

Solution structure of Phl p 3, a major allergen from timothy grass pollen

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Abstract

The major 97-aa timothy grass (*Phleum pratense*) allergen Phl p 3 was recently isolated from an extract of timothy grass pollen. Sequence comparison classifies this protein as a group 3 allergen. The solution structure of Phl p 3 as determined by nuclear magnetic resonance spectroscopy reveals that the protein consists of a core of hydrophobic amino-acid side chains from two β -sheets of five and four anti-parallel β -strands, respectively. This conformation is very similar to the crystal structure published for Phl p 2 and strongly resembles the known conformation of the carboxy-terminal domain of Phl p 1, the major difference being the loop orientations. Phl p 2 and Phl p 3 show virtually identical immunoreactivity, and comparison of the charged surface amino acids of the two proteins gives initial clues as to the IgE recognition epitopes of these proteins.

Keywords: allergen; epitopes; grass pollen; Phl p 3; *Phleum pratense*; timothy grass.

Introduction

Over 20% of the population of industrial countries suffer from seasonal allergic rhinitis, commonly known as hay fever (Kay, 2001a), and the allergens are most frequently proteins originating from pollen of grasses, trees, and weeds (Kay, 2001b). Recognition of an allergen by IgE is an important step in immunoreaction, and conformational similarities among allergens have been identified as the molecular and structural basis for immunologic cross-reactivity, explaining why allergic patients can exhibit clinical symptoms and IgE reactivity to various unrelated allergen sources (Neudecker et al., 2001). The cost to society caused by allergies runs into billions, and the only known safe strategy for allergic individuals is allergen avoidance, which is a highly unsatisfactory situation.

Thus, development of pharmaceuticals or vaccine-like approaches against allergies or, as an alternative route, development of hypoallergenic plants by genetic engineering, is of utmost importance. For all these strategies, knowledge of the IgE binding epitopes of allergens is highly desirable, if not mandatory, and the determination and comparison of conformations of allergens in atomic detail is a promising approach.

Grass pollen allergens are among the most frequently recognized allergens (Andersson and Lidholm, 2003). Of these, timothy grass (*Phleum pratense*) pollen is one of the most important allergen sources in northern and central Europe, and its set of allergens is the best characterized within grasses. The three-dimensional structures of several Timothy grass pollen allergens are known: Phl p 1 (pdb access code 1N10); Phl p 2 (pdb access codes 1BMW; De Marino et al., 1999; 1WHO, 1WHP); Phl p 5b (pdb access code 1L3P; Rajashankar et al., 2001); Phl p 6 (pdb access code 1NLX); and Phl p 7 (pdb access code 1K9U, Verdino et al., 2002). Phl p 3 was recently isolated from an extract of timothy grass pollen and the corresponding gene was cloned into *Escherichia coli* (Petersen et al., 2006). The protein sequence consists of 97 amino acids and shows high similarity with Phl p 2 and with the carboxy-terminal domain of Phl p 1 (Phl p 3.0101 vs. Phl p 1, carboxy-terminal domain: identity 31%, similarity 56%; Phl p 3.0101 vs. Phl p 2: identity 57%, similarity 78%; Figure 1). Homology modeling therefore predicts a similar tertiary structure among the three allergens (Petersen et al., 2006), a prediction substantiated for Phl p 2 and Phl p 3 by the observation of cross-reactivity between these proteins as determined by IgE prevalence. Phl p 1 IgE antibodies obtained by affinity purification, however, bind Phl p 1 exclusively, suggesting the existence of epitopes of this allergen outside of the carboxy-terminal Phl p 2-like domain (Petersen et al., 2006).

The surface regions of allergens representing immunoglobulin recognition epitopes are often a consecutive stretch of amino acids (Mirza et al., 2000; Padavattan et al., 2007). However, epitope mapping for Phl p 3 with overlapping peptides did not reveal such IgE-reactive linear epitopes (Petersen et al., 2006). This suggests that Phl p 3 requires an intact tertiary structure for IgE recognition, which has been consistently observed for numerous other allergens (Neudecker et al., 2003; Ball et al., 2005).

