



Structure in solution of a four-helix lipid binding protein

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Abstract

Because of the low solubility of lipids in water, intercellular and intracellular pathways of lipid transfer are necessary, e.g., for membrane formation. The mechanism by which lipids *in vivo* are transported from their site of biogenesis (endoplasmic reticulum and the chloroplasts) to their place of action is unknown. Several small plant proteins with the ability to mediate transfer of radiolabeled phospholipids *in vitro* from liposomal donor membranes to mitochondrial and chloroplast acceptor membranes have been isolated, and a protein with this ability, the nonspecific lipid transfer protein (nsLTP) isolated from barley seeds (bLTP), has been studied here. The structure and the protein lipid interactions of lipid transfer proteins are relevant for the understanding of their function, and here we present the three-dimensional structure in solution of bLTP as determined by NMR spectroscopy. The ¹H NMR spectrum of the 91-residue protein was assigned for more than 97% of the protein ¹H atoms, and the structure was calculated on the basis of 813 distance restraints from ¹H-¹H nuclear Overhauser effects, four disulfide bond restraints, from dihedral angle restraints for 66 ϕ -angles, 61 χ^1 angles, and 2 χ^2 angles, and from 31 sets of hydrogen bond restraints. The solution structure of bLTP consists of four well-defined α -helices A–D (A, Cys 3–Gly 19; B, Gly 25–Ala 38; C, Arg 44–Gly 57; D, Leu 63–Cys 73), separated by three short loops that are less well defined and concluded by a well defined C-terminal peptide segment with no observable regular secondary structure. For the 17 structures that are used to represent the solution structure of bLTP, the RMS deviation to an average structure is $0.63 \text{ \AA} \pm 0.04 \text{ \AA}$ for backbone atoms and $0.93 \text{ \AA} \pm 0.06 \text{ \AA}$ for all heavy atoms. The secondary structure elements and their locations in the sequence resemble those of nsLTP from two other plant species, wheat and maize, whose structures were previously determined (Gincel E et al, 1995, *Eur J Biochem* 226:413–422; Shin DH et al, 1995, *Structure* 3:189–199). In bLTP, the residues analogous to those in maize nsLTP that constitute the palmitate binding site are forming a similar hydrophobic cavity and a potential acyl group binding site. Analysis of the solution structure of bLTP and bLTP in complex with a ligand might provide information on the conformational changes in the protein upon ligand binding and subsequently provide information on the mode of ligand uptake and release. In this work, we hope to establish a foundation for further work of determining the solution structure of bLTP in complex with palmitoyl coenzyme A, which is a suitable ligand, and subsequently to outline the mode of ligand binding.

Keywords: barley nonspecific lipid transfer protein; NMR spectroscopy; solution structure

The plant nonspecific lipid transfer proteins (nsLTPs) are a group of homologous proteins with approximately 90 residues and high pIs. Nearly all nsLTPs have eight conserved Cys residues arranged in a Cys–Cys–Cys–Cys–Cys X Cys–Cys–Cys pattern (where X denotes one variable residue position), forming

four disulfide bridges (Desormeaux et al., 1992). They have been identified in a variety of plants, including barley (Mundy & Rogers, 1986; Breu et al., 1989), maize (Douady et al., 1982), wheat (Douady et al., 1985), spinach (Kader et al., 1984), and castor bean (Takishima et al., 1988). A number of very different *in vitro* capabilities of the nsLTPs has been established, including transfer of phosphatidylcholine from liposomes to mitochondria (Breu et al., 1989), inhibition of bacterial and fungal plant pathogens (Terras et al., 1992; Molina & Garcia-Olmedo, 1993; Molina et al., 1993), and enhancement of acyl-coenzyme A oxidase in a glyoxysomal fraction (Tsuboi et al., 1992). In addi-

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tion, there is evidence for nsLTP having an amino-terminal extension and being synthesized on membrane-bound polysomes (Tchang et al., 1988; Vergnolle et al., 1988). Also, spinach nsLTP contains a signal peptide and is cotranslationally inserted into microsomal membranes (Bernhard & Sommerville, 1989). This suggests a pathway for making nsLTP secreted or membrane bound.

Recently, the three-dimensional crystal structures of maize nsLTP (mLTP) and the complex with palmitate (Shin et al., 1995) were reported, and so was the solution structure of wheat nsLTP (wLTP) (Gincel et al., 1995). These LTPs are four-helix proteins, and mLTP is shown to bind palmitate in an extended form in a hydrophobic cavity in the four-helix protein. This binding mode of fatty acids in an almost linear conformation in the interior of the protein, fully protected from hydrophilic contacts, is similar to that seen in fatty acid binding protein (Sacchetti et al., 1989) and retinol binding protein (Newcomer et al., 1984), which, in contrast to nsLTPs, are both β -clamp fatty acid binding proteins. A similar mode of fatty acid binding, but in an all-helix environment, is seen in recoverin (Tanaka et al., 1995). A completely different binding concept for acyl groups of lipids is found in the four-helix bundle protein, acyl-coenzyme A binding protein (Andersen & Poulsen, 1992, 1993; Kragelund et al., 1993), where the long fatty acid chain binds in a coiled configuration between the protein and hydrophobic groups of the coenzyme A part of the ligand.

Here we are concerned with determining the solution structure of nsLTP from barley seeds (bLTP) by ^1H NMR spectroscopy. We have determined the three-dimensional structure of the protein and shown it to be closely related to the recently presented structures of nsLTPs of other plant species, forming an atypical four-helix protein motif with a long well-defined C-terminal tail that interacts with each of the four helices. In addition, we have found a hydrophobic cavity in the solution structure of bLTP that, by analogy, most likely is the acyl group binding site.

Results

A complete sequential assignment of the high-resolution and high-quality two-dimensional ^1H NMR spectra of bLTP was obtained using the Wagner-Wüthrich strategy (Wüthrich, 1986). The spin systems were identified primarily in DQF-COSY spectra, complemented by TOCSY spectra. bLTP gives excellent ^1H -NMR spectra (Fig. 1), as seen in the fingerprint region of the DQF-COSY spectrum (Fig. 1A), where 86 of 93 expected $\text{H}^N\text{-H}^\alpha$ cross peaks were immediately observed. All spin systems were fully assigned, except for the outermost protons of the lysines and one H^β of Pro 83. The results are shown in Table I and Figure 2. On the basis of these assignments, 813 NOEs, 66 ϕ -angles, 61 χ^1 -angles, and 2 χ^2 angles were determined as well as the four disulfide bonds of the eight cysteines. Of the 813 NOEs, 146 were intraresidual (i), 254 sequential ($|i-j|=1$), 241 short-range ($1 < |i-j| \leq 5$), and 172 long-range ($|i-j| > 5$), with the sequential distribution shown in Figure 3. Together, these represent the experimentally obtained restraints and the input for the structure calculations that resulted in a set of 17 structures of bLTP in solution.

