Chapter 6

Laetiporus sulphureus Lectin and Aerolysin Protein Family

José Miguel Mancheño,* Hiroaki Tateno, Daniel Sher and Irwin J. Goldstein

Abstract

The parasitic mushroom *Laetiporus sulphureus* produces a family of lectins (LSL′s) sharing 80-90% sequence identity that possesses a low but significant sequence similarity to the bacterial pore-forming toxins mosquitocidal toxin Mtx-2 from *Bacillus sphaericus* and α toxin from *Clostridium septicum*. The crystal structure of one member of the *L. sulphureus* lectins family (LSLa) reveals unexpected structural similarities to the β-pore-forming toxins from the aerolysin family, namely, aerolysin from the Gram-negative bacterium *Aeromonas hydrophila*, ε-toxin from *Clostridium perfringens* and parasporin from *B. thuringiensis*. This similarity presumably indicates that the hemolytic activity of LSLa proceeds through a molecular mechanism that involves the formation of oligomeric transmembrane β-barrels. Comparison of the crystal structures of the above mentioned proteins reveals common pore-forming modules, which are then distributed both in bacteria and fungi. Currently, it can be stated that the above three dimensional structures have been key in revealing structural similarities that were elusive at the sequence level. A potential corollary from this is that structural studies aimed at determining high resolution structures of aerolysin-like pore-forming toxins, whose biological activity involves large conformational changes, are mandatory to define protein domains or structural motifs with membrane-binding properties.

Introduction

The isolation and partial characterization of a lectin with hemolytic and hemagglutinating properties produced by the mushroom *Laetiporus sulphureus* were first reported by the Konska group.[1] The isolated lectin was stated to be a heterotetrameric species of 190 kDa composed of two distinct types of subunits (about 36 and 60 kDa, respectively). This analysis also revealed that the lectin was specific for N-acetyllactosamine residues and that both hemagglutinating and hemolysis activities were supported by the same site. More recently, the Goldstein group[2] undertook a detailed analysis on the sugar binding specificity of the hemolytic lectin and closely related lectins from *L. sulphureus* and also carried out the corresponding cDNA cloning, heterologous expression and characterization of the recombinant lectin proteins. Analysis of the protein sequences revealed that the *L. sulphureus* lectins contain three tandemly repeated subdomains at their N-termini. Each subdomain possesses the highly conserved QXF motif, which resembles the QXW motif present in the ricin B-chain. Therefore, the *L. sulphureus* lectins are members of the R-type lectins, the lectin family found in both prokaryotes (bacteria) and eukaryotes (*C. elegans*, Drosophila, vertebrates, plants). Due to the high degree of conservation in structure and

*Corresponding Author: José Miguel Mancheño—Grupo de Cristalografía Macromolecular y Biología Estructural. Instituto de Quimica Fisica Rocasolano, CSIC. Serrano 119, 28006 Madrid. Spain. Email: xjosemi@iqfr.csic.es

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in sugar-binding function, a gene encoding an R-type lectin has been thought to have moved laterally between species. Conversely, the sequence analysis of the C-termini of the lectins revealed the lack of close homologues and only a low sequence similarity was identified with sequences from bacterial pore-forming toxins, in particular with mosquitocidal toxin Mtx-2 from *Bacillus sphaericus* and α toxin from *Clostridium septicum*. Recent high resolution structural analysis on the hemolytic lectin finally rendered the novel crystal structure of the protein at 2.6 Å resolution.[3] The structure revealed that the lectin is a highly symmetrical hexameric species, with each monomeric subunit being composed of two functionally and structurally distinct domains: an N-terminal β-trefoil lectin domain and an elongated C-terminal domain which unexpectedly revealed clear similarities with domains present in members of the aerolysin family of β-pore-forming toxins. These findings permitted for the first time to delimit a minimum pore-forming domain in this family of toxins and also to reveal structural similarities in spite of very low levels of similarity in the primary amino acid sequences.

Pore-Forming Hemolytic Lectins

Lectins are defined as glycan-binding proteins or glycoproteins of non-immune origin,[4] which have ability to discriminate extremely diverse glycan structures; stereoisomers and branch numbers and positions. Though recognition of glycans on cell surfaces and glycoconjugates, lectins have ability to agglutinate cells and/or precipitate glycoconjugates. Some lectins are also known to have cytotoxicity as represented by ricin.[5] Ricin is a type II ribosome-inactivating protein isolated from *Ricinus communis*, consisting of two disulfide-linked polypeptides, A-chain and B-chain. Binding of ricin to β-linked galactose at the cell surface mediated by the two lectin $(QXW)_3$ domains of B-chain, is a prerequisite for the membrane translocation of the enzymatically active A-chain. Upon reaching the cytosol, active A-chain specifically inactivates the ribosomes. Conversely, other cytotoxic lectins have ability to lyse as well as agglutinate cells. Such lectins are called hemolytic lectins. Hemolytic lectins make pores within the membrane of the target cells, such as the one produced by the marine invertebrate *Cucumaria echinata* (CEL-III),[6,7] or those produced by the mushroom *L. sulphureus*[1,2] (LSL's). In both cases, the hemagglutinating and hemolysis activities are mediated by binding of the protein to specific carbohydrate chains of the cells, followed by the formation of discrete ion-permeable pores in the cell membrane of the target cells through oligomerization of the protein. After formation of the pores, erythrocytes are ruptured by colloid osmotic shock. Osmotic protection experiments indicated that LSLa formed pores with a functional diameter smaller than 3.8 nm in the cell membrane of human erythrocytes.[2]