Results and discussion

NMR spectra of the heterologously expressed timothy grass pollen allergen Phl p 3 (isoform Phl p 3.0101) show the signal dispersion characteristic of well-folded proteins. Application of standard double- and triple-reso-

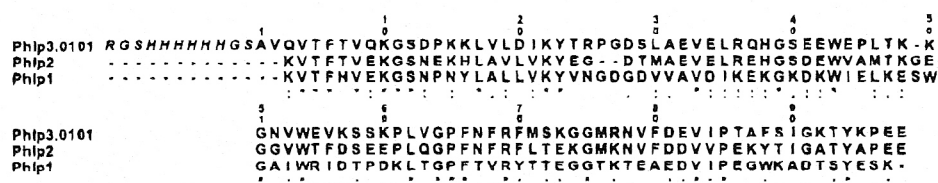


Figure 1 CLUSTAL W sequence alignment of Phl p 1 (carboxy-terminal domain), Phl p 2, and Phl p 3.

In the bottom line, identical amino acids are marked with asterisks, conserved amino acids are marked with colons, and semi-conserved amino acids are marked with dots. The additional cloned residues (His-Tag) at the amino-terminus are marked in italics.

nance NMR experiments yielded nearly complete assignment of all ^1H , ^{13}C , and ^{15}N resonances. Only the amino-terminal region Arg⁽¹⁰⁾–Ser⁽⁰⁾ containing the His-Tag had to be left unassigned as the signals for this amino acid sequence were undetectable in the ^1H , ^{15}N HSQC. It is likely that this region is unstructured and that conformational exchange or exchange with the solvent broadens the amide resonances beyond the limits of detection. In contrast, a complete backbone assignment was possible for Ala¹–Glu⁹⁷, and very few side-chain resonances, exclusively those for longer amino acids such as lysine, could not be assigned.

For the structure calculation, 2975 experimentally derived restraints including 138 residual dipolar couplings (RDCs) were obtained. The resulting ensemble displays high coordinate precision combined with low violations of experimental and geometrical restraints, as well as good stereochemical properties. Moreover, 90% of the residues are located in the most favored regions of the Ramachandran map (Table 1).

The solution structure of Phl p 3 (Figure 2) consists of nine anti-parallel β -strands ($\beta 1 = \text{Gln}^3\text{--Val}^8$; $\beta 2 = \text{Lys}^{16}\text{--Thr}^{24}$; $\beta 3 = \text{Leu}^{30}\text{--Arg}^{36}$; $\beta 4 = \text{Trp}^{43}\text{--Pro}^{45}$; $\beta 5 = \text{Thr}^{47}\text{--Lys}^{49}$; $\beta 6 = \text{Val}^{52}\text{--Lys}^{56}$; $\beta 7 = \text{Phe}^{65}\text{--Ser}^{71}$; $\beta 8 = \text{Met}^{75}\text{--Ile}^{81}$; and $\beta 9 = \text{Thr}^{92}\text{--Tyr}^{97}$) that form two β -sheets (sheet A: $\beta 5$, $\beta 6$, $\beta 2$, $\beta 1$, $\beta 9$; sheet B: $\beta 4$, $\beta 3$, $\beta 7$, $\beta 8$). Numerous hydrophobic interactions involving most of the residues with aromatic side chains between the two β -sheets stabilize the tertiary structure.

Pro⁶⁴ adopts a *cis* conformation, as evidenced by strong NOEs between its α -protons and the α -protons of Gly⁶³. All other prolines are found in the *trans* conformation, showing strong $\text{H}^{\beta}\text{,H}^{\alpha}(\text{i-1})$ NOEs, in strict accordance with the crystal structures of Phl p 2 and Phl p 1 (pdb access codes 1WHO, 1N10), but in contrast to the solution structure of Phl p 2 (De Marino et al., 1999), where a *trans* conformation is reported for the highly conserved Pro⁶⁴ residue. The chemical shifts of the preceding glycine H^{α} are remarkably upfield (3.28 and 2.00 ppm) and difficult to detect unambiguously in the homonuclear

Table 1 Statistics for structure calculations.

