online commentary

Mutational epitope analysis of Pru av 1 and Api g 1, the major allergens of cherry (*Prunus avium*) and celery (*Apium graveolens*): correlating IgE reactivity with three-dimensional structure

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Birch pollinosis is often accompanied by adverse reactions to food due to pollen-allergen specific IgE cross-reacting with homologous food allergens. The tertiary structure of Pru av 1, the major cherry (*Prunus avium*) allergen, for example, is nearly identical with Bet v 1, the major birch (*Betula verrucosa*) pollen allergen. In order to define cross-reactive IgE epitopes, we generated and analysed mutants of Pru av 1 and Api g 1.0101, the major celery (*Apium graveolens*) allergen, by immunoblotting, EAST (enzyme allergosorbent test), CD and NMR spectroscopy. The mutation of Glu^{45} to Trp^{45} in the P-loop region, a known IgE epitope of Bet v 1, significantly reduced IgE binding to Pru av 1 in a subgroup of cherry-allergic patients. The backbone conformation of Pru av 1 wild-type is conserved in the three-dimensional structure of Pru av 1 Trp^{45}, demonstrating that the side chain of Glu^{45} is involved in a cross-reactive IgE epitope. Accordingly, for a subgroup of celery-allergic patients, IgE binding to the homologous celery allergen Api g 1.0101 was enhanced by the mutation of Lys^{44} to Glu. The almost complete loss of IgE reactivity to the Pru av 1 Pro^{112} mutant is due to disruption of its tertiary structure. Neither the mutation Ala^{112} nor deletion of the C-terminal residues 155–159 influenced IgE binding to Pru av 1. In conclusion, the structure of the P-loop partially explains the cross-reactivity pattern, and modulation of IgE-binding by site-directed mutagenesis is a promising approach to develop hypo-allergenic variants for patient-tailored specific immunotherapy.

Key words: allergen structure, allergy, cross-reactivity, hypo-allergenic mutants, IgE-epitope analysis.

INTRODUCTION

Birch pollinosis belongs to the group of prevailing allergic diseases in Northern and Central Europe. Approx. 50–93 % of birch pollen-allergic patients suffer from accompanying adverse reactions to fruits and vegetables [1]. The symptoms of these type I allergies are mediated by cross-linking of receptor-bound allergen-specific IgE antibodies on the surface of mast cells or basophils by an otherwise harmless antigen, the so-called allergen. Pollen-related food allergies typically result from primary sensitization against pollen allergens, and subsequent cross-reaction of IgE antibodies raised against these pollen allergens with similar conformational IgE epitopes presented by homologous food proteins [2].

Previously, we showed that the tertiary structure of Pru av 1 (formerly Pru a 1) [3] from cherry (*Prunus avium*) in solution, determined by heteronuclear multidimensional NMR spectroscopy, is virtually identical with that of the major birch (*Betula verrucosa*) allergen Bet v 1 [4,5]. The main feature of the 3D (three-dimensional) structure is a folded seven-stranded antiparallel β-sheet and two short α-helices that wrap around a long C-terminal α-helix to form a basket-like structure with a large hydrophobic cavity. The second and third β-strand are connected by a P-loop motif, a common structural element in many nucleotide-binding proteins [6]. NMR spectroscopy, together with molecular modelling, fluorescence spectroscopy, X-ray crystallography and MS, provided strong evidence that the cavity of both Pru av 1 and Bet v 1 interacts with steroids and other lipids [7–9], but their exact physiological function remains unclear. The P-loop is one of three highly conserved regions on the surface of the Bet v 1 molecule proposed as IgE epitopes leading to cross-reactivity with pollen allergens of other trees of the Fagales order [5]. In the crystal structure of Bet v 1 in complex with a Fab fragment of the monoclonal murine IgG1 BV16, which was raised against Bet v 1 and partially inhibits binding of human IgE, the P-loop region constitutes the contact surface, with the side chain of Glu^{45} located in a positively charged binding pocket forming two hydrogen bonds with the CDR3 (complementary determining region 3) of the heavy-chain variable domain [10]. These findings suggest the P-loop region around Glu^{45} as one of the IgE epitopes of Bet v 1, and the introduction of four point mutations, including a mutation from Glu^{45} to Ser, indeed reduced the IgE-binding capacity of Bet v 1 [11].

IgE epitope differences between the cross-reactive food allergens of the Bet v 1 family are indicated by a weak inhibition of IgE binding to Pru av 1 after pre-incubation of patient sera with the major celery (*Apium graveolens*) allergen Api g 1.0101 [12]. Interestingly, the P-loop of the isoform Api g 1.0101 differs

Abbreviations used: 1D/2D/3D, one-/two-/three-dimensional; EAST, enzyme allergosorbent test; HMQC, heteronuclear multiple quantum correlation spectroscopy; HSQC, heteronuclear single-quantum coherence; mAb, monoclonal antibody; OAS, oral allergy syndrome; RMSD, root-mean-square deviation; SIT, specific immunotherapy.

