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Refined Solution Structure of a Liganded Type 2 Wheat Nonspecific Lipid Transfer Protein*

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The refined structure of a wheat type 2 nonspecific lipid transfer protein (ns-LTP2) liganded with l-α-palmitoylphosphatidylglycerol has been determined by NMR. The 15N-labeled protein was produced in Pichia pastoris. Physicochemical conditions and ligandation were intensively screened to obtain the best NMR spectra quality. This ns-LTP2 is a 67-residue globular protein with a diameter of about 30 Å. The structure is composed of five helices forming a right superhelix. The protein presents an inner cavity, which has been measured at 341 Å³. All of the helices display hydrophobic side chains oriented toward the cavity. The phospholipid is found in this cavity. Its fatty acid chain is completely inserted in the protein, the l-α-palmitoylphosphatidylglycerol glycerol moiety being located on a positively charged pocket on the surface of the protein. The superhelix structure of the protein is coiled around the fatty acid chain. The overall structure shows similarities with ns-LTP1. Nevertheless, large three-dimensional structural discrepancies are observed for the H3 and H4 α-helices, the C-terminal region, and the last turn of the H2 helix. The lipid is orthogonal to the orientation observed in ns-LTP1. The volume of the hydrophobic cavity appears to be in the same range as the one of ns-LTP1, despite the fact that ns-LTP2 is shorter by 24 residues.

Plant nonspecific lipid transfer proteins (ns-LTPs)³ were first isolated from spinach leaves and named based on their ability to mediate in vitro the transfer of phospholipids between membranes (1). ns-LTPs are widely distributed and form a superfamily of related proteins subdivided into two families: the type 1 ns-LTPs (ns-LTP1) and the type 2 ns-LTPs (ns-LTP2) (see Refs. 2 and 3 for review). Both families are multi-genic, and more than 150 sequences of plant ns-LTPs are listed in data bases. Only a limited number of proteins have been isolated from plant, and in vitro lipid transfer or binding has been demonstrated for an even more limited number of proteins.

The biological functions of ns-LTP1 have not yet been clearly determined, the most favored hypothesis being a role in the transport of cutin monomers (4, 5) or in plant defense mechanisms (6–8) for ns-LTP1. ns-LTP2 gene expression has been reported in the Zinnia elegans cell differentiation process (9, 10), in barley and rice developing seeds (11, 12), under abiotic stress conditions in barley roots (12), or during nodulation in Vigna unguiculata root hairs (13). However, there is no biological evidence of their function in these different contexts. The recent discovery that some ns-LTPs are pan-allergens of plant-derived foods has brought new interest for their study. Most of the ns-LTP allergens identified so far belong to the ns-LTP1 family (14–17), whereas ns-LTP2 has been reported only as a potent allergen of the pollen of Brassica rapa (18).

The three-dimensional structure of four cereal ns-LTP1s has been determined, i.e. wheat (19), barley (20), maize (21, 22), and rice (23, 24). In addition, seven structures of plant ns-LTP1 in complex with ligands have been determined, including those of maize ns-LTP1 with palmitate (21) or palmitoyl-lyso-phosphatidylcholine (22), barley ns-LTP1 with palmitoyl CoA (25) or palmitate (26), and wheat ns-LTP1 with di-myristoyl-phosphatidylglycerol (27), lyso-myristoyl-phosphatidylcholine (28), or prostaglandin B2 (29). All of these data showed that ns-LTP1 are compact single domain proteins whose fold is stabilized by four disulfide bonds. They are characterized by a four-α-helix bundle and a C-terminal region with no regular secondary structure. The most interesting feature of ns-LTP1 structure is the tunnel-like hydrophobic cavity that runs through the molecule and appears as a potential site for lipid binding. Although plant ns-LTP1s exhibit very similar global folds, the shape and size of this hydrophobic cavity vary considerably depending on the protein and/or on the ligand. This clearly indicates a high plasticity of the cavity that is able to accommodate a variety of hydrophobic molecules. In contrast, an antifungal protein extracted from onion seeds that showed a structure similar to those of ns-LTP1 except that the internal cavity, obstructed by several aromatic side chains, is unable to transfer lipids (30).