Analysis of the primary structures of LSLa and CEL-III revealed a negligible level of similarity between them. Nevertheless, in both cases a substructure of tandemly repeated subdomains at their N-termini was identified analogously to those from the B-chains of the toxic lectins ricin[5] and abrin[8] from *Abrus precatorius*: three subdomains in LSLa[2] and six in CEL-III.[9] The recently described crystal structures of both hemolytic lectins[3,10] supported this conclusion (Fig. 1). The structure of CEL-III reveals a three-domain architecture with two carbohydrate-binding domains at the N-terminus (domains 1 and 2, respectively) and a C-terminal pore-forming domain (domain 3) which has no structurally similar proteins in the Protein Data Bank. Domains 1 (residues 1-149) and 2 (residues 150-283) share an identical β-trefoil fold (r.m.s. deviation of 0.77 Å for 115 aligned C_α atoms), despite sharing a 33.8% sequence identity for 145 residues. The C_α backbones of the three basic motifs constituting the β-trefoil fold (see below) are almost identical (average pairwise r.m.s.d. 0.62 Å for 29 aligned C_α atoms) and with the exception of motif 1β, bound one Ca^{2+} ion through coordination by two conserved aspartic acid residues.[10] These complexes may help explaining the dependence of the carbohydrate-binding activity on Ca^{2+} concentration.

The pore-forming modules (PFM's) of LSLa (residues 151-314) and CEL-III (residues 284-432) are dissimilar, probably indicating unlike molecular mechanisms of membrane pore-formation. Whereas PFM from LSLa shows structural similarities to aerolysin-like proteins (see below), that from CEL-III has no related protein domain. This last domain comprises two α-helices and an eight-stranded β-sandwich[9] whose β-sheets are held together by hydrophobic

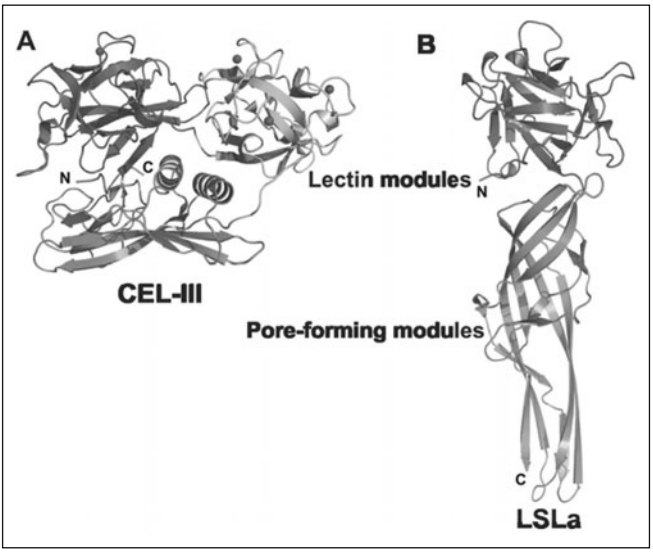

Figure 1. Modular architecture of the hemolytic lectins LSLa and CEL-III. Protein molecules are shown as cartoon representation. A) CEL-III from *C. echinata* possesses two lectin β-trefoil folds and a C-terminal pore-forming domain comprised by two α-helices and an eight-stranded β-sandwich whose β-sheets are held together by hydrophobic interactions. Calcium atoms are shown as spheres. B) LSLa from *L. sulphureus* possesses an N-terminal β-trefoil lectin domain and a C-terminal pore-forming domain which shows structural similarities with bacterial toxins from the aerolysin family.

interactions. The two helices lie on the surface of the β-sandwich in parallel with the β-sandwich surface. A C-terminal helical loop of 25 residues is stabilized by two disulfide bonds what clearly restraints its flexibility.

Two different pore-formation mechanisms have been proposed for CEL-III by the Hatakeyama group.[9] The first scenario considers that after an initial recognition of the carbohydrate molecular receptor mediated by the lectin domains 1 and 2, CEL-III would behave as a β-pore-forming toxin, with the α-helices from domain 3 making a conformational change to a 42-residue amphipathic β-hairpin. Six such hairpins then associate to make up a β-barrel with 1.7 nm in diameter within the erythrocyte membranes. After formation of the pores, erythrocytes are ruptured by colloid osmotic shock. Conversely, the second mechanism would involve insertion of the complete domain 3 into the erythrocyte membrane after a similar alpha-to-beta conformational transition.

