

The molecular basis for allergen cross-reactivity: crystal structure and IgE-epitope mapping of birch pollen profilin

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Background: The profilins are a group of ubiquitous actin monomer binding proteins that are responsible for regulating the normal distribution of filamentous actin networks in eukaryotic cells. Profilins also bind polyphosphoinositides, which can disrupt the profilin–actin complex, and proline-rich ligands which localize profilin to sites requiring extensive actin filament accumulation. Profilins represent cross-reactive allergens for almost 20% of all pollen allergic patients.

Results: We report the X-ray crystal structure of birch pollen profilin (BPP) at 2.4 Å resolution. The major IgE-reactive epitopes have been mapped and were found to cluster on the N- and C-terminal α helices and a segment of the protein containing two strands of the β sheet. The overall fold of this protein is similar to that of the mammalian and amoeba profilins, however, there is a significant change in the orientation of the N-terminal α helix in BPP. This change in orientation alters the topography of a hydrophobic patch on the surface of the molecule, which is thought to be involved in the binding of proline-rich ligands.

Conclusions: Profilin has been identified as an important cross-reactive allergen for patients suffering from multivalent type I allergy. The prevalent epitopic areas are located in regions with conserved sequence and secondary structure and overlap the binding sites for natural profilin ligands, indicating that the native ligand-free profilin acts as the original cross-sensitizing agent. Structural homology indicates that the basic features of the G actin–profilin interaction are conserved in all eukaryotic organisms, but suggests that mechanistic differences in the binding of proline-rich ligands may exist. The structure of BPP provides a molecular basis for understanding allergen cross-reactivity.

Introduction

Almost 20% of the population suffers from type I allergic symptoms which include rhinitis, conjunctivitis and bronchial asthma [1]. An allergic response is initiated when polyvalent allergen molecules direct the clustering of surface bound IgE receptors. This clustering is accompanied by the activation of one or more tyrosine kinases and subsequent release of anaphylactic mediators including histamine, leukotrienes, prostaglandins and cytokines. Profilins (molecular weight 12–15 kDa), are major ubiquitous actin monomer binding proteins involved in regulating the actin cytoskeleton. Patients suffering from multivalent allergies exhibit extensive IgE cross-reactivity towards a broad range of profilins extending from distantly related plants to human profilin. This wide spread cross-reactivity has led to the designation of profilins as ‘pan-allergens’ [2]. It is assumed that allergic symptoms in profilin-sensitized patients are triggered and maintained by the ubiquitous occurrence of these proteins. Using IgE antibodies, a cDNA coding for birch pollen profilin (BPP) was recently isolated from a pollen cDNA library [3].

In vitro, profilins bind monomeric actin (G actin) with an equilibrium dissociation constant (K_d) in the micromolar range [4]. *In vivo*, profilins have been proposed to regulate the pools of G and F actin by several mechanisms, including simple monomer sequestration [4], catalytic enhancement of actin-bound adenine nucleotide exchange [5–7] and the coupling of monomer addition to the growing filament with ATP hydrolysis [8]. The precise mechanism is likely to vary between species and, perhaps, physiological conditions.

Various profilins have been shown to bind lipid structures containing polyphosphoinositides such as phosphatidyl-inositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP₂) with K_d s in the 1–100 μ M range; *in vitro*, phosphoinositides can disrupt the association between profilin and actin [9–11]. Genetic [12,13] and structural studies [14,15] have identified a cluster of conserved basic amino acids as the PIP₂-binding site. These residues overlap with the known binding site for actin [15], suggesting that direct steric competition is the

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likely mechanism for disruption of the profilin-actin complex [14].

All cellular profilins bind polymers of L-proline in the 10–100 μM range. Based on the results of fluorescence [16–19], mutagenesis [12,20] and qualitative NMR [18,19] studies, the binding site has been mapped to a conserved patch of hydrophobic residues on the surface of the molecule. The binding of proline-rich ligands allows for the localization of profilin to sites requiring extensive actin filament formation [21,22].

We report here the structure of the wide spread allergen, BPP, and directly identify the major IgE-reactive epitopes. The sequence and location of these epitopes provides an explanation for the wide spread cross-reactivity of BPP, and indicates that the native unliganded profilin is responsible for the elicitation of the allergic response. Furthermore, BPP is the first actin-binding protein from plants to yield a structure and shows that all profilins display the same basic topology and structure.

Results

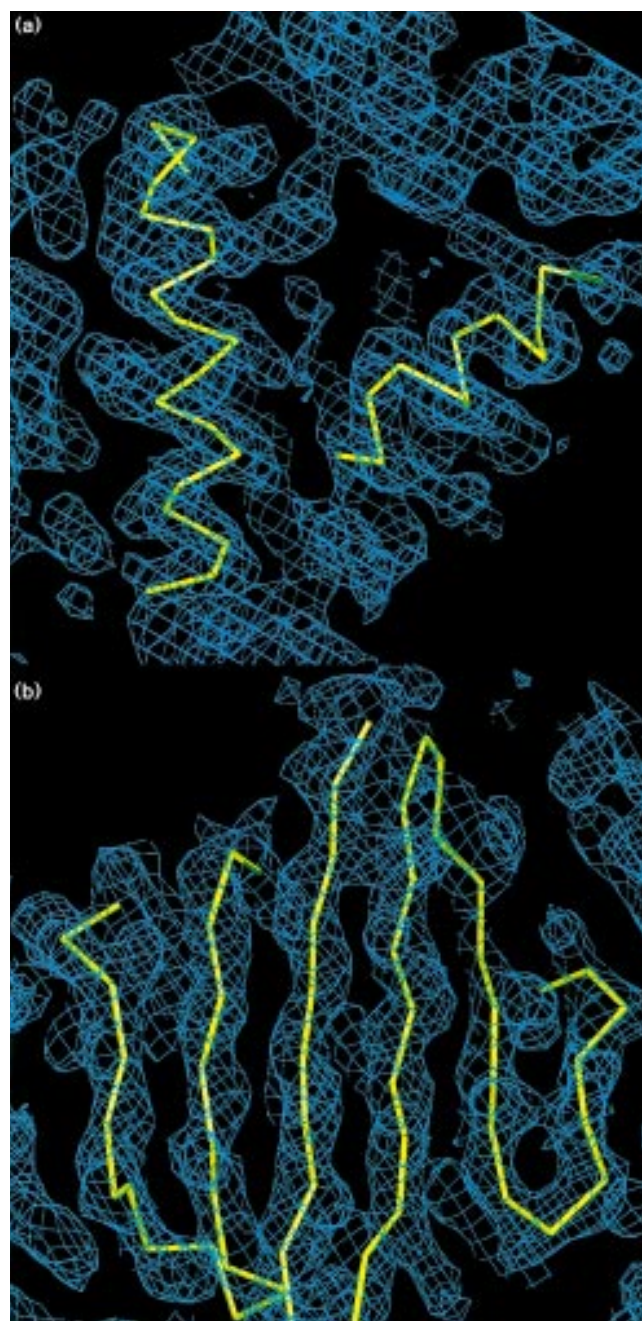
Quality of the refined structure

The structure of BPP was determined using a combination of single isomorphous replacement and molecular replacement techniques at 3.5 Å and refined to a crystallographic R factor of 0.178 at 2.4 Å resolution (Fig. 1; Tables 1,2). The refined model of BPP contains 123 amino acids, with Met1 and a nine residue loop (residues 13–21) being disordered, and 48 ordered solvent molecules. All residues are located in 'allowed' regions of the Ramachandran (ϕ, ψ) plot [23]. Based on a Luzzati analysis [24], the estimated overall error in the atomic positions is 0.23 Å. The average B factor for all non-hydrogen atoms in the BPP model is 19.8 Å² ($\langle B \rangle = 18.4 \text{ Å}^2$ for the mainchain atoms; $\langle B \rangle = 21.3 \text{ Å}^2$ for sidechain atoms).

