Structural Analysis of the Laetiporus sulphureus Hemolytic Pore-forming Lectin in Complex with Sugars*

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LSL is a lectin produced by the parasitic mushroom Laetiporus sulphureus, which exhibits hemolytic and hemagglutinating activities. Here, we report the crystal structure of LSL refined to 2.6-Å resolution determined by the single isomorphous replacement method with the anomalous scatter (SIRAS) signal of a platinum derivative. The structure reveals that LSL is hexameric, which was also shown by analytical ultracentrifugation. The monomeric protein (35 kDa) consists of two distinct modules: an N-terminal lectin module and a pore-forming module. The lectin module has a β-trefoil scaffold that bears structural similarities to those present in toxins known to interact with galactose-related carbohydrates such as the hemagglutinin component (HA1) of the progenitor toxin from Clostridium botulinum, abrin, and ricin. On the other hand, the C-terminal pore-forming module (composed of domains 2 and 3) exhibits three-dimensional structural resemblances with domains 3 and 4 of the β-pore-forming toxin aerolysin from the Gram-negative bacterium Aeromonas hydrophila, and domains 2 and 3 from the ε-toxin from Clostridium perfringens. This finding reveals the existence of common structural elements within the aerolysin-like family of toxins that could be directly involved in membrane-pore formation. The crystal structures of the complexes of LSL with lactose and N-acetyllactosamine reveal two disaccharide-binding sites per subunit and permits the identification of critical residues involved in sugar binding.

Hemolytic lectins are sugar-binding proteins that lyse and agglutinate cells. Recently, a novel toxic lectin from the parasitic mushroom Laetiporus sulphureus (LSL)1 that shows strong hemagglutination and hemolytic activity has been characterized (1). These activities are mediated by binding carbohydrates as demonstrated by the correlation found between both processes in sugar inhibition assays, which reveals LacNAc (Galβ14GlcNAc) as a potent inhibitor. The N-terminal module of LSL is a carbohydrate recognition domain as deduced from the C-terminal deletion mutant of LSL, LSLa-D1, which shows hemagglutination, but not hemolytic activity. Conversely, the C-terminal module shows structural similarity to pore-forming bacterial toxins suggesting a similar mode of biological activity. In this sense, LSL-induced hemolysis proceeds through a mechanism involving the formation of impermeable membrane pores with a functional diameter smaller than 3.8 nm, as revealed by osmotic protection experiments (1). After pore formation, erythrocytes are ruptured by a colloid-osmotic lysis mechanism. Thus, LSL appears to exhibit two functional modules: lectin and pore-forming. LSL is a non-covalent-linked oligomeric structure, in which the C-terminal domains play a crucial role as deduced by the monomeric and highly polar character of LSLa-D1.

Here we report the crystal structure of the novel eukaryotic pore-forming toxin present in the parasitic mushroom L. sulphureus. The two-module structure of LSL described herein suggests the existence of mechanisms of evolution involving the association of discrete stable functional modules into a final modular protein with toxic character. Additionally, the three-dimensional similarity between LSL and aerolysin from the bacterium Aeromonas hydrophila (2) and also ε-toxin from Clostridium perfringens (3), indicates a mechanism of pore formation in which amphipathic β-hairpins associate into a transmembrane β-barrel, as recently proposed for α-toxin of Clostridium septicum (4), a homologous toxin to aerolysin.

EXPERIMENTAL PROCEDURES

Materials—Lactose (Galβ14Glc) was purchased from Sigma. LacNAc was synthesized and available from other studies. The lectin from the mushroom L. sulphureus was prepared as described by Tateno and Goldstein (1).

Analytical Ultracentrifugation—Equilibrium ultracentrifugation experiments were performed at 10,500 and 12,500 × g at 20 °C, using a Beckman XL-A ultracentrifuge with an An-50Ti rotor and standard double sector centerpiece cells. Solvent density (1.005 g/ml) and the partial specific volume of LSL (0.722) were calculated from the buffer composition (137 mM NaCl, 3 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4) and from the predicted amino acid composition, respectively, with SEDNTERP (5). Data from sedimentation velocity and equilibrium experiments were analyzed using the programs Sedfit (6) and Sedphat (7), respectively.