A distinct feature of these two pore-forming hemolytic lectins when considered in the context of aerolysin family of proteins is that they can be defined as linear modular proteins, i.e., the modules that make up the final complex architecture of the protein are independent from each other at the primary level.

A New Member within the Aerolysin Family: The Crystal Structure of LSLa

The aerolysin family of β-pore-forming toxins unexpectedly received a new member as a result of the crystallographic studies on the hemolytic lectin LSLa produced by *L. sulphureus*.[3,11] The crystal structure revealed that LSLa is a homohexamer endowed with 32 point group symmetry, composed of noncovalently bound subunits of ~35 kDa. Sedimentation equilibrium analyses carried out in a wide range of protein concentrations confirmed this finding as the results indicate

that LSLa behaves in solution as a monomer-hexamer associative system essentially displaced to the oligomeric form, in perfect agreement with the crystallographic results.

The structure of each monomeric subunit consists of two different modules corresponding to the functional modules, lectin and pore-forming (Fig. 1). The lectin module (residues 1-150) has a globular structure ($39 \times 32 \times 32$ Å^3), consisting of a β-trefoil fold. Conversely, the pore-forming module (residues 151-314) has an elongated shape ($72 \times 23 \times 23$ Å^3) and can be effectively divided into two distinct domains: domain 2 is a twisted five-stranded antiparallel β-sheet and a long amphipathic loop and domain 3 consisting of a β-sandwich with one two-stranded and one three-stranded sheet. This last module reveals clear structural similarity between LSLa and the large lobe of aerolysin[12] a β-pore-forming toxin from the Gram-negative bacterium *Aeromonas hydrophila*, despite negligible sequence identity. This structural analogy together with previous results[1,2] suggests that LSLa is indeed a β-pore-forming toxin (β-PFT).

The N-Terminal Lectin Module

The N-terminal module of LSLa consists of a β-trefoil (Fig. 2), a well known protein fold within the lectin realm.[13-15] It is formed by a six-stranded antiparallel β-barrel capped on one end by three two-stranded hairpins (strand pairs β2 and β3, β6 and β7, β10 and β11). The global structure of the β-trefoil results from the tandem repetition of an underlying motif (called α, β, γ, respectively) composed of four strands separated by three loops, the third one containing a single-turn 3_{10} helix (Fig. 2). Each basic motif may have evolved from a primordial galactose-binding peptide of ~40 residues,[16] with the β-trefoil being the outcome of successive gene duplication cycles. Obviously, the β-trefoil architecture provides the structural basis of the subdomains previously found at the primary level.[2] Analysis of the primary and tertiary structure of these three motifs reveals that in effect they are homologous with each other, but also reveals the existence of notable divergences. Thus, pairwise sequence comparisons between motifs reveal identities around 20%, with only four residues being conserved in the three motifs. Yet, despite this degeneration, the three dimensional structure of the C_α backbone is essentially conserved. Structural comparison with known folds with DALI[17] reveals high homology with sugar-binding domains of toxins that exert their cytotoxic action by binding glycoproteins, such as *C. botulinum* cytotoxin[18,19] and the above mentioned ricin,[5] abrin[8] or CEL-III.[10] In all these cases, the level of similarity at the primary level is very low, which suggests that the β-trefoil is versatile protein architecture that withstands dramatic sequence departures.

Previous work demonstrated that lactose, N-acetyl-lactosamine and other galactose-related saccharides inhibited the hemagglutination and hemolytic activities of LSLa,[2] indicating that sugar-binding is involved in both molecular processes. Presumably, a specific recognition of carbohydrates would precede the formation of pores within the erythrocyte membrane.

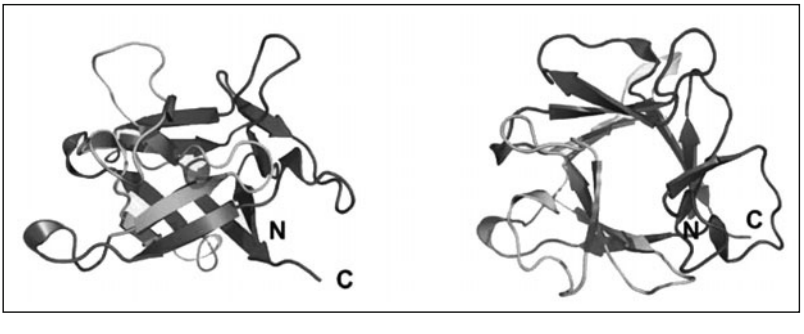

Figure 2. The lectin β-trefoil fold. Two orthogonal views of the N-terminal β-trefoil fold of LSLa are shown in cartoon representation. The structure of the LSLa β-trefoil is based on the tandem repetition of a basic motif composed of four strands separated by three loops. As a result, the structure exhibits a pseudo three-fold symmetry clearly identified when viewed along the molecular symmetry axis (*right panel*).

