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Structure of a Complex of Human Lactoferrin N-lobe with Pneumococcal Surface Protein A Provides Insight into Microbial Defense Mechanism

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Abstract

Human lactoferrin, a component of the innate immune system, kills a wide variety of microorganisms including the Gram positive bacteria *Streptococcus pneumoniae*. Pneumococcal surface protein A (PspA) efficiently inhibits this bactericidal action. The crystal structure of a complex of the lactoferrin-binding domain of PspA with the N-lobe of human lactoferrin reveals direct and specific interactions between the negatively charged surface of PspA helices and the highly cationic lactoferricin moiety of lactoferrin. Binding of PspA blocks surface accessibility of this bactericidal peptide preventing it from penetrating the bacterial membrane. Results of site-directed mutagenesis, *in vitro* protein binding assays and isothermal titration calorimetry measurements corroborate that the specific electrostatic interactions observed in the crystal structure represent major associations between PspA and lactoferrin. The structure provides a snapshot of the protective mechanism utilized by pathogens against the host's first line of defense. PspA represents a major virulence factor and a promising vaccine candidate. Insights from the structure of the complex have implications for designing therapeutic strategies for treatment and prevention of pneumococcal diseases that remain a major public health problem worldwide.

Keywords

host–pathogen interaction; immunity; lactoferricin; lactoferrin

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2007.04.075

Introduction

Survival of a microorganism inside the host depends to a large extent on its ability to avoid, escape or counter the host's defense mechanisms. The host's first line of defense against microbial infection relies on the mechanisms of its innate immunity. As part of the innate immune system the body fluids and organized tissues of animals contain a number of naturally produced antimicrobial agents that kill various microbes or inhibit their growth. A major component of this regimen is a multifunctional iron-binding glycoprotein, lactoferrin (LF), found in milk and other exocrine secretions, which plays an active role in host defense. LF's antimicrobial activity, both bacteriostatic and bactericidal, against various pathogens including bacteria, fungi and viruses has been clearly established.¹⁻³

LF is secreted into the mucosal fluids.⁴ Therefore, bacteria that colonize mucosal surfaces are exposed to LF and must protect themselves from this antimicrobial agent. Various pathogenic species that live in the mucosal environment have developed different mechanisms for adaptation. For example, Gram negative bacteria of the *Neisseriaceae* family use specific receptor systems for acquiring iron from LF^{5,6} while various strains of the Gram positive bacterium *Streptococcus pneumoniae* bind LF via a surface protein called pneumococcal surface protein A (PspA).^{7,8}

S. pneumoniae is one of the most important pathogens affecting human and is responsible for causing at least million deaths among children worldwide annually[†]. PspA is an important virulence factor expressed on the surface of all pathogenic strains of *S. pneumoniae*.⁹ PspA reduces the killing of pneumococci by LF.¹⁰ The protective role of PspA is apparent from the demonstration of increased susceptibility to LF in pneumococcal strains lacking PspA as compared to the wild-type bacteria.¹⁰ Furthermore, anti-PspA antibodies enhance the bactericidal effect of LF against wild-type pneumococci.¹⁰ Although LFs from various mammalian species are highly homologous in their amino acid sequence, and biochemical and structural properties, PspA exhibits preferential binding to human LF (hLF) as compared to the bovine homolog (bLF).⁸ Since *S. pneumoniae* is a human pathogen, the preference of PspA for hLF suggests a functional significance for this protein-protein interaction.

LF is a member of the transferrin family of proteins and, as in other transferrins, the N and C-terminal halves of LF form two homologous lobes referred to as the N and C-lobes. Each lobe contains one iron binding site situated in a deep cleft.¹¹ The low iron saturation level of naturally occurring LF and its extremely high binding affinity for iron allows it to sequester free iron from body secretions and neutrophils depriving the pathogen of this essential metal, thus giving rise to the bacteriostatic effect.¹²⁻¹⁴ However, the bactericidal activity of LF is independent of iron, but for reasons not well understood is restricted only to its iron-free or apo form.^{3,10,15} This function of apo-LF presumably results from direct interaction with the bacterial surface.

[†]<http://www.who.int/vaccines/en/pneumococcus.shtml>

Since Ellison *et al.* first demonstrated that LF damages the outer membrane of Gram negative bacteria¹⁶ it has been proposed that a highly cationic domain located at the N terminus of LF is responsible for its bactericidal activity.¹⁷ The N terminus of LF from human and other mammals contains positively charged bioactive peptides, collectively known as lactoferricins (Lfcn), that are released in the stomach and mucosal secretions through proteolytic digestion of LF.^{18,19} The composition of these peptides is characterized by a relatively large proportion of basic amino acids and a number of hydrophobic residues, especially tryptophan, that render them uniquely suitable for interacting with bacterial lipopolysaccharides and disrupting the membrane structure.^{20,21} Subsequent studies demonstrated that lactoferricin peptides, LfcnH and LfcnB, derived from hLF and bLF, respectively, exhibit potent antimicrobial activity against many bacteria, fungi and viruses^{22,23} although the exact mechanism of this action remains elusive. Recently we showed that a number of shorter peptides derived from LfcnH possess potent bactericidal activity against pneumococci.¹⁰ The central role of PspA in shielding the pneumococci from the lethal action of hLF is underscored by the observation that PspA, either as a component of the bacterial surface or as a recombinant protein, also protects the bacteria from killing by LfcnH peptides.¹⁰

PspA is a multidomain protein which remains anchored to the pneumococcal cell wall through a C-terminal choline binding domain.²⁴ The primary sequence of PspA shows no significant homology to any other protein in the database except a second choline binding surface protein of *S. pneumoniae*, PspC. The molecular mass of PspA ranges between 67 kDa and 98 kDa in various pneumococcal strains. The N-terminal half of mature PspA is predicted to be entirely α -helical.²⁵ A lactoferrin-binding region of PspA has been localized within residues 168–288 of this α -helical domain.⁸ However, the nature of molecular interactions between PspA and LF remains unknown. Moreover, the PspA binding site on LF is not known. It is also not known if PspA binds to one or both lobes of LF.

