Solution Structure of Plant Nonspecific Lipid Transfer Protein-2 from Rice (Oryza sativa)*

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The three-dimensional structure of rice nonspecific lipid transfer protein (nsLTP2) has been solved for the first time. The structure of nsLTP2 was obtained using 813 distance constraints, 30 hydrogen bond constraints, and 19 dihedral angle constraints. Fifteen of the 50 random simulated annealing structures satisfied all of the constraints and possessed good nonbonded contacts. The novel three-dimensional fold of rice nsLTP2 contains a triangular hydrophobic cavity formed by three prominent helices. The four disulfide bonds required for stabilization of the nsLTP2 structure show a different pattern of cysteine pairing compared with nsLTP1. The C terminus of the protein is very flexible and forms a cap over the hydrophobic cavity. Molecular modeling studies suggested that the hydrophobic cavity could accommodate large molecules with rigid structures, such as sterols. The positively charged residues on the molecular surface of nsLTP2 are structurally similar to other plant defense proteins.

Plant nonspecific lipid transfer proteins (nsLTPs)1 have been isolated from a number of plant species including wheat, rice, and barley (1). NsLTPs enhance the intermembrane exchange of glycosylated and reduced nsLTP fragments from beer and are involved in froth formation during the malting and brewing processes (7). This article must therefore be hereby marked "published, JBC Papers in Press, May 13, 2002".

Nuclear Magnetic Resonance Experiments and Structure Calculations—Rice nsLTP2 was purified from rice flour as described previously (11). All NMR experiments were carried out on a 3 mm sample of rice nsLTP2 dissolved in 50 mM phosphate buffer (90% H2O and 10% D2O, pH 6.4) containing sodium 3-(trimethylsilyl)[2,2,3,3-2H]propionate (d4-TSP) as the internal standard. A Bruker 600 MHz NMR spectrometer was used to record two-dimensional TOCSY and NOESY spectra with 512 t1 increments and 2048 t2 complex data points at 25 °C. All of the NMR spectra were processed using XWIN-nmr (Bruker) and analyzed using SPARKY (12). Distance constraints were derived from a y spectrum recorded with a 150-ms mixing time. A TOCSY spectrum was used to derive phi (φ) angle constraints (13). Hydrogen bonding information was obtained from amide exchange data. The experimental NOEs were classified as weak, weak medium, medium, strong medium, or strong according to standard procedures (14). Structure calculations were carried out using XPLOR, version 3.8 (15).

Lipid Transfer Assay—Fluorescence lipid transfer assays were performed as previously described (10, 16). 2 μl of pyrPtdCho in ethanol, 0.118 mM, and 2 μl of MyrPtdGro, 1 mg/ml, were added to a cuvette containing 0.8 μM protein in 2 ml of 20 mM Tris-HCl, 5 mM EDTA buffer (pH 7.4). Fluorescence intensities were monitored at 396 nm with excitation at 346 nm using an SLM 48000S spectrofluorometer at 25 °C.

Molecular Modeling—A geometric recognition algorithm (gramm), was used to dock ligands with the protein (17). Grid steps of 1.7 Å for translation and 10° for rotation were used to dock the ligands in hydrophobic mode. Optimal values for the repulsion (E) and attraction double range were chosen as 10 Å and 0.5 Å, respectively. After initial docking, the best complex structures were chosen for further refinement using the Discover 3 in Insight II package (Molecular Simulation Inc.). 500 energy minimization steps were carried out with the steepest decent method using the consistent valence force field to attain the most stable conformation. The dielectric constant was set to 1.0. A program for studying cavities of proteins, VOIDOO, was used to evaluate the hydrophobic...
RESULTS AND DISCUSSION

Structure Calculation of nsLTP2—Resonance assignments for most protons were achieved through conventional methods (14). Sequential assignments for a portion polypeptide chain are shown in Fig. 1A. Fig. 1B shows assignments for cross-peaks between various amide protons. The chemical shift data were deposited in the BioMagResBank (BMRB-5325). A total of 813 distance constraints were derived from cross-peaks in a 150-ms NOESY spectrum. Hydrogen bond constraints were
FIG. 2. Amino acid sequence and survey of sequential connectivities of rice nsLTP2. Differences in the NOE intensities are represented by block height. Various coupling constant values, 0–4, 4–8, and 8–12 Hz, are represented by different block intensities. *, indicates amide proton cross peaks present in the TOCSY spectrum recorded immediately after dissolving the lyophilized nsLTP2 in 99% D2O at 25°C. Various amino acids involved in the helical regions are denoted by the letter H.

