The structure of phytohemagglutinin-L (PHA-L), a leucoagglutinating seed lectin from Phaseolus vulgaris, has been solved with molecular replacement using the coordinates of lentil lectin as model, and refined to a resolution of 2.8 Å. The final R-factor of the structure is 20.0%. The quaternary structure of the PHA-L tetramer differs from the structures of the concanavalin A and peanut lectin tetramers, but resembles the structure of the soybean agglutinin tetramer. PHA-L consists of two canonical legume lectin dimers that pack together through the formation of a close contact between two β-strands. Of the two covalently bound oligosaccharides per monomer, only one GlcNAc residue per monomer is visible in the electron density. In this article we describe the structure of PHA-L, and we discuss the putative position of the high affinity adenine-binding site present in a number of legume lectins. A comparison with transthyretin, a protein that shows a remarkable resemblance to PHA-L, gives further ground to our proposal.

Lectins are proteins that bind carbohydrates in a reversible and specific manner, and often have hemagglutinating properties. At present, the legume lectins undoubtedly form the most extensively studied group of lectins (Sharon and Lis, 1991). Over 70 different seed lectins have been identified in various Leguminosae species. The function of these lectins in vivo remains unsure, but defense against predation (Chrispeels and Raikhel, 1991) and interaction with symbionts (Diaz et al., 1989) have been proposed. The X-ray structures of eight different legume lectins have been solved and refined: pea lectin (PSL, Einspahr et al. (1986)), lentil lectin (LCL, Loris et al. (1993)), Lathyrus ocrhus isolecitins I and II (LOL I, Bourne et al. (1990); LOL II, Bourne et al. (1994)), Griffonia simplicifolia lectin IV (GS4, Debaere et al. (1990)), Erythrina corallodendron lectin (ECorL, Shaanan et al. (1991)), concanavalin A (Con A, Becker et al. (1975)), peanut agglutinin (PNA, Banerjee et al. (1994)) and, recently, soybean agglutinin (SBA, Dessen et al. (1995)). The seeds of the common bean contain a protein fraction with sugar binding and hemagglutinating properties, called phytohemagglutinin (PHA). This fraction consists of five different tetramers, built out of two polypeptides (L and E) in all possible combinations (L4, L3E, L2E2, LE3, E4). The E-type and L-type subunits are, respectively, responsible for the erythroagglutinating and leucoagglutinating properties of the PHA fraction. The two polypeptides are both members of a family of four different polypeptides encoded by four tightly linked genes, generally referred to as the phytohemagglutinin family of bean proteins. This family, in addition to PHA-E and PHA-L, also contains arcelin (Romero et al., 1986; Hartweck et al., 1991; Goossens et al., 1994), which exists in at least six electrophoretic forms, and an α-amylase inhibitor (Moreno and Chrispeels, 1989). Arcelin and α-amylase inhibitor can be considered as truncated forms of PHA, in which, respectively, one and two loops that play a sugar binding role are missing, abolishing the sugar binding properties. Both arcelin (Osborn et al., 1988) and α-amylase inhibitor (Shade et al., 1994) protect bean seed against predation by pests although the precise origin of the toxicity of arcelin is as yet unknown. In this paper we present the crystal structure of PHA-L, the L4 tetramer. PHA-L is a glycoprotein: each subunit is N-glycosylated at two different sites, with consensus sequence Asn-X-Ser/Thr. The subunit possesses a high mannose type glycan attached at Asn-12, and a complex type glycan at Asn-60 (Sturm and Chrispeels, 1986). The minimal structural unit for high-affinity binding by PHA-L is the pentasaccharide Galβ1→4GlcNAcβ1→2Galβ1→4GlcNAcβ1→6Man, which is found in tetra- and tri-antennary complex type oligosaccharides of mammalian origin (Hammerstrøm et al., 1982). In addition to the sugar binding properties of the legume lectins, a number of legume lectins also bind adenine and related ligands. High affinity binding sites for adenine and its derivatives have been found for PHA-E, Dolichos biflorus seed lectin (DBL), D. biflorus stem and leaves lectin (DBS8), soybean agglutinin, and Phaseolus lunatus lectin (LBL) (Roberts and Goldstein, 1982; Roberts and Goldstein, 1983a; Gegg et al., 1992). In this article, we suggest the possible location of this site, based on photoaffinity labeling of the adenine site in PHA-E and LBL (Maliarik and Goldstein, 1988) and the common quaternary structure of PHA-L and SBA.