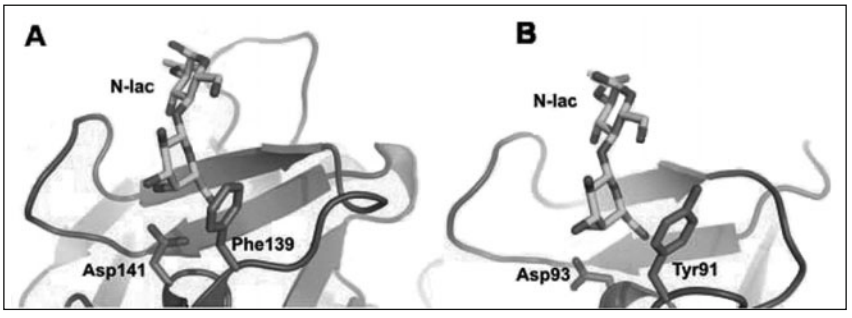

Figure 3. Sugar-binding to the lectin module of LSLa. A) Structure of the N-acetyllactosamine bound (A) to the γ-motif and (B) to the β-motif of LSLa. The two critical amino acid residues for sugar-binding from each motif are shown as *stick* representation. Tyr91 and Phe139 stack against the galactose ring of N-acetyllactosamine and Asp93 and Asp141 hydrogen bonds with the axial C4 hydroxyl group of galactose.

The crystal structures of LSLa complexed with lactose or N-acetyl-lactosamine show sugar binding at two (β and γ) of the three possible sites[3] (Fig. 3) and have permitted identifying residues directly participating in sugar-binding and also explaining the lack of sugar binding to the α site. This binding mode presents close structural similarities to others previously reported for lectin-lactose complexes.[20-23] Among others, the main common interactions identified are stacking interactions between the galactose ring and an aromatic side chain (Tyr-91 and Phe-139) and hydrogen bonds between the axial C4 hydroxyl group of galactose and an acidic lectin side chain (Asp-93 and Asp-141).

The C-Terminal Pore-Forming Module

The pore-forming module (PFM) of LSLa (residues 151-314) is elongated (70 Å long and 20-40 Å in thickness) and can be split into two domains containing mainly β-sheets (Fig. 4). Domain 2 is a five-stranded antiparallel β-sheet with an amphipathic loop on one side (residues 212-241). Besides, domain 3 is a β-sandwich with a two- and a three-stranded sheet, respectively. Although positive hits (in terms of Z-score) were not detected in structural comparisons carried out with the DALI server, structural similarities can be readily identified between domains 2 and 3 of LSLa with domains 3 and 4 of aerolysin,[12] domains 2 and 3 of ε-toxin from *C. prefringens*[24] and domains 2 and 3 of parasporin from *B. thuringiensis*[25] (Fig. 4). As can be observed in this last figure, all these proteins exhibit a virtually identical arrangement of the secondary structures within the compared domains despite a low sequence identity (<20%). Nonetheless, it is interesting to note that in contrast to the large lobe of aerolysin, ε-toxin or parasporin, the PFM of LSLa is composed of a continuous stretch of the protein sequence. We believe that this finding is remarkable as it may indicate the existence of an aerolysin-like pore-forming module structure whose defining characteristics are essentially topological (secondary level) and physico-chemical (primary level) and thus only loosely dependent on specific primary structures. Obviously, this finding raises important questions regarding protein folding and stability as to how essentially unrelated amino acid sequences yield a common protein fold both structurally and functionally. This last issue is especially relevant within the context of membrane pore-forming proteins as these macromolecules experience dramatic conformational changes between a (meta)stable water-soluble state of the toxin and a membrane-embedded functional protein.[26,27]

Regarding the specific role of the above mentioned structural elements of LSLa in pore formation, analysis carried out on the aerolysin-like *C. septicum* α toxin[28] and aerolysin[29] shows that the amphipathic loop in domain 2 lines the channel when inserted into the membrane. The loop presents an almost perfectly conserved alternating pattern of hydrophobic residues that is characteristic of amphipathic transmembrane β-hairpins.[3,29] The crystal structure of LSLa shows that the size