ns-LTP1s have been studied more extensively than ns-LTP2s that are distinct in terms of primary sequence with less than 30% homology (Fig. 1), size (7 kDa versus 9 kDa), and lipid transfer efficiency. In wheat, both ns-LTP1 and ns-LTP2 have been biochemically characterized, and ns-LTP2 exhibits a higher lipid transfer activity than ns-LTP1 (31). They share the same 8-cysteine skeleton, but their disulfide bond assignment has been shown to be different (32). Because the wheat ns-LTP2 exhibits a lipid transfer activity, one can hypothesize the presence of an hydrophobic cavity. However, whether ns-LTP2s have a fold that is similar to or different from that of ns-LTP1
remained to be elucidated at the initiation of the present work. This paper presents the refined solution structure of a recombinant wheat ns-LTP2 liganded with a C16 lysy-phosphopholipid. In the final stages of preparation of this manuscript, the structure of a rice ns-LTP2 was reported (33) (Protein Data Bank entry 1L6H).

EXPERIMENTAL PROCEDURES
Production/Purification—The Pichia pastoris transformant GS115-Tdlt18-tr5.2 (34) expressing a wheat ns-LTP2 was used for the production of the 15N-labeled protein. Production was carried out in an Applikon fermentor (400 ml of culture) with 99.4% 15N-labeled ammonium sulfate (Euroisotop) as nitrogen source. Labeled (15NH4)2SO4 (0.9% w/v) was added from the very beginning of the biomass production phase and during the induction phase at 0, 24, and 48 h (0.27, 0.27, and 0.36 w/v, respectively). Production of ns-LTP2 was induced by methanol after 22 h of culture and lasted for 77 h. The amount of secreted protein was estimated by densitometer analysis of SDS gels, using purified recombinant protein, 15% being glycosylated. The sugar moiety, assumed to be mannose, were located in the first 29-residue fragment. The protein was purified by iodoacetamide prior to digestion. The protein was digested for digestion analysis, the protein was reduced by dithiothreitol and alkylated by 100 pmol of the protein were solubilized in 10 mM or in 60 mM phosphate buffer), and lipid concentration (0 – 1.2 eq, with further lipid addition having no effect on the protein spectrum. The NMR spectra were recorded on a Bruker AMX 600 spectrometer, equipped with Hamatsu detector at 294 K in a 150-180 MHz probe with a field gradient unit on the z axis. All of the data processing was performed with the version 4 of the software (42).

NMR Spectroscopy—All of the NMR samples were prepared by dissolving the 15N-labeled and unlabeled recombinant wheat ns-LTP2 in H2O (10% D2O), at a concentration of 2.8 mM in presence of 70 mM L-

FIG. 1. Primary sequence alignment of various ns-LTP1 and ns-LTP2.

The fluorescence experiments were performed on a ISS instrument equipped with Hamatsu detector at 294 K in a 150-μl volume, excitation was set at 275 nm (300 W), and the spectra were recorded between 285 and 400 nm. Increasing amounts of ligand (0.1–100 equivalent) were added to a solution of ns-LTP2 (30 μM).

NOESY spectra were recorded for 12 h on a Bruker 600 MHz spectrometer at 300 K on a 2.7 mM ns-LTP2 solution in 60 mM phosphate buffer containing 1 eq of lipid. HSQC spectra were recorded for 30 min on a Bruker 400 MHz on a 1.1 mM ns-LTP2 solution, 10% D2O. The interaction with L-

The three-dimensional HSQC-NOESY (200-ms mixing time, 82-h acquisition 305.2 K), a three-dimensional experiment, 295.2 K) were performed. Three-dimensional experiments were conducted on BiFex III mass spectrometer (Bruker Daltonics, Bremen, Germany). 100 pmol of the protein were solubilized in 10 μl of water containing 0.1% trifluoroacetic acid. A 0.5-μl sample of this solution was mixed with 0.5 μl of matrix (a saturated solution of α-cyan-4-hydroxycinnamic acid in water/acetonitrile 50/50 v/v) and deposited on the target (dried droplet preparation). The spectra were recorded on the linear positive mode using the Pulsed Ion Extraction. For the tryptic digestion analysis, the protein was reduced by dithiothreitol and alkylated by iodoacetamide prior to digestion. The protein was digested for 4 h by trypsin (modified, sequencing grade from Roche Molecular Biochemicals) in 25 mM ammonium carbonate buffer (pH 8) at an enzyme/protein ratio of 1/20 (w/v). Proteolysis was stopped by adding diluted trifluoroacetic acid. The digest mixture was analyzed by MALDI-TOF mass spectrometry in the positive reflector mode. For the tryptic digestion analysis, the protein was reduced by dithiothreitol and alkylated by iodoacetamide prior to digestion.
Refined Solution Structure of a Liganded Wheat ns-LTP2