The Crystallographic Structure of Phytohemagglutinin-L*

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Crystallographic Structure of Phytohemagglutinin-L

EXPERIMENTAL PROCEDURES

Crystallization and Data Collection—Crystallization of PHA-L has been described elsewhere (Dao-Thi et al., 1996). Briefly, the crystals were grown at 4°C by vapor diffusion using the hanging drop method. The composition of the bottom solution was 8% (w/v) PEG 6 kDa (Janssen Pharmaceutical) and 100 mM Tris, pH 8.5. Droplets consisted of 5 µl of bottom solution and 5 µl of 5 mg/ml PHA-L solution. PHA-L was purchased from Sigma. PHA-L crystallized in the monoclinic space group C2, with cell parameters a = 106.3 Å, b = 121.2 Å, c = 90.8 Å, and β = 96.7°. The asymmetric unit contains one complete PHA-L tetramer. The data collection was performed on a single crystal at room temperature with an Enraf-Nonius FAST area detector, using CuKα radiation generated by a rotating anode x-ray generator (40 kV, 98 mA). Data reduction was done using the MADNESS software package (Pflugrath and Meserschmidt, 1989). The statistics of the data collection are given in Tables I and II.

Structure Determination and Interpretation—All calculations were done on a Silicon Graphics INDY workstation and on a Cray YMP supercomputer. Molecular replacement was carried out by the AMORE software package (Navaza, 1994). The structure of the lentil lectin dimer complexed with sucrose, solved at a resolution of 1.9 Å (Casset et al., 1995; entry 1L5S in the Brookhaven data base) was used as search model, after removal of the solvent molecules, the metal ions and the two bound sucrose molecules. Only two clear solutions were found that were consistent with a tetramer in the asymmetric unit while showing reasonable contacts with the symmetry mates. An initial rigid body refinement was performed using X-PLOR (Brünger, 1992), using data between 10.0 and 4.0 Å, which caused a drop of the R-factor from 52.5 to 41.0%. Model building was done with O, by making extensive use of the provided library of protein structures. Further refinement consisted of restrained individual B value refinement, POWERL positional refinement, and simulated annealing (4000 to 300 K, t = 0.0001 ps) with X-PLOR. All data between 10 and 2.8 Å were used. During the refinement, non-crystallographic symmetry restraints between the four monomers were applied (weight = 300.0 kcal mol⁻¹ Å⁻², σ₂₀₀ = 1.0 Å²). After each refinement step the quality of the model was checked using PROCHECK (Laskowski et al., 1993). We decided not to systematically add water molecules to the structure, as they can cause considerable model overfitting at 2.8 Å resolution. An exception was made for the four water molecules which are ligands of the Ca²⁺ and Mn²⁺ ions present in the monomer, since the electron density is clearly seen for this and since they are a very conserved feature of the legume lectin crystal structures (Loris et al., 1994). The final R-factor of the converged structure was 20.0% for 24850 reflections between 10 and 2.8 Å. The posterior free R value (Brünger, 1996), calculated after subjecting the final structure to simulated annealing refinement against the working set, was 22.9%. Hydrogen bonds were calculated using the HBPLUS program (McDonald et al., 1993). Entry 1SBA (Dessen et al., 1995) in the Brookhaven data base was used to compare PHA-L with SBA. Accessible surface areas were calculated with the GRASP program (Nicholls et al., 1991).