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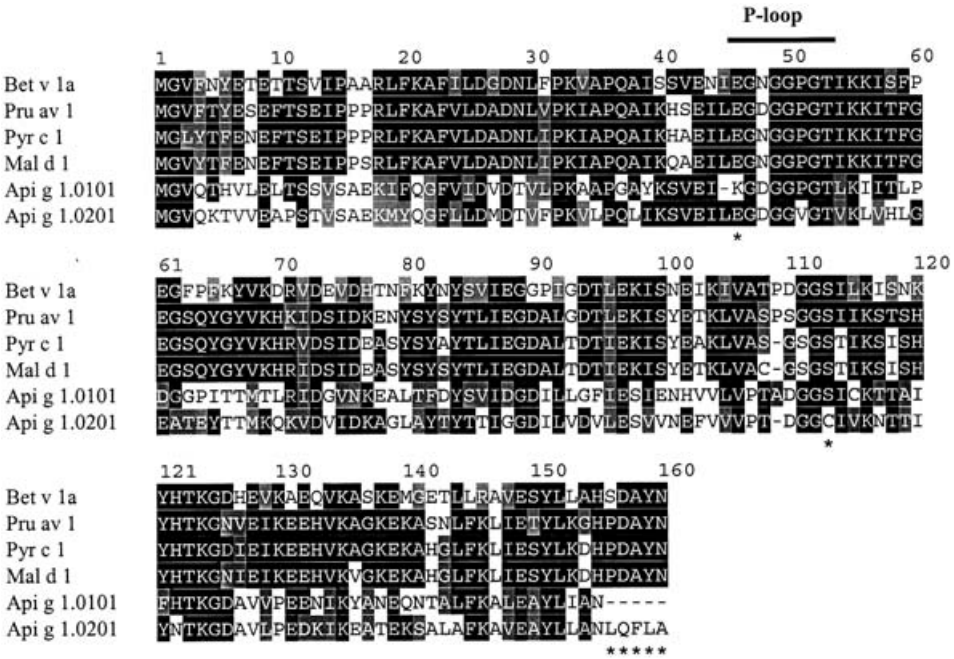

Figure 1 Structure-based sequence alignment of Bet v 1 isoform a (X15877), Pru av 1 (U66076), Pyr c 1 (AF057030) from pear (*Pyrus communis*), Mal d 1 (X83672) from apple (*Malus domestica*), Api g 1.0101 (P49372) and Api g 1.0201 (P92918) from celery (*Apium graveolens*)

The output of CLUSTALW 1.82 was edited manually and formatted with BOXSHADE 3.21. Black, consensus by identity; grey, consensus by similarity; mutation sites are indicated by an asterisk.

markedly to the isoform 1.0201 [13,14] and the other Bet v 1-related food allergens (Figure 1). This prompted us to postulate the P-loop around Glu[45] as one of at least two conformational cross-reactive IgE epitopes of Pru av 1 and hence a promising candidate for site-directed mutagenesis to alter the IgE-binding properties of Pru av 1 [7]. Based on mutational studies of the weakly IgE-binding isoform Bet v 1d [15], Mal d 1 [16] from apple (*Malus domestica*) and Api g 1.0101 [12], a region on the adjacent first and seventh β-strands around Thr[10] and Ser[112] respectively, was also proposed as a cross-reactive IgE epitope of Pru av 1 [14].

Since the identification of key residues for the IgE reactivity of the Bet v 1 family provides a more detailed understanding of the birch-fruit syndrome at the molecular level and may allow the development of hypo-allergenic variants for novel approaches to allergen-specific immunotherapy, we decided to verify our hypothesis that the P-loop around Glu[45] constitutes a cross-reactive epitope by producing and analysing the mutants Pru av 1 Trp[45], whose bulky hydrophobic side chain at position 45 is expected to negatively affect the affinity of IgE binding to the P-loop, and Api g 1.0101 Lys[44] mutated to Glu, whose negatively charged side chain at position 44 is expected to enhance IgE cross-reactivity within the Bet v 1 allergen family (Figure 1). To further assess the role of Ser[112], we included the mutants Pru av 1 Ala[112] and Pro[112] in this study, as well as a mutant of Pru av 1 with its five C-terminal residues 155–159 deleted, because, in addition to the differences in the P-loop, Api g 1.0101 also lacks the five C-terminal residues of Api g 1.0201 (Figure 1). Since a particular mutation can affect IgE binding by complete disruption of the tertiary structure, local modification of the tertiary structure or just alteration of the biophysical properties of the mutated side chain, the IgE binding features and the 3D structure of these mutants were characterized.

