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Conserved immunoglobulin-like features in a family of periplasmic pilus chaperones in bacteria

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Detailed structural analyses revealed a family of periplasmic chaperones in Gram-negative prokaryotes which are structurally and possibly evolutionarily related to the immunoglobulin superfamily and assist in the assembly of adhesive pili. The members of this family have similar structures consistent with the overall topology of an immunoglobulin fold. Seven pilus chaperone sequences from *Escherichia coli*, *Haemophilus influenzae* and *Klebsiella pneumoniae* were aligned and their consensus sequence was superimposed onto the known three-dimensional structure of PapD, a representative member of the family. The molecular details of the conserved and variable structural motifs in this family of periplasmic chaperones give important insight into their structure, function, mechanism of action and evolutionary relationship with the immunoglobulin superfamily.

Key words: crystal structure/immunoglobulin fold/pathogenesis/periplasmic chaperone/biogenesis of pili

Introduction

Most Gram-negative pathogenic and commensal bacteria assemble supramolecular structures on their surface that mediate attachment to specific receptors. The assembly of most adhesins into surface organelles called pili requires the function of specialized periplasmic chaperones. Understanding how pilus chaperones target protein protomers to specific assembly sites after their secretion across the cytoplasmic membrane is of great interest. PapD is the specific periplasmic chaperone required for the assembly of P pili (Hultgren *et al.*, 1989; Lindberg *et al.*, 1989). The crystal structure of PapD has revealed that it has the overall topology of an immunoglobulin fold (Holmgren and Brändén, 1989). The analysis of the structurally related features amongst seven periplasmic pilus chaperones has given important insight into their function, mechanism of action and evolutionary relationship with the immunoglobulin superfamily.