Experimental restraints	
NOE distance restraints	
Total	2746
Intraresidual	690
Sequential	715
medium range ($1 < \Delta i \leq 4$)	289
Long range ($4 < \Delta i$)	1052
Hydrogen bond restraints (2 per H-bond)	33
Dihedral restraints	25
Residual dipolar couplings	
Total	138
$^1\text{D}(\text{H}^{\alpha}, \text{N})$	73
$^1\text{D}(\text{H}^{\alpha}, \text{C}^{\alpha})$	65
Restraint violation	
Average distance restraint violation ($\text{\AA}$)	0.005 ± 0.0005
Maximum distance restraint violation ($\text{\AA}$)	0.14
Average dihedral restraint violation ($^{\circ}$)	0.28 ± 0.20
Maximum dihedral restraint violation ($^{\circ}$)	3.78
Average RDC restraint violation (Hz)	0.36 ± 0.01
Maximum RDC restraint violation (Hz)	1.53
Deviation from ideal geometry	
Bond length ($\text{\AA}$)	0.00092 ± 0.00004
Bond angle ($^{\circ}$)	0.234 ± 0.008
Coordinate precision (Ala ¹ –Pro ⁹⁵)	
Backbone heavy atoms ($\text{\AA}$)	0.29
All heavy atoms ($\text{\AA}$)	0.71
Ramachandran plot statistics	
	90.0%, 9.5%, 0.4%, 0.1%

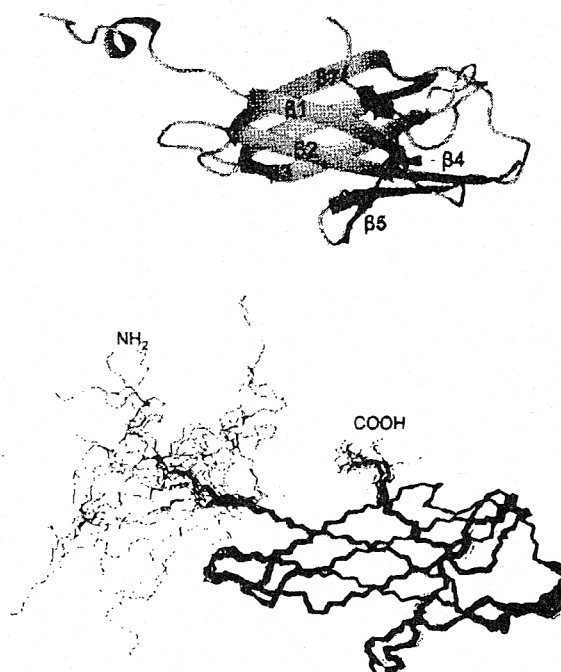


Figure 2 Solution structure of Phl p 3.

Backbone overlay of 20 accepted structures of Phl p 3 (left) and cartoon representation of the structure with the lowest target function (right).

NMR experiments used exclusively for the solution structure determination of Phl p 2.

The solution structure of Phl p 3 shows high similarity with the solution (pdb access code 1BMW) and crystal (pdb access code 1WHO) structures of Phl p 2, as indicated by rms deviation of the atomic backbone coordinates of approximately 2 Å (Table 2). According to the crystal structure, the carboxy-terminal domain of Phl p 1 (pdb access code 1N10) shows the same three-dimensional fold with a backbone rmsd of 2.1 Å compared to Phl p 3, with the main differences resulting from different orientations of the loop regions (Figure 3).

Two allergen:antibody complexes were solved by X-ray crystallography, revealing several common features of their conformational epitopes (Mirza et al., 2000; Padavattan et al., 2007): the binding regions of the allergens consist of a sequence of approximately ten amino acids located in a loop protruding from the center of the allergen. Surprisingly, however, linear peptides containing the corresponding sequences do not show any antibody binding (Mirza et al., 2000; Padavattan et al., 2007), and the overall context of the protein fold is thus important for adoption of the recognition conformation by the peptide sequence. In fact, the local conformation of the epitope region does not necessarily change on binding, as evidenced by the binding of Bet v 1 (Glu⁴²–Thr⁵²) to the F_{ab} fragment from a murine monoclonal IgG1 antibody, BV16, for which the allergen displays a backbone rmsd of 0.65 Å between free and bound forms, and by the