RESULTS AND DISCUSSION

Structure Description—In accordance with the currently accepted refinement protocols, the structure has been refined using non-crystallographic symmetry restraints between the four subunits (termed A, B, C, and D). Hence, the structures of the four subunits are virtually identical, with the exception of a few regions in which the quality of the electron density varies between the different subunits (see below). The r.m.s. differences between the α-carbon positions of the four subunits, calculated with the ISQ explicit command of the O program, vary between 0.11 and 0.16 Å. The structure of the PHA-L monomer contains 233 residues of the 252 residues of the mature monomer, based on the sequence of the PHA-L encoding gene (Hoffman and Donaldson, 1985): no electron density is observed for the final 19 C-terminal residues. The probable reasons for this absence are discussed below. Of the two covalently attached oligosaccharides per monomer (Sturm and Chrispeels, 1986) only the core GlcNAc residue of the high mannose glycan attached to Asn-12 is clearly visible in the electron density, probably due to the flexibility of these glycan moieties. No interpretable density is observed for the small complex type glycan attached to Asn-60. All residues are located in the allowed regions of the Ramachandran plot. A number of regions located in surface exposed loops suffer from rather poor electron density or breaks in the density for the main chain. Breaks in the density of the main chain were observed for regions A36-A38, C36-C38, and D35-D38. These regions were consequently removed from the model. Monomer B shows the best electron density: for this monomer, no main chain electron density breaks are observed, and the overall quality of the electron density is slightly higher. As is the case with the other solved legume lectin structures, the subunit structure in PHA-L consists of a flat, six-stranded β-sheet, and a curved, seven-stranded β-sheet.

Quaternary Structure—PHA-L is a tetramer with approximate dimensions 40 Å × 60 Å × 80 Å, consisting of four identical subunits (Fig. 1). Each monomer is involved in two different monomer-monomer interfaces. The first interface is a conventional β-sheet like contact between two strands, creating a continuous, curved, antiparallel 12-strand β-sheet spanning two monomers (interfaces A-B and C-D). The other interface is mainly formed by van der Waals interactions between two β-strands (interfaces A-C and B-D) (Fig. 2). In the tetramer, the two curved 12-strand β-sheets face each other, creating a large channel between them. The same tetrameric organization is also found in SBA (Dessen et al., 1995). As most tetramers (Miller, 1989), the PHA-L tetramer has internal 222 symmetry. At present, the x-ray structures of three other legume lectin tetramers (PNA, SBA, and ConA) and of three different types of dimers (GS4, EcorL and the “canonical dimer,” as represented by lentil lectin, the two L. ochrus isolectins, and pea lectin) are known. The structures of the tetramers solved so far can be described as classic “dimers of dimers” with a 222 symmetry, with the exception of PNA. The PNA tetramer is a dimer of two GS4-like dimers, but unlike ConA, SBA, and PHA-L, this protein does not possess 222 symmetry. Although SBA and PHA-L on the one hand and ConA on the other hand both possess 222 symmetry and both consist of two canonical dimers (dimers A-B and C-D in PHA-L), their quaternary structures are clearly different (Fig. 1). ConA consists of two canonical dimers that pack together through the formation of a dimer-dimer interface that involves the central part of both canonical dimers. The contact between the two dimers consists mainly of loop interactions. The two canonical dimers in PHA-L pack together mainly through close contacts between the two outmost strands of the 12-strand β-sheet of both dimers. The contacts mainly involve van der Waals interactions between the side chains of these two strands, from residues 181 to 192. Since the dimer interface consists of two strands in two β-sheets that face each other, the side chains of the residues in these strands are consecutively oriented toward the inside of
the monomer and toward the interface. Thus, the residues that mostly contribute to the van der Waals contact surface are residues Ser-186, Ile-188, and Ser-190 while Thr-185, Phe-187, Val-189, and Asp-191 only contribute marginally (see Table III). The fact that the protruding side chains are small (Ile and Ser), allows them to be intercalated in a “zipper-like” fashion (Fig. 2). This intercalation of side chains is normally not present in β-sheet face to face packing, where the side chains of the two sheets involved can be separated by a plane and the interface is essentially smooth (Chothia and Janin, 1981). This kind of architecture was first proposed for silk β-fibroin, and has been observed in pyruvoly-dependent histidine decarboxylase from Lactobacillus (Gallagher et al., 1993; Murzin, 1994). The intercalation of side chains also allows the two strands in a silk β-sandwich to be closer to each other than in the default packing; the backbone atoms are only 6 Å apart in pyruvoyl-dependent histidine decarboxylase, as opposed to 10 Å in a classic β-sheet. The backbone atoms of the two interacting β-strands in PHA-L are 6.5 Å apart. Eight hydrogen bonds are made in the interface between the two dimers, involving two serine side chain hydroxyl groups (Ser-186 and Ser-190) and a lysine main chain oxygen atom (Lys-184) in each monomer (see Table IV). This lysine 184 residue lies on the end of the β-strand and positions its side chain almost in parallel with the β-strand, making van der Waals contacts with three residues from the other strand (Ser-190, Asp-191, and Thr-192). The way in which the two dimers pack in PHA-L creates a large channel in the center of the molecule; the two 12-strand β-sheets facing each other are approximately 18 Å apart in the center. Similar channels are found in tetramers where the monomers, as in PHA-L and ConA, only pairwise interact (Miller, 1989).