The positions of the secondary structure elements of bLTP are very similar to the positions in the recently presented struc-

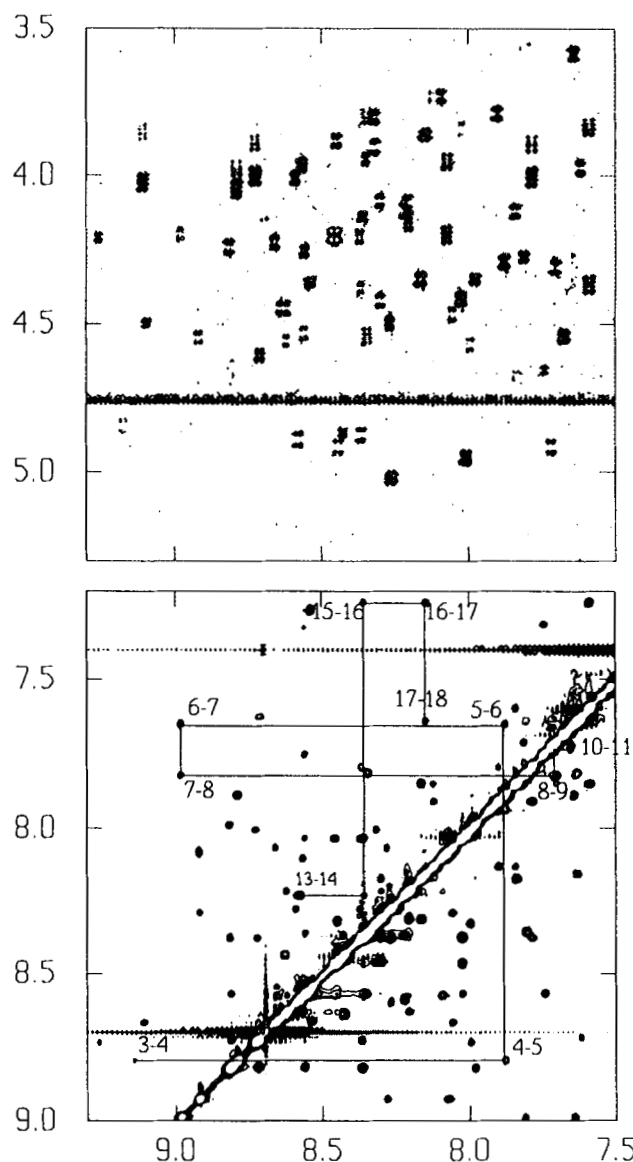


Fig. 1. Top: Part of the fingerprint region of the DQF-COSY spectrum of bLTP, 3 mM in 90:10 $\text{H}_2\text{O}/^2\text{H}_2\text{O}$, at pH 4.0 and 310 K. Bottom: Expanded region of the NOESY spectrum of bLTP (mixing time 200 ms), recorded at the same conditions as above. HN-HN trace is comfortably resolved in, e.g., helix A from Gly 3 to Gln 18 as shown, except for Pro 12.

tures of homologous nsLTPs. The structure of bLTP consists of four α -helices (A-D), A, Cys 3-Gly 19; B, Gly 25-Ala 38; C, Arg 44-Gly 57; D, Leu 63-Cys 73, with extended loop structures between the helices: the N-terminal Leu 1-Asn 2; Loop 1, Gly 20-Ser 24; Loop 2, Gln 39-Asp 43; Loop 3, Ile 58-Asn 62. The C-terminal polypeptide Asn 74-Ile 91, of 18 residues, is in an extended configuration with no obvious secondary structure. The locations and extensions of secondary structure elements in bLTP correspond to the locations of α -helices in mLTP: A_m, Cys 4-Ala 18; B_m, Ala 27-Ala 39; C_m, Thr 43-Ala 58; and D_m, Ala 65-Cys 75, with extended loop structures between the helices (Shin et al., 1995). In mLTP, there is also one full round of a 3_{10} helix of Cys 89-Arg 92 in addition to the four helices (Shin