epitope of hyaluronidase (Arg¹³⁸–Arg¹⁴³) as recognized by a murine monoclonal anti-Hyal IgG1 antibody (mAb 21E11), for which differences in backbone atomic coordinates between the free and bound forms result in an rmsd of only 0.32 Å. Furthermore, breakdown of the global fold of the major cherry allergen Pru av 1 on introduction of the S112A mutation abolishes the allergenic character of this protein (Neudecker et al., 2003), suggesting that the conformation of the binding region is stabilized in the tertiary structure of the allergen. Single amino acids in the epitope seem to be important for binding. The Bet v 1 Glu⁴²Ser mutant does not bind to an IgG1 antibody (Spangfort et al., 2003), and the Pru av 1 Glu⁴⁵Trp mutant clearly modulates IgE reactivity (Neudecker et al., 2003). This can be attributed to the important intermolecular hydrogen bonds involving the side chain of Glu⁴⁵ in the complex of the allergen with the corresponding antibody (Mirza et al., 2000). The crystal structure of hyaluronidase in complex with a specific antibody also revealed that hydrogen bonds of side-chain carboxyl groups of the allergens, as well as salt bridges, are important for binding (Padavattan et al., 2007). This conformational context obviously necessary for epitope formation makes it even more valuable to analyze the tertiary structure of free allergens in atomic detail, as these structures already provide substantial information about potential epitopes.

The epitopes of Phl p 2 and Phl p 3 could not be determined. The Phl p 1 construct rT147 containing the whole carboxy-terminal domain, which exhibits a region homologous to Phl p 3, was the most active peptide in immunological tests (Flicker et al., 2006). Immunological studies revealed that Phl p 2 and Phl p 3 display similar immunoreactivity, whereas Phl p 1 exclusively binds its affinity-purified IgE antibodies. Although the high sequence similarity explains the common fold of Phl p 2, Phl p 3, and the carboxy-terminal domain of Phl p 1, the differences at certain sequence positions result in remarkably different surface charge distributions (Figure 4). The high number of acidic residues in Phl p 2 results in large negatively charged regions on its surface, whereas Phl p 3 shows negatively and positively charged surface patches. Several charged residues at the protein surface, however, are common to both proteins: Lys10, Lys15, Glu41, Glu42, and Arg76. From other allergen structures it is known that charged residues are key for antibody recognition. Thus, we expect that mutations of these residues combined with extensive sera screening tests could allow identification of the conformational epitope.

Materials and methods

Protein expression and purification

Phl p 3 DNA was prepared as described by Petersen et al. (2006). After transformation, cells were grown on LB agar plates containing ampicillin and kanamycin for selection overnight at 37°C. LB medium (20 ml) containing ampicillin and kanamycin was inoculated with a single colony and grown at 37°C for 10 h. This pre-culture was used to inoculate 100 ml of minimal medium M9 consisting of 2 mM MgSO₄, 10 µM Fe(III) citrate, 0.1 mM

Table 2 Structure comparison of Phl p 3, Phl p 2 and the carboxy-terminal domain of Phl p 1.

	Atom rmsd (Å)		
	Phl p 2 (1BMW)	Phl p 2 (1WHO)	Phl p 1 (1N10)
Phl p 3 (1JNZ)	2.02 (1.39)	1.91 (0.96)	2.13 (1.38)
Phl p 2 (1BMW)		1.98 (1.08)	2.51 (1.48)
Phl p 2 (1WHO)			1.64 (1.13)

Values represent the overall heavy backbone atom rmsd (in parentheses for regions with defined secondary structure only).