The dynamical annealing protocol (anneal.inp) of the crystallography NMR (CNS) software (51) was used to generate the protein structure from the set of constraints. A first set of structures was obtained from the constraint list, without any constraint on the cysteine linkage and with no phospholipid in the topology. From this set of structures, the disulfide linkage could be unambiguously assigned, because all of the sulfur atoms were located in compatible distances. The observed disulfide bridges correspond to Cys2/Cys30, Cys10/Cys24, Cys2/Cys34, and Cys36/Cys67. Hβ-Hβ NOE contacts were further observed in the NOESY map, which confirmed this assignment.

The model of the liganded protein was constructed from the same set of constraints. Additional constraints were used, corresponding to the observed connectivity of the C16 terminal CH3 of the fatty acid chain (C16) with Hδ of Tyr-44 and the middle of the lipid chain (C8) with Hδ of Phe-35 with a long distance constraint. No additional constraint was used to force the exit of the phospholipid from the protein core. The distance constraints corresponding to the S-S bounds were then added to the constraint list. The dynamical annealing protocol was used for the calculation of 250 structures, from which the 10 structures with the lowest global energy were conserved. The obtained set of structures was deposited to the Protein Data Bank (code 1N89).

The Procheck (52) program was used to check the quality of the obtained structures, as well as to compute the Ramachandran maps. The Voodoo (53–55) was used to compute the volume of the inner cavity.

RESULTS AND DISCUSSION

Production and Purification—The recombinant protein preparations were obtained as previously described (34–36). The N-terminal sequence (ACQASQLAVC) of the recombinant ns-LTP2, determined by mass spectroscopy, is identical to those of the wheat-purified ns-LTP2 (56), indicating that the recombinant protein was correctly processed by the P. pastoris KEX2 protease.

The mass spectroscopy was performed on the 15N-labeled protein. A 7055-Da average molecular mass was measured, confirming a 15N isotopic labeling over 95%. The mass spectrum also revealed that approximately 15% of the protein has been glycosylated with one to four C6 sugar moieties, assumed to be mannose (162 Da). This is consistent with the fact that P. pastoris adds O-oligosaccharides composed solely of mannose residues. Peptide mass fingerprinting of the digested protein revealed that only the first tryptic fragment at the N terminus of the protein (fragment 1–29) is glycosylated.

N-Glycosylation requires the Asn-Xaa-Ser/Thr consensus sequence, whereas O-glycosylation requires the presence of a Ser or Thr residue. The wheat ns-LTP2 does not contain the Asn-Xaa-Ser/Thr sequence in its first 29 residues; however, it does contain four serine residues (at positions 5, 12, 16, and 21) and one threonine residue (at position 27), residues that are accessible for possible glycosylation. The presence of a proline residue in the vicinity of a serine or threonine residue can enhance O-mannosylation (57). Of the four serine residues available for O-linkage, residue 21 is the only one close to a proline residue. All of these results indicated that a small fraction of the wheat ns-LTP2 is expressed as a O-glycosylated protein. To our knowledge, no glycosylation has been reported for ns-LTP purified from plant.

Physicochemical Context Screening and Ligand Choice—Intensive screening of the physicochemical solution conditions was performed to find a set of conditions that provides NMR spectra of good quality. Ligand nature and ionic strength conditions were found to be critical.

Fluorescence screening experiments were performed on a series of phospholipids and fatty acids, highlighting a higher affinity for negatively charged phospholipids. We finally selected LPG with an average chain length (C16). The titration of the ns-LTP2 by LPG, monitored by NMR, shows that the ns-LTP2 becomes more structurally constrained in the presence of this ligand. A narrowing and a spreading out of the peaks are observed on the two-dimensional HSQC spectrum. This evolu-
tions and Secondary Structure Elements—The assignment of the protein resonances was performed from the set of $^{15}$N-edited NOESY and TOCSY spectra. The sequential strategy was used, aided by the $^{13}$C and natural abundance $^{13}$C HSQC spectra (Fig. 3).