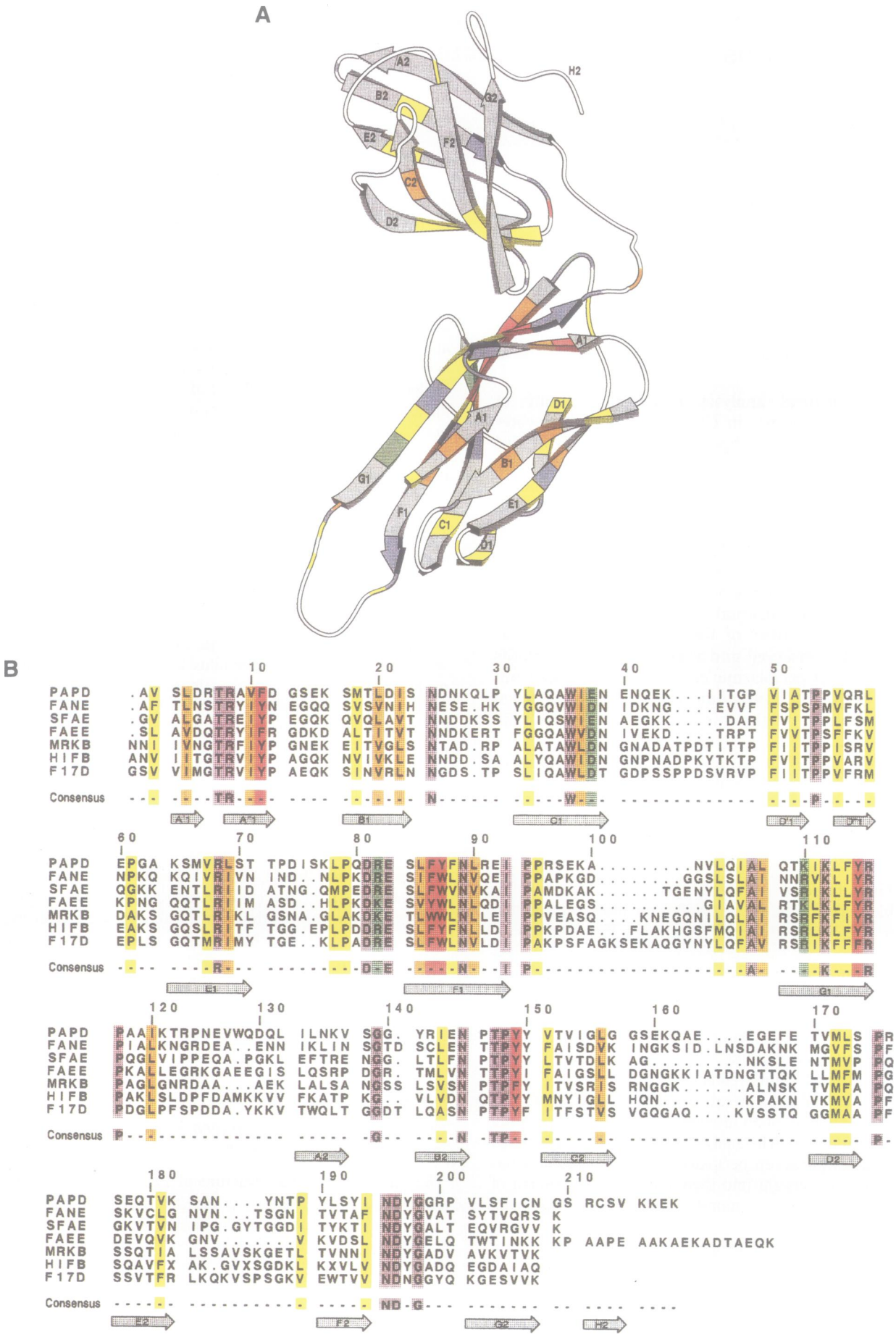
P pili, encoded by the *pap* operon, are composite structures consisting of a stalk and a thin adhesive tip fibrillum (Hultgren *et al.*, 1991; Kuehn *et al.*, in press). The stalk is composed of repeating PapA monomers probably arranged in a right handed helix (Brinton, 1965). The tip fibrillum is composed mostly of repeating subunits of PapE arranged in an open helical conformation with the α-D-galactopyranosyl-(1,4)-β-D-galactopyranose or Galα(1,4)Gal-binding adhesin, PapG, generally located at the distal ends of the tip fibrillae (Lindberg *et al.*, 1987; Lund *et al.*, 1987; Kuehn *et al.*, in press). The binding activity of PapG is thought to be an important virulence factor commonly associated with pyelonephritogenic *Escherichia coli* strains and is also frequently present in collections of cystitis and asymptomatic bacteruria strains (Hultgren *et al.*, 1986; Kallenius *et al.*, 1981; Leffler *et al.*, 1981). PapD modulates the assembly of both of the fibers which comprise the P pilus but is not part of the final structure (Hultgren *et al.*, 1989; Lindberg *et al.*, 1989). PapD binds to pilus proteins imported into the periplasmic space partitioning them into assembly competent complexes which prevents nonproductive aggregation of the subunits in the periplasm (Kuehn *et al.*, 1991). Chaperone-subunit complexes are then targeted to outer membrane assembly sites where the complexes are dissociated and the uncapped pilus subunits are polymerized to other pilus subunits correctly oriented in the pilus assembly site.

With the exception of the type IV class of pili, all other genetically well characterized pilus systems in Gram-negative prokaryotes contain a gene analogous to *papD* (Normark *et al.*, 1986; Hultgren *et al.*, 1991). *FanE*, *faeE*, *sfaE* and *f17-D* have been sequenced (Lintermans, 1990; Schmoll *et al.*, 1990; Bakker *et al.*, 1991) and encode pilus chaperones required for the assembly of K99, K88, S and F17 pili, respectively in *E.coli*. The assembly of *Klebsiella pneumoniae* type 3 pili and *Haemophilus influenzae* type b pili requires the *mrkb* and *hifB* gene products, respectively (Gerlach *et al.*, 1989; Allen *et al.*, 1991; A.Smith, L.Forney, M.Chanyangham, M.Kuehn, S.Lohrke, S.Moseley, S.Hultgren and T.Fsiker, in preparation). The structure–function relationships of all of these chaperones were analyzed using their amino acid sequences and information from the crystal structure of PapD. The results provided intriguing insight into the molecular intricacies that have been evolutionarily conserved in this class of proteins and suggested significant functional similarities to immunoglobulins.