Data Collection and Processing—Initial diffraction data were collected on hexagonal LSL crystals obtained as previously described (8). For derivatization, we soaked the crystals for 24 h in crystallization solution containing 20 mM K3Pt(CN)6. The crystals were then transferred to the cryoprotectant solution (soaking solution plus 30% [v/v] glycerol) for ~5 s and flash-frozen in the cold nitrogen stream (100 K).
All data were collected at beamline ID14-1 (Å = 0.934 Å) at the ESRF (Grenoble, France), using an ADSC Q4 CCD detector. Data were indexed, integrated, and scaled with the CCP4 suite (9). Data statistics are summarized in Table I. Diffraction data from crystals of LSL-LacNAc complexes were collected in-house on a Kappa 2000 CCD detector with Cu Kα X-rays generated by a Nonius FR-591 rotating-anode generator equipped with Montel mirrors and operated at 45 kV and 100 mA. Data were indexed, integrated, and scaled with Denzo and Scalepack (10). Subsequent data manipulation was carried out with the CCP4 software suite (9).

Structure Determination and Refinement—The structure of LSL with bound Lac (see below) was determined by the method of single isomorphous replacement using the anomalous scatter signal of a platinum derivative. The platinum atoms were located on both isomorphous and anomalous difference Patterson maps. Refinement of heavy atom parameters and phase calculations to 2.6-Å resolution were performed with SOLVE (11), MLPHARE (9), and SHARP (12). Solvent flattening and histogram matching were performed with DM (9) assuming a solvent content of 40%, i.e., two LSL molecules in the asymmetric unit, rendered a low-quality electron density map. On the contrary, the electron density map obtained considering one LSL molecule in the asymmetric unit (70% solvent content), was of excellent quality, and enabled the incorporation of a complete protein model using O (13). Only two long loops in the C-terminal module did not show interpretable density. After refinement, only two regions of the atomic model, those comprising residues 180–186 and 258–263, are not defined in the electron density map. The initial model had a crystallographic R factor of 40% for all reflections in the resolution range of 50–2.6 Å. Structure refinement was performed using the programs CNS (14) and REFMAC5 (15). Model building was done with O (11). The final model has an R factor of 22.7% and a free R factor of 27.9%, and included 312 amino acid residues, 1 Lac, 6 glycerols, and 77 solvent molecules. The structures of the LSL-LacNAc complexes were determined by the molecular replacement method with MOLREP (9) using the structure of LSL as search model. The quality of the models were checked using the program PROCHECK (16). Phasing and refinement statistics are summarized in Table I.

RESULTS AND DISCUSSION

Overall Description of the Structure—The monomeric LSL molecule consists of two different structural modules corresponding to the previously reported functional modules, lectin and pore-forming (Fig. 1). The lectin module (residues 3–150) has a globular structure, consisting of a β-trefoil with approximate dimensions 39 × 32 × 32 Å3. The structure of the complexes with Lac and LacNAc reveals two sugar binding sites. The pore-forming module (residues 151–314) consists of an elongated lobe with dimensions 72 × 23 × 23 Å3. This module can be effectively divided into two regions: domain 2 is formed by a twisted five-stranded anti-parallel β-sheet together with a long amphipathic loop located in one face of the sheet, and domain 3 consisting of a β-sandwich.