Topology of BPP

The BPP molecule is composed of three α helices, seven β strands and ten turns [25,26] and displays the same seven-stranded incomplete, antiparallel up-and-down β barrel structure observed in amoeba and mammalian profilins (Figs 2,3; Table 3). The two edge strands of the barrel, $\beta 2$ and $\beta 3$, are linked by the chain segment containing residues 38–66 that includes helix H2 and three reverse turns. As viewed in Figures 2 and 3a, the seven-stranded β barrel appears as two orthogonal β sheets [27], with the first sheet formed by strands $\beta 1$, $\beta 2$, $\beta 4$, $\beta 5$, $\beta 6$ and $\beta 7$ (six-stranded β sheet), and the smaller second sheet formed by strands $\beta 3$ and $\beta 4$ (two-stranded β sheet). Strand $\beta 4$ abruptly changes direction at Ile77, such that it is shared by both sheets. Helix H3 packs against the face of the six-stranded β sheet and runs approximately antiparallel to $\beta 7$. Helix H1 packs against both helix H3 and the central β sheet, and runs approximately perpendicular to these elements. The angle between these helices is $\sim -110^\circ$.

Figure 1



The α carbon chain trace of the final birch pollen profilin model (shown in yellow) and 3.5 Å experimental electron-density map: (a) the N- and C-terminal α helices; (b) the central six-stranded antiparallel β sheet. The experimental electron density is based on a single ethyl mercury phosphate derivative using only isomorphous contributions and solvent flattening and is displayed at 1σ .

Hydrophobic cores and secondary structure

The BPP molecule contains two hydrophobic cores which are separated by the central six-stranded β sheet (Fig. 3b). The major hydrophobic core is formed by residues contributed from the six-stranded β sheet on one side,

Table 1

Crystallographic structure determination.		
Crystal	Native*	Derivative†
Resolution range (Å)	2.4	3.5
No. of unique reflections	5973	2249
Completeness of data (%)	91.6	99.0
R _{merge} (%)††	8.4	11.6
R _{deriv} [§]	N/A	28.0
No. of sites		1
R _{cullis} for centric reflections‡		0.55

*Native crystals obtained from 2.0 M ammonium sulfate, 0.1 M MES pH = 6.0, 5 % t-BuOH. †Crystals soaked in 1 mM ethyl mercury phosphate 2 days, pH 6.0. ††R_{merge} = $\sum |I_{obs} - \langle I \rangle| / \sum \langle I \rangle$ summed over all observations of all reflections. §R_{deriv} = $\sum |F_{ph} - F_p| / \sum F_p$ summed over all unique reflections. ‡R_{cullis} = $\sum |F_{ph, obs} - F_{p, obs}| - F_{h, calc}| / \sum |F_{ph, obs} - F_{p, obs}|$.

and from the two-stranded β sheet and chain segment (residues 38–66, which includes helix H2, on the opposite side of the molecule. A smaller hydrophobic patch is located on the opposite side of the six-stranded β sheet, which is relatively open to solvent. This smaller patch is composed of hydrophobic residues from strands β 1, β 2 and β 7, and helices H1 and H3.

The hydrogen-bonding pattern of the seven-stranded β barrel is illustrated in Figure 4 and two irregularities can be noted. A classic β bulge, involving Trp35 and Ala36, occurs in strand β 2, with the carbonyl oxygen atom of Trp35 pointing towards bulk solvent instead of forming a hydrogen bond with the amide of Ile27. The second irregularity occurs in strand β 4, which runs antiparallel to strand β 5. The hydrogen bonding between these two strands is interrupted at residues Ile77 and Arg86 by the occurrence of a three-residue β bulge, involving Ile77, Gln78 and Gly79 [28]. The two residue β bulge orients strands β 1 and β 2 so as to close the barrel from one side, while the three residue β bulge imposes a strong local twist which allows for closure of the barrel from the other side. Most of the secondary structural elements in the X-ray derived structures of BPP, *Acanthamoeba* profilin and mammalian profilins are similar, though the precise positions of the termini vary.

A large number of polar interactions are critical for the structural integrity of the molecule. In particular, the salt bridge between Arg86 and Glu80 stabilizes the three residue β bulge in strand β 4 responsible for closing the β barrel. Several sidechain to mainchain interactions involving helix H2 provide the interactions required to close the incomplete seven-stranded β barrel. These interactions are normally present as standard mainchain to mainchain interstrand hydrogen bonds in structures containing a complete eight-stranded β barrel. A number of hydrogen bonds involving helix H1 and strands β 1 and β 2 stabilize the hydrophobic patch and serve to fix the orientation

Table 2

Crystallographic refinement.	
Resolution range (Å)	8–2.4
Reflections used ($F > 2\sigma(F)$)	5356
R factor*	0.178
Protein atoms in model (123 out of 133 residues)	930
No. of waters	48
Rms deviations	
bond length (Å)	0.009
bond angles (°)	2.19
dihedral angle (°)	25.72
planarity (°)	1.54
Average B factor (Å ²)	
all atoms	19.8
mainchain	18.4
sidechain	21.3

*R factor = $\sum |F_{obs} - F_c| / \sum F_{obs}$.

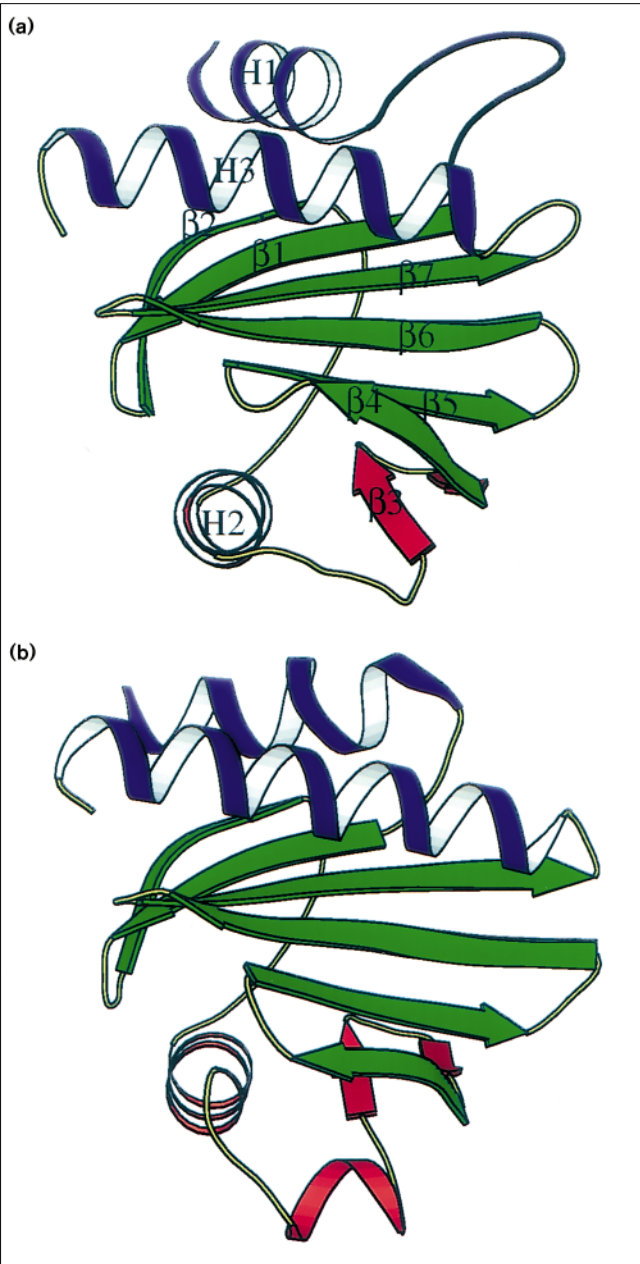
of helix H1. Several solvent molecules play important structural roles by stabilizing the three-stranded β bulge and participate in the closure of the incomplete seven-stranded β barrel.