The Metal Binding Site—All legume lectins possess two bound metal ions (one calcium ion and one transition metal ion, mainly Mn$^{2+}$) per monomer in the vicinity of the sugar binding site. The presence of these two bound metal ions is vital for the sugar binding capabilities of the legume lectins. The structure of the metal binding site is extremely conserved in the legume lectin structures solved up to now. The two metal ions are ligated by four water molecules and six amino acid residues: 1 His, 1 Glu, 1 Asn, 2 Asp, and 1 hydrophobic residue (Phe in LCL, PSL, LOL I and II, EcorL, SBA; Tyr in ConA and PNA; Trp in GS4). As expected, the metal binding site of PHA-L is highly similar to the metal binding sites found in the other legume lectin structures. The Mn$^{2+}$ ion is co-ordinated by His-137, Glu-122, Asp-124, and Asp-132. The Ca$^{2+}$ ion is co-ordinated by Leu-126, Asp-124, Asn-128, and Asp-132. In all the other legume lectin structures, the position of Leu-126 is occupied by a residue with an aromatic side chain (Phe, Tyr, or Trp, see above), that is involved in hydrophobic contacts with the bound sugar. The Ca$^{2+}$ ion interacts via a water molecule with an oxygen atom of the side chain of Asp-86, and thus stabilizes the conserved Ala-85, Asp-86 cis-peptide bond, that is present in all solved legume lectin structures.

Comparison between PHA-L and SBA—SBA and PHA-L share the same quaternary structure. To investigate the similarity of the interfaces of both proteins, we superimposed both dimer-dimer interfaces. The interface between the two canonical dimers in SBA is extremely similar to the PHA-L interface. As in PHA-L, the association of the two subunits occurs through intercalation of the side chain of 2 Ser (Ser-187 and Ser-191 in SBA) and 1 Ile residue (Ile-189 in SBA). The only substantial difference between the two interfaces is the con-
servative substitution of Lys-184 in PHA-L by Arg-185 in SBA. The long, hydrophobic part of the Arg-185 side chain in SBA is involved in similar van der Waals contacts, but the side chain also forms an additional salt bridge with the Asp-192 residue. The structure of the SBA dimer-dimer interface is shown in Fig. 3.

It has been suggested that in SBA, a C-truncated subunit (240 AA) and a non-truncated subunit (253 AA) have to face each other in the tetramer, because the C-terminal portion of the intact subunit positions itself between the two subunits while the space between the subunits is too small to allow for two C-terminal portions (Dessen et al., 1995). C-terminal truncation has also been reported for PHA-E (Young et al., 1995), but was not investigated for PHA-L. The intact PHA-E subunit consists of 254 AA, the truncated subunit consists of 244 AA. In PHA-L, no electron density is observed beyond 233 Å due to either C-terminal truncation or chain flexibility, or possibly a combination of both. In any case, inspection of the density between the two dimers did not clearly reveal the presence of the C-terminal end of the chain in the space between the two dimers as was reported for SBA, although some uninterpretable electron density was observed.