Data presented here show that PspA binds to only the N-lobe of hLF. In order to gain a detailed understanding of the molecular interactions that protect *S. pneumoniae* and possibly other pathogens from the bactericidal effect of LF, we have determined the crystal structure of the complex of the LF-binding domain of PspA with the N-lobe of human LF. This is the first structure of LF (or either of its lobes) bound to a protein molecule. The structure of the complex reveals that through a set of specific interactions PspA binds to the lactoferricin domain. Results of this structure analysis contradict a previously proposed model.²⁶ Using site-directed alanine mutations we confirmed that the protein–protein interactions identified in the crystal structure represent the major association between LF and PspA in solution and that the only binding site for PspA on hLF is located on the lactoferricin domain. The structure provides an insight into the mechanism by which this surface protein of pneumococci protects the bacteria from the host's first line of defense.

Results

In vitro binding of PspA and LF

We cloned, expressed, and purified two truncated PspA fragments and two recombinant versions of human lactoferrin, the N-lobe (NLF) and full length (rhLF), as specified in

Materials and Methods. PspA₁ represents the complete N-terminal α -helical domain of mature PspA from strain Rx1 (residues 1–288). PspA₂ contains the region (residues 168–288) that has been shown to be necessary for binding human LF.⁸ NLF consists of amino acid residues 1–343 of the mature hLF sequence, which include the N-lobe and the hinge domain between the N and C-lobes. Using an *in vitro* protein-binding assay with purified PspA₁ and PspA₂ and LF preparations, we conclude that both recombinant forms of PspA bind NLF as well as rhLF (Supplementary Data, Figure S1). Isothermal titration calorimetry (ITC) data for binding of PspA fragments with rhLF and NLF fit well to a single binding site model. The calculated stoichiometries for binding of PspA₁: rhLF, PspA₁:NLF and PspA₂:rhLF are 1:1 (0.93, 0.88 and 1.14, respectively). The dissociation constants computed for these three complexes (4.8(±1.6), 10.3(±3.2) and 6.0(±2.3) nM, respectively) are consistent with that determined for binding of radio-labeled LF to pneumococci.⁷ Based on the results of size exclusion chromatography and ITC, PspA₁ and PspA₂ each form a 1:1 complex with NLF and with hLF. Since PspA₂ and NLF represent the minimum binding domain of each component, a complex of PspA₂ with NLF was subjected to structural investigation.

Structure of PspA₂ in the complex with NLF

The PspA₂:NLF complex crystallized in the space group $P3_2$. The asymmetric unit contains two copies of a 1:1 complex of NLF and PspA₂ related by a non-crystallographic 2-fold symmetry (Figure 1(a)). The two molecules of each protein in the asymmetric unit are very similar to each other. The calculated r.m.s. deviations for all the C $^{\alpha}$ atoms of the NLF chains A and B, and of the PspA₂ chains C and D are 0.1 Å and 0.2 Å, respectively. Data collection and refinement statistics are presented in Table 1.

LF structure—The structure of the LF N-lobe in the complex with PspA₂ is very similar to the described structure of the iron-bound N-lobe²⁷ (calculated r.m.s. deviation: 0.5 Å for all C $^{\alpha}$ atoms). There are two seven-strand β -sheets surrounded by a total of 11 α -helices. The structure of the N-lobe in the truncated form is almost identical to that in the full length LF.^{28–30} In the intact hLF, helix 11 of the N-lobe is joined by a loop to the short helix that links the two lobes. In the crystal structures of the truncated N-lobe the secondary structure at the C terminus varies depending on the stability of this region. In the present complex, although residues of the hinge domain are not visible in the electron density map, residues 317–331 form a helical structure similar to the one seen in the full length protein. The N-terminal four or five residues of hLF show some flexibility in various crystal structures. In the present complex the first three residues (Arg3, Arg4 and Arg5) in both NLF molecules (A and B) move noticeably away from the positions of the corresponding residues in other structures (Figure 2). As seen in Figure 2, both the main-chain and side-chains of these terminal residues of NLF deviate significantly in the complex although the rest of the structure remains unaffected by PspA₂ binding. Compared to the previously reported hLF N-lobe structures,^{27,31} the only other significant difference is the conformation of His92, for which the side-chain assumes a different rotamer conformation. This new conformation allows the side-chain of His92 to form a hydrogen bond with the glycine residue (–4) in the N-terminal tag of a symmetry-related PspA₂ molecule.