Table 1. Chemical shift table for bLTP recorded at 310 K and pH 4.0

Residue	H ^N	H ^α	H ^β	Others	³ J _{HNHα} , φ, χ ^a
1 Leu		4.30	1.65, 1.57	1.60(H ^γ); 0.78 ¹ , 0.76 ² (H ^δ)	(-, -, -)
2 Asn	8.20	4.95	2.92 ³ , 3.14 ^{2b}	7.62, 6.91(H ^γ)	(10, -120, 60)
3 Cys	9.08	4.63	2.82, 3.40		(-, -, -180)
4 Gly	8.73	3.98, 3.91			(-, -, -)
5 Gln	7.82	4.22	2.02 ³ , 2.34 ²	2.50, 2.41(H ^γ); 7.41, 6.80(H ^{ε2})	(5, -57, -180)
6 Val	7.58	3.51	2.17	0.86 ³ , 1.01 ² (H ^γ)	(2, -57, 180)
7 Asp	8.92	4.12	2.77 ³ , 2.90 ²		(5, -57, 60)
8 Ser	7.75	4.20	4.05		(2, -57, -180)
9 Lys	7.64	4.23	2.07	1.78, 1.70(H ^γ)	(5, -57, -60)
10 Met	7.66	4.86	1.94 ³ , 2.03 ²	2.38, 2.52(H ^γ)	(6, -, 60)
11 Lys	7.60	4.21	2.12 ³ , 2.03 ²	3.74(H ^γ); 1.38(H ^δ); 2.53(H ^ε)	(-, -57, 60)
12 Pro		3.00	1.86, 1.69	1.77, 1.57(H ^γ), 3.01(H ^δ)	(-, -, -)
13 Cys	8.52	4.82	3.21 ³ , 3.24 ²		(6, -57, 60)
14 Leu	8.16	4.14	2.03, 1.50	1.98(H ^γ); 0.96, 0.72(H ^δ);	(-, -57, -60)
15 Thr	8.30	4.06	4.24	1.40(H ^{γ2}), 4.69(H ^γ)	(2, -57, -60)
16 Tyr	7.17	4.59	3.08, 3.53	6.98(H ^γ); 6.62(H ^ε)	(-, -57, -180)
17 Val	8.09	3.79	2.51	0.99 ³ , 1.07 ² (H ^γ)	(2, -57, -60)
18 Gln	7.57	4.71	2.06	2.30, 2.45(H ^γ); 6.82, 7.16(H ^{ε2})	(-, -, -)
19 Gly	8.28	4.47, 3.73			(-, -, -)
20 Gly	7.75	3.48, 4.62			(-, -, -)
21 Pro		4.68	1.90, 2.35	2.00, 2.05(H ^γ); 3.65, 3.58(H ^δ)	(-, -, -)
22 Gly	8.14	4.08, 3.20			(-, -, -)
23 Pro		3.90	1.81	1.48, 1.75(H ^γ); 3.33, 2.73(H ^δ)	(-, -, -)
24 Ser	9.04	4.43	4.15 ³ , 3.85 ²		(2, -57, -60)
25 Gly	9.05	3.79, 3.95			(-, -, -)
26 Glu	8.60	4.16	2.06, 1.99	2.49, 2.43(H ^γ)	(2, -57, 0)
27 Cys	8.00	4.40	2.87 ³ , 3.33 ²		(2, -57, -60)
28 Cys	8.22	4.69	2.83, 3.06		(-, -57, 120)
29 Asn	8.86	4.48	2.94 ³ , 2.99 ²	7.58, 6.80(H ^{γ2})	(<2, -57, 60)
30 Gly	8.01	4.13, 3.88			(-, -, -)
31 Val	8.03	3.66	2.21	0.91(H ^{γ1}); 1.12(H ^{γ2})	(5, -57, -180)
32 Arg	8.51	3.90	2.05 ³ , 1.97 ²	1.81, 1.61(H ^γ); 3.31, 3.23(H ^δ); 7.19(H ^ε)	(5, -57, -180)
33 Asp	8.56	4.49	3.00, 2.71		(2, -57, -60)
34 Leu	8.15	4.02	2.11 ³ , 1.57 ²	1.96(H ^γ); 0.86, 0.95(H ^δ)	(-, -57, 60)
35 His	8.15	4.51	3.56 ³ , 3.02 ²	8.24(H ^{δ2}); 7.33(H ^{δ1})	(<2, -57, -180)
36 Asn	8.24	4.35	2.93 ³ , 3.04 ²	6.95, 7.87(H ^{γ2})	(5, -57, -60)
37 Gln	8.10	4.28	2.14 ³ , 2.03 ²	2.41(H ^γ); 7.36, 6.50(H ^{ε2})	(8, -120, -60)
38 Ala	7.78	4.61	1.21		(7, -, -)
39 Gln	8.57	4.38	2.29 ³ , 1.93 ²	2.37, 2.25(H ^γ), 6.73, 7.41(H ^{ε2})	(8, -120, -60)
40 Ser	8.37	4.80	4.04, 4.31		(9, -120, 0)
41 Ser	9.20	4.14	3.96 ³ , 4.03 ²		(<2, -57, -180)
42 Gly	8.67	3.93, 3.82			(-, -, -)
43 Asp	7.93	4.50	2.47 ³ , 2.79 ²		(6, -, -60)
44 Arg	8.26	3.84	2.04 ³ , 1.74 ²	1.84(H ^γ); 3.03, 3.36(H ^δ); 7.85(H ^ε)	(5, -57, -)
45 Gln	8.39	3.82	2.06 ³ , 2.20 ²	2.60(H ^γ); 7.49, 6.67(H ^{ε2})	(2, -57, -60)
46 Thr	8.24	4.02	4.43	1.12(H ^{γ2})	(2, -57, -60)
47 Val	8.30	3.08	1.76	0.40(H ^{γ1}); 0.88(H ^{γ2})	(2, -57, -180)
48 Cys	7.96	3.78	3.43 ³ , 2.68 ²		(<2, -57, -180)
49 Asn	8.50	4.47	2.71, 2.91	7.20, 7.26(H ^{γ2})	(<2, -57, 0)
50 Cys	8.75	4.57	2.97		(<2, -57, -60)
51 Leu	8.66	3.96	1.35 ³ , 2.14 ²	1.77(H ^γ); 0.66(H ^δ)	(-, -57, -60)
52 Lys	8.31	4.30	2.03 ³ , 1.94 ²	1.44(H ^γ); 1.79(H ^δ)	(2, -57, -180)
53 Gly	7.73	3.94, 3.82			(-, -, -)
54 Ile	7.84	3.72	1.93	1.08, 1.80(H ^{γ1}); 0.80(H ^{γ2}); 0.77(H ^δ)	(2, -57, -)
55 Ala	8.04	3.66	1.24		(2, -57, -)
56 Arg	7.78	4.05	1.90	1.74(H ^γ); 3.28, 2.00(H ^δ); 7.33(H ^ε)	(2, -57, -)
57 Gly	7.53	4.29, 3.77			(-, -, -)
58 Ile	7.17	4.16	1.88	1.63, 1.16(H ^{γ1}); 0.79(H ^{γ2}); 0.69(H ^δ)	(9, -120, -180)
59 His	8.75	4.63	3.26, 3.19	8.64(H ^{δ2}); 7.34(H ^{δ1})	(5, -57, -)
60 Asn	8.65	4.54	2.87, 2.80	7.53, 6.81(H ^{γ2})	(9, -120, -)
61 Leu	7.56	3.90	1.71 ³ , 1.70 ²	1.37(H ^γ); 0.93, 0.89(H ^δ)	(5, -57, -180)

(continued)

Table 1. Continued

Residue	H ^N	H ^α	H ^β	Others	³ J _{H^NH^α} , φ, χ ^a
62 Asn	9.11	4.79	2.76, 2.42	7.56, 6.81(H ^{γ2})	(8, -120, -180)
63 Leu	8.53	3.94	1.64 ³ , 1.70 ²	1.73(H ^γ); 0.94, 0.99(H ^δ)	(5, -57, -60)
64 Asn	8.21	4.43	2.86	7.57, 6.94(H ^{γ2})	(3, -57, -)
65 Asn	8.31	4.13	0.99 ³ , 2.36 ²	7.02, 6.83(H ^{γ2})	(<2, -57, -60)
66 Ala	8.29	3.88	1.61		(5, -57, -)
67 Ala	8.50	4.18	1.58		(5, -57, -)
68 Ser	7.68	4.59	4.34 ³ , 4.11 ²		(-, -120, -180)
69 Ile	7.24	3.75	2.13	1.06, 1.10(H ^{γ1}); 0.96(H ^{γ2}); 1.57(H ^δ)	(5, -57, -60)
70 Pro		4.07	2.07, 2.70	1.71, 2.16(H ^γ); 3.78, 3.62(H ^δ)	(-, -, -)
71 Ser	7.92	4.28	3.96		(2, -57, -)
72 Lys	8.75	4.17	1.84 ³ , 1.64 ²	2.72, 1.56(H ^γ)	(5, -57, 180)
73 Cys	8.30	4.82	3.01 ³ , 2.73 ²		(-, -, -60)
74 Asn	7.96	4.34	3.18 ³ , 2.66 ²	6.74, 7.42(H ^{γ2})	(3, -57, -60)
75 Val	8.40	4.13	1.59	0.71(H ^{γ1}); 0.65(H ^{γ2})	(8, -120, -120)
76 Asn	8.50	4.65	2.81 ³ , 2.58 ²	7.46, 6.82(H ^{γ2})	(-, -, -60)
77 Val	7.21	4.52	1.05	0.77(H ^{γ1}); 0.61(H ^{γ2})	(9, -120, 60)
78 Pro			1.59	1.96, 2.12(H ^γ); 3.61, 3.07(H ^δ)	(-, -, -)
79 Tyr	6.10	4.76	2.75, 3.10	6.71(H ^δ); 6.67(H ^γ)	(4, -57, 60)
80 Thr	6.98	4.46	4.14	1.04(H ^{γ2})	(7, -, 60)
81 Ile	8.26	3.74	1.75	0.84(H ^{γ1})	(2, -57, -)
82 Ser	6.91	4.80	3.40, 3.86		(6, -, -180)
83 Pro		4.57	2.15	2.00(H ^γ); 3.90, 3.40(H ^δ)	(-, -, -)
84 Asp	7.95	4.88	2.45 ³ , 2.81 ²		(9, -120, -180)
85 Ile	6.79	3.99	1.78	1.05(H ^{γ1}); 1.18(H ^{γ2}); 1.57(H ^δ)	(2, -57, 60)
86 Asp	8.39	4.85	2.61 ³ , 2.93 ²		(9, -120, -180)
87 Cys	8.60	4.64	3.19 ³ , 2.92 ²		(5, -57, -60)
88 Ser	8.48	4.29	4.03, 3.95		(<2, -57, -60)
89 Arg	7.19	4.45	2.10, 1.66	1.57(H ^γ); 3.22(H ^δ); 7.15(H ^γ)	(9, -120, -)
90 Ile	7.09	4.12	2.02	1.52, 1.44(H ^{γ1}); 0.91(H ^{γ2}); 0.89(H ^δ)	(8, -120, -60)
91 Tyr	7.62	4.47	3.12, 2.89	7.11(H ^δ); 6.82(H ^γ)	(8, -120, -)

^a H chemical shifts are accurate within 0.01 ppm and measured in ppm relative to TSP.