The solvent protection experiments indicate that the secondary structure is quite strongly established, because about 40% of the amide protons remain unexchanged during the first hours of the D$_2$O exchange experiment at 1 °C, and eight remain unexchanged after 5 days at room temperature. The solvent protection patterns, as well as J-coupling and NOESY patterns, are indicative of a structure mostly helical. On the other hand, the secondary structure prediction program Jpred (59) anticipates two helical zones, ranging from residues 22 to 29 and from residues 33 to 39.

Chemical assays as well as mass spectroscopy have shown that all eight cysteines of the protein are engaged in disulfide bridges. We have not been able to unambiguously assign the Hβ-Hi NOE contacts characteristic of this structure because of the crowding of this spectral region. However, after the first run of structure generation, all of the obtained structures exhibited side chain proximities permitting the disulfide skeleton based on these prestructures to be assigned. The disulfide bridges thus found are: Cys-25/Cys-60, Cys-10/Cys-24, Cys-2/Cys-34, and Cys-36/Cys-67. This is in agreement with the chemically determined assignment (32).

The $^1$H assignments of the LPG in the complexed form were obtained from two-dimensional COSY homonuclear experiments on the free LPG and by comparing the homonuclear spectra obtained from the LPG-ns-LTP2 complex and from the DPC-ns-LTP2 complex. A few chemical shifts of the fatty acid chain were clearly identified: the terminal methyl group (C16), its vicinal methylene (C15), as well as the proximal methylene (C2); the glycerol moiety attached to the fatty acid chains was also assigned. Some intermolecular NOE contacts were observed.

Structure Determination—From the complete set of geometric constraints extracted from the NMR spectra, a set of 10 structures has been obtained. They do not display any important constraint violation, and all of the residues are localized in the allowed regions of the Ramachandran plot. The ensemble of the 10 best structures present a root mean square deviation computed on residues 2–67 of 0.9 Å for all of the heavy atoms and a root mean square deviation of 0.67 Å for the backbone atoms. All of the statistics of the geometrical constraints and the structure reconstruction are given in Table I. The set of solution structures of ns-LTP2 liganded with LPG as obtained from this experimental work is presented in Fig. 4.

The protein is observed as a globular protein with a diameter of about 30 Å. The structure is composed of five helices arranged in a superhelix tertiary structure. Helix 1 is a 3–10 helix, encompassing residues 7–15. All of the other helices are $\alpha$-helices. Helix 2 includes residues 22–31, helix 3 includes residues 34–40, helix 4 includes residues 44–49, and helix 5 includes residues 51–60. The overall fold is a right superhelix. The localization of the helices is in good agreement with the proton exchange experiment, which has shown that there are five main zones in which the amide protons are protected against solvent exchange: residues 14 and 15, residues 26–31,


The NMR restraints and structural statistics of the ensemble calculated for LPG-liganded wheat ns-LTP2 (10 structures)

| Distance & angles restraints                  | 403 |
| NOE intraresidual                             |     |
| NOE sequential \((i, i+1)\)                   | 188 |
| NOE medium range \((i-i) \leq 3\)             | 72  |
| NOE long range \((i-i) > 3\)                 | 95  |
| NOE Intermolecular                            | 5   |
| S-S bridges                                   | 4   |
| Dihedral angle \(\phi\)                      | 29  |
| Dihedral angle \(\chi\)                      | 8   |
| Total                                         | 800 |

Deviation from idealized geometry

| Bonds (Å)                                      | 0.00323 ± 0.00013 |
| Angles                                         | 0.372 ± 0.012     |
| Improper angles                                | 0.187 ± 0.017     |
| Dihedral angles                                | 30.16 ± 3.04      |

Deviation from restraints

| NOE restraints                                 | 0.029 ± 0.001 |
| Dihedral restraints                            | 0.45 ± 0.1    |

Ramachandran plot (see Ref. 52)

| Most favored regions                           | 74.2% |
| Allowed regions                                | 23.5% |
| Generously allowed regions                     | 2.4%  |
| Disallowed regions                             | 0.0%  |

