown to be required for PapD to bind to pilus subunits and mediate pilus assembly *in vivo*.[7,8] Thus, the subunit binding site of PapD-like chaperones is highly conserved. A characteristic feature of a large number of pilus proteins is their conserved *C*-terminus. Using synthetic peptides we revealed that this conserved region is essential for complex formation of the P pilus subunits with PapD.[8] Furthermore, the crystal structure of PapD bound to the *C*-terminal 19-mer peptide from PapG (G1'-19') was determined at 3.0 Å resolution thereby providing the first "snapshot" of the interaction of a chaperone with a pilus subunit (Figure 1).[8,9] Interestingly, in the crystal the *C*-terminus of the peptide was anchored to the invariant cleft residues Arg^8 and Lys^{112} which were found to be critical for chaperone function. The amino acids in G1'-19' and

related peptides in the present paper were numbered from the *C*-terminus towards the *N*-terminus, thereby maintaining the *C*-terminal proline in PapG as a point of reference at 1'.

Based on the crystal structure of the PapD - G1'-19' complex the following model was proposed[8] for the initial steps in the binding of peptides and pilus subunits to PapD; [1)]the *C*-terminal carboxylate group of the polypeptide is anchored by critical hydrogen and ionic bonding to the invariant Arg^{8} and Lys^{112}, [2)]hydrogen bonding occurs between the eight *C*-terminal polypeptide residues and the G1 strand in PapD to create a parallel β-strand interaction, and [3)]hydrophobic and van der Waals interactions are formed between the four nonpolar side chains of residues 2', 4', 6' and 8' in the polypeptide and complementary surfaces on PapD. The hydrophobic interactions were proposed to provide part of the explanation for the specificity of PapD for pilus subunits and related peptides. However, in the crystal, the β-sheet composed of part of PapD and its bound peptide is extended as a second PapD-peptide complex is placed adjacent to the first so that the two bound peptides interact as antiparallel β-strands (Figure 1). The dimerization might to some extent have influenced the structural details of the PapD - peptide complex in the crystal, and prompted further investigations of complex formation in solution under physiological conditions. In addition, it was important to establish if peptides are able to inhibit complex formation between PapD and pilus subunits, and potentially also pilus assembly.

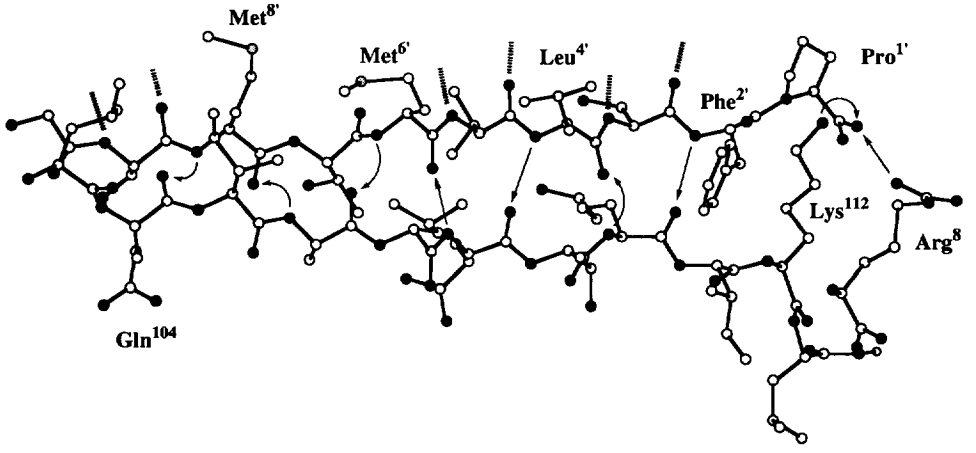

Figure 1. Part of the crystal structure of the PapD chaperone bound to the peptide G1'-19' derived from the *C*-terminus of the pilus adhesin PapG.[8,9] The *C*-terminal 10 amino acids of G1'-19' ($Gly^{10'}$-Ser-Met-Thr-Met-Val-Leu-Ser-Phe-$Pro^{1'}$) that interact with Arg^{8} and with residues Gln^{104}-Lys^{112} in the G1 β-strand of PapD are shown with intermolecular hydrogen bonds indicated with arrows. Hydrogen bonding between G1'-19' and another PapD - G1'-19' complex placed adjacent to the first in the crystal is indicated with dotted lines. Hydrogen atoms have been omitted for clarity, oxygen atoms are black, whereas nitrogen and sulfur atoms are dark and light grey, respectively.