^b Stereospecific assignment is indicated by the superscript ² and ³ as recommended by IUPAC-IUB. Dihedral angle restraints are listed as middle values and are restrained as shown in Figure 5 for φ and χ¹, respectively.

et al., 1995). In bLTP, $d_{\alpha N}(i, i+2)$ NOEs are present between Asp 86–Ser 88 and Ser 88–Ile 90 (Table 1), indicating 3_{10} -helix type turns for these residues, but an inspection of the structure reveals no 3_{10} -helix. The four disulfide bonds in bLTP were identified by the presence of NOEs between the β -protons of the cysteines of residues Cys 3–Cys 50, Cys 12–Cys 27, Cys 28–Cys 73, and Cys 48–Cys 87, corresponding to similar sites in mLTP: Cys 4–Cys 52, Cys 14–Cys 29, Cys 30–Cys 75, Cys 50–Cys 89. When superimposed on an average structure, the RMS deviation (RMSD) for the C^α, C, O, and N atoms of the peptide backbone is 0.63 ± 0.04 Å and for all atoms except hydrogens, the RMSD is 0.93 ± 0.06 Å. A more detailed comparison is shown in Table 2, Figure 4, and Kinemage 1, and the sequential distribution of the RMSD is shown in Figure 5. The non-hydrogen atoms of the four α -helices are well defined, as seen from the number of sequential and short-range NOEs (Fig. 2), from the ϕ -angles obtained for these residues (Fig. 6), and as reflected in the structures by the atomic and angular RMSDs for the residues (Table 3). Less well defined are the peptide segments in the N- and C-terminal ends and in the loop regions, especially Loop 2.

The atomic coordinates of the maize structure are not yet available, therefore, initially we have been confined to a rather

qualitative comparison to this structure. The coordinates of the wheat structure, however, are available in the Protein Data Bank (PDB) (Bernstein et al., 1977) (access code: 1lpt) and a comparison superimposing the C^α, N, and C atoms of each of the 11 deposited wheat structures on each of the 17 bLTP structures results in a RMSD 3.02 ± 0.01 Å (Fig. 7).

Table 2. RMSD of the different sets of atoms in the 17 structures of bLTP to a common average structure

RMSD (Å)	Backbone heavy atoms ^a	All heavy atoms ^b
Helix A	0.46 ± 0.03	0.69 ± 0.05
Helix B	0.54 ± 0.04	0.77 ± 0.08
Helix C	0.38 ± 0.03	0.73 ± 0.13
Helix D	0.50 ± 0.03	0.74 ± 0.09
bLTP	0.63 ± 0.04	0.93 ± 0.06

^a Backbone heavy atoms includes N, C, C^α, and O.

^b All heavy atoms includes all atoms except Hs.

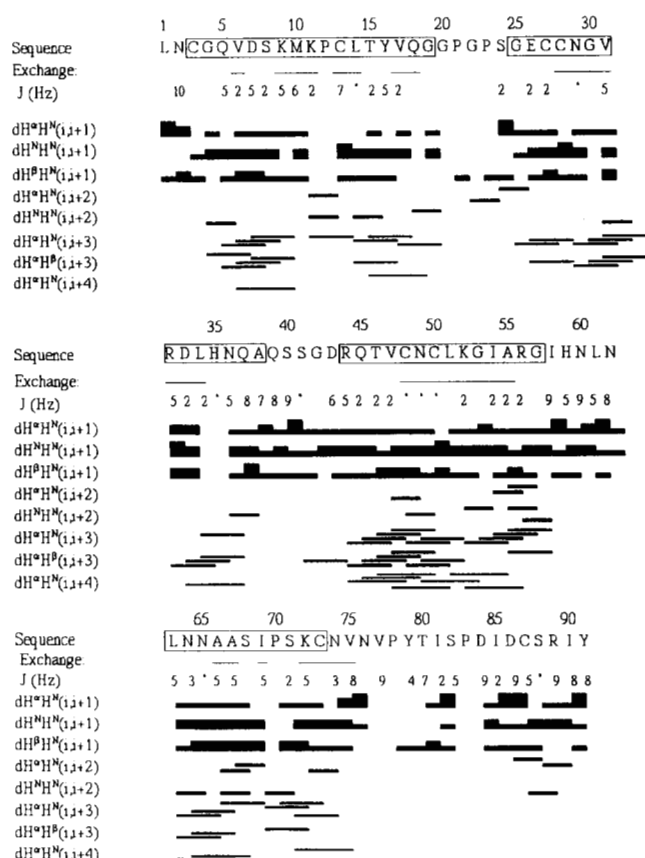


Fig. 2. Summary of the sequential NOEs, amide proton exchange, and elements of secondary structure found in bLTP. Amide proton exchange is classified as slow by presence of a horizontal line. Coupling constants are given in Hz, as are the $^3J_{\text{HNH}\alpha}$ coupling constants, where * indicates a coupling constant below 2 Hz. The NOEs have been categorized as either strong, medium, or weak intensities, and displayed accordingly by the thickness of the lines. Regions of α -helices are framed at the assigned sequence positions.

Discussion

The information of the mode of lipid uptake, binding, and release in cells is sparse. For the plant LTPs, so far only one crystal structure and one solution structure of other homologous proteins have been determined. Furthermore, the crystal structure of a nsLTP with a ligand has been determined (Shin et al., 1995). Here we present the three-dimensional solution structure of bLTP, which consists of four helices interspaced by four short loops and concluded by a large well-defined C-terminal peptide segment, with no observable secondary structure. In addition, we show the presence of a hydrophobic cavity, provided by hydrophobic side chains facing the interior of the protein, possibly affording a binding site for a lipid ligand by hydrophobic interactions between the fatty acid chain and the hydrophobic residues.

bLTP is a four-helix protein (Fig. 8 and Kinemage 1) with three of the helices in a regular and consecutive up-down-up motif, where the central helix A makes contacts with the two helices B and C, on each side (Fig. 9). Helix D extends from helix C and makes contacts to helix A and C. The NMR data suggest that helix A starts at Cys 3 and ends at Gly 19. Helix A is an amphi-

Table 3. Persistent hydrogen bonds in the 17 structures of bLTP in solution^a

Acceptor residue	Donor residue
3	7
4	8
5	9*
6	10*
7	11*
11	15
12	16
13	17*
14	18*
15	19
24	28*
25	29*
26	30*
27	31*
28	32*
30	34*
31	35
32	36
33	37
34	38
44	48*
45	49*
46	50*
47	51*
48	52*
49	53*
50	54*
51	55*
52	56
62	17
64	68
68	72*
69	73*

^a The first column lists the acceptor residues and the second column lists the donor residues. Each hydrogen bond is utilized as a distance restraint between the carbonyl oxygen at the acceptor residue and the backbone amide proton and the backbone amide nitrogen, respectively, of the donor residue. In the second column, entries marked with an asterisk mark donor residues with slow ^1H - ^2H amide exchange.