Mean energies (kcal/mol)

| 10.29 ± 0.61                                   |
| 37.14 ± 2.5                                    |
| 2.67 ± 0.54                                    |
| 23.5 ± 3.8                                     |
| 48.5 ± 3.93                                    |
| 0.94 ± 0.39                                    |

Root mean square deviation from average structure (Å) (residues 2–67)

| Backbone                                       | 0.67 |
| Heavy atom                                    | 0.90 |

Helices 1 and 2 are organized in a near anti-parallel conformation. The contact between helices 1 and 2 is tightened by the Cys-10/Cys-24 disulfide bridge. They are linked by an extended strand from Ser-16 to Gly-22. Lys-19 appears to be oriented toward the solvent and does not display any NOE contact with other residue. It should be noted that in a previous dynamic study, it has been observed that the H-N vector of Lys-19 is highly mobile in the ns range (58). Helices 3–5 form a square configuration. Helix 3 contains a characteristic Cys-34, Phe-35, Cys-36 pattern, with Phe-35 buried into the structure and contributing to the hydrophobic core of the structure. Cys-34 and Cys-36 are respectively engaged in disulfide bridges with Cys-2 and Cys-67, forming two diametrically opposed bonds relative to the helix axis. All of the prolines are observed in trans conformation, as confirmed by the H\(\alpha\)-(i+1) contacts observed in the NOESY spectra, for all of the proline residues (Pro-20, Pro-42, Pro-51, and Pro-65).

Several additional secondary structure elements can be observed in the structure. The C-terminal of helix 1 presents an unusual hydrogen bond pattern, with the carboxyl moiety of Ser-12 being engaged with the NH of Ala-18. This structure is found in the 10 structures generated, and the NH of residue Ala-18 is found to be protected against solvent exchange, thus confirming this result. A type-1 \(\gamma\) turn is observed between helices 2 and 3, corresponding to residues Gln-31, Gln-32, and Gly-33. A type-1 \(\beta\) turn is observed between helices 3 and 4, and the NH of Gln-32 bonded to the CO of Arg-29; and the N-capping of helix 2 with the acidic head of Asp-41 engaged in a hydrogen bond with the NH of Thr-43. Three classic helix cappings can also be observed: the N-capping of helix 2 with the OH of Ser-21 hydrogen-bonded to the NH of Glu-23; the C-capping of helix 2 with the side chain of Gln-32 bonded to the CO of Arg-29; and the N-capping of helix 5, with the OH of Ser-50 bonded to the NH of His-52. Finally, a transient salt bridge between the amide moiety of Lys-40 and the C-terminus of the backbone can be observed in several structures of the NMR ensemble.

The protein presents an inner cavity, which has been measured at 341 Å\(^3\). All of the helices present hydrophobic side chains directed toward the cavity. The phospholipid is found in this cavity. Only one unique phospholipid position is observed in the cavity for all 10 retained structures. The fatty acid chains are completely embedded in the protein structure. Its axis is aligned with the axis of the superhelix and is orthogonal to the \(\alpha\)-helix axes (Fig. 4c). The terminal methyl group is positioned between the H1 and H4 helices. The fatty acid chain is inserted in the cavity constituted by the hydrophobic residues (Leu-7, Leu-28, Phe-35, Tyr-38, Tyr-44, Tyr-47, Ile-48, Ala-53, Leu-57, Val-64, and Pro-65). The chain presents a turn near carbon 8 and exits the cavity in a cleft between helices H4 and H5 and the C-terminal residue. The inner glycerol moiety...
is located on a pocket on the surface of the protein. This pocket presents a basic environment constituted by the Arg-49, Arg-54, and His-66 side chains. These basic residues are observed in close proximity with the phosphate moiety, equilibrating the phosphate charge (Fig. 5). The cavity of ns-LTP2 is asymmetric. The proximal entrance of the cavity, where the phosphate group is found, presents several hydrophilic and basic groups: Arg-49, Arg-54, Thr-58, and His-66. The distal opening of the cavity is characterized with hydrophobic residues, such as Leu-7, Tyr-38, Tyr-44, and Tyr-47.