The N-terminal Lectin Module—The N-terminal module of LSL consists of a β-trefoil (Fig. 2), a characteristic fold previously described in other lectins (17–19). Only two long loops in the C-terminal module did not show interpretable density. After refinement, only two regions of the atomic model, those comprising residues 180–186 and 258–263, are not defined in the electron density map. The initial model had a crystallographic R factor of 40% for all reflections in the resolution range of 50–2.6 Å. Structure refinement was performed using the programs CNS (14) and REFMAC5 (15). Model building was done with O (11). The final model has an R factor of 22.7% and a free R factor of 27.9%, and included 312 amino acid residues, 1 Lac, 6 glycerols, and 77 solvent molecules. The structures of the LSL-LacNAc complexes were determined by the molecular replacement method with MOLREP (9) using the structure of LSL as search model. The quality of the models were checked using the program PROCHECK (16). Phasing and refinement statistics are summarized in Table I.
plet, arrange in a triangular array that gives rise to a pseudo 3-fold symmetry. The global structure of the LSL β-trefoil is based on tandem repetition of a basic motif (called α, β, and γ, respectively) composed of four β-strands separated by three loops, the third one containing a single-turn 310 helix (Fig. 2B). In this regard, it has been previously suggested that the existence of a primordial galactose-binding peptide of 40 residues would be the ancestor of the basic motifs of the LSL β-trefoil scaffold (20). Analysis of the primary (Fig. 2C) and tertiary structure (Fig. 2D) of the three motifs reveals that in effect they are homologous with each other, but also reveal the existence of clear divergences. Thus, although pairwise sequence comparisons between motifs reveal identities around 20%, only four conserved residues are contributed by each motif (Fig. 2C); yet, despite the primary degeneration, the three-dimensional structure of the peptide backbone of the motifs is essentially conserved (Fig. 2D). Doubtless, the interplay between three-dimensional conservation and primary departures should provide the structural basis for the different sugar binding properties of each motif (see below).

Structural comparison of the N-terminal module of LSL, with known folds using the DALI algorithm (21), revealed a high homology with proteins known to interact with sugars, for example, the hemagglutinin component (HA1) of the progenitor toxin from Clostridium botulinum (22) (1qxm-A, Z = 17.6), basic fibroblast growth factor (23) (1bgf-A, Z = 16.8), Amaranthus caudatus agglutinin (24) (1jly-A, Z = 15.4), inositol 1,4,5-triphosphate receptor (25) (1n4k-A, Z = 14.7), mannose receptor fragment (26) (1dqg-A, Z = 13.7), hisactophilin (27) (1hce-A, Z = 13.2), the toxin abrin (28) (1abr-B, Z = 12.8) and the tetanus neurotoxin fragment (29) (1a8d, Z = 10.4). Perhaps, the most notable of the DALI results is the remarkable resem-
The N-terminal module of LSL with sugar-binding domains of toxins that exert their cytotoxic action by binding glycoproteins; particularly, the hemagglutinin component (HA1), which has been shown to increase the toxicity of C. botulinum neurotoxin by binding to oligosaccharides lining the intestine (30, 31), or abrin and the closely related deadly plant toxin ricin (20), which bind their target membranes through a β-trefoil motif. The emergence of this structural motif in toxins from diverse sources could suggest a mechanism of evolution involving the association of discrete stable functional modules into a final modular protein with toxic character.

Sugar-binding Sites—Previous work demonstrated that Lac, LacNAc, and other galactose-related saccharides inhibited the hemagglutination and hemolytic activity of LSL (1). The correlation between both processes indicates that sugar binding is involved in both molecular mechanisms. Because experiments of osmotic protection of erythrocytes showed that hemolysis results from the formation of discrete pores in the membranes, it can be inferred that the molecular mechanism of pore formation by LSL involves the initial, specific recognition of carbohydrates.

The x-ray crystal structures of LSL complexed with Lac or LacNAc show sugar binding at two (β and γ) of the three possible sites. Moreover, the soaking experiments considered herein reveal significantly different sugar affinities between the above two binding sites. The color coding of secondary structure elements: α-motif, blue; β-motif, red, γ-motif, green. D, molecular surface of the β-trefoil scaffold, with the LacNAc molecules (as stick models) bound to the β- and γ-sites. The surface is colored according to the electrostatic potential, blue for positive and red for negative. E, structure of Lac bound to one binding site in ricin B-chain (Protein Data Bank code 2AAI; Ref. 40). A–C were prepared with MOLSCRIPT (54) and RASTER3D (53); the molecular surface was built with the program GRASP (55).