Results and Discussion

Three series of peptides corresponding to the *C*-terminus of PapG; a length series, a substitution series and a deletion series (Figure 2), were prepared in order to investigate the submolecular details of the interactions between the chaperone PapD and polypeptides. The peptides were synthesized in an automatic peptide synthesizer, or by multiple peptide synthesis techniques, using the Fmoc solid-phase strategy and were then purified by reversed-phase HPLC.[10] Evaluation was performed in an enzyme linked imunosorbent assay (ELISA) using the peptides as inhibitors of complex formation between PapD and a fusion protein containing the 140 *C*-terminal amino acids of PapG linked to the Maltose Binding Protein (MaltBP-G140).[11,12] The complex between PapD and the *C*-terminal PapG truncate in MaltBP-G140 has been shown to be as stable as the native PapD-PapG complex[11], arguing that PapD - MaltBP-G140 complex formation is a relevant model for the native PapD - PapG complex. This is in good agreement with previous observations showing the PapG *C*-terminus to mediate complex formation with PapD[13], and the *N*-terminus to be responsible for binding to the Galα(1-4)Gal disaccharide moiety[13,14].

The **length series** consisted of peptides derived from the *C*-terminus of PapG ranging in length from G1'-6' to G1'-19'. Evaluation of the peptides in this series revealed a low inhibitory power[15] for the short G1'-6' and G1'-7', a significant, 3-4 fold increase in inhibition for the octamer G1'-8' but no further increase in inhibition for peptides longer than eight amino acids (Figure 2a). Importantly, this showed that peptides as small as octamers are able to inhibit complex formation between the PapD chaperone and pilus subunits. In addition, the data supported that the hydrogen bonding pattern between PapD and G1'-19' seen in the crystal occurred also in solution. The almost equal inhibitory power displayed by G1'-6' and G1'-7' can be explained by hydrogen bonding of $Pro^{1'}$, $Phe^{2'}$, $Leu^{4'}$, and $Met^{6'}$ to PapD, and by lack of important contacts between $Thr^{7'}$ and PapD, as observed in the crystal (Figure 1). An additional two hydrogen bonds are formed by $Met^{8'}$ of the more potent inhibitor G1'-8', but since hydrogen bonding in the crystal breaks after $Met^{8'}$ no further increase in inhibitory power was expected for the longer peptides.

Since the octamer G1'-8' was the shortest peptide that retained the inhibitory power of the longer peptides the roles of the side chains of this PapG fragment in binding to PapD were probed in detail. In the crystal the hydrophobic $Phe^{2'}$, $Leu^{4'}$, $Met^{6'}$ and $Met^{8'}$ of G1'-19' make important contacts with PapD.[8] Interestingly, these residues are part of a conserved pattern of alternating hydrophobic and hydrophilic residues characteristic for pilus subunits.[8] In the **substitution series** the hydrophobic residues in G1'-8' were replaced, one by one, with the polar serine, whereas $Ser^{3'}$ and $Thr^{7'}$ were replaced by alanine. Examination of the inhibitory powers of the peptides in the substitution series indicated that residues $Leu^{4'}$, $Val^{5'}$ and $Met^{6'}$ in G1'-8' are essential for interactions with the chaperone PapD, whereas residues 2' and 8' are less important than expected from the crystal structure (Figure 2b).

The model for binding of polypeptides to PapD is based on critical anchoring of the *C*-terminal carboxylate group of the peptide to PapD, followed by "zippering" of the peptide to PapD by hydrophobic interactions and hydrogen bonds. The **deletion series** of G1'-8' analogues, in which the residues in G1'-9' were successively deleted, one by one, was used to probe the importance of the anchoring in relation to the role of the side chains. For the deletion series, *C*-terminal anchoring followed by zippering was expected to result in successively increasing inhibitory powers as the deletion was moved from the *C*-terminus of the peptide towards the *N*-terminus. As shown in Figure 2c this expectation was not confirmed on evaluation of the deletion series. Instead the peptides that had the lowest inhibitory powers were those lacking residues $Leu^{4'}$,

Val$^{5'}$ and Met$^{6'}$. This result may be explained by a less critical role for *C*-terminal anchoring of the peptide to PapD and by the fact that the side chains of residues Leu$^{4'}$, Val$^{5'}$ and Met$^{6'}$ provide the most important contacts with PapD. In the PapD - G1'-19' complex the side chain of Thr$^{7'}$ is adjacent to a hydrophobic patch on PapD. Hydrophobic interactions between Met$^{8'}$ in G1'-9'/-T$^{7'}$ and this patch may explain the high inhibitory power displayed by G1'-9'/-T$^{7'}$.