FIG. 3. A, stereo representation of the 15 best superimposed NMR structures of rice nsLTP2 (only the backbone atoms are shown for clarity). B, solution structure of rice nsLTP2. Disulfide bonds involved in the three-dimensional structure are shown in ball-and-stick representation. Helix I (green) and helix II (red) are connected through a loop (Gly17–Pro21). Helix II and helix III (purple) are joined by a sharp 90° turn. The region containing two single-turn helices and the C terminus are shown in orange and brown, respectively.

| Experimental constraints | Total | Intraresidue | Sequential | Medium range | Long range | Hydrogen bond restraints | Dihedral angle restraint | r.s.m.d. from the average structure (Å) |
|--------------------------|-------|--------------|------------|--------------|-----------|-------------------------|-------------------------|-----------------------------|
|                          | 862   | 353          | 197        | 203          | 70        | 30                      | 19                      | Backbone: 1.54 ± 0.25, All heavy atoms: 1.09 ± 0.20 |
|                          |      |              |            |              |           |                         |                         | Residues 1–69: 0.95 ± 0.15, Residues 1–40: 0.65 ± 0.10 |
|                          |      |              |            |              |           |                         |                         | E_{sum} measure from Xplor (KJ.mol⁻¹): 300 ± 100 |

ProCheck analysis:

- Residues in most favored regions: 36 (65.5)%
- Residues in additional allowed regions: 16 (29.1)%
- Residues in generously allowed regions: 1 (1.8)%
- Residues in disallowed regions: 2 (3.6)%

* Result of minimized average structure through Ramachandran plot.
* Numbers in parentheses are percent of total.
generated for 30 slowly exchanging backbone amides observed in a TOCSY spectrum measured immediately after dissolving the lyophilized sample in 99% D2O, and 19 dihedral angle constraints were obtained from a TOCSY spectrum measured according to the procedure of Wang et al. (13). A total of 862 constraints were used for the structure calculations. Intensities of all sequential NOEs are summarized in Fig. 2. Because no crystal or theoretical structures are available for nsLTP2, ambiguous NOE constraints were added in an iterative manner over the course of the refinement. The final ensemble of 15 structures was chosen from 50 randomly simulated annealing structures (Table I). The set of final structures contains no violations greater than 0.5 Å for the NOE interproton distances or 5° for the dihedral angle constraints. The unminimized average structure was compared with the accepted structures to calculate atomic r.m.s.d. Although the disulfide bond constraints were available from biophysical studies, they were not incorporated into the structure calculations, to avoid biasing the process of refinement against experimental NMR data. Interestingly, in all 50 of the calculated structures, the pairs of cysteine residues that were near enough to form disulfide bonds were identical. We concluded that the disulfide bond pattern of the rice nsLTP2 is Cys3–Cys35, Cys11–Cys25, Cys37–Cys61, and Cys26–Cys68, which agrees with our enzymatic digestion results (9).
Structure of Rice nsLTP2—The rice nsLTP2 is a predominantly $\alpha$-helical protein consisting of three prominent helices within the N-terminal 40 amino acids. The well conserved cysteine residues form four disulfide bonds to stabilize the three-dimensional fold of the protein. The C-terminal amino acid residues, Lys41–His69, constitute a less structured region of the molecule with a high density of positively charged residues. The r.m.s.d. values for the backbone and all heavy atoms were 1.09 ± 0.20 and 1.54 ± 0.25 Å, respectively. The first 40 amino acids (Ala1–Ala40), constituting the rigid portion of the molecule, have r.m.s.d. values of 0.65 ± 0.1 Å for the backbone and 0.95 ± 0.15 Å for all heavy atoms. Superposition of the 15 NMR structures are shown as a stereo representation in Fig. 3A. Three helices of rice nsLTP2 positioned at Cys3–Ala14, Thr22–Ala31, and Gln38–Ala40 are colored green, red, and purple, respectively. Helices II and III are connected by a 90° turn to form a very rigid and unique structural motif. The curved helix I accommodates two disulfide bonds (Cys3–Cys35 and Cys11–Cys25). The flexible portion of the polypeptide contains two single-turn helices at positions Tyr45–Tyr48 and Ala54–Val58. A series of hydrophobic residues distributed throughout the nsLTP2 sequence combine to form a hydrophobic cavity. A continuous stretch of hydrophobic residues, Cys51–Ile55, near the C terminus forms a flexible cap over the hydrophobic cavity. The C-terminal region also contains two cysteines bridged to the rigid portion of the molecule (Cys50–Cys61 and Cys37–Cys68). These two disulfide bonds help to maintain the correct orientation of the hydrophobic cap. The final energy-minimized average structure of rice nsLTP2 is shown in Fig. 3B. A ProCheck analysis of the three-dimensional structure revealed that only Ser59 and Ser60 are in the disallowed region, corresponding to 3.6% of the residues in the protein (19). These residues constitute a portion in the flexible C terminus that makes a very sharp turn to cover the hydrophobic cavity.