Fig. 3. Lactose and N-acetyllactosamine binding to the lectin module of LSL. A, structure of the Lac molecule bound to the γ-motif of LSL. B, three-dimensional structure of LacNAc bound to the γ-motif of LSL. The models show the F – F, electron density maps contoured at 2.5σ around the Lac molecule initially calculated without including the disaccharide. C, LacNAc bound to the β-motif. The F – F, electron density map contoured at 3σ calculated without including the disaccharide is shown in blue around the bound carbohydrate. Broken lines indicate hydrogen bonds between sugar and protein residues. Color coding of secondary structure elements: α-motif, blue; β-motif, red, γ-motif, green. D, molecular surface of the β-trefoil scaffold, with the LacNAc molecules (as stick models) bound to the β- and γ-sites. The surface is colored according to the electrostatic potential, blue for positive and red for negative. E, structure of Lac bound to one binding site in ricin B-chain (Protein Data Bank code 2AAI; Ref. 40). A–C were prepared with MOLSCRIPT (54) and RASTER3D (53); the molecular surface was built with the program GRASP (55).
source of this molecule (1). Additionally, a well ordered glycerol molecule is found in each of the other two motifs, these molecules coming from the short soak in the cryoprotectant solution that contained 30% (v/v) glycerol. In this sense, it has been suggested that this compound together with ethylene glycol is good at mimicking carbohydrate binding (22).

The γ site binds Lac at its nonreducing galactose (Gal) moiety (Fig. 3A). The aromatic ring of Phe-139 makes van der Waals interactions with the plane formed by the C-3, C-4, C-5, and C-6 carbon atoms of the Gal residue in a similar fashion to that seen in other protein-carbohydrate complexes (32, 39). The axial C4 hydroxyl group can form hydrogen bonds with the ring N-ε of His-125 and with the carboxyl oxygen atoms of Asp-141. On the other hand, the C-6 hydroxyl group hydrogen bonds to the ring oxygen atoms of Asp-93, as there is no amino acid residue equivalent to His-133 in the γ site. The only interaction involving the glucose residue is that observed between the C3’ hydroxyl group and the NH1 and NH2 atoms of Arg-123. Consistent with this pattern of toxin-Lac interactions, the x-ray structures of some bacterial toxins complexed with Lac show that interaction with the disaccharide is mediated essentially by the galactose (34–36).

Recent studies revealed the higher efficiency of LacNAc relative to Lac in inhibiting both hemagglutination and hemolysis (1). Quick soaking experiments using cryoprotectant solution containing 20 mM LacNAc results in the incorporation of this molecule into the γ site of LSL as revealed by the unambiguous difference electron density (Fig. 3B). The structure of the complex was determined at 2.58 Å resolution (Table I) by molecular replacement (see “Experimental Procedures”). Whereas the Lac portion of LacNAc can perfectly be superposed to the Lac ligand present in the LSL-Lac complex, the presence of the acetamido moiety promotes several new interactions between sugar and protein. Thus, the carbonyl oxygen of the N-acetyl moiety of LacNAc can form hydrogen bonds with the ring Nε-1 atom of Trp-131 and with nitrogen atoms NH1 and NH2 of Arg-76; additionally, a water molecule mediates a hydrogen bond between the nitrogen atom of the acetamido moiety and the peptidic oxygen of Gln-127 (Fig. 3B). No additional sugar binding was observed in the other two potential LSL sites apart from the presence of a glycerol molecule in each motif. In addition to the γ site of LSL, the β site also binds LacNAc as revealed by overnight soaking experiments in the presence of 0.1 mM LacNAc. Under these experimental conditions, LSL binds two LacNAc molecules, one at the γ site and one at the β site. In this second binding site, the aromatic ring of Tyr-91 and the side chains of Asp-93 and Asn-94 play similar roles to those of Phe-139, Asp-141, and Gln-142 present in the γ motif, respectively (Fig. 3C). In this case, however, the axial C4 hydroxyl group can only form hydrogen bonds with the carboxyl oxygen atoms of Asp-93, as there is no amino acid residue equivalent to His-125 in the γ site. An important difference with respect to the γ site is the absence of water molecules mediating interactions between sugar and protein presumably because of critical amino acid changes. Thus, the presence of Ile-85 (equivalent to His-133) and the absence of a residue equivalent to Arg-76 would prevent the stabilization of a water molecule that similarly to the γ site would coordinate the O-5 atom of the galactose residue. On the other hand, the bulky side chain of Phe-73 (equivalent to Val-121), which interacts with Arg-75 through cation-π interactions, sterically hinders the presence of a solvent molecule that would stabilize the acetamido moiety as observed in the γ motif. Finally, no further