Results

Structural relationship between PapD and immunoglobulin superfamily

The three-dimensional structure of PapD, the periplasmic protein that forms transient complexes with the adhesin and the other pilus subunit proteins (Hultgren *et al.*, 1989; Lindberg *et al.*, 1989), has been solved by Holmgren and Brändén to a resolution of 2.5 Å and a crystallographic R-factor of 19.7% (Holmgren and Brändén, 1989; see Figure 1A). PapD consists of two globular domains oriented towards one another in a way which gives the molecule the overall shape of a boomerang. Each domain is a β-barrel


A
A2
H2
B2
G2
E2
F2
C2
D2
A1
D1
A1
B1
G1
E1
F1
C1
D1
B
10
20
30
40
50
PAPD .AV SLDRTRAVFD GSEK SMTLDIS NDNKQLP YLAQAWIEN ENQEK...IITGP VIATPPVQRL
FANE .AF TLNSTRYIYN EGQQ SVSVNIH NESE.HK YGGQVWIDN IDKNG....EVVF FSPSPMVFKL
SFAE .GV ALGATREIYP EGQK QVQLAVT NNDDKSS YLIQSWIEN AEGKK.....DAR FVITPPLFSM
FAEE .SL AVDQTRYIFR GDKD ALTITVT NNDKERT FGGQAWVDN IVEKD....TRPT FVVTPSFFKV
MRKB NNI IVNGTRFIYP GNEK EITVGLS NTAD.RP ALATAWLDN GNADATPDTITTP FIITPPISRV
HIFB ANV IITGTRVIYP AGQK NVIVKLE NNDD.SA ALYQAWIDN GNPNADPKYTKTP FVITPPVARV
F17D GSV VIMGTRVIYP AEQK SINVRLN NGDS.TP SLIQAWLDT GDPSSPPDSVRVP FIITPPVFRM
Consensus
A1
A2
B1
C1
D1
E1
60
70
80
90
100
110
PAPD EPGA KSMVRLST TPDISKLPQDRE SLFYFNLRE I PPRSEKA.....NVLQIAL QTKIKLFYR
FANE NPKQ KQIVRIVN IND..NLPKDRE SIFWLNVQE I PPAPKGD.....GGSLSLAI NNRVKLIYR
SFAE QGKK ENTLRIID ATNG.QMPEDRE SLFWVNVKA I PAMDKAK.....TGENYLQFAI VSRIKLLYR
FAEE KPNG QQTLRIIM ASD..HLPKDKE SVYWLNLQD I PPALEGS.....GIAVAL RTKLKLFYR
MRKB DAKS GQTLRIKL GSNA.GLAKDKE TLWWLNLLE I PPVEASQ....KNEGQNILQLAI RSRFKFIYR
HIFB EAKS GQSLRITF TGG.EPLPDDRE SLFYFNLLD I PPKPDAE...FLAKHGSFMQIAI RSRLKLFYR
F17D EPLS GQTMRIMY TGE..KLPADRE SLFWLNVLD I PAKPSFAGKSEKAQGYNYLQFAV RSRIKFFFR
Consensus
E1
F1
G1
120
130
140
150
160
170
PAPD PAAIKTRPNEVWQDQL ILNKV SGG. YRIEN PTPYY VTVIGLG GSEKQAE....EGEFE TVMLS PR
FANE PIALKNGRDEA..ENN IKLIN SGTD SCLEN TTPYY FAISDVK INGKSID.LNSDAKNK MGVFS PF
SFAE PQGLVIPPEQA.PGKL EFTRE NGG. LTLFN PTPYY LTVTDLK AG.....NKSLE NTMVP PQ
FAEE PKALLEGRKGAEEGIS LQSRP DGR. TMLVN TTPYI FAIGSLL DGNGKKIATDNGTTQK LLMFM PG
MRKB PAGLGNRDAA...AEK LALSA NGSS LSVSN PTPFY ITVSRIS RNGGK.....ALNSK TVMFA PQ
HIFB PAKLSLDPFDAMKKVV FKATP KG.. VLVDN QTPYY MNYIGLL HQN.....KPAKN VKMVA PF
F17D PDGLPFSPDDA.YKKV TWQLT GGDT LQASN PTPYF ITFSTVS VGQGAQ....KVSSTQ GGMAA PF
Consensus
A2
B2
C2
D2
180
190
200
210
PAPD SEQTVK SAN....YNTP YLSYI NDYGGRP VLSFICN GS RCSV KKEK
FANE SKVCLG NVN...TSGNI TVTAF NDYGVAT SYTVQRS K
SFAE GKVTVN IPG.GYTGGDI TYKTI NDYGALT EQVRGVV K
FAEE DEVQVK GNV.....V KVDSL NDYGELQ TWTINKK KP AAPE AAKAEKADTAEQK
MRKB SSQTIA LSSAVSKGETL TVNNI NDYGADV AVKVTVK
HIFB SQAVFX AK.GVXSGDKL KXVLV NDYGADQ EGDAIAQ
F17D SSVTFR LKQKVSPSGKV EWTVV NDNGGYQ KGESVVK
Consensus
E2
F2
G2
H2