Although the complex was purified and crystallized without adding iron, electron density for one Fe^{3+} in the iron binding site of each NLF molecule (in the asymmetric unit) was clearly identifiable in the $F_o - F_c$ map. Six chelating atoms are contributed by two carbonate oxygen atoms, two hydroxyl oxygen atoms from Tyr93 and Tyr193, one carboxyl oxygen atom from Asp61 and the NE2 from His254. The average Fe^{3+} -O distance is 2.10 Å (range 1.74 Å–2.58 Å); the two Fe^{3+} -N distances are 2.07 Å and 2.03 Å. There are three sulfate ions (possibly from the crystallization reagents) bound to the surface of each molecule of NLF. One *N*-acetyl glucosamine molecule has also been modeled into the electron density near the known glycosylation site, Asp138, in each N-lobe in the asymmetric unit.

PspA structure—The PspA₂ molecule consists of four α -helices. Immediately following the vector-derived tetra-peptide (GSHM, numbered –4 to –1) at the N terminus there is a short helix (helix 1, residues 168–172) followed by three long helical segments (helix 2, residues 174–191; helix 3, 212–248; and helix 4, 258–288) connected by mobile loops (Figure 1(b)). Only the loop between the first two helices is visible in the structure; the other two are disordered. The three long helices are amphipathic with numerous hydrophobic interactions between neighboring antiparallel helices. The calculated inter-helix angle between helix 2 and helix 3 is -144° and between helix 3 and helix 4 is -158° [‡]. A plot of correlation coefficients of PspA₂ residues is provided in Supplementary Data, Figure S2.

Structural comparison with the DALI database³² shows that the overall structure of PspA₂ has similarity with the coiled-coil segments of many functionally unrelated proteins that are involved in various protein–protein interactions. In the SCOP database³³ similar proteins containing at least two long antiparallel helices are classified as “antiparallel coiled coil”. The superfamilies of proteins in this class include among others the N-terminal domains of variant surface glycoproteins, colicins and some colicin receptors.

There was one unexplained peak ($>5\sigma$) in the electron density map near Asp168 in each PspA₂ molecule at a site suitable for metal ion binding. After consideration of the coordination geometry and crystallographic parameters, we placed a Zn^{2+} at this site. It should be noted that LF is known to bind Zn^{2+} (among other metal ions)³⁴ and the culture media from which recombinant NLF was purified contained Zn^{2+} . Although zinc binding sites have been found in the C-lobe of bLF³⁵ no such site has been identified in hLF. The quality of electron density at this site in both molecules is excellent (Supplementary Data, Figure S3). The octahedral coordination of each Zn^{2+} is satisfied by residues from PspA₂ and NLF molecules (Supplementary Data, Figure S3). The biological relevance of this apparent zinc binding is not known.

Protein–protein interactions—The PspA₂ molecule binds to the surface of NLF. Helices 2 and 3 of PspA₂ fit into two grooves on the N-lobe as shown in Figure 3(a). Side-chains from a number of NLF residues create the surface for binding the PspA₂ helices; these residues include Arg3, Arg4, Arg28 and Arg31 for helix 3 and Ser36, Ile38, Lys39 and the aromatic ring of Phe21 for helix 2. Calculated buried surface areas for the two complexes (A:C and B:D) in the asymmetric unit are 1643 and 1650 Å², respectively. The protein

[‡]<http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/>

interface accessible surface area on the NLF molecules is $\sim 818 \text{ \AA}^2$ while that on the PspA₂ molecules is $\sim 843 \text{ \AA}^2$. These represent approximately 5.35% and 9.99% of NLF and PspA₂ surface, respectively. The protein–protein interface is formed by approximately 57% polar residues and 43% non-polar residues. The protein–protein interaction server[§] identifies a total of 25 residues of PspA₂ spanning the range 170–279 and 21 residues of NLF spanning the range of 3 to 54 as participating in the formation of the interface.

As shown in Table 2 there are a total of 19 interactions between NLF(A) and PspA₂(C) and 21 interactions between NLF(B) and PspA₂(D). Residues from all three long helices of PspA₂ contribute to binding (Figure 3(b) and (c)) with helix 2 providing six contacts (Table 2). The majority of the PspA residues involved in binding to hLF are acidic. However, three contacts are noticed between amide nitrogen atoms on PspA with oxygen atoms from residues on LfcnH domain (Table 2).

Interaction sites on the LF N-lobe are almost entirely confined to the lactoferricin region. Since the structure of the Lfcn domain remains unchanged in the apo-LF and holo-LF,^{36,37} similar protein–protein interactions are expected between PspA and both forms of LF. In the crystal structure of intact LF or its N-lobe, the Lfcn domain consists of two β -sheets flanked by two α -helices. There is no major change in the overall structure of N-lobe or Lfcn domain due to binding of PspA₂ except for the first three N-terminal residues that move considerably with respect to their positions in uncomplexed LF (see Figure 2). Since residues Arg4 and Arg5 are involved in direct interactions with PspA₂ this observed deviation may be induced by PspA₂ binding. The side-chain of Arg4 is inserted between helix 2 and helix 3 to form two contacts with Ser230 and Glu227 in helix 3, while Arg5 of the Lfcn domain interacts with carboxyl group of Asp231 at the polar end of helix 3 of PspA₂. It has been noted earlier that these three residues together with Arg25, Arg28, Arg29 and Arg31 from the C-terminal end of the first LF helix render this region a hot spot for binding to negatively charged surfaces of a variety of molecules including DNA and lipopolysaccharides.^{20,21}

A total of six positively charged residues (Arg4, 5, 25, 28 and 40 and Lys39) of NLF directly interact with various residues in PspA₂. In addition, the side-chain of Gln24 and the peptide oxygen atoms of Lys39 and Phe21 also interact with PspA₂. Three water molecules are involved in bridging each NLF molecule with its binding partner PspA₂ (Table 2). There are incidental contacts between the NLF(A) molecule and the N-terminal residues His-4, Ser-3 and Asp168 of PspA₂(D) molecule. Two of these terminal residues of PspA₂ (His-4 and Asp168) are also involved in coordination with the Zn^{2+} .