Structure based alignment

Profilins from birch pollen, *Acanthamoeba* [14,29] and mammalian sources [30,31] display the same basic tertiary structure indicating that, despite low sequence homology of ~25 % identity, all profilins adopt the same fold. The structures were compared pairwise by a least squares fitting algorithm [32] using α carbon positions. When the *Acanthamoeba* profilin is superimposed on the BPP model (Fig. 5), the positions of 100 equivalent α carbons in the two molecules can be aligned with a root mean square (rms) difference of 1.39 Å. If only those residues belonging to the seven-stranded β barrel are superimposed, the rms deviation based on α carbon positions decreases to 0.75 Å. BPP and human platelet profilin have an rms deviation of 1.28 Å, for 91 superimposed pairs. Strands β 5, β 6 and β 7 and the C-terminal helix of BPP and *Acanthamoeba* profilin show the greatest structural similarity, as this continuous stretch of sequence (residues 82–133, BPP numbering) has no insertions or deletions and displays high sequence homology. Previous alignments of the profilin family, based only on sequence information, have been limited due to the low degree of homology across the profilin family; however, the extensive structural data base now allows for an accurate structure-based alignment (Fig. 6).

The most striking structural difference between BPP and the *Acanthamoeba* and mammalian profilins, which could not be predicted on the basis of sequence alignments, is the orientation of the N-terminal helix with respect to the central β sheet and the C-terminal helix (Figs 2,3 and 7). The N-terminal helix in *Acanthamoeba* profilin runs approximately antiparallel to the C-terminal helix with an angle of ~–170°, whereas in BPP the N-terminal helix is

Figure 2



The overall fold of birch pollen and *Acanthamoeba* profilins. (a) Ribbon diagram showing the fold of birch pollen profilin. The molecule is built around a central six-stranded antiparallel β sheet (green). One face of the sheet is occupied by the N-terminal (H1) and C-terminal (H3) α helices (blue), while the other face is packed against a smaller two-stranded sheet and a single helix (H2) (red). The black segment represents the nine amino acid loop which is not visible in the electron density. This orientation shows the actin-binding surface of profilin [14,15], which is predominately composed of the C-terminal helix (H3) and the bottom three strands ($\beta 4$, $\beta 5$ and $\beta 6$) of the central sheet. Strands $\beta 3$ and the beginning of $\beta 4$ are shown in red to highlight the fact that they are approximately perpendicular to the main six-stranded β sheet. (b) Ribbon diagram of the *Acanthamoeba* profilin showing that the general architectural features of the actin binding-site are preserved. (This figure was prepared with MOLSCRIPT [55].)

Table 3

The secondary structure elements of birch pollen profilin.

Residue	Element	Comments
2–12	α Helix 1 (H1)	
24–29	β Strand 1 ($\beta 1$)	
29–32	Reverse turn I	
33–38	β Strand 2 ($\beta 2$)	Trp35, Ala36: β bulge
38–41	Reverse turn I	
46–57	α Helix 2 (H2)	
58–61	Near reverse turn II	Gly60 in position 3
62–65	Near reverse turn III	
67–69	β Strand 3 ($\beta 3$)	
69–72	Reverse turn I'	Gly70, Gly71 in positions 2,3
72–80	β Strand 4 ($\beta 4$)	Ile77, Gln78, Gly79: β bulge
80–83	Reverse turn II	Gly82 in position 3
84–89	β Strand 5 ($\beta 5$)	
89–92	Near reverse turn II'	Gly90 in position 2
92–99	β Strand 6 ($\beta 6$)	
99–102	Near reverse turn I	
102–109	β Strand 7 ($\beta 7$)	
109–112	<i>cis</i> -Proline turn VIa	<i>cis</i> -Pro111 in position 3
114–130	α Helix 3 (H3)	
128–133	π -Turn	

almost perpendicular to both the C-terminal helix and the β sheet with an angle of $\sim -110^\circ$.

Epitope mapping

The prevalent IgE-reactive epitopes of BPP were identified directly by cloning random fragments of the BPP cDNA into an expression library and probing with the serum IgE from a single allergic patient who mounted an extremely broad allergic response (i.e. serum which cross-reacted with a large number of allergens) (Figs 8,9). The IgE-binding capacity of the individual clones was confirmed with the sera of eight additional profilin allergic patients. The initial library was also rescreened with the sera from other individuals and, so far, no additional epitope clones have been isolated. The distribution of the positive clones shows a clustering of reactive epitopes at the N- and C-terminal α helices and within a two-stranded segment roughly composed of residues 30–50 (Fig. 9). The N- and C-terminal helices have also been identified as IgE-reactive epitopes using synthetic peptide dodecamers in solid phase binding studies (RV, unpublished results).

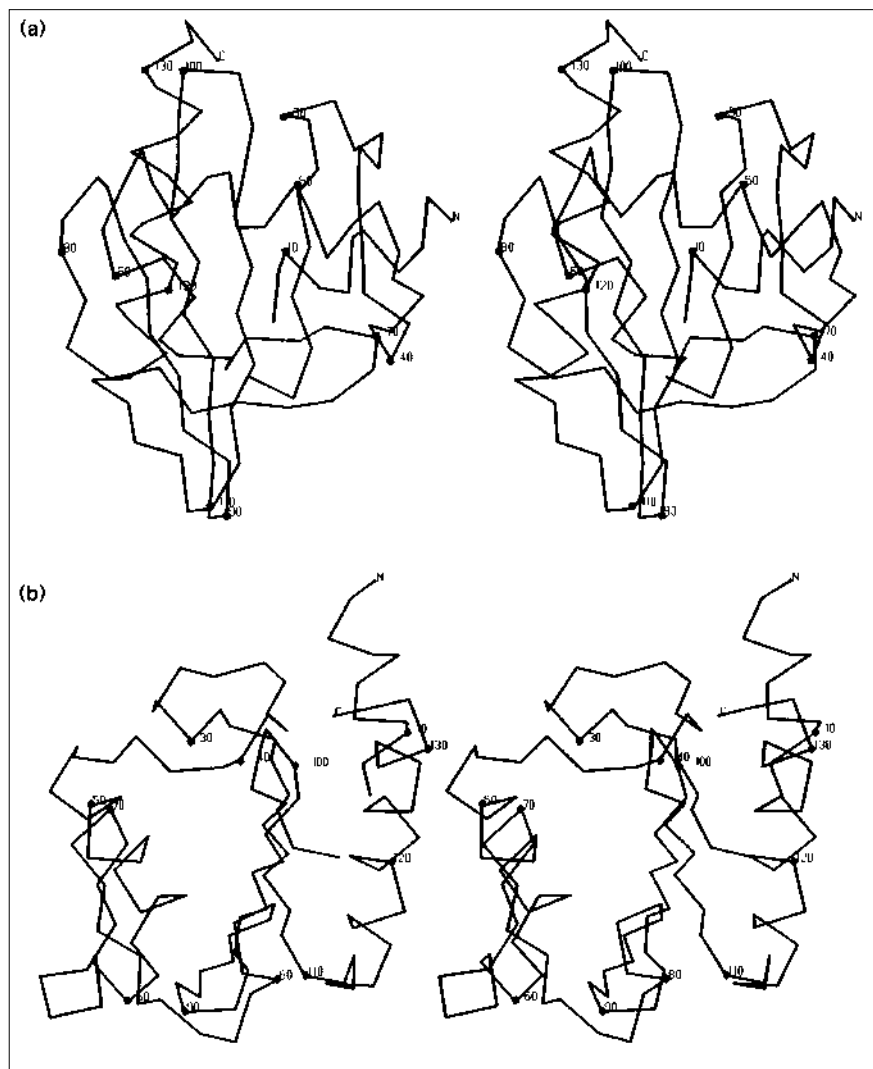
Discussion

Structure of the profilins

The comparison of profilins from diverse sources such as mammals, amoeba and plants shows that they have similar folds and indicates that all profilins share a common tertiary structure. The structures of these divergent profilins allow for a structure-based alignment, which is more accurate and therefore more informative than a sequence based alignment, for this divergent family of proteins. The availability of this alignment provides a powerful tool to assess the functional importance of specific residues and

Figure 3

Stereoview α carbon traces of the refined birch pollen profilin structure. The traces show (a) the orthogonal β sheets and (b) the view into one end of the incomplete seven-stranded barrel; every tenth residue is labeled.