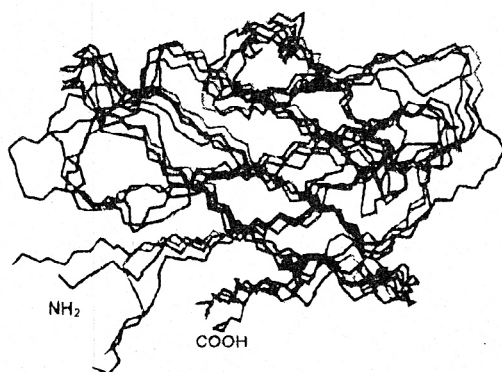


Figure 3 Structure comparison according to the data given in Table 2.

Phl p 3 (pdb access code 1JNZ, red), Phl p 2 (X-ray, pdb access code 1WHO, light green; NMR, pdb access code 1BMW, dark green), and the carboxy-terminal domain of Phl p 1 (pdb access code 1N10, blue).

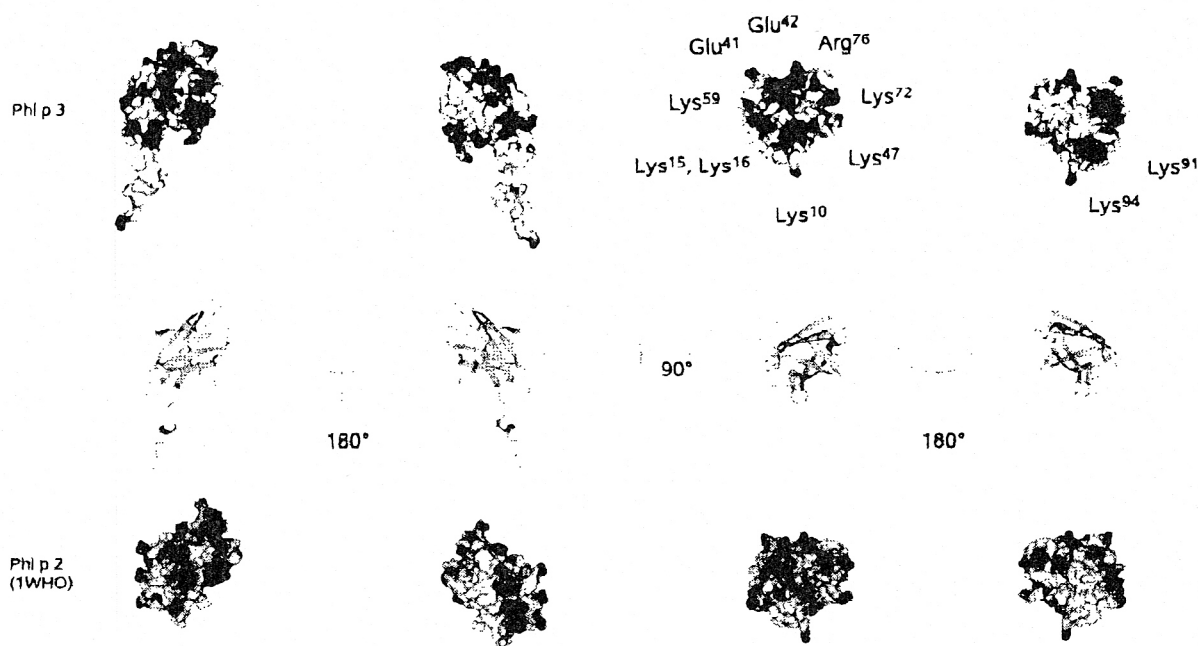


Figure 4 Comparison of the electrostatic potential of Phl p 3 and Phl p 2. Negatively charged regions are colored in red, positively charged regions are colored in blue. The cartoon representation in the middle row shows the corresponding orientation. The Figure was generated using MOLMOL.