This location is in good agreement with the following spectroscopic observations: In the fatty acid chain only three unambiguous NOE intermolecular contacts have been identified. Four NOE contacts connect the LPG-terminal methyl groups (C16) with the Tyr-44 and Tyr-47 aromatic part. The other NOE connects the one of the methylenes of the fatty acid chain with the Hβ of Phe-35. All of these residues are found in the hydrophobic cavity. No NOE contacts were found between the protein and LPG glycerol moiety. The chemical shifts of the phospholipid glycerol moiety do not present much shift upon complexation with the protein. On the other hand, the fatty acyl chain chemical shift differences between isolated and liganded LPG are important.

The structure of the minor form present in solution has not been studied, even though it appears to be structured. About one-third of the amino acids seem to be involved in the conformation equilibrium; no attempt was made to assign the residues involved in this equilibrium. Previous dynamic study (58) has shown that the major form is predominantly rigid, with $S^2$ ranging from 0.8 to 0.9, except for Lys-19, exposed to the solvent.

**ns-LTP2 versus ns-LTP1 Comparison**—The three-dimensional structure is known for several ns-LTP1 from different species (19–29). They are very similar among plant and consist in four $\alpha$-helices organized in a superhelix structure and connected by four disulfide bridges. We compared the structure we present here with wheat ns-LTP1 (Protein Data Bank code 1gh1) (19) using the Visual Molecular Dynamics software (46) (Fig. 6).

The best superposition is obtained when the H1, H2, and H5 (residues 3–16, 22–32, and 50–63) helices in ns-LTP2 are superimposed with the H1, H2, and H4 helices (residues 5–8, 10–
H2 helix is also different in both structures. Charged residues are colored yellow and how the basic residues surround the phosphate group (in red). The orientation is similar to that in Fig. 4a.

The two short H3 and H4 α-helices orthogonal to each other in ns-LTP2 are replaced by the longer H3 helix in ns-LTP1. The H3 and H4 (respectively H3) α-helices and the C-terminal region. The last turn of the H2 helix is also different in both structures.

The present work presents the refined structure of the wheat ns-LTP2 protein, liganded with L-palmitoylphosphatidylglycerol, as determined by NMR spectroscopy. The protein was observed as being composed of five helices, structured as a right superhelix, surrounding the phospholipid. This structure presents some homologies with other lipid transfer proteins such as ns-LTP1; however, the phospholipid was found in a quite different location than in most LTP1s. An hydrophobic cavity was also observed, with a volume equivalent to the one found in ns-LTP1 but with a different geometry. The orientation of the phospholipid main chain, observed in ns-LTP2, is roughly orthogonal to the α-helix axes, and the chain runs from helices H1 to H5. This is in contrast with most described ns-LTP1 cavities found with a main axis parallel to the α-helix axes.

Conclusion—The present work presents the refined structure of the wheat ns-LTP2 protein, liganded with L-palmitoylphosphatidylglycerol, as determined by NMR spectroscopy. The protein was observed as being composed of five helices, structured as a right superhelix, surrounding the phospholipid. This structure presents some homologies with other lipid transfer proteins such as ns-LTP1; however, the phospholipid was found in a quite different location than in most LTP1s. An hydrophobic cavity was also observed, with a volume equivalent to the one found in ns-LTP1 but with a different geometry. The ns-LTP2 protein structure also presents homology with the one soy bean hydrophobic protein that does not exhibit any lipid transfer activity. This permits us to devise a protein family encompassing ns-LTP1 and ns-LTP2 but also soy bean hydrophobic protein and other related vegetal proteins, based on structural homologies, rather than on the function of primary sequence homologies.

Extensive ligand screening had to be undertaken to find a set of conditions allowing structural study. This indicates that even if the ns-LTP2 is able to adapt a large number of hydrophobic ligands in its hydrophobic pocket, it certainly presents varying affinities depending on the nature of the ligand. The better affinity for single chain phospholipid, negatively charged,
can be explained a postmortem, by the size of the pocket and by the patch of positively charged residues located around its entrance. Further comparative studies on this protein and on homologous proteins will have to be undertaken to improve the understanding on the phospholipid binding affinity and of the phospholipid transfer activity.

Finally it remains to be explained how the structural differences observed in the plant LTP family might be related to the various in vivo activities putatively assigned to its members. Comparative structural and dynamical studies on several isoforms of wheat ns-LTPs are currently in progress in our group. This complementary work should permit us to draw stronger links between structural features and biological functions.

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