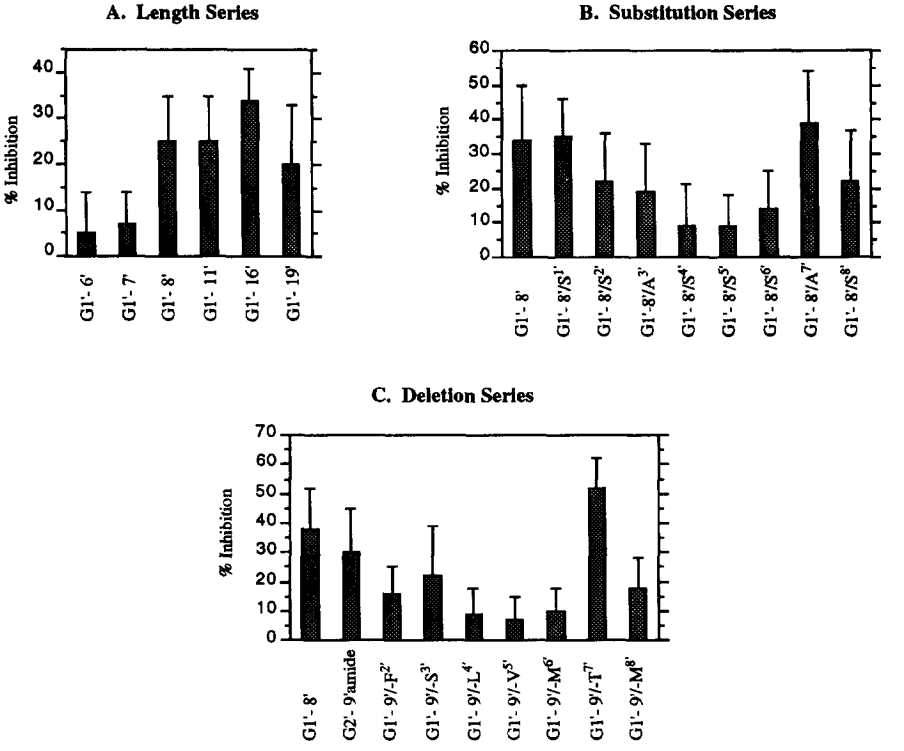

Figure 2. Inhibition of the binding of the PapD chaperone to the MaltBP-G140 fusion protein with the peptide G1'-19' from the *C*-terminus of the pilus adhesin PapG (Gly$^{19'}$-Lys-Arg-Lys-Pro-Gly-Glu-Leu-Ser-Gly-Ser-Met-Thr-Met-Val-Leu-Ser-Phe-Pro$^{1'}$) and shorter analogues.[12,15] The inhibitory powers were determined using identical concentrations for all peptides. **A:** Inhibition with a **length series** of *C*-terminal PapG peptides ranging from the hexamer G1'-6' to the 19-mer G1'-19'. **B:** Inhibition with G1'-8' and a **substitution series** of analogues having single Ser and Ala substitutions (*e.g.* G1'-8'/S$^{2'}$ represents G1'-8' in which Phe$^{2'}$ has been substituted by a Ser). **C:** Inhibition with G1'-8' and a **deletion series** of analogues obtained by deletion, one by one, of the residues in G1'-9' (*e.g.* G1'-9'/-F$^{2'}$ represents G1'-9' in which Phe$^{2'}$ has been deleted).

In conclusion, peptides as small as octamers were found to inhibit protein-protein complex formation between the PapD chaperone and the pilus adhesin PapG. Furthermore, the intermolecular hydrogen bonding pattern occurring in the crystalline complex between PapD and a *C*-terminal peptide from the adhesin PapG was

revealed to be important also for complex formation in solution. The roles of the side chains of the peptide in complex formation was indicated to be slightly different in solution as compared to the situation in the crystal. These results suggest that low molecular weight compounds which bind to bacterial chaperones could function as pilus assembly inhibitors *in vivo*. Design and synthesis of such inhibitors, which constitute potential novel antibiotics, is now in progress in our laboratories.