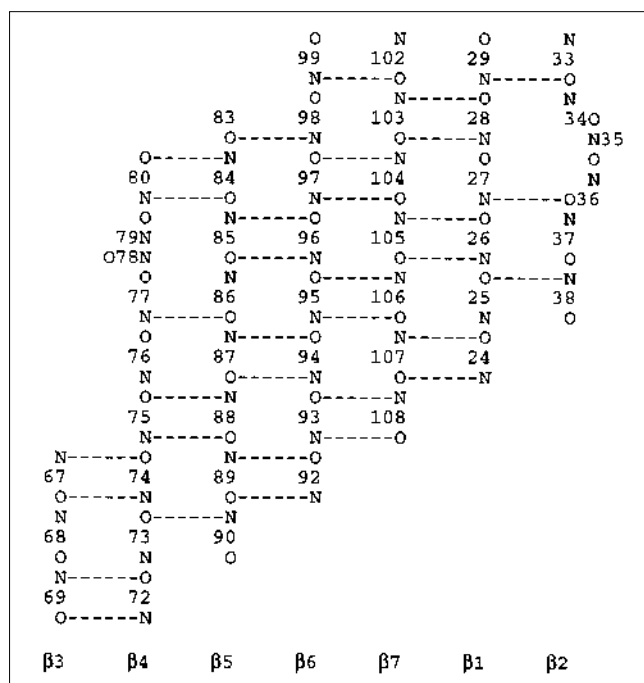
regions for actin and lipid binding. This alignment emphasizes that most of the covalent differences are located in solvent-exposed loops.

There are three insertions in BPP relative to *Acanthamoeba* profilin, which correspond to residues 13–18, 42 and 80 (Figs 2,6). The first insertion is located in the solvent-exposed loop between helix H1 and strand β 1 and should be easily accommodated by the BPP structure. The second insertion, Pro42, occurs in the loop (residues 41–46) between reverse turn I and helix H2. This insertion contributes to the specific conformation of this loop, which is the epitope responsible for binding the mouse monoclonal antibody 4A6 [33]. The insertion of Glu80 in strand β 4 alters the hydrogen-bonding pattern between strands β 4 and β 5, and results in the formation of a three-residue β bulge composed of Ile77, Asn78 and Gly79.

In *Acanthamoeba* profilin this segment corresponds to a ‘classic’ two-residue β bulge, composed of residues Leu70 and Arg71 (*Acanthamoeba* numbering).

There is a single deletion in the BPP structure, corresponding to residue 7 in *Acanthamoeba* profilin, which leads to detailed differences of helix H1 in the two structures (Figs 2,6). In BPP, helix H1 (comprising residues 3–12) is identified as pure α helix with strict $i, i+4$ backbone hydrogen bonding, while in *Acanthamoeba* the extra residue is accommodated by a change in hydrogen bonding which results in a mixture of both α and π helical character with $i, i+4$ and $i, i+5$ hydrogen bonding, respectively [14]. The mammalian profilins, which are covalently equivalent to BPP in this region, display a pure α helix, and based on sequence considerations the same is predicted to be true of the profilins from other plants and *Saccharomyces*

Figure 4



Schematic representation of the hydrogen-bonding pattern of the antiparallel β strands in birch pollen profilin. The dashed lines represent hydrogen bonds. The β sheet contains two irregularities in the hydrogen-bonding pattern: a 'classic' β bulge (residues Trp35 and Ala36) and a three-residue β bulge (Ile77, Gln78, Gly79).

cerevisiae. Also based on sequence alignments, the profilins from *Physarum*, *Saccharomyces pombe* and *Drosophila* are predicted to have a mixed $\alpha+\pi$ N-terminal helix.

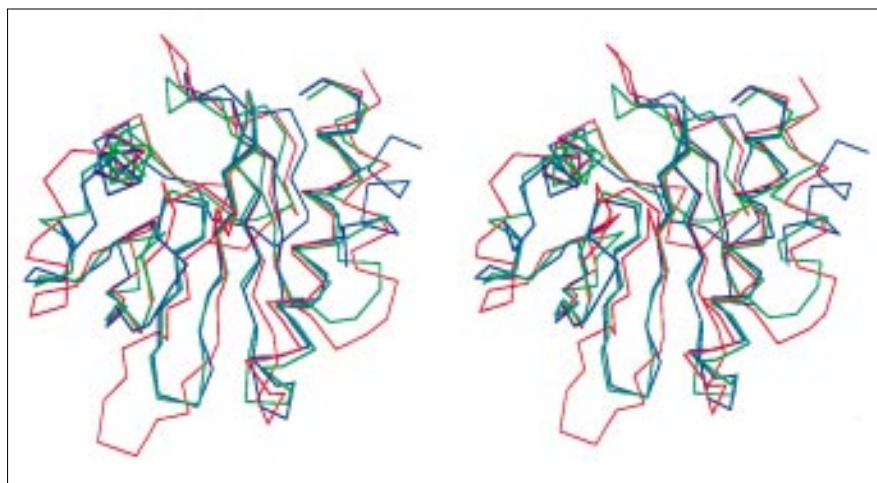
A short helix in the *Acanthamoeba* profilin structure (residues 53–58 in *Acanthamoeba* numbering) [14] is absent

in BPP. The corresponding amino acids in BPP (residues 60–65) belong to an extended loop (residues 58–66) which lacks periodic secondary structure (Fig. 10). This loop contains two near reverse turns at residues 58–61 (type II) and 62–65 (type III), and is stabilized by a network of direct and water mediated hydrogen bonds. Asp55 O δ 1 forms direct hydrogen bonds to the amide nitrogens of residues His61, Leu62 and water-mediated hydrogen bonds to Ala63 N. The structurally important water molecule, W206, further stabilizes this loop by forming hydrogen bonds to the amide nitrogens of Leu62 and Ala63. This region has also been identified as α helical in the X-ray and NMR structures of the human and bovine profilins. By contrast, the solution NMR structure of the *Acanthamoeba* profilin did not unambiguously define α helix, most likely due to the lack of sufficient hydrogen exchange data and NOE restraints needed to define secondary structure.