Comparison of nsLTP2 with nsLTP1—the biophysical properties of the two subfamilies of nsLTP are very different. A higher concentration of GdnHCl is required to denature NaLTP2 ($C_m = 4.2$ M) than NaLTP1 ($C_m = 3.0$ M). NaLTP1 has unusual thermal stability ($T_m = 95$ °C), but nsLTP2 could not be thermally denatured even at temperatures approaching...
100 °C (data not shown). A primary sequence analysis using CLUSTAL W revealed a close relationship between these two subfamilies (20). The locations of cysteines, hydrophobic amino acids, and important positively charged residues are well conserved. There are, however, notable differences. In the -CXC-motif, an asparagine between the two cysteines in nsLTP1 is replaced by a hydrophobic amino acid, phenylalanine, in nsLTP2 (Fig. 4). The disulfide bond pattern in nsLTP2 differs from nsLTP1 at the -CXC-motif (Fig. 5A). The hydrophobic residue in the -CXC-motif of nsLTP2 is buried inside the molecule, whereas the hydrophilic residue of nsLTP1 is at the surface (Fig. 5B). These observations suggest that the central residue of the -CXC-motif may govern the cysteine pairing and influence the overall fold of the protein.

The backbone folds of nsLTP1 and nsLTP2 show structural similarities. The superimposed three-dimensional structures of nsLTP1 (red) and nsLTP2 (green) are represented with a ribbon diagram in Fig. 6. Portions of helix I and helix II of nsLTP2 are closely aligned with nsLTP1. Helices II and III of nsLTP1 are connected through a loop to form a gradual bend. In nsLTP2, helix III joins helix II with a 90° angle (the region labeled 31 and 38 in the Fig. 6). The backbone r.m.s.d. values for the Val^{10}–Ala^{22} and the perfectly aligned helix II (Ala^{23}–Gln^{31}) are 1.10 and 0.52 Å, respectively. The residues Gln^{38}–Gly^{62}, constituting the flexible region of nsLTP2, are aligned with helix III and helix IV of nsLTP1 but are quite divergent (r.m.s.d. = 4.62 Å). The C-terminal residues of nsLTP1 and nsLTP2 are not aligned as they form a supple cap over their hydrophobic cavities. The interior hydrophobic cavity of nsLTP2 is significantly different from nsLTP1 and important for its biological functions. The hydrophobic cavity of nsLTP1 is a tunnel (19), whereas the hydrophobic cavity of nsLTP2 is a triangular hollow box (Fig. 7). The three faces of the hydrophobic cavity are designated as face I, formed by Ala^{14}, Val^{48}, and Ala^{84}, face II, formed by Cys^{25}, Cys^{26}, Leu^{29}, and Cys^{31}, and face III, formed by Phe^{36} and Phe^{39}. All three faces are covered at the bottom by Leu^{8}, Ala^{14}, and Ile^{15} and at the top by Val^{58}, Cys^{61}, Gly^{62}, Ile^{63}, Ala^{64}, and Leu^{65} residues. In nsLTP1, helices II and IV do not contribute to the hydrophobic cavity directly except for the terminal Leu^{34}, Ala^{38}, and Ile^{69} residues, whereas in the case of nsLTP2 all of the helices, including single helical turns at positions Tyr^{45}, Tyr^{48} and Ala^{54}–Val^{58}, contribute to the hydrophobic cavity.