Results of our crystal structure analysis contradict a previously published model²⁶ which proposed that a positive surface potential of PspA is responsible for LF binding and that the PspA binding grooves on LF are in the vicinity of the iron binding site. In fact, PspA binds far from the iron binding pocket (see Figure 1(a)) and its negatively charged surface interacts with LF thereby neutralizing the cationic Lfcn domain (Figure 4).

[§]<http://www.biochem.ucl.ac.uk/bsm/PP/server/>

Mutational analysis of PspA–LF interaction

In order to understand the relative contribution of different regions of PspA to the overall binding to LF, we conducted site-directed mutagenesis by selectively replacing one or more residues in each of the helices 2, 3 and 4 of PspA₂ with alanine. Each residue selected for mutation was identified by the crystal structure as forming one or more contacts with NLF. Mutations were introduced in the DNA sequence for PspA₁ (residues 1–288) and PspA₂ (168–288) fragments. We expressed and purified mutant forms of both PspA₁ and PspA₂ and examined their ability to bind the N-lobe and full length rhLF.

After incubation of mutant proteins with purified NLF and rhLF, binding of PspA fragments with these proteins was monitored by electrophoresis on non-denaturing gels and Western blot analysis using an anti-PspA monoclonal antibody (Xi126).³⁹ Due to high carbohydrate content and basic pI (8.4–9),^{2,11} LF does not migrate as a band on non-denaturing gel (Figure 5(a), lanes 7, N-lobe and 8, full length). However, when bound to PspA, both NLF and rhLF migrate as a single band representing the complex (Figure 5(a), lanes 2 and 3). A quadruple mutant of PspA₁ (lane 4) containing two mutations in helix 2 (Glu180Ala, Glu182Ala) and one mutation in each of helix 3 (Asp237Ala) and helix 4 (Glu279Ala) does not form a complex with either NLF (lane 5) or intact rhLF (lane 6). The absence of binding interaction between PspA₁ mutant and LF (both NLF and rhLF) was confirmed by Western blot (Figure 5(b)).

In contrast, double mutants containing two mutations in helix 2 (Glu180Ala and Glu182Ala) or one mutation in helix 3 (Asp237Ala) and helix 4 (Glu279Ala), were still able to form a complex with rhLF and NLF (Supplementary Data, Figure S4). Three triple mutants (Glu180Ala, Glu182Ala, Asp237Ala), (Glu180Ala, Glu182Ala, Glu279Ala) and (Glu182Ala, Asp237Ala, Glu279Ala) lost the ability to form a complex, but another triple mutant (Glu180Ala, Asp237Ala, Glu279Ala) retained some binding capacity (Supplementary Data, Figure S4).

For quantitative assessment of binding interaction between rhLF or NLF with wild-type or mutant PspA fragments we performed ITC. There is no significant difference in thermodynamic parameters for binding of wild-type PspA₁ with rhLF and NLF as shown in Figure 6(a) and (b), respectively. On the other hand, no interaction was observed when binding of rhLF with quadruple mutant of PspA₁ (Glu180Ala, Glu182Ala, Asp237Ala, Glu279Ala) was measured under similar conditions. The titration curve in this case (Figure 6(c)) was comparable to that obtained for rhLF titration by buffer (Figure 6(d)). The titration curve for rhLF and PspA₂ triple mutant (Glu180Ala, Asp237Ala, Glu279Ala) suggested some residual binding capacity though the interaction parameters could not be calculated accurately (data not shown). Therefore, ITC data are in good agreement with the results of gel shift experiments.

Results of our protein binding assays using various PspA mutants show that contributions from multiple helices are necessary for binding to LF and binding is not prevented by mutation of one binding residue in any helix of PspA. Moreover, mutation of the two binding residues on helix 2 (Glu180 and Glu182) or one binding residue on each of the PspA helices 3 (Asp237) and 4 (Glu279) to alanine, did not inhibit LF binding, showing that

the protein–protein interactions between PspA and LF can withstand some degree of alterations on the PspA surface. However, replacing three binding residues (two in helix 2 and one in helix 3 or helix 4) with alanine was detrimental to binding. Similarly, mutation of Glu182 in helix 2 and one LF-binding residue in helices 3 and 4 inhibited complex formation between the triple mutant and LF. When Glu180 was mutated to alanine (instead of Glu182) the resulting triple mutant was still capable of binding LF, suggesting a relatively important contribution of Glu182, which is involved in multiple interactions with NLF (see Table 2).