Actin and PIP₂ interactions

Based on the structure of BPP (Fig. 2) and the sequence alignment (Fig. 6), it is evident that BPP maintains the gross geometric features of the actin-binding surface previously identified in the mammalian [15] and amoeba [34] profilin structures. Several of the residues located at the actin-binding interface are conserved in BPP, including residues 73, 77, 88 and 115 (BPP numbering). It is important to note that the conformation of the N-terminal helix in BPP should not influence the interactions with G-actin, as the N-terminal helix does not participate in the profilin-actin interface thought to be relevant in solution [15,34]. These observations are consistent with the finding that all profilins examined, including BPP [35], show affinities for rabbit skeletal actin in the 1–10 μ M range, and indicate that the profilin-actin complex has similarities in plants, protista and mammals. The structure-based alignment also shows many of the basic residues implicated in binding

Figure 5



Stereoview comparing the α carbon traces of birch pollen profilin (BPP) (blue), *Acanthamoeba* profilin (green) and human platelet profilin (red). The comparison between BPP and the *Acanthamoeba* profilin is based on the superposition of the following set of α carbon atoms: 21–33, 35–37, 39–51, 54–70, 73–125 (from *Acanthamoeba*); and 27–39, 41, 43–44, 46–58, 61–77, 81–133 (from BPP). The root mean square (rms) deviation for these 100 pairs of atoms is 1.39 Å. The comparison between BPP and human platelet profilin is based on the superposition of α carbon atoms: 21–24, 28–35, 41–52, 58–74, 83–89, 91–133 (from human platelet profilin) and 27–30, 32, 43–54, 62–78, 83–89 and 91–133 (from BPP). The rms deviation between these 91 pairs of atoms is 1.28 Å. (This figure was prepared with MOLSCRIPT [55].)

Figure 6

Structure-based sequence alignment. The amino acid sequence alignment of birch pollen profilin (BPP), *Acanthamoeba* profilin I (AMP I), *Acanthamoeba* profilin II (AMP II), human platelet profilin (HPP) and native bovine profilin (pdb1pnl.ent: BOVP). Amino acids occupying equivalent positions were aligned after superposition of the refined models.

	1	10	20	30	40	50	60	
BPP	MSWQTYV-DEHLMCDIDGQGEELAA	SI	VGH--DGSVWAQSSS--FPQFKPQ	EITGIMKDFE	FGHLAPTGL			
AMP I	SWQTYVDTNLVG-----TGAVTQA	AILGL--DGNTWATSAG--F-AVT	PAQGGQTLASAFNNADPI	RASGF				
AMP II	SWQTYVDTNLVG-----TGAVTQA	AILGH--DGNTWATSAG--F-AV	SPANGAALANAFKDATAIR	SNGF				
HPP	AGWNAYI-DNLMA-----DGTCDAA	IVGYKDSPSVWAAVPGKTFVNIT	PAEVGVLVG--KDRSS	FVYVNGL				
BOVP	AGWNAYI-DNLMA-----DGTCDAA	IVGYKDSPSVWAAVPGKTFVNIT	PAEVGILVG--KDRSS	FPVYVNGL				
	70	80	90	100	110	120	130	
HLGGIKY	MVIQGEAG-----AVIRGKKG-----	SGGITIKKTGQALVF	GIYEBFVTPGQCNM	VVERLG	DYLDQGL			
DLAGVHY	VTLEA-DD-----RSIYGKKG-----	SAGVITVKT	SKSILVG	YNEKI	QPGTAANV	VEKLADY	LIGQGF	
ELAGTRY	VTIRA-DD-----RSVYGKKG-----	SAGVITVKT	SKAILIG	VYNEKI	QPGTAANV	VEKLADY	LIGQGF	
TLGGQKC	SVIRD-SLLQDGEF	SMDLRTKSTGGAPT	FNVT	TKTDKTLV	LLMGKEGV	HGGLINK	KCYEMASH	LRRSQY
TLGGQKC	SVIRD-SLLQDGEF	TMDLRTKSTGGAPT	FNITVT	TMTAKTLV	LLMGKEGV	HGGMINK	KCYEMASH	LRRSQY

acidic phospholipid headgroups are not conserved. The distribution of charged residues suggests that the interaction between BPP and PIP₂ is weaker than observed for the

mammalian or amoeba homologs. Thus, the PIP₂ mediated disruption of the profilin–actin complex is likely to be less efficient in plants, or may occur by a different mechanism.

Figure 7

Placement of the N-terminal α helix and hydrophobic patch in birch pollen profilin and *Acanthamoeba* profilin. (a) Stereoview ribbon diagram of birch pollen profilin looking into the face of the six-stranded β sheet. The N- (right) and C- (left) terminal helices run approximately perpendicular and parallel to the β sheet direction, respectively. The conserved hydrophobic residues (Trp3, Tyr6, Trp35, Tyr127, Leu128 and Leu133) implicated as part of the proline-rich ligand binding site are shown in blue. The disordered nine amino acid loop is displayed in magenta; other loops are in yellow. The α helices are shown in red and the β strands in turquoise. (b) *Acanthamoeba* profilin in the same orientation as (a) showing the alteration in the position of the N-terminal helix and the disposition of the conserved hydrophobic patch (Trp2, Tyr5, Trp29, Tyr119, Leu120 and Phe125). The color scheme is as in (a). (Figure prepared using ICM [56].)

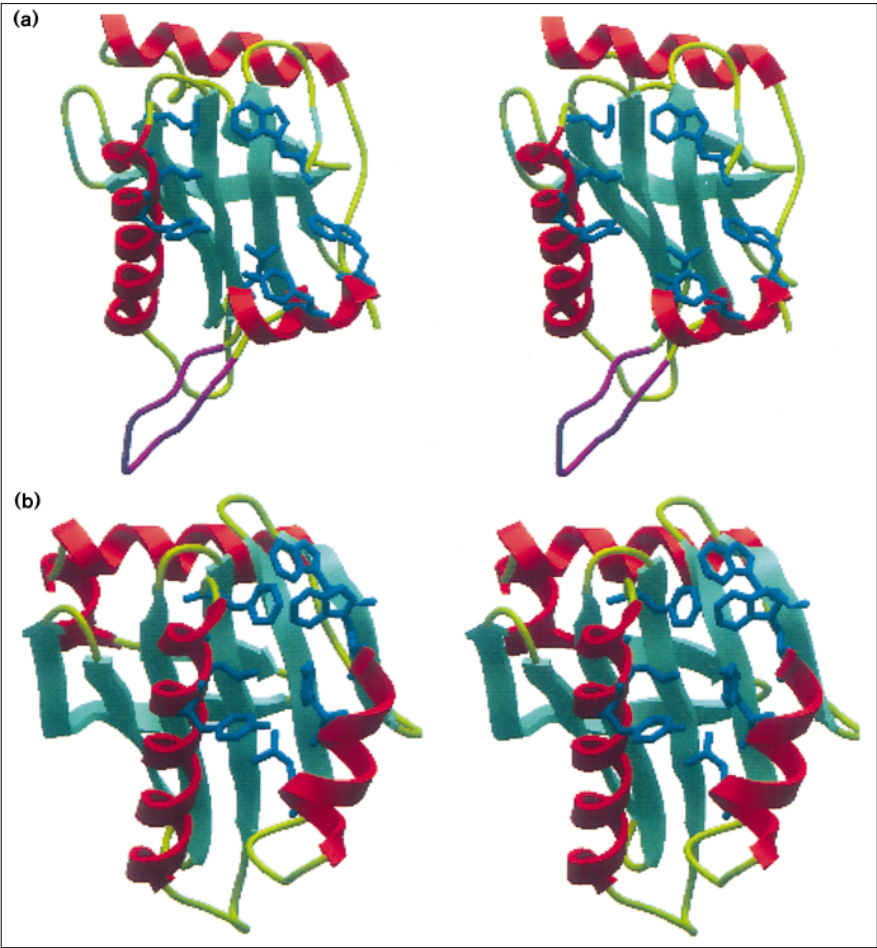
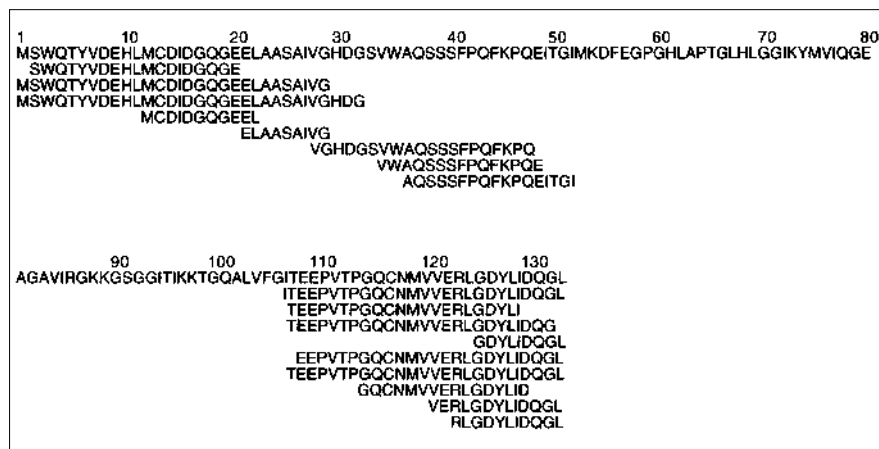


Figure 8



IgE-binding sites. The full length birch pollen profilin amino acid sequence is displayed on the top line (single letter code) and the residue numbers are given. The experimentally determined IgE-reactive epitopes are shown below.