structure formed by two anti-parallel β pleated sheets and has a topology identical to an immunoglobulin fold. The C-terminal domain, domain 2, has structural features analagous to the second domain of the HIV receptor, CD4 (Ryu *et al.*, 1990; Wang *et al.*, 1990). In both of these immunoglobulin-like domains the upper sheet is comprised of three strands, E, B and A, and the lower sheet of four strands, G, F, C and D. In addition, the C-terminal domain of PapD also has a short eighth strand, H, which is linked to strand G by a disulfide bridge between cysteines 207 and 212. In the classical immunoglobulin fold of a constant domain, strand D belongs to the upper sheet (Williams and Barclay, 1988). The strand orders in these domains are D,E,B,A for the upper sheet and G,F,C for the lower sheet. In the variable domain of immunoglobulins, strand A is shared between the sheets so that the strand order is D,E,B,a1 and a2,G,F,C,C′,C″ (Williams and Barclay, 1988). Domain 1 of PapD is a different variation of the immunoglobulin domains. In domain 1 of PapD, both strands D and A are shared between the two sheets, giving the strand order d1,E,B,a1 and a2,G,F,C,d2, making the framework more similar to variable domains than to constant domains.

Consensus sequence of the periplasmic chaperone family

The structures of FanE, FaeE, SfaE, F17-D, HifB and MrkB were analyzed using their amino acid sequences and the known PapD crystal structure. All seven proteins were found to be 30−40% identical in sequence and ~60% similar considering conservative substitutions. This alignment strongly indicates that all of these proteins have similar functions and confirms that they belong to the same family of proteins.

Overall, 23 of 218 residues in PapD were found to be identical in all seven chaperones (see Figure 1A and B). 58 additional residues are identical in at least four of the seven proteins. All residues that form the hydrophobic core in PapD are conservatively substituted in all members of the family. Domain 1 and domain 2 of the chaperones are 49% and 20% identical, respectively, in at least four of the seven sequences, with the β strand residues of domain 1 being 66% identical in a majority of the sequences. In contrast, 70% of all of the loop residues are variable. This analysis was used to compile a consensus sequence (Figure 1B) which was superimposed onto the three-dimensional structure of PapD to investigate the structural and functional significances of the conserved and variable features. Most of the conserved residues were found to participate in maintaining the overall structure of the domains.

Structural analysis of invariant residues in pilus chaperones

One class of invariant residues includes those that occupy critical points in loops or are involved in intramolecular interactions which serve to orient loops. For example, the loop regions between strands B and C of the first domain and strands B and C, as well as F and G of the second domain, are positioned by hydrogen bonds formed by 24N, 145N and 195N, respectively, to main chain atoms (see Figure 2A). Similarly, residue 39N in the loop between strands C and D of domain 1 is invariant in six of the seven chaperones. Invariant residues were also found at some of the bends or where β strands are disrupted. 7T and 54P are positioned at bends in domain 1 where strand A and strand D shift from one sheet to another, respectively (Figure 2B). 117P is located at the elbow bend between the domains, and 147T and 148P are within a short internal loop region between strands B and C of the second domain. 148P is followed by two conserved aromatic residues which contribute to the hydrophobic core of the molecule between the domains. Invariant glycine residue 198 is part of a reverse turn between strands F and G in the second domain.