EXPERIMENTAL

Patients' sera and mAbs (monoclonal antibodies)

All sera were from the sera collection of the Paul-Ehrlich-Institut (Langen, Germany) or provided by Dr H. Aulepp (Borkum Riff Hospital, Borkum, Germany). Fifteen sera were taken from patients allergic to birch pollen who reported OAS (oral allergy syndrome) after ingestion of fresh cherries. A positive case history of these allergies was confirmed by a positive skin prick test to birch pollen and cherry extract and/or by demonstration of specific serum IgE with EAST (enzyme allergosorbent test) classes in the range 2–4. Most patients also reported oral symptoms upon ingestion of other fruits and vegetables. Celery-allergic patients ($n = 15$) were selected on the basis of a positive case history. Nine of these patients reported mild OAS, the six remaining patients reporting systemic reactions. All these celery-allergic patients were sensitized against birch pollen. The mAb BV16 raised against Bet v 1 was provided by Dr M. Spangfort (ALK-Abelló, Hørsholm, Denmark), and additional mAbs were raised against Pru av 1 and Bet v 1 by conventionally hybridoma techniques [17].

Cloning and site-directed mutagenesis of Pru av 1 and Api g 1.0101

Mutagenesis of Pru av 1 wt (wild-type) (accession number O24248) to Pru av 1 Trp[45], Ala[112], Pro[112], and Δ155–159 was performed by PCR using Pru av 1-pET11a or Pru av 1-pBluescript KS as templates. Mutagenesis of Glu[45] and Ser[112] was performed in a two-step PCR [18]. The initial PCRs were primed by 3′-Pruav1E45W(−) and 5′-M13R2(+) and by 5′-Pruav1S112A(+) and 3′-Pruav1(−) respectively (Table 1). The purified PCR products and the oligonucleotides 5′-Pruav1(+) for mutation to Pru av 1 Ala[112] and 3′-M13F2(−) for Pru av 1 Trp[45] were

Table 1 Oligonucleotide primers for cloning and site-directed mutagenesis of Pru av 1 and Api g 1.0101

Mutated regions are underlined.

Oligonucleotide primer	Nucleotide sequence
3′-Pruav1E45W(−)	5′-C TCT GAA ATC CTT TGG GGA GAT GGC GGC C-3′
5′-Pruav1S112A(+)	5′-GC GGA GGA GCT ATC ATC AAG AGC ACC-3′
3′-Pruav1Δ155-159(−)	5′- GGA GGA TCT AGA GCT CAG CTT AGT GGC CCT TAA GGT AGG-3′
5′-Pruav1(+)	5′-GGA GGA CTC GAG CAT ATG GGT GTC TTC ACC TAT GAG AGC G-3′
3′-Pruav1(−)	5′-GGA GGA TCT AGA GCT CAG CTT AGT TGT AGG CAT CGG GGT GGC-3′
Apig1.01(+)	5′-ATG GGA GTG CAG ACA CAT GTG TTG GAG CTC ACC-3′
Apig1.01(−)	5′-TTA ATT AGC GAT GAG ATA GGC CTC GAG AGC CTT-3′
5′-Apig1.01K44E(+)	5′-GCT TAC AAG AGT GTA GAA ATC GAG GGA GAT GGT GGA CC-3′
3′-Apig1.01K44E(−)	5′-CC AGG TCC ACC ATC TCC CTC GAT TTC GAC ACT CTT G-3′
5′-M13R2(+)	5-GGA AAC AGC TAT GAC CAT G-3′
M13F2(−)	5′-GTT TTC CCA GTC ACG AC-3′

taken as primers in a second PCR. Pru av 1 Δ155–159 was constructed by a single PCR using the oligonucleotides 3′-Pruav1Δ155–159(−) and 5′-Pruav1(+) (Table 1). The mutated Pru av 1 sequences were finally inserted in the T7 promoter driven expression vector pET-11a (Novagen, Madison, WI, U.S.A.) and transformed into BL21(DE3) cells for protein expression. Api g 1.0101 cDNA (accession number Z48967) was amplified by reverse-transcriptase PCR using the gene-specific terminal oligonucleotide primers Apig1.01(+) and Apig 1.01(−), cloned into the pCRII-TOPO vector (Invitrogen, Groningen, The Netherlands) and into pET-11a vector for protein expression. Mutagenesis of Api g 1.0101 was performed with the QuikChange™ site-directed-mutagenesis kit (Stratagene, Amsterdam, Netherlands) using the Api g 1.0101-pET11a construct as a template. The mutagenesis reaction was primed by the oligonucleotides 5′-Apig1.01K44E(+) and 3′-Apig1.01K44E(−). Briefly, amplified plasmids were treated with *Dpn*I, an endonuclease specific for methylated DNA, to digest the parenteral plasmid DNA template from bacteria. Mutated and unmethylated plasmids were transformed into XL1-Blue Supercompetent cells (provided with the kit), sequenced and subsequently transformed into BL21(DE3) cells for protein expression.