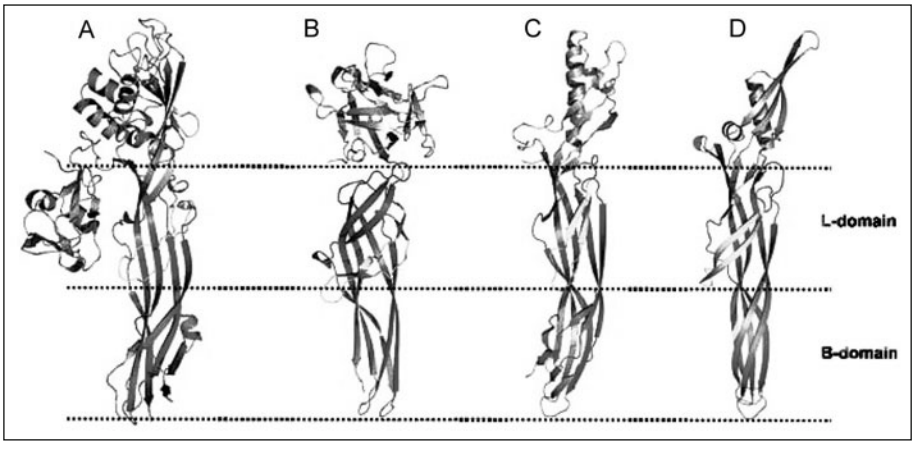

Figure 4. Three dimensional modular architecture of β-pore-forming proteins of the aerolysin family. The protein structures are shown in cartoon representation. A) Proaerolysin[12] from *A. hydrophila,* (B) LSLa[3] from the mushroom *L. sulphureus*, (C) ε-toxin[24] from *C. perfringens* and (D) parasporin from *B. thuringiensis*.[25] The structural similarity appears when the corresponding pore-forming modules are compared; in all cases this module can be split into a L-domain (see the text) composed of a five-stranded β-sheet and an amphipathic loop that lies on it and a B-domain based on a β-sandwich fold.

of the amphipathic β-hairpin, ~30 residues, is similar to *Staphylococcus aureus* α-hemolysin,[30] the anthrax protective antigen[31] and the two transmembrane hairpins of the cholesterol-dependent cytolysin perfringolysin O.[32,33]

Oligomeric State of Water-Soluble LSLa

The crystal structure of LSLa shows that it is a homohexamer endowed with 32 point group symmetry, i.e., LSLa subunits are first organized around 3-fold symmetry axes forming tripoid-like trimers which in turn associate forming a dimer of trimers which are organized around a 2-fold symmetry axis (Fig. 5). The overall dimensions of the hexameric assembly are 70 × 80 × 150 Å^3 which implies that LSLa has an overall cylindrical shape. Conversely, sedimentation equilibrium analyses in a wide range of protein concentrations indicate that LSLa is a

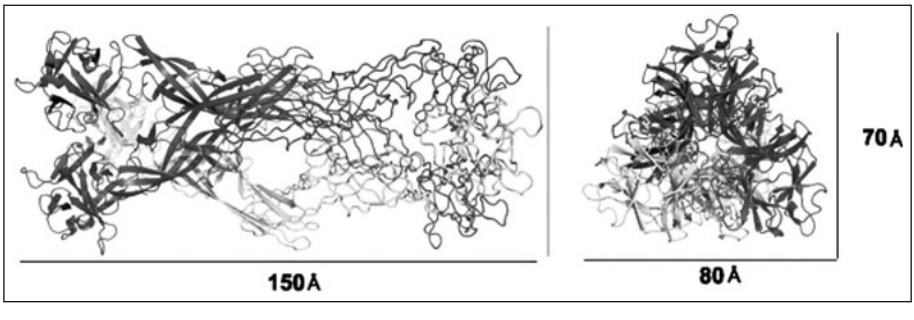

Figure 5. Oligomeric structure of LSLa. Two orthogonal views of the hexameric assembly of LSLa are shown. The three subunits that make up one tripoid-like trimer are shown as cartoon representation and the other three as ribbon models. The dimensions of the oligomer are also shown. The *left panel* shows the hexamer when viewed perpendicular to the molecular two-fold symmetry axis and the right panel when viewed along the three-fold symmetry axis.

monomer-hexamer associative system, essentially displaced to the oligomeric form (equilibrium dissociation constant 10 μM[5]).

Previous biochemical characterization of LSL has shown that both native and recombinant LSL are tetramers in solution with subunits of ~35 kDa as determined by gel-filtration combined with SDS-PAGE.[1,2] The discrepancy between these results and the crystallographic studies may be explained by the clearly nonglobular hydrodynamic behavior of LSLa.