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9. The crystal structure of the complex between PapD and the peptide K1'-19' from the pilus adaptor protein PapK was recently solved and displayed almost identical intermolecular interactions as found in the PapD - G1'-19' complex (Ogg, D. J., results to be published).
10. Peptide Synthesis: The peptides were synthesized as *C*-terminal carboxylic acids using the 9-fluorenylmethoxycarbonyl (Fmoc) solid phase synthesis strategy, except for G2'-9'amide which was prepared as a *C*-terminal amide. Peptides having a *C*-terminal carboxyl group were prepared on a polystyrene-based 2-chlorotritylchloride resin (Novabiochem, Switzerland) to which Fmoc-Pro-OH was coupled to give a loading of 0.6 mmol/g (Barlos, K. *Int. J. Pept. Protein Res.* **1991**, *37*, 513). The peptide G2'-9'amide was syntesized on an aminomethylated polystyrene resin functionalized with the Rink linker, [*p*-[α-[(9-fluorenylmethoxy)formamido]-2,4-dimethoxybenzyl]phenoxy]acetic acid (Novabiochem, Switzerland).

    Peptides belonging to the length series were synthesized using a fully automated continuous flow peptide synthesizer constructed in our laboratory essentially as described previously by others (Cameron, L. R.; Holder, J. L.; Meldal, M.; Sheppard, R. C. *J. Chem. Soc. Perkin Trans. I* **1988**, 2895-2901). In the synthesizer N^{α}-Fmoc amino acids (Bachem Feinchemikalien AG, Switzerland) with standard side chain protective groups were activated in DMF by 1,3-diisopropylcarbodiimide mediated conversion to benzotriazolyl (HOBt) esters and couplings were monitored spectrophotmetrically using bromophenol blue.

Peptides belonging to the deletion and substituiton series were synthesized by multiple peptide synthesis in a simple, manually operated apparatus (Holm, A.; Meldal, M. *Peptides 1988, Proc. Eur. Pept. Symp., 20th*; Jung, G.; Bayer, E., Eds.; de Gruyter: Berlin, 1989; 208-210). N^{α}-Fmoc amino acid pentafluorophenyl esters (Bachem Feinchemikalien AG, Switzerland) with standard side chain protective groups were used in the syntheses.

For all peptides Fmoc-deprotection was affected with 20% piperidine in DMF whereas cleavage from the resin and side chain deprotection was performed using trifluoroacetic acid and cation scavengers. The peptides were purified to more than 95% homogeneity by reversed-phase high-performance liquid chromatography (HPLC). Structures were confirmed by fast atom bombardment mass spectroscopy (FAB-MS) and by amino acid analysis for G1'-8', G1'-11', G1'-16' and G1'-19'.

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12. PapD-peptide inhibition ELISA: Microtiter wells (Nunc-Immuno Plate Maxisorp) were coated overnight at 4 °C with 50 µl of the Maltose Binding Protein - PapG1'-140' fusion protein at 5 pmol/50 µl in phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 8.3 mM phosphate, pH 7.4). The solutions in the plates were discarded, and the wells were blocked with 200 µl of 3 percent bovine serum albumin (BSA, Sigma) in PBS for 2-4 h at 25 °C. The plates were washed vigorously three times with PBS. Each test peptide (3.1 nmol, 5 µl of a 0.625 nmol/µl solution in DMSO) was preincubated with 125 pmol PapD in 5 µL KMES buffer for 30 min. The PapD-peptide solution was diluted 50 times with PBS containing 3 % BSA, 50 µl of the solution was added to the coated wells and incubated at 25 °C for 45 min. After three washings with PBS, the wells were incubated with 50 µL of a 1:500 dilution of rabbit antiserum to PapD in 3 % BSA-PBS for 45 min at 25 °C. After three washings with PBS, the wells were incubated with 50 µL of a 1:1000 dilution of goat antiserum to rabbit IgG coupled to alkaline phosphatase (Sigma) in 3 % BSA-PBS for 45 min at 25 °C. After three washings with PBS and three washings with developing buffer (10 mM diethanolamine, pH 9.5, 0.5 mM $MgCl_2$), 50 µl of filtered *p*-nitrophenyl phosphate substrate (10 mg/ml, Sigma) in developing buffer was added; the reaction was incubated for 2 h in the dark at 25 °C, and the absorbance at 405 nm was read.

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15. The inhibitory powers (% inhibition) are means of 4 - 7 runs performed in triplicate. Data was subjected to analyses of variance with two sources of variation (inhibitor and run) and error bars represent 95% confidence limits.

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