The N-terminal α helix and proline-rich peptide interactions

The most significant difference between BPP and the other profilin structures is the large displacement of the N-terminal α helix, which alters the packing in the solvent accessible hydrophobic patch (Fig. 7). In the mammalian and *Acanthamoeba* profilins, the N- and C-terminal helices and strands β 1, β 2 and β 7 contribute hydrophobic residues (including Trp2, Tyr5, Trp29, Tyr119, Leu120 and Phe125; *Acanthamoeba* numbering) to the hydrophobic patch. The different orientation of the N-terminal helix in BPP disrupts this hydrophobic patch, leading to an open configuration which resembles a hydrophobic surface more than a compact core. The structure of human platelet profilin bound to a proline oligomer shows little change in the orientation of the terminal α helices which remain approximately antiparallel (NMM and SCA, unpublished results). The altered placement of the hydrophobic residues in BPP suggests either a different binding mode for proline-rich ligands, or a reorientation of the N-terminal α helix upon binding.

Several structural features are likely to contribute to the stability of the observed conformation in BPP. In BPP, the buried hydrophobic leucine residues present at position 10 of the *Acanthamoeba* and mammalian profilins is replaced by a polar histidine residue. This substitution may be accommodated in BPP by the reorientation of the N-terminal helix, which exposes the sidechain of His10 to solvent, while allowing for the burial of the sidechains of Trp3, Val7 and Leu11. In the *Acanthamoeba* structure, the corresponding sidechains (Trp2, Val6 and Leu10) also occupy buried positions. The presence of Ser25 in the hydrophobic patch may also contribute to the observed orientation of the N-terminal helix, which prevents burial of this hydrophilic residue.

Another factor relevant to the shift of the N-terminal helix is the loop connecting this helix to the first β strand, which

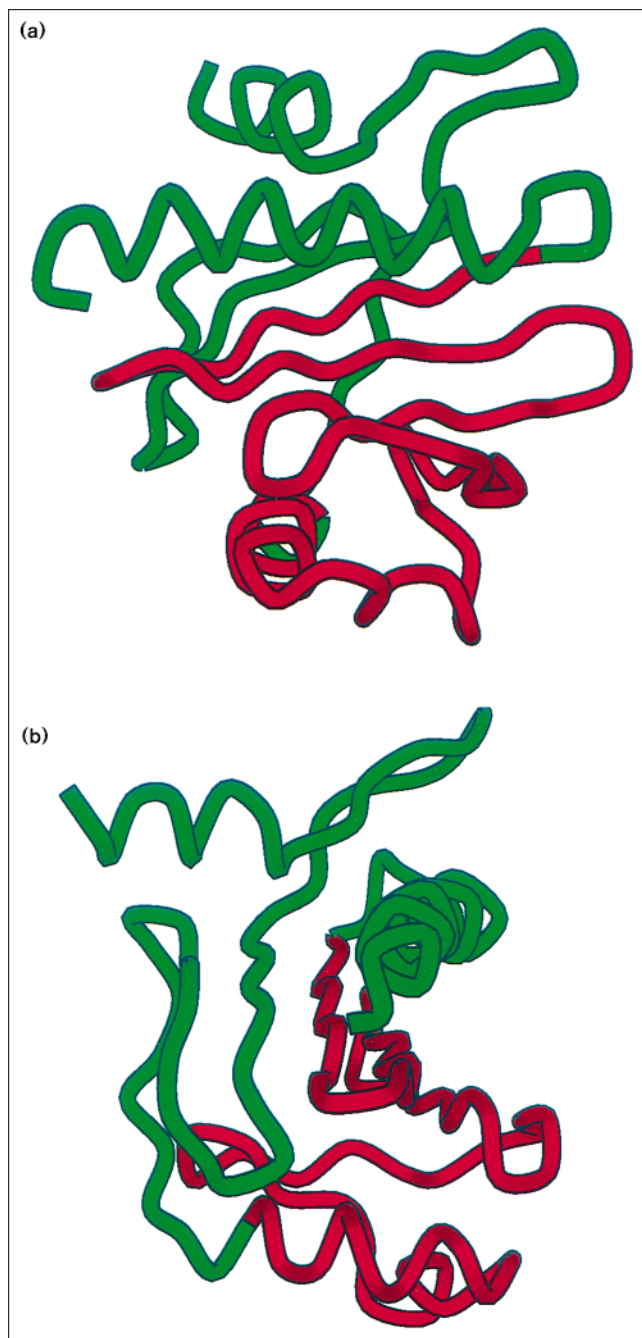
is larger (11 residues versus 5) and more flexible in BPP than in either the *Acanthamoeba* or mammalian profilins. The additional conformational freedom of the extended BPP loop may more readily accommodate the observed conformation. Helix H1 in BPP is involved in an extensive network of intramolecular hydrogen bonds and packing interactions, which appear to stabilize this conformation and rigidly fix the observed orientation.

The observed orientation could be the consequence of crystal packing and solvent conditions, however, these effects are generally considered to be weak and are not likely to be responsible for a displacement of this magnitude. Fluorescence measurements show that ammonium sulfate and t-BuOH, the reagents used for crystallization, do not promote a conformational transition in BPP or human platelet profilin (NMM, unpublished data).

Mechanism of profilin allergenicity

The direct experimental mapping of the prominent IgE-reactive epitopes onto the BPP structure (Figs 8,9) is relevant to several aspects of the allergic response. Computer modeling studies, using an Fab fragment, suggest that IgE antibodies directed against the three epitopes can approach these epitopes without interpenetration (SCA, unpublished data). This is consistent with the requirement for individual allergen molecules to simultaneously bind multiple IgE antibodies with different epitope specificities to facilitate efficient receptor aggregation.

A fundamental issue is whether different allergens share common structural and/or functional features that are relevant to stimulating the allergic response. Solution NMR studies show the major ragweed allergens (Amb a 5 and Amb t 5) are compact structures with many disulfide cross-links, the structures are composed of a short α helix, a small antiparallel β sheet and several connecting loops

Figure 9

Backbone representation of birch pollen profilin showing the clustering of continuous IgE-reactive epitopes. The sequenced epitopes are displayed in green with the remainder of the backbone in red. (a) This is the same view as in Figure 2a displaying the actin-binding surface. (b) The same representation as in (a) rotated 90° about the vertical axis. (Figure was rendered with MOLSCRIPT [55].)