The main contacting interface between subunits (~680 Å^2) is contributed by their domains 3, particularly through interactions between strands β-21 and β-23, which acting as "sticky" adapters between the two contacting sandwiches determine the formation of a large intersubunit β-sandwich. The contacting interface is mainly hydrophobic yet polar residues, in particular threonine and serine residues, are also identified. In this regard, it is remarkable the abundance of these small and polar residues in the region flanking the amphipathic loop, constituting the 42% and 46% of the total residues of strands β16 and β21. As shown below, this feature is shared by the other members of the aerolysin-like proteins which suggests this physicochemical property may be a requisite for pore-forming activity. It is obvious that the oligomeric state of LSLa in solution raises questions regarding the structural rearrangements required for pore-formation and the nature of the factor(s) that trigger such process. In this sense, the magnitude of the area of the contacting interface between protomers (~680 Å^2) and its hydrophobic nature agrees well with strong and non-obligate complexes.[34,35]

A Common Aerolysin-Like Pore-Forming Module Structure?

As above mentioned, the structural results described so far point to a structural similarity at the secondary level between domains 3 and 4 of aerolysin[12] from *A. hydrophila*, ε-toxin[24] from *C. prefringens*, domains 2 and 3 of parasporin from *B. thuringiensis*[25] and domains 2 and 3 of LSLa.[3] For the sake of simplicity, we define as L-domains (from loop-containing domain) those analogous to domain 3 of aerolysin and B-domains (from β-sandwich-containing domain) those analogous to domain 4 of aerolysin.

L-Domains

The L-domains of the members of the aerolysin-like pore-forming toxins are formed by a four or five-stranded β-sheet on one face and a long loop on the other face (Fig. 6). As can be observed in the figure the topology of the sheet is strictly conserved in all the proteins. Two main features are easily identified within this region: first, a cluster of aromatic residues located in the contacting interface between the inner face of the sheet and the amphipathic loop and secondly a predominance of serine and threonine residues mainly distributed in the outer face of the sheet (Table 1). It is noteworthy that with the exception of LSLa where the PFM is continuous in the primary structure, the rest of the PFM's are not delineated on the primary structure[36] but results from the three dimensional arrangement of the corresponding secondary regular elements and therefore are necessarily interconnected with other protein modules. This observation points to the working hypothesis that this group of β-pore-forming toxins may represent the evolutionary outcome of structural convergence to a common module notably efficient in membrane-binding and pore-formation. Although the possibility of a common ancestor cannot be discarded the discontinuous nature of the PFM architecture of aerolysin, ε-toxin and parasporin does not fit well with recent studies that strongly support the notion that multidomain proteins have evolved mechanisms to minimize the problems of interdomain misfolding.[37] On the contrary, the continuous modular nature of LSLa agrees well with the protein having evolved from shuffling of domains with individual functions whose association renders a protein with a new level of functional complexity. The modular structure of LSLa suggests that the PFM may be an autonomous folding unit, a hypothesis that is partially reinforced by the fact that the domain interface is small[3] and also because the lectin module of LSLa properly folds when heterologously produced in *E. coli* (unpublished results).

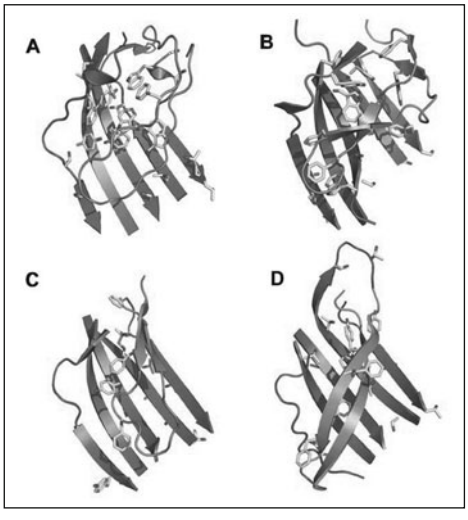

Figure 6. Structure of the L-domains of the areolysin pore-forming module. The Cα backbone is shown as cartoon representation. The scaffold of the L-domain is based on an antiparallel five-stranded sheet with a long loop lying on one of its faces. Aromatic and serine/threonine residues present in the domain are shown as *stick* models. A) L-domain from proaerolysin from *A. hydrophila,* B) from LSLa from *L. sulphureus,* C) from greek epsilon toxin from *C. perfringens* and D) from parasporin from *B. thuringiensis*.

As can be observed in Figure 6, both the length and the three dimensional structure of the long loop that lies on the β-sheet of the L-domains is highly variable. The structural complexity of these regions makes the very definition of its boundaries a difficult and rather arbitrary issue which somehow explains the different alignments reported in the literature.[3,29,38] Thus, we herein operatively define these boundaries in terms of their three dimensional structure as the sequence stretches comprised between residues: V239-W265 (aerolysin); A215-N239 (LSLa); A127-S147 (ε-toxin) and G102-E122 (parasporin). In Table 2 are shown the residues from these loops whose side chains are mostly buried. It is obvious that an imperfect, alternating pattern of buried hydrophobic residues is observed what would parallel only partially the behavior expected for a membrane-spanning β-hairpin.[30] As above mentioned, it has been reported for α toxin[28] from *C. septicum* and aerolysin[29] that this loop becomes the transmembrane β-hairpin when inserted