A second group of conserved residues is involved in internal salt bridge formation. 196D and 116R form an internal salt bridge in association with 83E between the two domains (Figure 3). This internal bridge probably serves to orient the two domains towards one another to stabilize the cleft region between the domains that may be important for chaperone activity.

The last group of invariant residues are not conserved for any obvious structural reasons, unlike the previous two groups. They are surface exposed with their side chains oriented towards the solvent (Figure 4). 93I, 94P and 106A comprise part of a surface exposed hydrophobic patch in the first domain. 36W is close to this hydrophobic region and is locked into an unusual rotamer conformation by the formation of a hydrogen bond between its ND atom and OD1 of the invariant residue 89N which is also surface exposed. 36W is also intriguing from the standpoint that it is located in the same relative position as the characteristic tryptophan found in immunoglobulins (Kabat *et al.*, 1987). 8R, 112K and 172M are surface exposed and oriented towards the cleft between the domains. Interestingly, 172M was the major heavy atom site in PapD. It is frequently found that the heavy atoms used for phasing the X-ray data bind to the protein within the active site. 68R from strand E in the middle of the first domain protrudes towards the solvent but away from the cleft. 81D is located in the EF loop of domain 1 at almost the same position as the aspartic acid typically found in the variable domains of immunoglobulins (Williams and Barclay, 1988). These conserved hydrophobic and charged surfaces may be important for chaperone activity.

Disulfide bridges

The polypeptide chain in five of the seven chaperones stops after strand G in domain 2. PapD and FaeE have longer chains. In PapD these extra residues form an eighth antiparallel β strand H in domain 2. This strand is thus not

Fig. 1. A: Ribbon model of the crystal structure of PapD. Positions of invariant (purple) and conserved residues (yellow, orange, red and green) are indicated according to the color scheme in panel B. **B**: Amino acid sequence alignment of PapD, FanE, SfaE, FaeE, MrkB, HifB and F17D. Arrows indicate β strands. The consensus sequence consists of 23 invariant residues. Conserved amino acid substitutions occur at 44 additional positions. The conserved character of the residues at each position are indicated in color. Color key: purple, invariant residues; yellow, hydrophobic character conserved (G, A, P, V, I, L and F); orange, branched aliphatic residues conserved (V, I and L); red, aromatic ring conserved (F, Y and W); green, charge of residue conserved (K and R or D and E). The proteins were aligned without signal sequences. Residues in the alignment are numbered according to PapD.