[36]. On the basis of modeling, it has been proposed that the α -helical segment is a major epitope and is responsible for cross-reactivity observed with different ragweed species [37]. The X-ray structure of the 98 amino acid Phl

p 2, a major grass pollen allergen [38,39], shows a compact β barrel structure (AAF and SCA, unpublished results). Therefore, the IgE-reactive epitopes in the native allergen must adopt either β strand or loop conformations. Because native allergens are delivered to the mucosal membranes, it is the native conformation of the epitopes which are relevant to the allergic response. This is well documented in the case of profilin, as BPP extracted directly from pollen can bind both actin and poly-L-proline [40]. The accumulated structural data on the native conformation of epitopes shows that there is no unique structural motif involved in the elicitation of allergic responses.

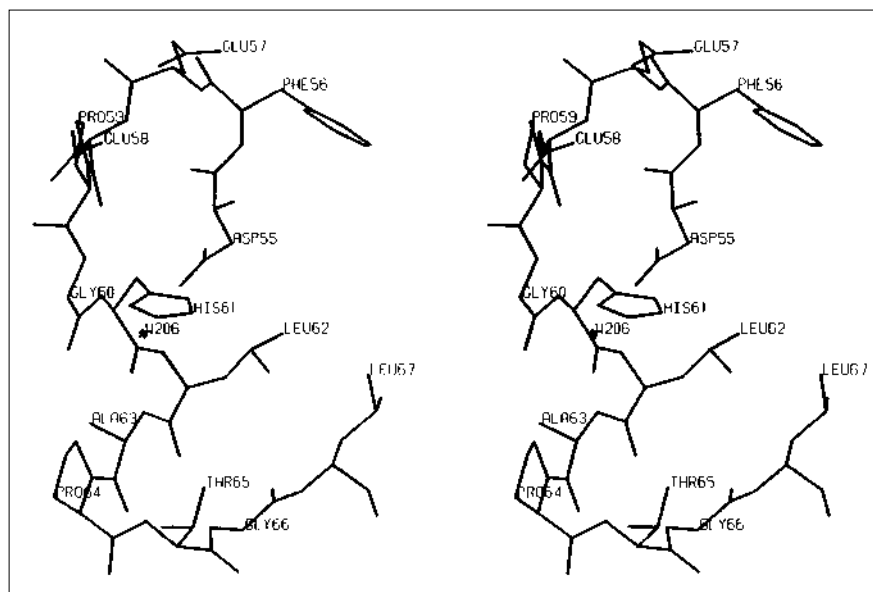
Some allergens have been suggested to have protease [41,42] or nuclease activity [43], while others contain motifs consistent with calcium binding [44]. There is considerable speculation that functional aspects may contribute to the allergenic potential of proteins, perhaps due to the involvement of allergen–ligand interactions (i.e. enzyme–substrate interactions) in sensitization and activation. Two of the three major regions of IgE-reactive epitopes, the N- and C-terminal helices, directly overlap with the binding sites for proline-rich ligands and actin, respectively. Steric considerations seem to preclude the simultaneous binding of these ligands and antibodies, indicating that the functional properties of this particular allergen are not necessary for allergenicity, and that native ligand-free profilin is the species responsible for sensitization and subsequent stimulation of the allergic response. This is consistent with the observations that during pollen hydration and germination the profilin–actin complex dissociates and that profilin, in contrast to actin, is easily extracted from pollen by aqueous solution, a situation which mimics contact with mucosal surfaces [45].

The nature of the epitopes which have been identified provide an explanation for the clinical observation that 20% of all pollen allergic patients are cross-sensitized towards pollen profilins from distantly related trees, grasses and weeds [1–3]. The N- and C-terminal α -helical segments display a very high degree of sequence homology throughout the plant profilins (AAF and SCA, unpublished data). Sensitization against these particular regions readily affords cross-sensitivity against homologous proteins from very distant plant species due to the conserved structure and sequence of these epitopes. This may also provide the structural basis for cross-reactivity of allergic individuals to a wide variety of foods and fruits [46,47]. This proposed mechanism of cross-reactivity is consistent with the extensive cross-reactivity towards diverse plant profilins exhibited by rabbit polyclonal sera raised against the C-terminal 25 amino acids of BPP [40].

The N-terminal helical region and the two-stranded segment (~residues 30–50), which show modest homology between the human and birch pollen profilins, are the

Figure 10

Stereoview of residues 56–67 in birch pollen profilin. The segment includes residues 60–65 which form a short α helix in the *Acanthamoeba* and mammalian profilins. Although these residues do not adopt a well defined periodic secondary structure they are represented by strong electron density.



likely cause of the extensive, albeit weaker, cross-reactivity exhibited by the serum IgE of individuals allergic to plant profilins towards human profilin [3]. This cross-reactivity suggests that at least some of the allergic symptoms caused by initial sensitization to plant profilins may be exacerbated or prolonged by endogenous human profilin. Despite the relatively short lifetime of serum IgE, individuals allergic to pollen profilin maintain a high and constant titer of profilin-reactive IgEs throughout the year, even outside the pollen season [3]. This is likely to be a consequence of the profilin sequence homology which allows persistent low levels of endogenous profilin to act as a booster. Consistent with this is the observation that the titer of IgEs specific for other allergens, which do not have a human homolog, show distinct seasonal variation.

A very common therapy for allergy is immunotherapy, in which the patient is directly challenged with subcutaneous injections of the offending allergen in an effort to switch from the production of membrane-bound IgE antibodies to soluble IgG antibodies. Increased IgG titer will block the binding of allergens to cell-surface bound receptors and lead to a reduced allergic response. The efficacy of the therapy differs among individuals and may have only temporary effects. The results presented here provide the detailed chemical and physical description of IgE-reactive epitopes required to design conformationally restricted, tight-binding monovalent allergen fragments (haptens) with the appropriate placement of functional groups for optimal antibody binding. Monovalent IgE-binding haptens which do not cross-link effector cell-bound IgE could be used for local blocking therapy as

well as for active immunization [48]. Due to the lack of anaphylactic activity, high doses of these monovalent IgE-binding haptens could be utilized for the induction of blocking IgG antibodies that interfere with the allergen–IgE interaction. This hapten therapy has the potential to be tailored to the specific allergic spectrum of individual patients and represents a novel alternative to traditional approaches.

Biological implications

Type I allergy afflicts 20% of the individuals of industrialized populations and thus represents a major health care issue. An allergic response is initiated when polyvalent allergen molecules bind multiple cell surface-bound IgE antibodies, each recognizing a different epitope, and cause the aggregation of IgE receptors. This aggregation initiates a number of protein phosphorylation events, which activate multiple signal transduction pathways resulting in the synthesis and release of mediators of the allergic response, including histamine, growth factors and inflammatory agents. This work provides the first high resolution structural information about wide spread allergens and allows a number of issues regarding the biology of allergy to be addressed.

We have solved the structure of the wide spread allergen, birch pollen profilin, and have directly identified the prominent IgE-reactive epitopes and mapped these three regions onto the three-dimensional structure. The spatial distribution of the major epitopes should allow for the productive interaction with IgE antibodies, of different epitope specificities, required for efficient signal

transduction and initiation of the allergic response. The major epitopes correspond to those regions of the profilin molecule which display extensive structural and sequence homology with other profilins thus providing the molecular basis for allergen cross-sensitivity. In light of steric considerations, the involvement of these epitopic regions in the binding of physiologically relevant profilin ligands indicates that the unliganded native profilin is the species responsible for eliciting the allergic response. The detailed chemical and physical description of the major reactive epitopes provide a data base for the design of tight binding monovalent ligands which can prevent receptor aggregation and thereby reduce the allergic response. This novel therapy for allergy can be customized to address the specific allergic responses of individual patients.

A detailed structural comparison of the different profilin homologs shows that they are all expected to form roughly similar complexes with G actin. However, the placement of the N-terminal helix in birch pollen profilin suggests that mechanistic differences in the binding of proline-rich ligands may exist among the profilins.