Table 1. Aromatic and hydroxylated amino acid residues present in L-domain of aerolysin-like proteins

Protein	Aromatic Residues	Serine/Threonine
Aerolysin	F184; W227; W247; W265; Y304; Y306; F404	T190; S228, T230; T232; S264; S267; S272; T274
LSLa	Y158; F202; F216; F223; F228; W238; W240; F246; F279; W290; W304	S167; S168; T169; T197; T199; S200; T205; T245; S247; S302; S303;
ε-toxin	Y79; F131; F135; Y146; F-148; Y244	S76; T110; T112; T114; T116; T151; T153; T155; S157; S245
Parasporin	Y104; F105; F109; F110; F121; Y131; F164	T51; T83; S84; T86; S87; S88; T90; T98; T101; S115; T132; T136; T168; T228; S230

Table 2. Buried residues from the loop within the L-domain of aerolysin-like proteins

Protein	Buried Residues
Aerolysin	T241; F245; W247; P248; V250; T253; L255; I257; I259
LSLa	F216; L220; P221; F223; F228; V234; W238
ε-toxin	A127; F131; V133; P134; F135; V140; L142; T144; Y146
Parasporin	Y104; A106; L108; F110; I119; F121

into the lipid bilayer what is consistent with a mechanism involving the formation of a β-barrel within the membrane similarly to α-hemolysin from *S. aureus*.[30] If this is the case, the mechanism of pore-formation by aerolysin-like proteins would necessarily involve large-scale conformational changes of the loop that should translocate to the lipid bilayer in order for oligomer contacts to be established. In this sense, the need for the loop movement for aerolysin's toxic activity has been demonstrated by mutagenesis studies that incorporated a disulfide-bridge between the loop and the wall of the sheet.[38] The reduced form of the double mutant behave as wild-type toxin but the oxidized form could not oligomerize. Furthermore, the movement of the loop would also demand the existence of a hinge region that supposedly should be located around the boundaries of this structural motif. The identity of this hinge region remains nowadays unknown for aerolysin-like proteins and in this regard, it is noteworthy that no region around the loop shows sequence similarity to the putative hinge region of α-hemolysin (around residues 103-111 and 147-152) and temperature factors of the structures do not permit to deduce highly flexible or dynamic regions around the loops.

B-Domains

The basic structural fold of the B-domains is based on a β-sandwich scaffold. The structures of the sandwiches shown in Figure 7 reveal a minimum common β-sandwich core composed of a three-stranded and a two-stranded antiparallel β-sheets, respectively together with additional specific elements present in proaerolysin and ε-toxin which are related to the proteolytic step required for their respective activation. In the case of aerolysin, the C-terminal activation peptide adopts a strand-helix-strand, with the proteolytic reaction taking place in the loop between residues 422-440.[38] Conversely, in the case of ε-toxin the position of the last two strands lie on opposite sides of the sheets.[24] Doubtless, one of the most conspicuous characteristics of the B-domains is the abundance of serine and threonine residues (Table 3) which are mainly located in the exposed face of the two-stranded β-sheet (Fig. 7). Precisely, these last two strands are the ones which flank the amphipathic loop from the L-domain. A potential role for these residues in oligomerization may come from the detailed analysis of the crystal structure of LSLa, in particular, from the analysis of the subunit interactions within the hexamer. LSLa subunits essentially interact through their B-domains, specifically through interactions between strands β-21 and β-23

Table 3. Hydroxylated amino acid residues present in B-domain of aerolysin-like proteins

Protein	Serine/Threonine
Aerolysin	T218; T223; T275; S276, S278; S280; T284; S461; S463
LSLa	T175; T177; T181; S182; T185; S189; S193; T195; T196; T249; T251; T253; S255; T263; S268; T269; S270; T309; T311; S312; T314
ε-toxin	T91; T94; S102; S104; T106; T161; S166; S287; S289
Parasporin	S65; T67; S69; S71; S77; S79; S142; T144

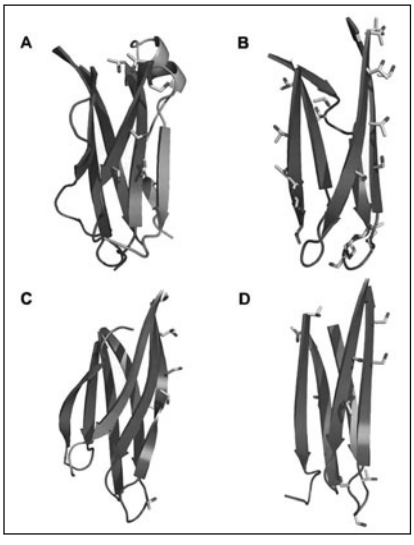

Figure 7. Structure of the B-domains of the areolysin pore-forming module. The Cα backbone is shown as cartoon representation. The scaffold of the B-domain is based on a β-sandwich fold. A conspicuous feature of this domain is that they are rich in serine/threonine residues (*stick* model), mainly located at the two-stranded sheet of the β-sandwich (see the text). A) B-domain from proaerolysin from *A. hydrophila*, B) from LSLa from *L. sulphureus*, C) from ε-toxin from *C. perfingens* and D) from parasporin from *B. thuringiensis*.