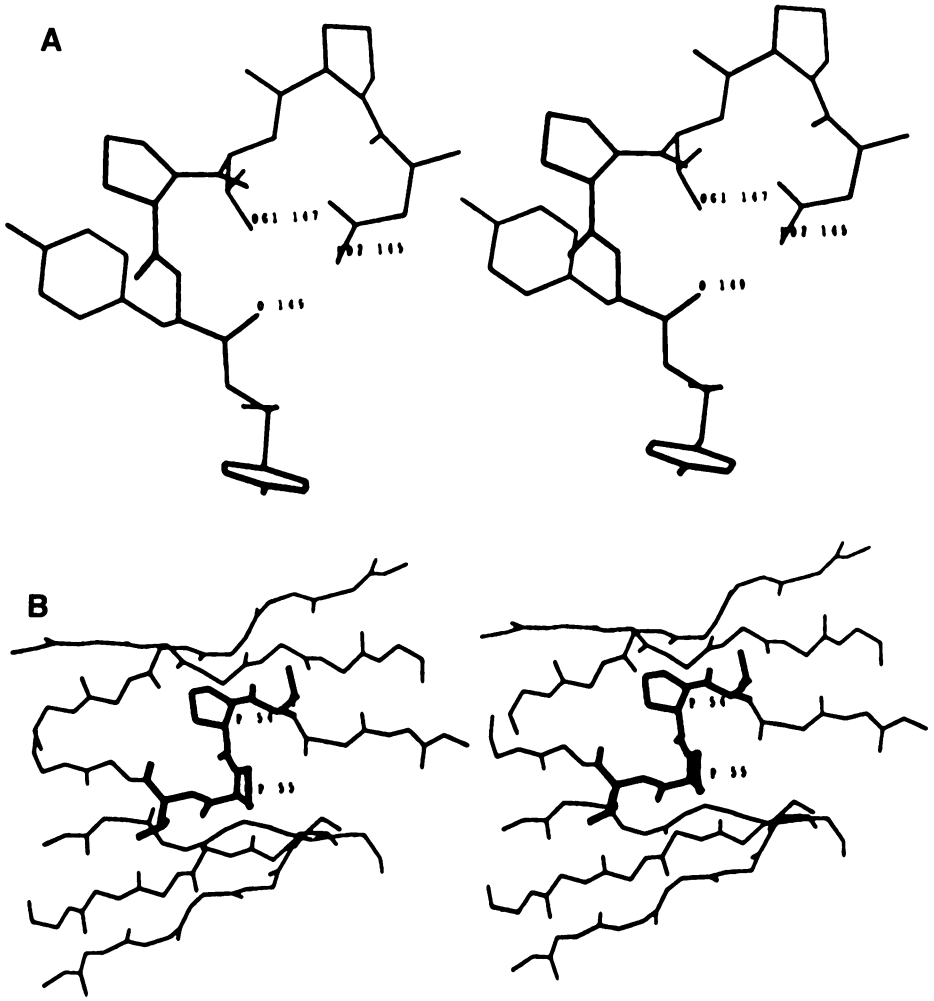


Fig. 2. A: Stereo diagram of the conserved loop region between strands B and C in the second domain. This loop is positioned by hydrogen bonds between side chain N and O atoms of 145N and the main chain O atom of residue 149 and side chain O atom of 147T, respectively. This arrangement locks the loop region into a specific conformation. The use of asparagine residues to position loop regions occurs frequently throughout the chaperone protein family. **B**: Stereo diagram showing the β-barrel of the first domain in PapD from one side. A proline residue (54P) which is conserved throughout the chaperone family disrupts β strand D1 and makes it shift from one sheet to the other. 55P is conserved in five out of seven members of the family.

a conserved feature of PapD homologues. Cysteine residues are rare in the chaperone family and only present in PapD and FanE each of which contain two cysteines. In PapD we know that these form a disulfide bridge (Holmgren and Brändén, 1989) and model building suggests that they also do so in FanE. With respect to the immunoglobulin fold, both PapD and FanE have unconventional disulfide bridges in that they are formed between two strands in the same sheet (between strands G and H of PapD and strands B and E of FanE). Usually, disulfide bonds in immunoglobulin domains occur between cysteines in strands B and F linking the two different β sheets together. However, CD4 and CD8 also have immunoglobulin-like domains with unconventional disulfide linkages between strands in the same sheet (Wang *et al.*, 1990).

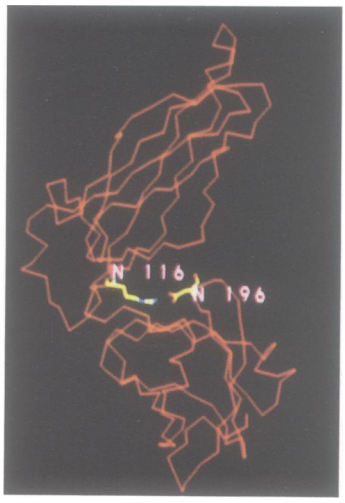


Fig. 3. The internal salt bridge found in all chaperone proteins is highlighted on the C-α backbone of PapD. In PapD it consists of R116 of domain 1 which interacts with D196 of domain 2, linking the two domains.

Chaperone variable loop regions

The majority of conserved residues are concentrated within the β strands in the region between the domains (see Figure 5A). Most of the loop regions between β strands are