**Lipid Transferring Activity of nsLTP2—**In vitro studies have shown that nsLTP1 could transfer and/or exchange various phospholipid and glycolipid molecules across membranes (2). Lipid transfer activity of nsLTP2 was tested in vitro with fluorescence spectroscopy (Fig. 8). A solution consisting of two populations of homogeneous vesicles, one population containing fluorescent lipid molecules (pyrPtdCho) and the other population containing nonfluorescent lipid molecules (Myr2PtdGro) fluoresced very little. Because of the interactions between the pyrene moieties, fluorescence intensity was quenched (shown by an arrow in Fig. 8) (16). The fluorescence intensity increased after the addition of protein (nsLTP1 or nsLTP2, 0.8 μM) at 200 s. The protein molecules catalyzed the transfer of lipid molecules between vesicles. The fluorescence intensities plateaued when the lipid molecules containing the pyrene moiety were evenly distributed between the vesicles (16). The lipid transfer efficiency is monitored by the time required for the fluorescence intensity to plateau. The lipid transfer profiles of nsLTPs shown in Fig. 8 imply that the transfer efficiencies of both proteins are comparable. However, the size of the hydrophobic cavity in nsLTP1 and nsLTP2 are different. NaLTP2 efficiently transferred lipid molecules, despite its smaller hydrophobic cavity. A similar trend was also observed for wheat nsLTPs (10). The van der Waals volume and probe-occupied volume of the hydrophobic cavities measured by VOIDOO were 569.2 and 140 Å^3 for nsLTP1 and 200 and 38 Å^3 for nsLTP2.
for nsLTP2, respectively. Molecular modeling studies suggested that the hydrophobic cavity of nsLTP2 is more flexible than nsLTP1 (Fig. 7). When binding a molecule of stearic acid, cavity sizes of nsLTP1 and nsLTP2 increased, respectively, to 882.45 and 824.6 Å³ for the probe-occupied volume. The fatty acid molecule was oriented more compactly into the hydrophobic cavity of nsLTP1, but the triangular hollow box of nsLTP2 distorted to accommodate the fatty acid molecule. A molecular model of the nsLTP-ergosterol complex was analyzed to evaluate the flexibility of the hydrophobic cavity. The hydrophobic cavity of nsLTP1 was not flexible enough to accommodate the sterol molecule (21). However, the ergosterol molecule fit into cavity of nsLTP1, but the triangular hollow box of nsLTP2 was oriented more compactly into the hydrophobic cavity of nsLTP1, but the triangular hollow box of nsLTP2 was oriented more compactly into the hydrophobic cavity of nsLTP1. The cavity volume of sterol molecule (21). However, the ergosterol molecule fit into cavity of nsLTP1, but the triangular hollow box of nsLTP2 was oriented more compactly into the hydrophobic cavity of nsLTP1. The cavity volume of nsLTP2 increased to 1099 and 1389 Å³ in van der Waals volume and probe-accessible volume, respectively. The plasticity of the nsLTP2 cavity might facilitate more rapid binding or release of the lipid molecule. The smaller molecular size may also increase the lipid transfer efficiency of the nsLTP2.

Probable Role of nsLTP2 as Plant Defensin—Plant defense peptides bind to membrane surfaces of microorganisms through a positively charged face and inhibit bacterial growth by blocking a key process or by lysing the cells (22, 23). Plants attacked by pathogens express large amount nsLTPs (24). nsLTP1 from maize, spinach, arabidopsis, radish, onion, and broccoli exhibit antimicrobial activity (25, 26). nsLTPs are complemented by thionin, a plant defense peptide, to impart antimicrobial action. nsLTPs are more active against bacterial pathogens, whereas thionins act on fungal pathogens (25). Although nsLTPs play an important role in plant response to pathogens, the mechanism of action is not known. The three-dimensional structure of nsLTP2 shows a high concentration of positively charged residues on the flexible face of the molecule. nsLTP1 also has a large number of positively charged residues, but these are evenly distributed over the entire molecular surface (27). Many plant defense peptides also have a large concentration of positively charged residues on one surface of the molecule. Examination of the charge distributions of structures of the antifungal protein from pokeweed (PDB code 1dkc), antifungal protein from radish (PDB code 1ajy), thionin from wheat (PDB code 1gps), and rice nsLTP2 (PDB code 1l6h) shows a concentration of basic residues on one molecular face (Fig. 9). The asymmetric distribution of positive charges may be necessary for effective antimicrobial activity in defense proteins. nsLTP2 may serve two functions for the plant. Initially the protein rapidly transports lipids to the plasma membrane, which may then remain at the cell surface to defend against pathogens.

CONCLUSION

The three-dimensional structure of nsLTP2 has been solved for the first time. Its unique structural features provide new insights into the biological function and significance of nsLTP2. The plasticity of the hydrophobic cavity suggests that the nsLTP2 could bind and transport a variety of molecular shapes and sizes. Although the nsLTP1 protein has been proposed for drug delivery, nsLTP2 may represent a more suitable candidate because of its flexible hydrophobic cavity (28). The identity of the central residue of the -CXC- motif governs a variable cysteine pairing and may affect the overall fold of the protein. The influence of various amino acid substitutions at this position on the overall fold of the protein is presently under study in our laboratory. Despite the lack of experimental evidence, the distribution of basic residues in the nsLTP2 three-dimensional structure strongly suggests a determinant for antimicrobial activity. Current investigations are under way to elucidate the relationship of nsLTP structure to the biological functions of nsLTP2.

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