CaCl₂, trace element solution TS2[™], MEM vitamin solution (Gibco, Invitrogen, Karlsruhe, Germany), (NH₄)₂SO₄ as the only source of nitrogen, 2 g/l glucose, and antibiotics. The cells were incubated at 37°C overnight, and this overnight culture was used to inoculate 2 l of M9 medium to an OD₆₀₀ of 0.1. The cells were incubated at 37°C until the OD₆₀₀ reached 0.7 and then induced with 1 mM IPTG. The soluble His-tagged protein was purified by immobilized metal-ion affinity chromatography on an ÄKTA purifier 10-FPLC system (Amersham Biosciences, Freiburg, Germany). The protein was eluted with 100–150 mM imidazole. Fractions were analyzed by 15% SDS-PAGE, and fractions containing pure protein were combined and dialyzed against 2×5 l of Millipore water. After shock freezing in liquid nitrogen, the protein was lyophilized and stored at 4°C. Expression of ¹⁵N-labeled and ¹⁵N,¹³C-labeled protein was achieved by replacing (NH₄)₂SO₄ with (¹⁵NH₄)₂SO₄ and glucose with ¹³C-glucose. For protein expression and purification, the same protocol was used.

NMR samples were prepared by dissolving 15 mg of ¹³C,¹⁵N-labeled lyophilized protein in 600 µl of NMR buffer (20 mM sodium phosphate, pH 7, 100 mM NaCl, 1 mM DTT, 1 mM NaN₃) containing 10% D₂O.

NMR measurements

All NMR experiments were conducted at 298 K on a Bruker DRX 600 MHz or Bruker Avance 700 MHz (equipped with a cryogenic probe) spectrometer. ¹H,¹⁵N HSQC, ¹H,¹³C ct-HSQC, HNCO, HNCA, HNCACB, CBCA(CO)NH, HBHA(CO)NH, C(CO)NH, and HCCH-TOCSY spectra were recorded for assignment of chemical shifts (Sattler et al., 1999). HNHB, ¹³CO and ¹⁵N spin-echo difference experiments were performed for χ 1-restraints (Bax et al., 1994). ¹⁵N- and ¹³C-edited NOESY experiments (mixing time 120 ms) were acquired to obtain distance restraints.

Residual dipolar couplings were measured using a sample of 1 mM Phl p 3 in 10 mg/ml Pf1 phage solution (Hansen et al., 1998). ¹D(H¹,N) were determined using the IPAP methodology (Ottiger et al., 1998) and ¹D(H¹,C¹) were determined in a HA(CA)CONH experiment (Hu et al., 2003). Reference scalar

couplings were obtained from a sample containing no alignment media. NMR data were processed using in-house software and visualized with NMRView (Johnson and Blevins, 1994).

Structure calculation

Distance restraints for structure calculation were derived from ¹⁵N-NOESY-HSQC and ¹³C-NOESY-HSQC spectra. NOESY cross peaks were classified according to their relative intensities and converted to distance restraints with upper limits of 3.0 Å (strong), 4.0 Å (medium), 5.0 Å (weak), and 6.0 Å (very weak). For ambiguous distance restraints, the r^6 summation over all assigned possibilities defined the upper distance limit. Experimental NOESY spectra were validated semi-quantitatively against back-calculated spectra to confirm the assignment and to avoid bias of upper distance restraints due to spin diffusion.

Hydrogen bonds were included for backbone amide protons in the regular secondary structure if the amide proton did not show a water exchange cross-peak in the ¹⁵N-edited NOESY spectrum. Dihedral restraints for χ 1-angles based on ³J(N,H¹), ³J(N,CH3), and ³J(CO,CH3) coupling constants were included as restraints for the most probable rotamer with 20° tolerance. Structure calculations were performed using the XPLOR-NIH 1.2.1 program (Tjandra et al., 1997) with a three-step simulated annealing protocol and floating assignment of prochiral groups, including a conformational database potential.

In a first step, 120 structures were calculated (Table 1) using distance and dihedral angle restraints only. The 30 structures with the lowest total energy were then refined using the RDCs with a harmonic potential (Tjandra et al., 1997). Values of D₃ and R were estimated from the distribution of ¹D(H¹,N) and ¹D(H¹,C¹) (Clare et al., 1998).

The 20 structures showing the lowest values of the target function excluding the database potential were further analyzed using X-PLOR (Tjandra et al., 1997), MolMol (Koradi et al., 1996), and PROCHECK 3.5.4 (Laskowski et al., 1996). The structure coordinates were deposited in the protein data bank (pdb acces-

sion code 1JNZ). and chemical shift assignments were deposited in the BioMagResBank (BMRB accession code 15134).

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