All PspA₁ and PspA₂ mutants showed identical binding properties with full length rhLF and NLF, suggesting that LF C-lobe or PspA residues 1–167 did not contribute to binding. Results of binding assays were also similar when purified native human LF (Sigma) was used instead of recombinant protein, indicating that the difference in the carbohydrate composition of various LF preparations has no influence on binding to the PspA₁ and PspA₂ fragments (data not shown). Therefore, only specific protein–protein interactions between PspA₂ and NLF domain are responsible for the binding affinity between these proteins.

Discussion

In spite of the availability of antibiotic treatment and a number of vaccines, pneumococcal infection still remains one of the main causes of morbidity and mortality especially among high risk groups.^{40,41} Emergence of antibiotic resistance⁴² and limitations of the currently available polysaccharide-based vaccines^{43,44} prompted a search for new therapeutic avenues, including identification of cross-reactive protein antigens conserved across capsular serotypes for vaccine preparation.^{45,46} PspA is the most intensively studied potential vaccine candidate.^{46–48} The multi-faceted biological activity of PspA includes inhibition of complement activation and deposition on the pneumococcal surface⁴⁹ and retardation of clearance of pneumococci from the blood.⁴⁸ However, the most intriguing property of PspA is its ability to protect pneumococci from the bactericidal action of LF and the Lfcn peptides.¹⁰

LF's antibacterial activity involves complex interactions of LF, bacterial cells, bacterial products, and host cells.^{1,2} The stretch of N-terminal 49 residues of human LF which constitutes the LfcnH is crucial for the bactericidal functions.⁵⁰ This effect is mediated through direct interaction with the bacterial surface, either as released peptide/peptides or while within the N-lobe of LF. Several lines of evidence established that PspA quite effectively inhibits the bactericidal action of apo-LF. LF is more lethal in the absence of PspA (in PspA[−] strain), and anti-PspA antibodies enhance the killing. Moreover, addition of recombinant PspA reduces killing of pneumococci by apo-LF and LfcnH peptides.¹⁰ These data combined with the fact that PspA is the only protein on the surface of *S. pneumoniae* which is known to bind LF^{7,8} emphasize the intimate relationship between PspA's interaction with LF and its ability to protect the bacterium from attack by the latter. However, the exact mechanism of this defense is not known.

The crystal structure of PspA₂ with NLF offers insight on how PspA's ability to bind lactoferrin can be protective for the bacteria. First, the structure shows that PspA specifically

binds to the Lfcn domain, thereby blocking its access to the bacterial surface. Second, although it is not known if *in vivo* the lactoferrin-mediated killing of *S. pneumoniae* is caused entirely by Lfcn peptides released from human LF, the crystal structure presented here shows that binding of PspA hinders the surface accessibility of the LF N-lobe. It is therefore reasonable to assume that PspA prevents protease access to the cleavage site and thereby inhibits the release of bactericidal peptides from LF. Finally, even in the incidence of proteolysis, PspA is likely to be able to scavenge cationic Lfcn peptide/peptides and thus inhibit their effect against pneumococci. The recently resolved NMR structure of LfcnH reveals that the helical structure of this domain resembles that in the intact LF.⁵⁰ As shown in Figure 7(a), the first helix in the free LfcnH (yellow) and in the Lfcn domain (green) from the NLF:PspA₂ complex superposes well while the β -sheets are completely unfolded in the solution structure. As this NLF helix contributes many interactions with PspA₂ (Figure 7(b)), it is expected that PspA can efficiently bind released 49 residue LfcnH peptide. The unfolded regions of LfcnH may provide additional contacts to PspA *via* exposed residues. Shorter peptides generated by proteolysis from LfcnH may involve other binding interactions than those seen in the crystal structure.

Bovine LF and the corresponding lactoferricin peptides also show strong bactericidal properties.^{23,51} bLF also binds to PspA but the binding efficiency is approximately 10–15-fold less than hLF.⁸ This may reflect the evolutionary history of *S. pneumoniae*, which is a human pathogen. Within the Lfcn domain there are seven differences between bovine and human proteins. Among these, the three most striking differences are Phe21 (human) Arg (bovine), Arg25Trp and Asp41Ala. All other differences between human and bovine LF are conservative changes (Arg5, 28, 54 of hLF to Lys residues in bLF and Lys39 to Arg). A careful look at the sites of the non-conservative mutations provides some clues to the observed difference in the binding efficiency of bovine and human LF to PspA. First, the oxygen atom Asp41OD2 of hLF forms contacts with PspA (Asn183 and Arg187) through a water molecule (see Figure 8(a)). Replacement of the negatively charged side-chain of this acidic residue with the aliphatic side-chain of alanine in bLF would abolish these interactions. Second, side-chain NH1 of Arg25 in hLF forms a hydrogen bond, though weak, with Gln175. This hydrogen bond would be absent in bLF where a bulky aromatic side-chain is present. Finally, replacing Phe21 in hLF with an Arg residue would alter the packing environment near the side-chain (Figure 8(b)). The crystal structure therefore provides clear and testable hypotheses to explain the decreased ability of bLF to bind PspA.

Although targeting protein–protein interactions is more challenging than traditional approaches of identification of small molecule inhibitors for protein targets, inhibitors of protein–protein interaction are becoming increasingly popular in the discovery of lead compounds.^{52,53} The crystal structure of PspA₂: NLF offers an interesting possibility for screening small molecule inhibitors. Inhibitors blocking PspA–LF interactions may allow the natural bactericidal effect of lactoferrin to protect the host from colonization and infection by *S. pneumoniae*.