phatic helix with the side chains of the nonpolar residues Val 6, Met 10, Leu 14, and Val 17 facing the interior side of the helix, making hydrophobic contacts mainly to helix C. The side chains of Cys 3 and Cys 13 are facing helix C and B, respectively, where they are linked by disulfide bonds to Cys 50 and Cys 27, respectively. These bonds, in combination with the hydrophobic interactions, adhere helix A to helix B and C, constituting the core of the structure. Helix A is slightly bend at Pro 12. This results in the lack of hydrogen bonds between Lys 9-Cys 13 and Met 10-Leu 14 (Table 3), and it is consistent with the large $^3J_{\text{HNH}\alpha}$ coupling constants of Met 10 and Cys 13 (Fig. 2), which are atypical of α -helices (Barlow & Thornton, 1988). A similar discontinuity is seen in the analogous helix in the structure of mLTP as determined by X-ray crystallography (Shin et al., 1995). The linear α -helix helix B from Gly 25 to Ala 38 is amphipathic, like helix A, with the nonpolar residues Val 31, Leu 34, and Ala 38

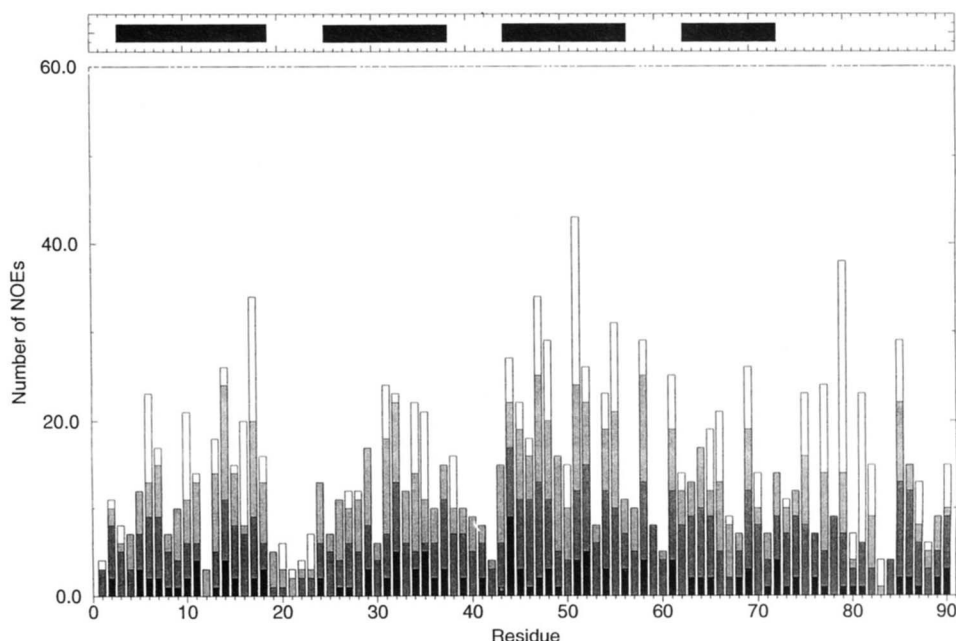


Fig. 3. Distribution of NOEs by residue. Black bars are intraresidual NOEs (i), dark grey bars are sequential NOEs ($|i-j|=1$), light grey bars are short-range NOEs ($1 < |i-j| \leq 5$), and open bars are long-range NOEs ($|i-j| > 5$). α -Helical regions are indicated by filled frames at the top.

making hydrophobic contacts to the interior of the protein. In the N-terminal of helix B, Cys 27 and Cys 28 form disulfide bonds with Cys 12 and Cys 73, respectively, connecting helix B with helix A and helix D, respectively. Like helix B, helix C is a linear α -helix from Arg 44 to Gly 57. Helix C is amphiphatic also, with the nonpolar residues Val 47, Leu 51, Ile 54, and Ala 55 facing the interior of the protein, making hydrophobic interactions mainly to helix A and the C-terminal. These hydrophobic interactions are supported by two disulfide bonds between Cys 48–Cys 87 and Cys 50–Cys 3, which connect helix C with

the C-terminal and helix A, respectively. Helix D, of residues Leu 63–Cys 73, is irregular, like helix A, with a bend in the middle at Pro 70. Helix D is amphiphatic, with the nonpolar residues Leu 63, Ala 66, Ala 67, Ile 69, and Pro 70 facing the interior of the protein, making contacts mainly to helix A, helix B, and the C-terminal. The bend in helix D causes the absence of several hydrogen bonds in this helix, consistent with a bend seen in the analogous helix in mLTP. The C-terminal region of 18 residues has extended loop structure, where residues Val 75, Val 77, Ile 81, Ile 85, and Ile 90 make contacts to all four helices by hy-

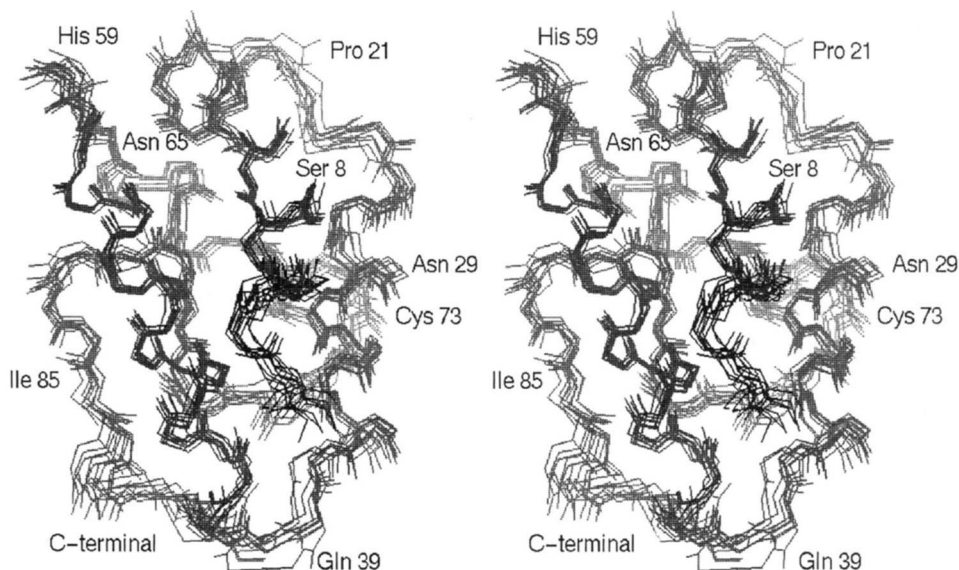


Fig. 4. An overlay stereo presentation of C^α , C, N, and O atoms of ten bLTP structures. Locations of selected residues are indicated.