### Table I

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* Values in parentheses are for the highest resolution shell.

b $R_{	ext{sym}} = \frac{\sum_{h} \left| I_h \right| - \langle I_h \rangle \sum_{h} \left| I_h \right|}{\sum_{h} \left| I_h \right|}$, where $h$ represents a unique reflection and $j$ symmetry-equivalent indices. $I$ is the observed intensity and $\langle I \rangle$ is the mean value of $I$.

c $R_{\text{calc}} = \sum_{\text{centric}} \left| F_{\text{calc}} - F_{\text{obs}} \right|$, summed over centric reflections.
stabilization of this last group is observed as there is no homologous side chain to Trp-131. As a result of the above amino acid changes between both sugar-binding sites, the electrostatic properties of the surface of both sites are different (Fig. 3D). These results clearly indicate the reduced number of stabilizing interactions between the disaccharide LacNAc in the β site with respect to the γ site that may explain their differences in affinity for this sugar. This correlates well with the average values of the atomic B-factors of the bound ligands in the LSL/Aerolysin complex that indicate a lower occupancy of the β site as compared with the γ site (62 Å²; average value for protein atoms in both sites ~40 Å²).

Finally, it is worth noting that although conserving the same structural framework of the β- and γ-sites, the α-site does not bind sugars in our experimental conditions. In this regard, the presence of Ala-43 instead of an aromatic residue equivalent to Tyr-91 (β-site) or Phe-137 (γ-site) may be critical for explaining the lack of sugar binding.

The structure of the above described complexes between LSL and Lac or LacNAc present close structural similarities to others previously reported for lectin-lactose complexes (33, 37–39). Thus, the existence of stacking interactions between the Gal ring and an aromatic side chain, together with hydrogen bonds between the axial C4 hydroxyl group of Gal and an acidic lectin side chain, are strictly conserved. This finding is in agreement with the lack of sugar binding by LSL in the α-site. In the particular case of the ricin B-chain (20, 40), which is composed of two β-trefoils each one with a galactose-binding site, Gal ring hydrophobically stacks against Trp-37 or Tyr-248, and also interacts through its C4 hydroxyl group with the carboxyl group of Asp-22 and Asp-234. For comparison purposes, Fig. 3E shows the structure of the Gal-binding site of the ricin B-chain.

The C-terminal Pore-forming Module—The pore-forming module (PFM) of LSL (residues 149–314) has an elongated shape, 70 Å long and 20–40 Å in thickness (Figs. 1 and 4). Apart from a single-turn 3₁₀ helix, the β-structure is the only regular secondary structure in the module, consisting of 11 β-strands (β13 to β23), the last two strands crossing it from tip to tip. The PFM of LSL can be suitably divided into two domains (Fig. 4). Domain 2 is a highly twisted five-stranded anti-parallel β-sheet capped on one side by an amphipathic loop (residues 212–241). Besides, domain 3 consists of a β-sandwich formed by a two- and a three-stranded anti-parallel sheets, respectively. In contrast to the results obtained with the lectin module, structural comparison of the PFM of LSL using the DALI algorithm rendered no positive hits. Still, LSL sequence comparison searches showed structural similarity to mosquitocidal toxin (MTX2) and α-toxin from C. septicum (1), this last belonging to the aerolysin-like family of pore-forming toxins (4, 41). Interestingly, comparison of the structure of LSL with those of aerolysin (2) and α-toxin from C. perfringens (3), the only members of the family with known three-dimensional structure, reveals structural similarities between domains 2 and 3 of LSL with domains 3 and 4 of aerolysin, and domains 2 and 3 of α-toxin, respectively (Fig. 4A). Markedly, this comparison permits the inference for the presence of common structural elements that could be directly involved in membrane pore formation. On the other hand, these common structural features are distinct from the aerolysin-like family of toxins: a set composed of a five-stranded β-sheet and an amphipathic loop, and a distal β-sandwich. According to the low sequence identity between these proteins (~20%), an evident feature of these elements is their versatility in the sense that they accommodate very different sequences without significant structural departures (Fig. 4A). Furthermore, it is interesting to note that in contrast to aerolysin and α-toxin, the PFM of LSL constitutes a structural module that is composed of a continuous stretch of the protein sequence. Yet, it is remarkable that the topology or connectivity between the above common structural elements and the other structural elements is identical for the three proteins.