Expression and purification of recombinant allergens

The protein synthesis of Pru av 1 Trp^{45}, Ala^{112} and Δ155–159 was induced by adding isopropyl β-D-thiogalactoside to a final concentration of 1 mM at an D_{600} of 0.8–0.9. Cultures were incubated at 37 °C for 3 to 4 h or overnight at 24 °C for the celery proteins Api g 1.0101 wt and the Glu^{44} mutant. Cells were harvested by centrifugation, resuspended in 20 mM imidazole (pH 7.5) containing one tablet of protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and disrupted by repeated freezing and thawing. The extract was clarified by centrifugation for 30 min at 24966 ***g*** and 4 °C. The allergens were purified from the soluble fraction by chromatofocusing [15] (Mono P HR 5/20; Amersham Biosciences, Freiburg, Germany) and anion-exchange chromatography [19] (Mono Q 10/10, Q-Sepharose Fast Flow Resin; Amersham Pharmacia Biotech, Freiburg, Germany). Fractions containing pure allergens were pooled, dialysed against water and lyophilized.

SDS/PAGE and IgE immunoblotting

Recombinant allergens (2 μg) were separated by SDS/PAGE under non-reducing conditions according to Laemmli [20], using the Mini-Protean II cell (Bio-Rad, Munich, Germany) and anlaysed by Coomassie Brilliant Blue staining. For IgE-immunoblot analysis, purified allergens (0.5 μg per cm slot; diameter = 1.5 mm) were transferred on to nitrocellulose membranes (0.45 μm pore size; Schleicher and Schuell, Dassel, Germany) by tank blotting using the Bio-Rad equipment. For the detection of specific IgE antibodies, patients' sera were diluted 1:10 in a total volume of 600 μl. Immunodetection was performed with an alkaline phosphatase-labelled anti-human IgE mAb (1:750; Becton Dickinson, Heidelberg, Germany) and the AP Conjugate Substrate Kit from Bio-Rad.

EAST and EAST inhibition

Binding of allergen-specific IgE was semi-quantified (IgE ⩽ 0.35 kunits/l = class 0; 0.35 < IgE ⩽ 0.7 kunits/l = class 1; 0.7 < IgE ⩽ 3.5 kunits/l = class 2; 3.5 < IgE ⩽ 17.5 kunits/l = class 3; IgE > 17.5 kunits/l = class 4) by the EAST according to the manufacturer's instructions (Allergopharma Spez. IgE ELISA; Allergopharma, Reinbek, Germany). Recombinant Pru av 1 wt, Trp^{45}, Ala^{112}, Pro^{112} and Δ155–159 were coupled to CNBr-activated filter paper discs (Hycor, Kassel, Germany) at a protein concentration of 1.5 μg/ml [21]. Dose-related EAST inhibition experiments with 0.5 μg of recombinant Pru av 1 immobilized on paper discs were performed as described previously [22]. Pooled serum samples were diluted 1:2 and subsequently pre-incubated with dilutions of recombinant Pru av 1 wt, Trp^{45}, Ala^{112} and Δ155–159 ranging from 0.3125 to 20 μg/ml. Inhibition values were calculated by measurement of *A*s as follows [where $A(B_0)$, serum; $A(P)$, serum with inhibitor; $A(NSB)$: non-specific binding]:

$$\text{Inhibition}\ (\%) = [A(B_0) - A(P)]/[A(B_0) - A(NSB)] \times 100\%$$

Measurement of IgE reactivity by ELISA

Maxisorb plates (96 wells) were coated with 50 ng of protein/100 μl of PBS per well (overnight at 4 °C), blocked with 200 μl of PBS/1 % BSA for 1 h and incubated with 100 μl of serum (diluted 1:2) (overnight at 23 °C). Determination of specific IgE was performed with a rabbit anti-human IgE antiserum (DAKO, Hamburg, Germany) (100 μl, 1:4000, 90 min at 23 °C) and a biotin-conjugated goat anti-rabbit IgG (DAKO) as a secondary antibody (100 μl, 1:6000, 1 h at 23 °C). For visualization, streptavidin–horseradish peroxidase (Calbiochem, Darmstadt, Germany) (100 μl, 1:10000, 30 min at 23 °C) was applied using 3,3′,5,5′-tetramethylbenzidine (100 μl, 20–30 min at 23 °C in the

dark) as a substrate. The reaction was stopped with 50 μl of 3 M H_2SO_4, and D was measured at 450 nm.