which are exceptionally rich in serine plus threonine residues (β-21: 249-TYTATFSVRA-258; β-23: 306-LRHTLTSVTA-314). We believe that the overall hydrophobicity of these sequences together with the presence of small and polar residues (potential hydrogen bond acceptors and donors) makes them suitable adapters for intermolecular interactions. In fact, this mechanism is the one proposed for the aggregation of β-barrels into amyloid-like fibrils by the Dobson[39,40] group. Interestingly and in agreement with this hypothesis, the intermolecular interactions between parasporin molecules identified within the crystal structure are established exclusively through β-strand 5 from the B-domain which is both hydrophobic and contains two serine residues. Additionally, this proposal would agree with the scenario proposed for the proteolytic activation of proaerolysin[38] where the loss of the activation peptide would determine the exposure of a large hydrophobic surface which then would promote protein oligomerization. Also, it has been shown that in *C. septicum* α toxin the C-terminal peptide has an inhibitory effect on oligomerization when applied in excess, indicating the importance of removal of the C terminus for successful oligomerization.[41]

Other New Members in the Aerolysin Family: Basic Aerolysin Pore-Forming Motifs?

Considering the above results that indicate structural similarity despite very low sequence similarity between the members of the aerolysin family, the search for new homologous β-pore-forming proteins exclusively by sequence analyses is not a straightforward task. In this regard, the work carried out by the Zlotkin[42-44] group on hydralysins (Hln's) from the cnidarian *Chlorohydra viridissima* has been especially valuable. Hydralysins are β-pore-forming proteins which make oligomeric pores within the erythrocyte membrane, with an internal diameter of ~1.2 nm.[42] Unlike other cnidarian toxins which are found in nematocytes, hydralysins are produced by digestive cells surrounding the gastrovascular cavity of *C. viridissima* and have been suggested to be involved in prey digestion.[44] Once the pore-forming activity of hydralysins had been demonstrated, an exhaustive search of related

Figure 8. Multiple sequence alignments of putative, novel members of the aerolysin-like family. A) The multiple alignments reveal the presence of homologous regions which reinforces the hypothesis of a minimum aerolysin-like pore-forming motif. B) The structural interpretation of this pore-forming motif within the LSLa structure indicates that the minimum, common regions in these proteins are restricted to the flanking regions of the amphipathic loop. For clarity, the common regions are shown as *cartoon* model and the rest of the molecule as *ribbon* model.

proteins within public databases permitted to conclude that Hln's are indeed β-pore-forming proteins, revealing similarities with members of the aerolysin family (Fig. 8). The same search also detected additional proteins sharing what can be defined as a putative "pore-forming motif". This motif can be described as a region with an alternating pattern of hydrophobic residues flanked by two regions exceedingly rich in serine/threonine residues. In addition, the C-terminal flanking Ser/Thr-rich region is followed by another region rich in hydrophobic residues, with a highly conserved diad of proline residues in the center.[43] The three dimensional interpretation of these results indicates that the *aerolysin pore-forming motif* identified would be made up of the complete two-stranded sheet from the B-domain together with two strands from the other sheet and also by the loop from the L-domain. It is interesting to note that according to the scenario proposed previously, these regions would provide structural elements directly participating in pore-formation (the loop from the L-domain) and oligomerization (β-strands from the B-domain) which reinforces the suggestions that this alignment reveals key features of pore-forming proteins.

Conclusion

One promising future avenue for research is elucidating the biological roles played by pore-forming proteins, in the context of the producing organism and the ecosystem in which it lives. Pore-forming proteins produced by pathogenic bacteria are often part of the chemical armament used to establish infection.[45] However, the role of other pore-forming proteins is unclear—proteins suggested to contain the aerolysin pore forming motif[42] (as described above) are found in the skin and gut of amphibians,[46,47] as subunits of moth yolk protein[48] and as components of earthworm innate immunity.[49] Even the role LSL's play in the biology of the *L. sulphureus* fruiting body is unclear. The ability of pore-forming proteins to create de-novo transmembrane channels in a receptor-mediated, spatially and temporally-restricted manner enables them to perform many different tasks, including the formation of intracellular chloride[50] and calcium channels[51,52] and participation in apoptosis, e.g., Bcl-2 family.[53,54] Having learned how different pore-forming proteins contain similar structural motifs, further research will help elucidate the way the structures of the proteins have evolved to enable them to fulfill what we believe to be diverse biological roles.

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