Regarding the specific role of the above mentioned structural elements in pore formation, an exhaustive analysis carried out recently on C. septicum α-toxin has shown that the equivalent region to the amphipathic loop becomes an amphipathic trans-

**FIG. 4. Three-dimensional similarities between aerolysin, ε-toxin, and LSL.** A, ribbon diagrams of the bacterial β-pore-forming toxins aerolysin from *A. hydrophila* (2), ε-toxin *C. perfringens* (3), and LSL from *L. sulphureus*. The five-stranded β-sheets are depicted in blue, and the β-sandwiches in red; amphipathic loops are indicated in yellow. d₁–d₄ are for the corresponding domains. Domains 1 and 2 from aerolysin, and domain 1 from both ε-toxin and LSL are shown in gray. B, sequence alignment of the putative transmembrane domains of the aerolysin-like family (LSL, aerolysin, ε-toxin, α-toxin, and enterolobin). Residues conserved in at least three members are shown in red. Positions with a negative value of average free energy of transfer (ΔG kcal/mol) from water to a membrane interface according to Wimley et al. (42) are indicated with the blue background.
membrane β-hairpin when the C. septicum α toxin oligomer was inserted into the membrane (4). An analysis of the putative transmembrane domains of the aerolysin-like family in terms of position-dependent average free energy of transfer (ΔG kcal/mol) from water to either n-octanol or to a membrane interface calculated according to Wimley et al. (42) demonstrates the presence of an almost perfectly conserved alternating pattern of hydrophobic residues that is characteristic of amphipathic transmembrane β-hairpins. The crystal structure of LSL shows that the size of the amphipathic β-hairpin, ~30 residues, is similar to Staphylococcus aureus α-hemolysin (43), the anthrax protective antigen (44), and the two transmembrane hairpins of perfringolysin O (45, 46). In summary, these results indicate that LSL is a β pore-forming toxin (47) capable of forming a transmembrane β-barrel by contributing individual β-hairpins.

Oligomeric State—The biochemical characterization of LSL has shown that both native and recombinant LSL are oligomeric structures (1). Estimation of the molecular mass by SDS-PAGE and gel filtration chromatography indicated that LSL exists as a tetramer of subunits of ~35 kDa. Unexpectedly, the crystal structure of LSL reveals the existence of hexameric LSL assemblies (Fig. 5). Considering the discrepancy between the different experimental approaches, we carried out analytical ultracentrifugation assays (Fig. 6). The sedimentation velocity analysis of LSL in the 0.18–1.10 mg/ml concentration range demonstrated that the protein is very homogeneous, with a sedimentation coefficient of 8.56 S (data not shown). Additionally, sedimentation equilibrium analyses indicate that the data are well described by a monomer-hexamer association equilibrium (Fig. 6), which is essentially displaced to the oligomeric form. A fit to a monomer-dimer or monomer-tetramer equilibrium gave much less satisfactory fits. The average molecular mass for 0.18 and 1.10 mg/ml samples were 204.8 and 204.5 kDa, respectively. The hexamer-monomer equilibrium dissociation constant is about 10^5 M. Thus, these results demonstrate that LSL associates as a hexamer in solution, in agreement with the crystal structure.