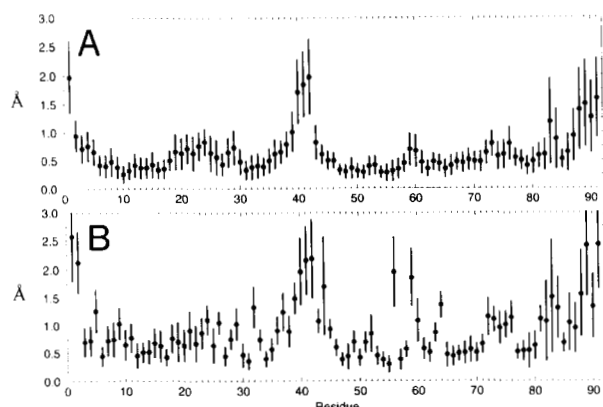


Fig. 5. Atomic RMS deviation values in Ångström per residue in relation to a geometrical average of the 17 structures representing bLTP, (A) for the peptide backbone atoms C α , C, N, and O in each residue, (B) for all non-hydrogen atoms in each residue.

drophobic interactions supported by Cys 48–Cys 87 disulfide bond. This is illustrated by the NOE contact map (Fig. 9).

The crystal structure of mLTP has a hydrophobic cavity, formed by the C-terminal loop placed over a “channel” of hydrophobic side chains of residues at the interface of helices A, B, and C, in the interior of the protein (Shin et al., 1995). This tunnel-like cavity is 5 Å wide and 15 Å long, and binds in the complexed form approximately 12 carbon atoms of palmitate by hydrophobic interactions, whereas the carboxylate group of palmitate is exposed to the solvent near loop 2. The hydrophobic residues contributing to the interaction of the fatty acid tail with the protein are located on the inward side of all four helices, in loop 2 and 3 and in the C-terminal region. The same type of cavity can be found in the structure of bLTP in solution (Figs. 10, 11), made up by Met 10, Leu 14, and Val 17 of helix A, Val 31 and Leu 34 of helix B, Val 47, Leu 51, and Ala 55 of

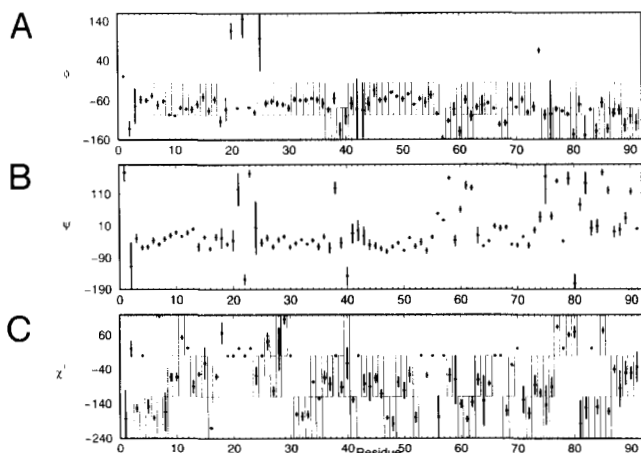


Fig. 6. Comparison of the ϕ , ψ , and χ^1 dihedral angles, respectively, for each residue in the set of 17 structures representing bLTP. A: ϕ -Angles. B: ψ -Angles. C: χ^1 -Angles. Vertical bars show the angular standard deviations, dots show the values of the average angles, and vertical boxes show the dihedral angle restraint limitations used in the structure calculations.

helix C, Ala 66 and Ile 69 of helix D, and Ile 81 and Val 77 of the C-terminal region. These residues form a potential acyl group binding site. In Figure 10 it is seen that Tyr 79 is placed over the end of the hydrophobic cavity, and it is possible that this residue has a function as a molecular cover in the uncomplexed form (Fig. 10). The aromatic ring of Tyr 79 is tightly held in place by many NOEs to the surrounding residues. The hydrophobic cavity is occupying the central part of the protein, from one end to the other, as shown in Figure 11.

In the uncomplexed form, the hydrophobic cavity is solvent filled, as determined by the crystallographic data from mLTP (Shin et al., 1995). This seems to be the case also for the solution structure of bLTP, where the low number of observed NOEs between the four helices (Fig. 9) is in accordance with this. In order to assess the covalent structure geometry independently of the experimental data, each of the 24 structures representing the preliminary solution structure of bLTP were analyzed using PROCHECK (Laskowski et al., 1993). PROCHECK applies published structures in PDB to create a set of standard values for bond angles and bond lengths. These standard values are compared with the values of the structure being analyzed. In addition, PROCHECK examines the structure for secondary structure elements, on the basis of Kabsch and Sanders assignments (Kabsch & Sander, 1983). In the ϕ, ψ check, as performed by PROCHECK, only four residues have ϕ, ψ values that are in disallowed regions: Cys 3, Asp 43, Asn 74, and Ser 82, respectively, in 2, 3, 1, and 1 of the 24 structures. Neither ϕ nor ψ angles of these residues are restrained in the structure calculation (Fig. 6). Hence, the 7 structures with angles in disallowed regions could be disregarded, leaving 17 structures to represent the solution structure of bLTP. In these structures, 13 residues have ϕ, ψ angles in generously allowed regions, but none of these are located in helices and none occur with a frequency higher than 4 of 17 structures. This means that all 17 structures in this respect satisfy the standard values applied by PROCHECK. The location of the secondary structure elements proposed by PROCHECK resembles the locations determined by hydrogen exchange and NOEs. PROCHECK finds four segments of helix and no β -strand, where the four sequences of helices comprise residues Cys 3–Val 17 (except Met 10–Cys 13), Gly 25–Gln 37, Arg 44–Ala 55, and Asn 65–Cys 73 (except Ser 68). These locations are found in all 17 structures and they are, by and large, the same as the helix segments of bLTP as defined by the NMR data (Fig. 2): helix A, Cys 3–Gly 19; helix B, Gly 25–Ala 38; helix C, Arg 44–Gly 57; and helix D, Leu 63–Cys 73. The exceptions in helix A and D corresponds to the described bends imposed by the presence of Pro 12 and Pro 70. In two of the analyzed structures, an additional stretch of helix is proposed by PROCHECK, Cys 87–Arg 89, corresponding to the single turn of a 3_{10} -helix in mLTP.

PROCHECK has also been used to analyze 17 structures derived from restrained molecular dynamics, as calculated on the basis of the 17 simulated annealing structures of bLTP. In general, the results obtained from the restrained molecular dynamics structures complement the results from the analysis of the simulated annealing structures with the consistent exceptions that the helices are longer, the number of bad contacts per structure are lower, the number of residues with generously allowed ϕ, ψ angles are higher, and residues with disallowed ϕ, ψ angles are introduced. The helices in the structures derived from molecular dynamics on average include the residues Cys 3–Gly 19,