Screening of allergen-specific mAbs by ELISA

Maxisorb plates (96 wells) were coated with 50 ng of protein/50 μl of TBS per well (1 h at 23 °C), blocked with 200 μl of TBS/0.05 % Tween 20/1 % BSA for 1 h and incubated with 50 μl of antibody (final dilution: 200 ng/well, 1 h at 23 °C). Visualization was performed with a conjugate of goat anti-mouse IgG linked to horseradish peroxidase (Sigma–Aldrich, München, Germany) (50 μl, 1:7000, 1 h at 23 °C). Further procedure was performed as described above.

CD spectroscopy

CD spectra from 185 nm to 250 nm were recorded on a Jasco J-810 Spectropolarimeter using a step width of 0.2 nm, a band width of 1 nm and a scanning speed of 50 nm/min in cuvettes with a light path of 1 mm (Hellma, Müllheim, Germany) at a temperature of 25 °C. To increase the signal-to-noise ratio, 8 to 10 scans were recorded. The protein concentrations were between 7.4 μM and 18.8 μM in 10 mM potassium phosphate (pH 7.0). The mean residue ellipticity ($[\theta]_{\mathrm{m.r.w.}}$) was calculated from the measured ellipticity $[\theta]$ according to

$$[\theta]_{\mathrm{m.r.w.}} = [\theta]/cdN$$

where c denotes the protein concentration, d the light path and N the number of residues [23].

NMR spectroscopy

NMR spectra were recorded on Bruker Avance400, DRX600, and DMX750 NMR spectrometers with pulsed-field gradient capabilities at a temperature of 25 °C in 10–50 mM potassium phosphate (pH 7.0) in $H_2O/^2H_2O$ (9:1). For 1D (one-dimensional) ^{1}H NMR spectra, 16384 real data points were acquired with a spectral width of 6410 Hz and 8389 Hz or 9615 Hz at 400 MHz and 600 MHz respectively, zero-filled to 16384 complex points, and apodized by multiplication with an exponential causing 2 Hz (full width at half maximum) line broadening. To increase the signal-to-noise ratio 1024 transients were collected with a recycle delay of 1.5 s, except for Pru av 1 Pro^{112} with 16384 transients. For the structure determination the following experiments were conducted on a sample of 0.7 mM uniformly ^{15}N-labelled Pru av 1 Trp^{45} and processed as described previously [7,24]: ^{15}N-HSQC (heteronuclear single-quantum coherence) [25], HNHA [26], ^{15}N-TOCSY-HSQC [27] (60 ms mixing time), ^{15}N-NOESY-HSQC [28] (120 ms mixing time), 3D ^{15}N-HMQC (heteronuclear multiple quantum correlation spectroscopy)-NOESY-HSQC [29,30] (150 ms mixing time), and ^{15}N-filtered 2D (two-dimensional) [^{1}H, ^{1}H] NOESY [31] (120 ms mixing time). The water resonance was suppressed with excitation sculpting [32], gradient coherence selection [33] and a binomial 3-9-19 WATERGATE sequence [34] with water flip-back [35] in the 1D ^{1}H, the ^{15}N-TOCSY-HSQC and the remaining NMR experiments respectively. Quadrature detection in the indirect dimensions was achieved by the States time-proportional phase incrementation method [36] or by the echo–antiecho method [37] if gradient coherence selection was employed. ^{1}H chemical shifts were referenced with respect to external DSS (2,2-dimethyl-2-silapentane-5-sulphonate) in ^2H_2O, and ^{15}N chemical shifts were referenced indirectly [38].

Sequence-specific resonance assignments and scalar coupling constants

Sequence-specific backbone amide resonance assignments by the standard procedure [39] based on the ^{15}N-TOCSY-HSQC, ^{15}N-NOESY-HSQC and 3D ^{15}N-HMQC-NOESY-HSQC spectra revealed that only the resonances of Trp^{45}, Gly^{46}, Asp^{47}, Gly^{48}, Gly^{51}, Thr^{52} and Ile^{53} of Pru av 1 Trp^{45} deviated significantly from those observed for Pru av 1 wt [7,24]. Aliphatic side-chain resonance assignments for these residues were obtained from the ^{15}N-TOCSY-HSQC and ^{15}N-NOESY-HSQC spectra, aromatic side-chain resonance assignments for Trp^{45} from the ^{15}N-NOESY-HSQC and ^{15}N-filtered 2D [^{1}H, ^{1}H] NOESY spectra, and $^3J_{\mathrm{HNH}\alpha}$ scalar coupling constants from the HNHA spectrum. ^{1}H, ^{13}C, and ^{15}N chemical shifts and scalar coupling constants of Pru av 1 Trp^{45} have been deposited with the BioMagResBank (accession number 5490).