In summary, the crystal structure of the lactoferrin-binding domain of PspA in complex with LF N-lobe shows details of the specific interaction between human LF and PspA. The structure reveals how PspA acts as a barrier between the bacterial outer membrane and LF

(or Lfcn) and thereby protects pneumococci from exposure to the highly cationic lactoferricin domain, and provides the first glimpse of the exposed surface epitopes of the PspA₂ region. This domain is known to elicit the most cross-protective immunity^{54,55} and therefore knowledge of the three-dimensional structure will aid design of vaccines based on peptides within this region.

Materials and Methods

Cloning, expression, and purification of PspA fragments

The coding sequences of truncated *S. pneumoniae* surface protein A constructs PspA₁ (amino acid residues 1–288 of mature sequence) and PspA₂ (168–288) were amplified by polymerase chain reaction (PCR) from plasmid pUAB103 containing amino acid residues 1–370 of mature PspA sequence of the strain Rx1 using Triple Master polymerase mix (Eppendorf, Germany) according to the manufacturer's protocol. Amplified DNAs were cloned into NdeI and XhoI sites of pET15b vector (Novagen). Recombinant PspAs were expressed in *Escherichia coli* strain BL21(DE3) (Stratagene) via induction of the culture with 0.4 mM isopropyl thio- β -D-galactoside when A_{600} = 0.8 and subsequent overnight growth at room temperature. Initially, N-terminally His₆-tagged proteins were purified from cell-free extracts by metal-chelate affinity chromatography on Talon column (Clontech) followed by removing the His-tag by proteolysis with thrombin. Additional purification steps included anion-exchange chromatography on a Q Sepharose column (GE Healthcare) and size exclusion chromatography on a Superdex 75 (16/60) column (GE Healthcare).

Point mutations in plasmids containing PspA₁ and PspA₂ were performed using QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. Mutated PspA fragments were expressed and purified by the same protocol as wild-type proteins.

Cloning, expression, and purification of LF and its N-lobe

The coding sequences of human LF and its N-lobe (NLF, amino acid residues 1–343 of mature hLF sequence) were amplified from plasmid pNUT:hLF by PCR using triple master polymerase mix. Amplified DNAs were subcloned into the BamHI site of baculovirus transfer vector pAcGP67A (BD Biosciences). Positive clones were checked for the correct orientation of inserts by restriction digestion of plasmid with SmaI enzyme. The transfer vector containing the rhLF or NLF sequence and linearized BD BaculoGold Bright Baculovirus DNA (BD Biosciences) were co-transfected into *Spodoptera frugiperda* insect cell line (Sf9) using cellfectin reagent (Invitrogen) according to the manufacturer's instructions. For rhLF and NLF expression, insect cell line BTI-TN-5B1-4 (High Five cells) were infected with recombinant baculovirus and grown at 27 °C for four to five days. Recombinant hLF and NLF were purified from the supernatant of the insect cells using cation exchange chromatography on SP Sepharose.

Isothermal titration calorimetry measurements

Calorimetric titrations were carried out with VP-ITC microcalorimeter (MicroCal, Inc., Northampton, MA). The solution of rhLF (Agennix Inc.) or NLF at concentration of 25–38

μM was loaded into the sample cell and the injection syringe contained PspA₁ or PspA₂ (wild-type or mutant) at concentration of 176–295 μM (all concentrations were calculated assuming that proteins are monomers). PspA was delivered to stirred cell (300 rpm) at 210–240 s intervals by 31 injections of 8 μl each. Experiments were conducted in 50 mM sodium phosphate buffer (pH 7.5) at $25(\pm 0.02)$ °C. Data were fitted to a single binding site model⁵⁶ using Origin software (version 5.0, MicroCal, Inc.). The binding enthalpy (ΔH), and entropy (ΔS), association constant (K_A) and stoichiometry were obtained.

Complex preparation, crystallization, and structure solution

For complex preparation, purified PspA₂ and NLF were mixed in ~1:1 molar ratio. The PspA₂:NLF complex was separated from unbound proteins by size exclusion chromatography on a Superdex 75 column. Finally, complex was dialyzed against 50 mM Tris-HCl (pH 7.5), 50 mM NaCl and concentrated to 45 mg/ml by ultrafiltration using a 10 kDa-cutoff membrane (Amicon). Crystals were grown using sitting drop vapor diffusion at 4 °C. The drops contained 1:1 mixture of the complex and the reservoir solution composed of 30% (v/v) PEG 400, 0.2 M lithium sulfate and 0.1 M sodium cacodylate (pH 6.5). For data collection a crystal was flash frozen in paraffin oil.⁵⁷ X-ray data were collected from a single crystal at 100 K on a Mar165 image plate at IMCA-CAT beamline (Advanced Photon Source, Argonne National Laboratory). Crystallographic data processing was performed using HKL 2000 (Table 1). The crystal belongs to space group $P3_2$ with unit cell dimensions $a=b=130.18$ Å and $c=80.80$ Å. There are two 1:1 complexes per asymmetric unit, and the value of V_m is 3.79 Å³/Da, which corresponds to a solvent volume fraction of 68%.