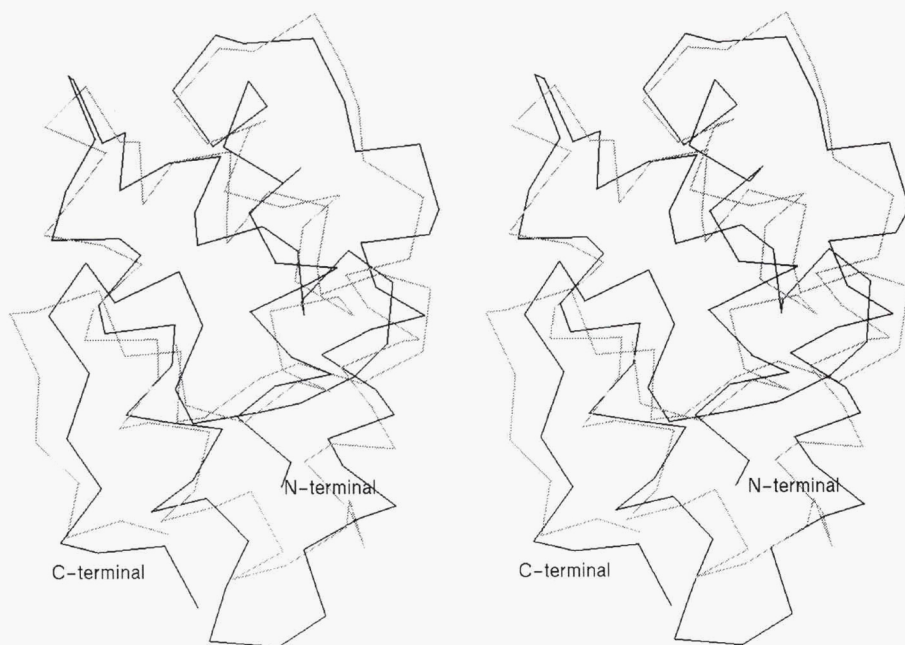


Fig. 7. A superimposed image of C^α , N, and C atoms of a bLTP structure (bold) on a wheat structure. When superimposing each of the 17 bLTP structures on each of the 11 wheat structures, the RMS deviation is 3.02 ± 0.01 .

Glu 26–Gln 37, Arg 44–Ala 55, and Leu 63–Cys 73. In addition there is, in 6 of 17 structures, a short patch of helix in residues Cys 87–Arg 89, identical to the patch found in two of the simulated annealing structures and in the homologous position in mLTP. In the structures derived by simulated annealing, the mean number of bad contacts per structure is 22.7 ± 5.1 , whereas the mean number in the molecular dynamics structures is 8.5 ± 2.7 bad contacts per structure. This large difference, combined with the higher number of generously allowed and disallowed ϕ, ψ angles, reflects a difference in the forcefield used in the two structure calculation methods.

In general, stereochemical analysis of the 17 structures of bLTP in solution verifies the location and amount of secondary structure elements, and the reported deviations from “ideality” illustrate the fluctuations between the structures.

Materials and methods

Sample preparation

bLTP was purified from barley (*Hordeum vulgare*) seeds as described previously (Sørensen et al., 1993), with some adjustments to the procedure described: After ion exchange chromatography on a column of S-Sepharose (5 cm $\times$ 15 cm, 300 mL) equilibrated with 20 mM Na-phosphate, pH 7.0, bLTP was eluted by a NaCl gradient from 0 to 0.1 M. This gradient resulted in two peaks of bLTP, where the fractions of the first peak were pooled. The pooled fractions were desalted on a Sephadex G25 column in 20 mM ammoniumbicarbonate, filtered through a 22- μ m sterile filter, and freeze dried. The bLTP NMR samples were prepared from lyophilized material to make 600- μ L 3-mM solutions of

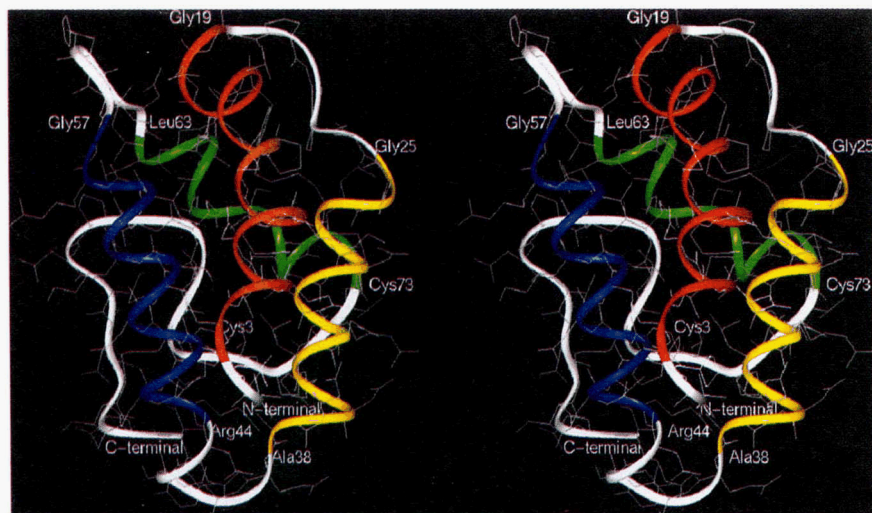


Fig. 8. Solution structure of bLTP. The structure shown is the one with the lowest energy of the 17 simulated annealing structures. All heavy atoms are shown, along with a smoothed ribbon drawn through the C^α , C, and N atoms of each residue. Helices are marked in colors: helix A, red; helix B, yellow; helix C, blue; helix D, green. The first and last residues of the helices and N- and C-terminals are labeled.

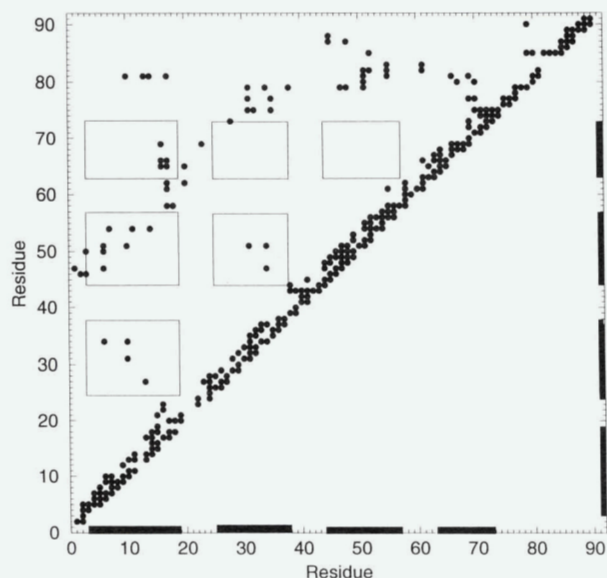


Fig. 9. NOE contact map. Upper left part of the plot shows the distribution of observed NOEs along the primary structure. α -Helix regions are indicated by filled frames at the axis, and by open frames in the plot.

bLTP in water with 10% $^2\text{H}_2\text{O}$ or in 99.96% $^2\text{H}_2\text{O}$. Both NMR samples were adjusted to pH 4.0 as recorded by pH-meter reading.

Recording and processing of NMR data

All spectra were recorded at 14.1 Tesla on a Bruker AMX600 spectrometer operating at 600.13 MHz. Two-dimensional DQF-COSY spectra (Piantini et al., 1982), TOCSY spectra with the DIPSI-2 mixing sequence (Braunschweiler & Ernst, 1983; Bax & Davis, 1985), and NOESY spectra (Jeener, 1971; Anil-Kumar et al., 1981) in 90/10 $\text{H}_2\text{O}/^2\text{H}_2\text{O}$ at pH 4.0, of bLTP at 298 K and 310 K, respectively, were recorded and processed according to the States-TPPI scheme (Marion et al., 1989). Mixing times in the NOESY spectra were 50, 100, or 200 ms; for the 50-ms mixing time, NOE magnetization transfer via zero quantum coherence was minimized by the method of Otting (1990). In the TOCSY experiment, the mixing time was 31.2 ms. Also,

a double quantum spectrum (Braunschweiler et al., 1983) with mixing time of 15 ms was recorded on the sample dissolved in 90/10 $\text{H}_2\text{O}/^2\text{H}_2\text{O}$. For measurements of the ^1H - ^2H exchange of amides, NOESY spectra with 200-ms mixing time were recorded with the fully protonated bLTP sample at 310 K, immediately after and 12 h after solvation in $^2\text{H}_2\text{O}$. The applied sweep width was 17.003 ppm for all spectra and ^1H -chemical shifts were measured relative to trimethyl silyl propionate (TSP). All spectra were recorded with 512 and 2,048 complex points in t_1 and t_2 , respectively, and zero-filled to 1,024 and 4,096 complex points in the two directions, except for the spectra used to measure the ^1H - ^2H exchange. NMR data were Fourier transformed with appropriate window functions using MNMR (Kjær et al., 1994). The NMR spectra of bLTP were assigned by applying the Wagner-Wüthrich strategy (Wüthrich, 1986) and using the computer program Pronto (Kjær et al., 1994).