Structure calculation

NOE cross-peaks and $^3J_{\mathrm{HNH}\alpha}$ scalar coupling constants were converted into distance and Φ backbone torsion angle restraints respectively, as described previously [7]. In addition to those 2174 NOE distance, 68 Φ backbone torsion angle, and 68 hydrogen bond distance restraints used for the structure calculation of Pru av 1 wt [7], which did not involve Glu^{45}, Gly^{46}, Asp^{47}, Gly^{48}, Gly^{51}, Thr^{52} or Ile^{53}, another 147 distance restraints could be derived from the 2D and 3D NOESY spectra in an iterative procedure and another 3 Φ backbone torsion angle restraints from the $^3J_{\mathrm{HNH}\alpha}$ scalar coupling constants measured. These experimental restraints served as an input for the calculation of 60 structures, as described previously [7]. The Gaussian conformational database potential [40] with a cut-off of 10.0 S.D.s [41] was included in the target function in order to improve the stereochemical properties of the structures. The 24 structures showing the lowest energy values (excluding conformational database potential) were selected for further characterization using X-PLOR 3.851 [42] and PROCHECK 3.4 [43]. Together with the experimental restraints, the atomic coordinates of this set of 24 structures have been deposited with the Protein Data Bank (accession number 1H2O).

RESULTS

Expression and purification of recombinant Pru av 1, Api g 1 and mutant proteins

The amino-acid sequence of the P-loop of Bet v 1 is highly conserved in Bet v 1-related food allergens, such as Pru av 1, Pyr c 1 [44] from pear (*Pyrus communis*), Mal d 1 [45] from apple (*Malus domestica*) and Api g 1.0201 [13]. In contrast, Api g 1.0101 [12] lacks Leu^{44} and the negatively charged Glu^{45} is substituted by the positively charged Lys^{44} (Figure 1). Furthermore, the C-terminus of Api g 1.0101 is truncated by five amino acids. Hence, the amino acids Glu^{45}, Ser^{112} and 155–159 of Pru av 1 and Lys^{44} of Api g 1.0101 were selected for site-directed mutagenesis and recombinant proteins were expressed in *Escherichia coli*. SDS/PAGE analysis revealed that all recombinant cherry proteins were obtained with a high degree of purity (> 98 %; Figure 2), except for Pru av 1 Ala^{112} with a purity of approx. 88 % according to densitometric gel analysis (GelScan 5.0, BioScitec, Frankfurt, Germany). Pru av 1 Arg^{45} underwent proteolysis after expression and was therefore not investigated any further. Api g 1.0101 wt and Glu^{44} were also prepared with high purity, analysed by SDS/PAGE and Coomassie Blue staining (results not shown).

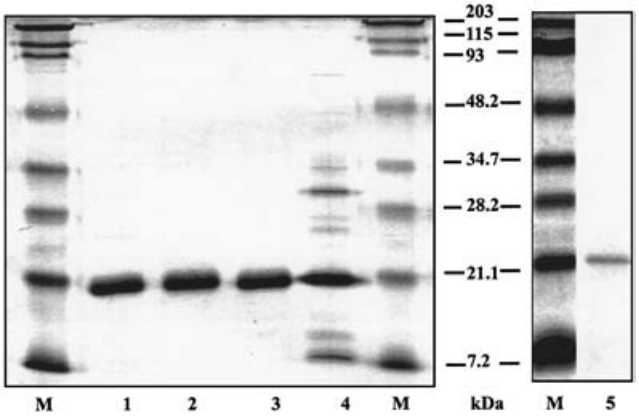

Figure 2 Analysis of purified recombinant Pru av 1 wt and its mutants

2 μg of protein was used per slot and subjected to SDS/PAGE (total acrylamide concentration, 15 %; non-reducing conditions) and Coomassie Blue staining. Lane 1, Pru av 1 wt; lane 2, Pru av 1 Δ155–159; lane 3, Pru av 1 Trp^{45}; lane 4, Pru av 1 Ala^{112}; lane 5, Pru av 1 Pro^{112}. M, molecular-mass markers (Broad Range, Bio-Rad).