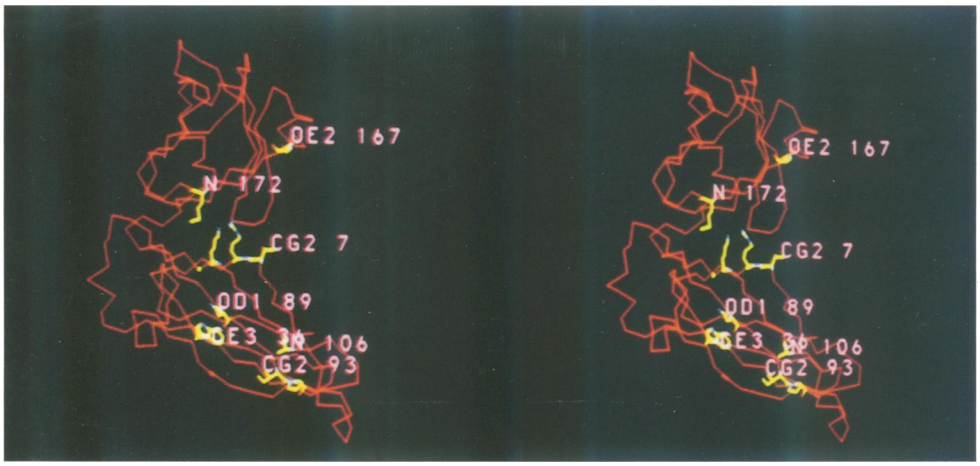


Fig. 4. Stereo diagram showing some of the surface exposed residues that are conserved throughout the chaperone family. In domain 1 there is a hydrophobic surface comprised of the side chains of residues 36W, 93I, 94P and 106A as the main constituents. 89N, 7T and 172M are also highly conserved as discussed in the text.

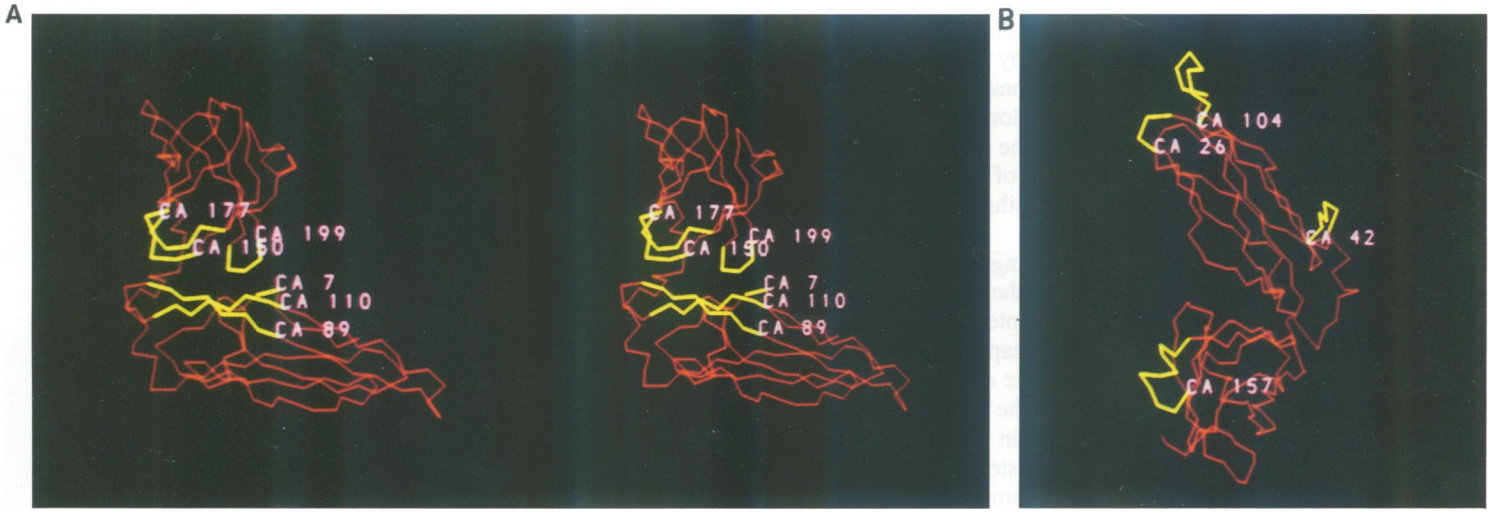


Fig. 5. A: Strongly conserved regions of the chaperone proteins (in yellow) superimposed on the C-α backbone of PapD is shown in stereo. These regions are concentrated in the interaction surface between the domains and in the cleft between the domains. Many of these residues are required for correct folding and domain orientation. **B**: Four variable loop regions of the chaperone protein family (in yellow) superimposed on the C-α backbone of PapD. Three of these are located at the tips of the protein and all of them occur between the β strands. In the first domain, two loops (highlighted by CA 104 and CA 26, respectively) are in structurally equivalent positions to immunoglobulin variable regions.