Structure determination and refinement

The structure of the N-terminal lobe of LF was solved by molecular replacement, using the human N-terminal lobe as a search model (PDB entry 1H45). The two highest peaks in the cross-rotation function corresponded to the two molecules in the asymmetric unit. A difference electron density map calculated with phases from the partial structure showed three long stretches of α -helical density, and idealized polyaniline helices were built and fit to the density using Coot.⁵⁸ Subsequent electron density maps calculated with phases from the partial structure allowed alignment of the sequence and fitting of the side-chains.

Refinement of the structure was carried out by simulated annealing using CNS.⁵⁹ No sigma cutoff was applied to the data. Five percent of the data were randomly selected and removed prior to refinement for analysis of the free R factor. The two complexes were restrained by the non-crystallographic symmetry during the refinement. The progress of the refinement was guided by the decrease in both the conventional and free R factors. Individual B -factors were included in the final refinement. Two final rounds of TLS restrained refinement (without any non-crystallographic symmetry restraints) were carried out using REFMAC5.0.⁶⁰ The refinement statistics are shown in Table 1. The final model includes residues 3–333 of NLF, residues 168–288 of PspA (plus the four residue N-terminal tag), two Fe ions, two carbonate ions, six sulfate ions, two molecules of N-acetylglucosamine, two Zn ions and 24 water molecules. Overall quality of the model is excellent with 99.4% residues in the allowed regions of Ramachandran plot, two NLF residues (Ser192 and

Leu300) in each complex in the disallowed region. These residues are in the disallowed region in previously described structures.

Data deposition

Atomic coordinates have been deposited to the RCSB Protein Data Bank (PDB ID 2PMS).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

LF	lactoferrin
hLF	human lactoferrin
bLF	bovine lactoferrin
rhLF	recombinant human lactoferrin (full length)
NLF	recombinant human lactoferrin N-lobe
Lfcn	lactoferricin
LfcnH	human lactoferricin
LfcnB	bovine lactoferricin
PspA	pneumococcal surface protein A
ITC	isothermal titration calorimetry

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Figure 1. Structure of the PspA₂:NLF complex. (a) Asymmetric unit content. LF N-lobes are shown in green (Lfcn peptide is colored blue), PspA₂ molecules in red. One Fe ion (orange) is bound to each NLF. The coordinating carbonate ligand is shown in sticks. One Zn ion (cyan) is bound at the N-terminal end of each PspA₂ molecule. The *N*-acetylglucosamine molecule is labeled NAG. (b) PspA₂ structure. Residue numbering for PspA₂ helices are shown.

Figure 2. Superposition of LF structures showing the movement of N-terminal residues in the PspA₂: NLF complex. NLF chains (A and B, shown in yellow and green, respectively) are superposed on the full length hLF structure (PDB ID, 1FCK, shown in brown). The N-terminal 15 amino acid residues of each chain are shown in stick model. While terminal residues 3–5 of NLF in the complex shift considerably from the corresponding residues in free hLF, the remaining residues show no movement.

Figure 3.

Binding of PspA₂ with the LF N-lobe. (a) Grooves on NLF for binding PspA₂ helices. NLF is shown in surface drawing and PspA₂ in ribbons. Several LF residues around the grooves are labeled. Helix 2 has the most interactions with NLF. (b) and (c) Close-up views of the intermolecular interactions between PspA₂ and NLF. Amino acid residues for both PspA₂ (salmon, labeled C) and NLF (green, labeled A) participating in binding are shown in sticks. Water molecules involved in bridging PspA₂ and NLF are shown as red spheres.

Figure 4.

Surface potentials: (a) NLF, (b) PspA₂ and (c) PspA₂:NLF complex. Surface potentials were calculated using the program GRASP2.³⁸ Positive potential is depicted in blue and negative potential in red. Uneven distribution of positive charge is seen on NLF with a high concentration at the N-terminal end of the molecule. The PspA₂ molecule shows a highly negatively charged surface.

Figure 5.

Analysis of binding of PspA₁ mutant to LF. Various PspA₁ residues (Glu180, Glu182, Asp237 and Glu279) involved in binding to NLF were mutated to alanine. The resulting mutant protein was expressed and used in binding studies. After incubation of PspA₁ (wild-type and mutant) with LF (NLF and rhLF), the mixtures were subjected to gel electrophoresis on a non-denaturing gel. For Western blot analysis proteins were then transferred to a nitrocellulose membrane and detected using monoclonal mouse IgG Xi126 (as primary antibody) and alkaline phosphate-conjugated anti-mouse IgG antibody (as secondary ones). (a) Non-denaturing gel. (b) Western blot. 1, PspA₁ (residues 1–288); 2,

PspA₁+ NLF; 3, PspA₁+ rhLF; 4, PspA₁ mutant; 5, PspA₁ mutant+ NLF; 6, PspA₁ mutant+ rhLF; 7, NLF; 8, rhLF.

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Figure 6.