Materials and methods

Expression and purification

BPP was expressed in *Escherichia coli* using a T7 expression system [49]. Appropriate restriction sites were introduced by PCR and the coding sequence of BPP ligated into the *NdeI*–*EcoRI* sites of pMW172. This construct was used to transform the *E. coli* strain BL21(DE3). Single colonies were used to inoculate large scale cultures, which were grown overnight at 30–32°C, without the addition of isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation, and lysed by sonication in 10 mM Tris, 40 mM KCl, 1 mM dithiothreitol, pH=8.0 (TK8), containing 8 M urea. The lysate was clarified by centrifugation and dialyzed against TK8 to remove the urea and promote folding. The protein was loaded onto a poly-L-proline affinity column, equilibrated in TK8 and the column was washed in TK8 containing 2.0 M urea; the profilin was stepped off with 8 M urea in TK8. After dialysis against TK8, the profilin was concentrated to 5–10 mg ml⁻¹ by Amicon ultrafiltration. Typical yields were in the range of 20–50 mg l⁻¹ and the material was greater than 98% pure as judged by Coomassie stained SDS-PAGE; cDNA sequencing and mass spectrometry confirmed that the recombinant BPP was full length.

Crystals and derivatives

Recombinant BPP crystallized from 2.0 M ammonium sulfate, 100 mM MES pH=6.0 at room temperature, yielded needles several millimeters long but only 10 μ m in diameter. Despite the very small volume of these crystals we could observe diffraction to 5 Å on a rotating-anode generator. The diffraction from these crystals was consistent with a hexagonal space group, $a=36.7$, $c=128$ Å. The observed systematic absences were consistent with the space group $P6_3$, but due to the limited resolution this should be regarded as tentative. A series of related conditions was screened and the addition of 5% t-BuOH at 4°C yielded chunky crystals which diffracted to at least 2.0 Å. Surprisingly, these crystals belong to the tetragonal system, space group $P4_12_12$ (the enantiomorph was established during structure determination), with unit cell dimensions $a=59.90$ Å, $c=85.37$ Å. Each crystallographic asymmetric unit contains one molecule of BPP. One heavy-atom derivative was prepared by soaking native tetragonal crystals in a 1 mM solution of the ethyl mercury phosphate (EMP) for 48 h at pH=6.0. Native and derivative data sets were collected on a Siemens X-1000 multiwire

area detector, using a Rigaku RU-200 rotating anode X-ray source operating in fine focus at 55 kV and 85 mA and were reduced using XDS [50].

Structure solution

The structure of BPP was determined using a combination of single isomorphous replacement (SIR) and molecular replacement techniques. The refined structure of *Acanthamoeba* profilin I [14] was used for rotation and translation function searches, which were performed with the program X-PLOR [51], using diffraction data in the resolution range 15–4 Å. The 3000 largest Patterson vectors between 40.0–5.0 Å of the model map were chosen for the rotation search. The rotation function solution was relatively featureless, with no outstanding solution. The highest peaks in the rotation function were subjected to Patterson correlation refinement. The two highest peaks from Patterson correlation refinement were 0.079 and 0.068. However, regardless of resolution range, the translation function map was very noisy with no outstanding solution. Several potential solutions were selected to provide initial phase sets which were used to calculate difference Fourier maps with derivative and native amplitudes. Only the phases from the correct molecular replacement solution exhibited a large peak at the single mercury-binding site, independently determined by the difference Patterson method. This peak was located close to the sidechain of Cys117, one of the two cysteine residues in BPP, and this served to fix the origin between the two phase sets. Heavy-atom refinement, SIR phase calculation, phase combination with the model phases and solvent flattening were performed with PHASES [52]. The summary of the SIR phasing is provided in Table 1. The resulting 3.5 Å resolution electron-density map clearly showed the central six-stranded antiparallel β sheet, three helices and some loops. It was immediately apparent that although *Acanthamoeba* profilin I and BPP structures have the same basic fold, large portions of the structures do not match well. This explained the failure of attempts to cleanly solve the structure of BPP by molecular replacement alone. A new partial polyaniline model was built *de novo* using the programs XCHAIN and TOM, a derivative of FRODO [53]. Phase combination subsequently allowed the entire molecule to be traced.

The BPP model was initially refined using the simulated annealing protocol implemented in X-PLOR [51]. Combined and difference ($2|F_o|-|F_c|$), ($|F_o|-|F_c|$) electron-density maps using the refined atomic coordinates were of high quality and readily allowed for improvement of the model. Manual corrections of the model were performed during the refinement and the data gradually extended to 2.4 Å resolution. Solvent molecules were inserted at stereochemically reasonable positions where the difference electron density exceeded 4σ . The last stages of refinement utilized least squares positional and temperature-factor refinement as implemented in PROLSQ [54], using data between 8.0 and 2.4 Å resolution. The current refined BPP model includes 930 non-hydrogen protein atoms, and 48 solvent molecules. This represents 123 of the 133 amino acids, with the N-terminal residue and a nine residue solvent exposed loop not being visible in the electron density. The results of the BPP refinement are summarized in Table 2.

Mapping of IgE-binding sites

The cDNA coding for BPP was randomly digested with DNase I and fragments shorter than 300 bp isolated by preparative agarose gel electrophoresis. Fragment ends were repaired with T4 polymerase and tailed with 5'-phosphorylated *EcoRI* linkers. After digestion with *EcoRI*, the fragments were purified with a nick column (Pharmacia, Uppsala, Sweden), ligated into dephosphorylated λ gt11 arms and phage DNA was packaged *in vitro* (Amersham, Buckinghamshire, UK).

Immunoscreening and epitope analysis was performed using the following procedure. 50 000 phage of the profilin epitope library were used to infect *E. coli* Y1090 at a density of 1000 phage per plate (140 mm diameter). Synthesis of recombinant protein was induced by overlaying the plates with nitrocellulose filters soaked in 10 mM IPTG. 48 profilin epitope clones were isolated using serum IgE from a profilin allergic

patient and ^{125}I -labeled antihuman IgE antibodies (Pharmacia, Uppsala, Sweden). The epitope clones were characterized by hybridization with three synthetic oligonucleotides spanning the profilin cDNA: oligo A, 5'-CCAATGTTTCATCCACGTACGTTTGCC-3' (bp 8–33); oligo B, 5'-CATGTATTTATGCCCCCAAGGTG-3' (bp 202–225); oligo C, 5'-CTACAGGCCCTGGTCAATAAGGTAATCC-3' (bp 375–402). All three of these oligonucleotides hybridized to the epitope library, indicating that the library spans the entire length of the BPP gene. The cDNAs coding for the epitopes were amplified using the polymerase chain reaction (PCR). Two primers, complimentary to the $\lambda\text{gt}11$ sequences flanking the *EcoRI* site, were used for the PCR reaction. The primers contained *Bam*HI sites to facilitate subcloning: oligo λ forward, 5'-CGGGATCC-CGGTTTCCATATGGGGATTGGTGGC-3'; oligo λ reverse, 5'-CGC-GGATCCCGTTGACACACAGACCACTGGTAATG-3'. PCR amplified fragments were digested with *Bam*HI, gel purified, subcloned into pUC18 and both strands were sequenced using the above PCR primers, ^{35}S -dCTP and a T7 sequencing kit (Pharmacia, Uppsala, Sweden). Forty-two independent clones were sequenced and the shortest sequences are presented for clarity in Figure 8. The method utilized in this study will exclusively identify continuous epitopes, however, these sequences bind with sufficient affinity to cross-react with IgE antibodies directed against the relevant conformational epitopes present in the native profilin allergen.

Accession numbers

The final BPP atomic coordinates have been deposited with the Brookhaven Protein Data Bank (entry code 1CQA).

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