### Table III

van der Waals contacts between the two canonical dimers in PHA-L for the A-C dimer interface. Atom distances below 4 Å were considered as belonging to atoms involved in van der Waals contacts. The B-D interface is virtually identical.

| Monomer A | Monomer C | Number of contacts |
|-----------|-----------|--------------------|
| Lys-149   | Lys-184   | 1                  |
| Leu-173   | Val-179   | 1                  |
| Pro-181   | Leu-173   | 1                  |
| Lys-184   | Lys-149, Ser-190, Asp-191, Thr-192 | 11 |
| Thr-185   | Ser-190   | 2                  |
| Ser-186   | Ile-188, Val-189, Ser-190 | 9 |
| Phe-187   | Ile-188   | 1                  |
| Ile-188   | Ser-186, Phe-187, Ile-188 | 9 |
| Val-189   | Ser-186   | 1                  |
| Ser-190   | Lys-184, Thr-185, Ser-186 | 10 |
| Asp-191   | Lys-184   | 1                  |
| Thr-192   | Lys-184   | 2                  |

### Table IV

Hydrogen bonds between the two canonical dimers in the PHA-L tetramer.

| Dimer 1 | Dimer 2 | Distance Å |
|---------|---------|------------|
| Lys-184A O | Ser-190C OG | 2.94 |
| Ser-186A OG | Ser-190C OG | 2.70 |
| Ser-190A OG | Lys-184C O | 3.02 |
| Ser-190A OG | Ser-186C OG | 2.57 |
| Lys-184B O | Ser-190D OG | 2.81 |
| Ser-186B OG | Ser-190D OG | 2.83 |
| Ser-190B OG | Lys-184D O | 3.06 |
| Ser-190B OG | Ser-186C OG | 2.76 |

Fig. 2. Stereoview of the two strands that form the 2-fold symmetrical interface between the two canonical dimers in PHA-L. The 2-fold axis is approximately positioned in the center of the figure. The side chains of residues Ser-186, Ile-188, and Ser-190 intercalate, as is clearly visible in the figure. Four hydrogen bonds (Lys-184A O-Ser-190C OG, Ser-186A OG-Ser-190C OG, Ser-190A OG-Lys-184C O, and Ser-190A OG-Ser-186C OG) across the interface are visualized as dashed lines. The hydrophobic part of the side chain of Lys-184 makes extensive van der Waals contacts with Ser-190, Asp-191, and Thr-192 from the other strand. Carbon atoms are white, nitrogen atoms are light gray, oxygen atoms are dark gray.
N-6 derivatives, including a number of adenine-derived plant hormones. This site also binds TNS, but not ANS. High affinity binding of adenine has been shown for SBA, DBL, DB58, PHA-E, and LBL. All these lectins are tetramers, except DB58, which is a dimer. There has been some controversy concerning the number of adenine binding sites per tetramer. A single site per tetramer has been reported for LBL and DBL (Roberts and Goldstein, 1982, 1983b), but more recent research yielded two adenine binding sites for the DBL tetramer (Gegg et al., 1992), suggesting a symmetric binding site at the interface between two subunits, as pointed out by Gegg et al. (1992). The sequences of the adenine binding sites in LBL and PHA-E have been determined by photoaffinity labeling (Maliarik and Goldstein, 1988). The two determined sequences had an overlapping five residues in common, with sequence Val-Leu-Ile-Thr-Tyr (residues 165 to 169 in PHA-E, residues 164 to 168 in LBL (Jordan and Goldstein, 1994)). This sequence is situated in a highly conserved region among the adenine binding legume lectins, and is also present in PHA-L. Furthermore, PHA-E and PHA-L (82% homology) can exchange subunits and must therefore have the same quaternary structure. Investigating the position of this sequence in the PHA-L structure may therefore give us an indication about the global position of the adenine binding site in certain legume lectins. In the PHA-L structure, this sequence is found in the third strand of the flat 6-strand $\beta$-sheet, from residue 163 to 167 (Fig. 4). This $\beta$-sheet is located in the central channel present in PHA-L. If this region is indeed involved in adenine binding, then localization of this region in the solved PHA-L structure suggests that there are two adenine binding sites per tetramer, located at both ends of the protein, in agreement with the work done by Gegg et al. (1992).