The LSL monomers associate as a highly symmetrical hexamer, in which protomers essentially interact through their β-sandwich domains forming three extended intersubunit six-stranded anti-parallel β-sheets (Fig. 5). LSL monomers are organized around 3-fold crystallographic axes with the β23 strand of each protomer contacting the C-terminal fraction of the β23 strand of the adjacent protomer. Tripoid-like trimers related by 2-fold crystallographic axes strongly interact exclusively through their β-sandwiches (β21 and β23 strands) to form hexamers endowed with three intersubunit large β-sandwiches (Fig. 5B). Lattice contacts between hexamers are mainly mediated by water molecules, and with the exception of a hydrogen bond between the carboxyl oxygen of Asp-56 and the amide group of Gly-207, involve exclusively the N-terminal domains of neighboring molecules. The limited number of lattice contacts and the 70% solvent content closely resemble a solution environment. Analyses of solvent accessible areas bur-
The best fit to the data set (solid line curves) is plotted against the radial position from the center of the rotor. The best fit to the data set (solid line curves) is plotted against the radial position from the center of the rotor.

ied upon hexamer formation reveal some striking features. Thus, the formation of each of the three intersubunit β-sandwiches within the hexamer involves a contact area of ~680 Å² per monomer. Although this contact area is relatively small, it is essentially hydrophobic (in fact, an important hydrophobic core is formed upon oligomerization involving, Ala-252, Phe-254, Val-256, Ala-258, Leu-306, and Leu-310 of each protomer), which agrees well with a relatively strong and non-obligate complex (48, 49). Additionally, as the two β-sandwiches involved are related by a 2-fold crystallographic axis (i.e. isologous association), this region is highly symmetric, which would make the building of the oligomeric structure more economic (50). Interestingly, the polar residues contributed by the two β-strands involved in oligomerization (β21 and β23) are Thr residues, which may indicate a possible role for small and hydroxylated residues in protomer association. In this regard, LSL has a remarkably high percentage of Ser and Thr residues in the PFM (27.3 versus 12.9% in the lectin module). These residues are mainly concentrated in the region flanking the amphipathic loop, constituting the 42% (8 of 19) and 46% (6 of 13) of the total residues of β16 and β21, respectively. On the other hand, although the association of the lectin modules involves a contact area of ~730 Å² per monomer, this region is essentially polar, which is typical of a very weak association (48, 49), in agreement with the monomeric character of the C-terminal LSL deletion mutant lacking the region between residues 187 and 314 (LSLα-D1) (1). Finally, the oligomeric character of LSL in solution raises important questions regarding the structural rearrangements required for membrane pore formation, which are currently under study.

Interestingly, Uchida et al. (51) have recently reported the crystal structure of the Ca²⁺-dependent hemolytic lectin CEL-III from Cucumaria echinata. CEL-III is organized in three domains: two N-terminal β-trefoil domains (domains 1 and 2), and a C-terminal pore-forming domain (domain 3). As is the case for LSL, this modular design is consistent with evolution processes involving the association of discrete stable functional modules into a final modular protein with toxic character. Although sequence comparison analysis (not shown) reveals no significant similarity between β-trefoils from CEL-III and LSL, their three-dimensional structure is highly conserved, with root mean square Ca deviations of 1.53 Å (for 103 atoms) and 1.61 Å (for 102 atoms) when comparing LSL domain 1 with CEL-III domains 1 and 2, respectively. Nevertheless, despite this structural similarity a different mechanism of β-galactoside binding can be expected between these proteins as the environment of the sugar-binding motifs are clearly dissimilar. Thus, although an acidic residue (equivalent to Asp-93 or Asp-141 in LSL) is present in all except one of the CEL-III motifs (Asp-43, Asp-141, Asp-188, Asp-229, Asp-276), there are no aromatic residues equivalent to Tyr-91 or Phe-139, which in LSL stack against the Gal ring. This comparison in turn reveals two additional aspects: first, it is precisely in the motif of CEL-III that lacks the acidic residue equivalent to that of LSL where no Ca²⁺ binding is observed (51) and second, the above mentioned equivalent acidic residues are accompanied by two additional aspartic residues (except motif 1γ, which has only one) and an aromatic residue (Tyr-36, Tyr-133, Tyr-181, Tyr-222, and Trp-269), which may be involved in sugar binding as suggested (51). Finally, regarding the PFM of both hemolytic lectins despite the absence of three-dimensional similarity between them, it is remarkable that similarly to CEL-III the region proposed by the above authors as responsible for pore formation (α-helices H-8 and H-9) is very rich in Ser and Thr residues (~34% for the sequence stretch comprising residues 318–360), indicating a potential functional role for these residues in the pore formation mechanism.

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