composed of variable residues based on the chaperone alignment (see Figure 1B and 5B). The variable regions in domain 1 between strands B and C, and F and G, occur at the same relative positions in a chaperone molecule as hypervariable regions occur in an immunoglobulin molecule. In addition, the variable loop region between strands C and D occurs sequentially in the same position as hypervariable region 2 in immunoglobulins, but is structurally in a different region from the other two immunoglobulin-like loops since the PapD domain lacks β strands C′ and C″ Therefore, this loop region is not located at the tip of the domain but rather in one side of the cleft region (Figure 5B). The second domain of PapD also contains two variable loops, one of which is located between strands C and D at the lip of the cleft. Residues in the variable loop regions may be important in providing chaperone binding specificity as is the case in immunoglobulins.

Discussion

The assembly of bacterial pili in Gram-negative bacteria is a complex process involving specific molecular interactions in the periplasm between structural subunits and chaperone proteins. It has been suggested that PapD functions as a reversible capping protein which modulates polymerization of pilus subunits (Kuehn *et al.*, 1991). It binds to nascently translocated subunits to make nonproductive interactions unfavorable, thus maintaining each subunit in an assembly-competent conformation. Each chaperone–subunit complex is then targeted to outer membrane assembly sites where the subunit is released from the chaperone and polymerized into the growing pilus rod. The work presented here demonstrates that the entire pilus chaperone family has a common structural framework with binding regions that are reminiscent of immunoglobulin domains. These immunoglobulin-like features seemingly make the

chaperones well suited for their role in recognizing families of related pilus proteins that are imported into the periplasmic space. It is proposed that the chaperone cleft contains the binding site and its conserved nature suggests a common mechanism of action amongst this class of proteins.

Many surface exposed invariant residues in the chaperone family have been conserved for no apparent structural reason but may be critical for chaperone function. Some of these invariant residues are located in the cleft of PapD and may form part of a pilus protein binding pocket (Figures 4 and 5). Structure–function studies that have been done on the PapD chaperone support a model where the cleft of PapD may form part of a pilus protein binding pocket (Figures 4 and 5; Slonim,L., Pinkner,J., Brändén,C.-I. and Hultgren,S.J., submitted). Variable residues within this cleft, on the other hand, may play a role in binding specificity, which would be similar in principle to the specificity provided by residues in variable loops of the antigen combining site of immunoglobulins. In addition, it has been suggested previously that PapD recognizes, in part, the carboxyl terminus of its pilus protein targets (Hultgren *et al.*, 1989). Interestingly, the carboxyl terminal region of almost all known pilus proteins produced by the Enterobacteriaceae family and *H.influenzae* is highly conserved (Lindberg *et al.*, 1986; Normark *et al.*, 1986; Gilsdorf *et al.*, 1990; Simons *et al.*, 1990) arguing that all of the pilus chaperones may bind to similar motifs. The degree of discrimination of each pilus chaperone for its family of pilus proteins is currently being investigated.

In conclusion, there exist intriguing structural and possible evolutionary relationships between the pilus chaperone family and immunoglobulins. The GC content and codon usage of the *E.coli* and the *H.influenzae* chaperones is reflective of their respective species (Marmur *et al.*, 1963) arguing that these are ancient bacterial genes. The possibility of divergent evolution of the immunoglobulin superfamily from a common ancient bacterial ancestor thus needs to be reconsidered. The role of periplasmic chaperones in post-secretional assembly of surface organelles is most likely a general phenomenon in the biology of Gram-negative bacteria.

Materials and methods

Amino acid sequence alignment

A consensus sequence was derived by superimposing the sequences onto the known crystal structure of PapD introducing gaps within loop regions in order to preserve the integrity of the β strands. Secondary structure has been assigned from the coordinates of the current model according to the method of Kabsch and Sander (Kabsch and Sander, 1983). The proteins were aligned without signal sequences and the residues in the alignment are numbered according to PapD. Use of this numbering system is suggested for future analysis of this family of proteins.

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