![Fig. 3. A stereo figure of the dimer-dimer interface in the SBA tetramer.](image1)

![Fig. 4. A view from the center of the molecule to the dimer-dimer interface on the putative adenine binding site between dimers A and C.](image2)

The organization of the PHA-L tetramer is such that the labeled 5-residue sequences of two monomers are close together, and are related by the 2-fold axis along the length of the molecule, thus creating a binding site with a 2-fold symmetry. Apparently, the adenine binding site is located near the inter-
face between the two canonical dimers that form a tetramer, suggesting an interplay between quaternary structure and function. The two possible binding sites are located approximately 30 Å from each other, far too great to cause direct steric hindrance between two bound adenine molecules. Possibly one bound adenine molecule impairs binding of a second one by an allosteric mechanism, resulting in only one observed adenine binding site. In this respect, it is useful to mention that the binding of certain ligands in the adenine binding site can enhance or diminish the affinity of the hydrophobic binding site for ANS (Roberts and Goldstein, 1983a).

Similar ligand binding in a symmetric binding site, created by the packing of the subunits in a tetramer, also occurs in the human serum protein transthyretin (formerly known as prealbumin). In transthyretin, two monomers associate into a dimer through the formation of a continuous antiparallel β-sheet (Blake et al., 1978) (Fig. 5). Two such dimers pack face to face to form a tetramer, forming a channel between the two β-sheets, as in PHA-L. This channel accommodates two identical binding sites for thyroxine, a steroid hormone (Blake and Oatley, 1977; Ciszak et al., 1992; Wojtczak et al., 1992). Although at first only one binding site for thyroxine was reported, further research showed that binding of thyroxine in one of the binding sites (10^8 M^-1) decreases the affinity of the other site (10^9 M^-1) (Ferguson et al., 1975). Again as in PHA-L, these sites show internal 2-fold symmetry. In addition, transthyretin binds ANS in the thyroxine binding sites. Although the adenine binding site of PHA-E does not bind ANS, it does bind the related ligand TNS. In both transthyretin and PHA-L, the central channel is lined with small, hydrophobic residues (Val, Ala, Ile, and Leu) and Ser or Thr side chains. Inspection of the channel in SBA, which also binds adenine and TNS, revealed a very similar pattern of small, hydrophobic and hydroxyl containing side chains. These striking similarities between PHA-L and transthyretin provide further support for the model of adenine binding in legume lectins proposed here.

Concluding Remarks—In this paper we described the structure of PHA-L. PHA-L is a tetrameric protein, in which two canonical legume lectin dimers pack together through the formation of a close contact between the two outmost β-strands. This way of packing creates a large channel in the center of the protein, lined mainly with Ser, Thr, and small, apolar residues (Val, Ala, Ile, and Leu). We suggest that this channel forms the adenine binding site, based on photoaffinity labeling (Maliarik and Goldstein, 1988) and comparison with transthyretin. The parallels between our model of PHA-L and transthyretin are striking: (i) both consist of similar dimers, (ii) the packing of both dimers in the tetramer is similar, (iii) they both contain a large channel, lined with mainly Ser, Thr, and residues with small hydrophobic side chains, created by the face to face positioning of the two β-sheets, (iv) both transthyretin and PHA-L bind similar ligands (ANS and TNS). Our results indicate that PHA-L and transthyretin apparently converged toward a similar framework for binding small ligands with aromatic groups.

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