Isothermal titration calorimetry data for the binding of different PspA and LF fragments. (a) rhLF (38 μM) was titrated by PspA₁ (251 μM), (b) NLF (30 μM) was titrated by PspA₁ (295 μM), (c) rhLF (29 μM) was titrated by PspA₁ mutant (Glu180Ala, Glu182Ala, Asp237Ala, Glu279Ala) (233 μM), (d) rhLF (32 μM) was titrated by 50 mM sodium phosphate buffer (pH 7.5). The top panels of (a) and (b) and (c) and (d) show the heat released per second during the addition of 31×8 μl of PspA₁ (native or mutant) or buffer to the cell containing rhLF or NLF. The bottom panels of (a) and (b) show the integrated binding isotherms with the continuous lines representing the fit of the data to a single site binding model. Binding enthalpy, H : (a) $-13.2(\pm 0.1)$, (b) $-11.0(\pm 0.1)$ kcal/mol; binding entropy, $-S$: (a) -6.15 , (b) -0.3 cal/(K mol); stoichiometry: (a) 0.932 ± 0.002 , (b) 0.875 ± 0.003 , and dissociation constant, K_D : (a) $4.8(\pm 1.6)$, (b) $10.3(\pm 3.2)$ nM.

Figure 7.

(a) Superposition of the NMR structure of intact LfcnH peptide (yellow) on the lactoferricin domain (residues 3–49) of the NLF (green) from the PspA₂:NLF complex. The conformation of the first helix of the Lfcn domain is very similar in both structures although the β -sheet is unfolded in the LfcnH peptide. (b) Residues in the first helix of NLF (green) that interact with various residues of PspA₂ (salmon) are shown in stick model. This helix of NLF provides many contacts with PspA. The corresponding section of LfcnH peptide is shown in yellow.

Figure 8.

Comparison of Lfc domain of bLF and hLF. (a) There are three non-conservative differences in the Lfc domains of human and bovine LF. These residues (Phe21, Asp41 and Arg48) of NLF (green), shown in stick model, provide important interactions for binding PspA (red). (b) Interactions between PspA₂ (surface drawing, grey) and the LF N-lobe (ribbons, green). Sites for three non-conservative mutations are shown in stick representation (bovine residues in yellow, human residues in green). These residues are Asp41 (human) to Ala (bovine), Phe21 to Arg and Arg25 to Trp. Mesh (rose) was calculated after mutating the residues according to the bovine sequence. These mutations are expected to alter the nature of interactions considerably.

Table 1

Crystal parameters, X-ray data collection and refinement statistics

Space group	$P3_2$
Cell dimensions	
a, b, c (Å)	130.18, 130.18, 80.80
α, β, γ (°)	90, 90, 120
<i>Data collection statistics</i>	
Resolution (Å)	15.0–2.91 (3.0–2.91) ^a
No. unique reflections (total set)	33,481 (3355)
R_{sym}	0.098 (0.36)
I/σ	7.0 (3.0)
Completeness (%)	99.9 (100)
Redundancy	2.9 (2.9)
<i>Refinement and model statistics</i>	
Resolution (Å)	15.0–2.91 (3.0–2.91)
No. reflections used (working set)	30, 285
R_{work}	20.3 (26.2)
R_{free}	24.9 (33.3)
r.m.s.d. bond lengths (Å)	0.009
r.m.s.d. bond angles (°)	1.16
<i>Average B-factor (Å²) (main-chain/side-chains/overall)</i>	
Molecule A	29.5/30.7/30.1
Molecule B	29.5/30.7/30.1
Molecule C	36.7/38.3/37.5
Molecule D	36.8/38.4/37.5
<i>Ramachandran statistics (%)</i>	
Core region	90.0
Additional allowed region	9.5
Generously allowed	0.3
Disallowed	0.3

^aNumbers in the parentheses are for the highest resolution bin.

Table 2

Intermolecular contacts between PspA and lactoferrin N-lobe

		Distance (Å)		
PspA residue/atom	NLF residue/atom	Complex A:C	Complex B:D	
Glu171 OE2	Gln14 NE2	–	3.15	
Gln175 NE2	Phe21 O	3.04	3.25	
Gln175 NE2	Gln24 OE1	2.68	2.53	
Gln175 OE1	Arg25 NH1	3.14	3.06	
Glu180 OE1	Lys39 NZ	3.05	2.92	
Glu182 OE2	Arg40 NH1	2.45	2.52	
Asn183 ND2	Lys39 O	2.76	2.81	
Glu227 OE1	Arg4 NH2	3.04	–	
Glu227 OE1	Arg4 NH1	3.17	2.79	
Glu227 OE	Arg4 NH1	–	3.17	
Ser230 OG	Arg4 NH2	3.12	3.16	
Asp231 OD1	Arg5 NE	3.23	3.14	
Asp231 OD1	Arg5 N	–	3.15	
Asp231 OD2	Arg5 NE	3.00	3.25	
Asp231 OD2	Arg5 NH2	3.27	2.91	
Asp237 OD2	Arg28 NE	2.77	2.76	
Glu279 OE1	Arg28 NH2	2.97	3.10	
Glu279 OE2	Arg28 NH1	3.07	3.02	
Gln 279 OE2	Arg28 NH2	–	3.07	
Contacts through bridging water molecules				
			Distance (Å)	
PspA residue/atom	Water	NLF residue/atom	PspA ₂ ... O	O ... NLF
Glu182 OE2(C)	404	Lys39 N(A)	2.70	2.73
Glu182 OE1(C)	406	Arg54 O(A)	2.96	2.64
Asn183 ND2(C)	402	Asp41 OD2(A)	3.11	2.96
Arg187 NH2(C)	402		2.82	
Ala179 O(D)	403	Lys39 N(B)	2.73	2.85
Glu182 OE1(D)	405	Arg54 O(B)	2.83	2.71
Asn183 ND2(D)	401	Asp41 OD1(B)	3.31	3.21