Analysis of the IgE reactivity

The IgE reactivity of sera from 15 cherry allergic patients to Pru av 1 wt, Trp^{45}, Ala^{112}, Pro^{112} and Δ155–159 was determined by EAST (Figure 3A). All patients were sensitized to Pru av 1 with EAST classes of 1 ($n = 2$), 2 ($n = 8$) or 3 ($n = 5$). The IgE reactivity to Pru av 1 Pro^{112} was completely abolished for seven sera (EAST class 0) and strongly reduced for additional seven sera tested, and the IgE reactivity of one serum was unmodified. In contrast, the IgE reactivity to Pru av 1 Trp^{45} was reduced for nine patients, unaffected for 4 patients and enhanced for two patients. Neither substitution of alanine by serine in position 112 nor deletion of the C-terminal residues altered the IgE reactivity of Pru av 1. The reduced IgE-binding capacity to the modified P-loop region was confirmed by inhibition of IgE binding to Pru av 1 wt after pre-incubation of pooled patient sera with Pru av 1 Trp^{45}, Ala^{112} and Δ155–159. For these inhibition assays three patients' sera (Bo111, PEI82 and PEI97) with reduced IgE reactivity to the Trp^{45} mutant were selected to verify the antibody reactivity in a dose-dependent manner. Pru av 1 Ala^{112} and Δ155–159 showed nearly identical IgE reactivity compared with Pru av 1 wt; the inhibition curves are highly superimposable, with up to 85 % IgE inhibition at the highest inhibitor concentration tested (Figure 4). The slightly reduced IgE-binding reactivity observed for Pru av 1 Ala^{112} is most likely due to its lower purity of approx. 88 % (Figure 2). In contrast, with Pru av 1 Trp^{45} a maximum inhibition of 32 % was obtained. Hence, amino-acid substitution of Glu^{45} in the P-loop region reduced IgE binding by approx. 68 % for a subset of cherry allergic patients. The experiments were extended by comparing the IgE reactivity of the celery allergens Api g 1.0101 wt and Glu^{44}. Nine out of 15 celery allergic patients were sensitized to Api g 1.0101. For three out of these nine sera the IgE reactivity to Api g 1.0101 Glu^{44} was clearly enhanced in comparison with Api g 1.0101 wt, for three sera IgE binding was reduced and for an additional three sera it remained unaffected (Figure 3B).

Application of murine mAbs

To investigate the effects of site-directed mutation on the tertiary structure and to select mAbs recognizing the P-loop region of Bet v 1-related food allergens as a tool for inhibition of IgE-binding, a panel of mAbs was screened with the wild-type and mutant proteins. Previously, the mAb BV16 raised against Bet v 1 was shown to compete with IgE binding to Bet v 1 [10]. Since BV16 recognizes homologous pollen allergens, but surprisingly did not cross-react with the homologous food allergens Pru av 1 and Api g 1.0101 (results not shown) in spite of the highly conserved interface [7,10], a panel of mAbs was raised against Pru av 1 and Bet v 1. The screening of their reactivity focused on binding to the P-loop region. Four mAbs raised against Pru av 1 wt were tested with Pru av 1 wt and Trp^{45}. None of them displayed an altered reactivity upon substitution in the P-loop region. Furthermore, a panel of anti-Bet v 1 mAbs was tested with Pru av 1 wt, Trp^{45} and Pro^{112}, as well as Api g 1.0101 wt and Glu^{44}. Two out of eight anti-Bet v 1 mAbs cross-reacted with Pru av 1 wt, and with similar reactivity with Pru av 1 Trp^{45}. Two other anti-Bet v 1 mAbs showed strong binding to Api g 1.0101 Glu^{44}. In contrast, none of the anti-Bet v 1 mAbs recognized Api g 1.0101. As to the patient sera (Figure 3A) the IgE reactivity of all monoclonals to Pru av 1 Pro^{112} was almost completely abolished.

Secondary and tertiary structure analysis

The CD (Figure 5) and 1D ^{1}H NMR (Figure 6) spectra of Pru av 1 wt, Ala^{112}, Trp^{45} and Δ155–159 are virtually superimposable and consistent with their mixed α/β secondary structure, demonstrating that they are all natively folded and share a common secondary and overall tertiary structure. The mutation Pro^{112}, however, was detrimental to the native tertiary structure of Pru av 1, resulting in CD and 1D ^{1}H NMR spectra typical for a largely unstructured protein, although the protein was soluble and purified as a monomer (Figure 2). The 1D ^{1}H NMR spectrum of Api g 1.0101 (Figure 7) also indicates a natively folded protein and is consistent with a secondary and tertiary structure similar to Bet v 1 and Pru av 1.

The NMR spectra recorded on uniformly ^{15}N-labelled Pru av 1 Trp^{45}, used to assess the structural changes upon the mutation Trp^{45} in more detail, are superimposable with those of Pru av 1 wt for all but seven residues in the P-loop (Figure 8), and therefore the 3D structure of Pru av 1 Trp^{45} only had to be re-determined locally. Similar to Pru av 1 wt, Pru av 1 Trp^{45} shows a well-defined structure in solution (Figure 9), with average atomic RMSDs (root-mean-square deviations) from the average structure of 0.60 Å for the backbone and 0.91 Å for all heavy atoms (Table 2). The average backbone atomic RMSDs from the average structure of Pru av 1 wt of 0.73 Å for all residues and 0.70 Å for the P-loop only (Table 2) are similar to the average backbone atomic RMSDs from the average structure of Pru av 1 Trp^{45} itself, and the two families of structures are accordingly perceived as a single set of structures rather than as two distinct sets of structures in a backbone overlay even for the P-loop itself (Figure 9). The backbone conformation of Pru av 1 is therefore obviously not disturbed by the mutation Trp^{45}. This also holds for the side chains with average heavy atomic RMSDs from the average structure of Pru av 1 wt of 1.07 Å for all residues and 0.93 Å for the P-loop only (Table 2), even the side-chain position of Glu^{45} and Trp^{45} is similar (Figure 9).