Structure calculations

Structure calculations were accomplished using the hybrid methods of distance geometry and simulated annealing. The program DIANA (Güntert et al., 1991) was used to identify and remove redundant structure information. Subsequently, the standard protocol in X-PLOR 3.1 (Brünger, 1992) for protein structure calculations was applied using, first, distance geometry; second, 3 ps of simulated annealing at 2,000 K, followed by 5 ps of simulated annealing while cooling to 100 K (Nilges et al., 1988) in the simulated annealing procedure; and third, 4.7 ps of simulated annealing while cooling from 1,000 K to 100 K in the refinement procedure. Initially, 50 structures were calculated and a subset of 20 structures with the smallest number of restraint violations was selected to represent the initial structure of bLTP. The location of hydrogen bonds in these structures were identified, and subsequently a new set of 50 structures was calculated with the identified hydrogen bonds as an additional set of restraints. From the new set of 50 calculated structures, 24 were selected to represent the preliminary solution structure of bLTP. These 24 structures all adhere to the NMR restraints by having no distance violations above 0.5 Å and no angle violations above 10° from the input data. These 24 structures were examined using the computer program PROCHECK (Laskowski et al., 1993). Of these 24 structures, 7 structures had ϕ, ψ angles in disallowed regions and were eliminated. A restrained molecular dy-

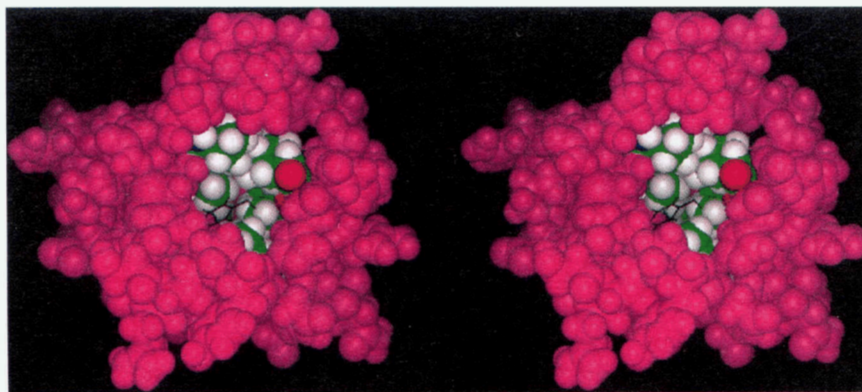


Fig. 10. CPK representation of the solution structure of bLTP. Residues that constitute the hydrophobic cavity (see text) are colored according to atoms, and the rest of the protein is colored purple. Residues from Gln 37 to Gln 45 are removed to give a better view of the cavity. The aromatic ring of Tyr 79 is colored black and shown as a pin drawing. The structure is viewed parallel to helix B and C, from the Loop 2 end. Purple atoms visible at the end of the cavity are H^α of Gly 22 and a H^γ of Pro 23.

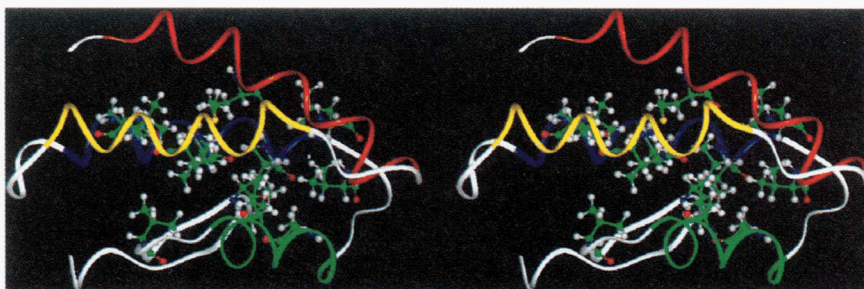


Fig. 11. Backbone trace representation of bLTP in white, with the residues that constitutes the hydrophobic cavity drawn as ball-and-stick representation, colored according to atoms. The structure is viewed perpendicular to helix B and C with helices colored as in Figure 6. Helix A is extending toward left in the top of the structure and the C-terminal region is in the bottom.

namics calculation was performed for the 17 simulated annealing structures selected to represent the solution structure of bLTP, including 2.0 ps of restrained molecular dynamics (Clare et al., 1986) at 300 K, followed by 500 cycles of restrained conjugate Powell minimization at full CHARMM potential (Brooks et al., 1983). Both sets of structures were stereochemically analyzed by the computer program PROCHECK. Hydrogen bonds were included in the structure calculations if they were formed consistently in the structures, and if they showed slow ^1H - ^2H amide hydrogen exchange.

The intensity of the NOEs were measured in 50-, 100-, and 200-ms NOESY spectra. The NOEs were divided into three groups with a common lower limit of 1.8 Å and an upper limit of 2.7, 3.3, and 5.0 Å, corresponding to strong, medium, and weak NOEs, respectively. When the distance restraint included a methyl group, the upper limit boundary was increased by 0.5 Å (Clare et al., 1986). ϕ -Angles were restrained between -17° and -97° for $^3J_{\text{H}\alpha\text{N}}\alpha$ coupling constants less than 5 Hz and between -80° and -160° . For $^3J_{\text{H}\alpha\text{N}}\alpha$ coupling constants larger than 8 Hz, the $^3J_{\text{H}\alpha\text{N}}\alpha$ coupling constants were measured by Pronto (Ludvigsen et al., 1991). Stereospecific assignment and determination of χ^1 angles and χ^2 angles were obtained from estimates of $^3J_{\text{H}\alpha\text{H}\beta}$ coupling constants in the DQF-COSY spectrum and the relevant NOEs in the short mixing time NOE experiments. The angle restraints, the NOE restraints, and the four disulfide bridges were used as input for the first round of structure calculation, generating 50 structures after simulated annealing refinement. These 50 structures were analyzed for hydrogen bond formation, yielding a set of 26 hydrogen bonds. These bonds were included as a set of distance restraints in a new cycle of structure calculation. The distance restraints used were 1.8 ± 0.5 Å for $\text{H}^{\text{N}}\text{-O}$ and 2.8 ± 0.5 Å for N-O distances. After the second round of structure calculation, a total set of 31 hydrogen bonds was formed and subsequently used as distance restraints. All calculations were performed on a Silicon Graphics Indigo2 computer, and structure displays were performed using the computer program Insight from Biosym Technologies (San Diego, California).

The coordinates of 4 structures representing bLTP have been deposited with the Protein Data Bank, Chemistry Department, Brookhaven National Library, Upton, New York 11973, accession code 1LiP.

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