DISCUSSION

The nearly complete loss of the IgE reactivity of Pru av 1 upon the mutation of Pro^{112} (Figure 3A) originates from disruption of the native tertiary structure (Figures 5 and 6), supporting the notion that the cross-reactive IgE-binding epitopes of Pru av 1 are predominantly conformational rather than sequential. Pru av 1 Ala^{112} exhibited an IgE-binding capacity similar to that of Pru

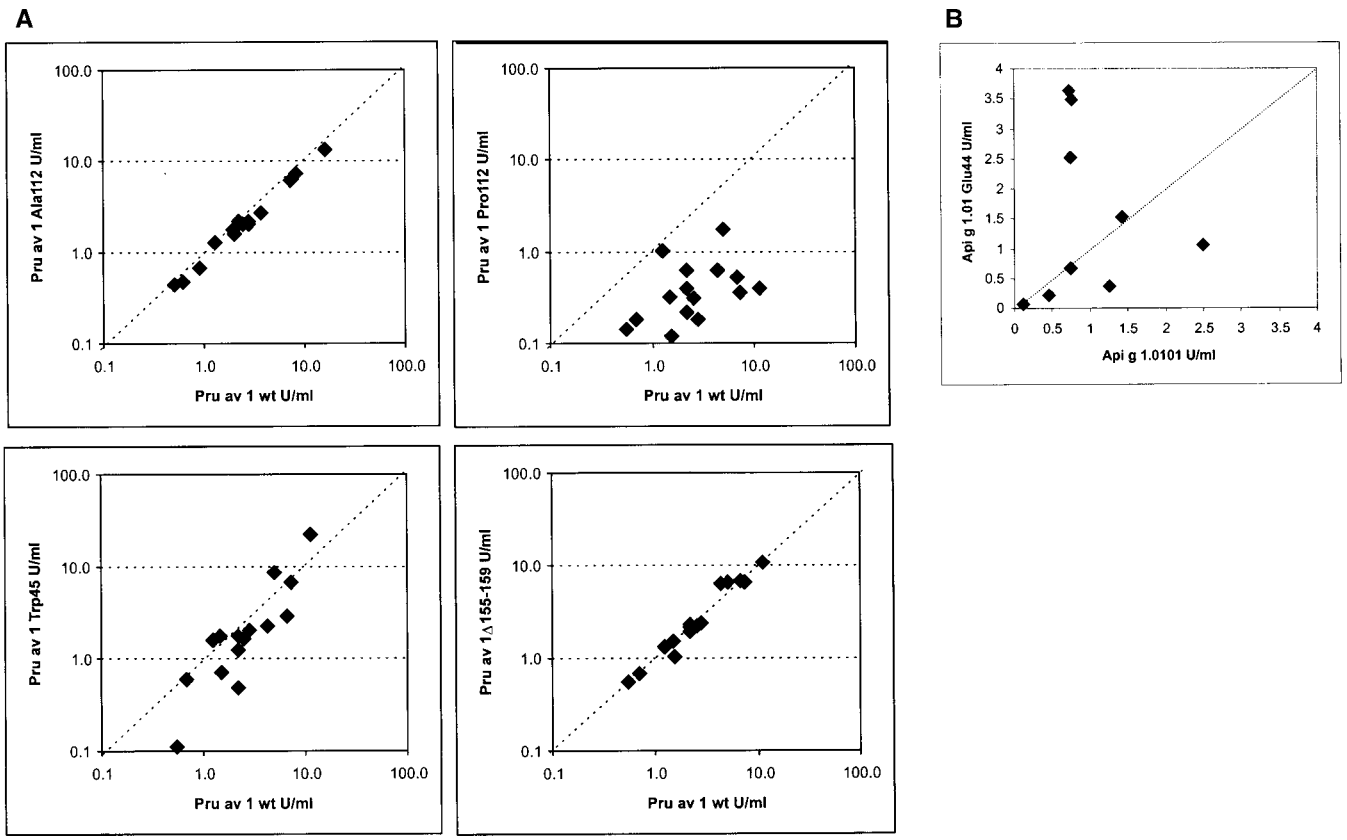

Figure 3 EAST comparison of the IgE-binding capacity of (A) Pru av 1 wt and its mutants and (B) Api g 1.0101 and its Glu^{44} mutant by IgE ELISA

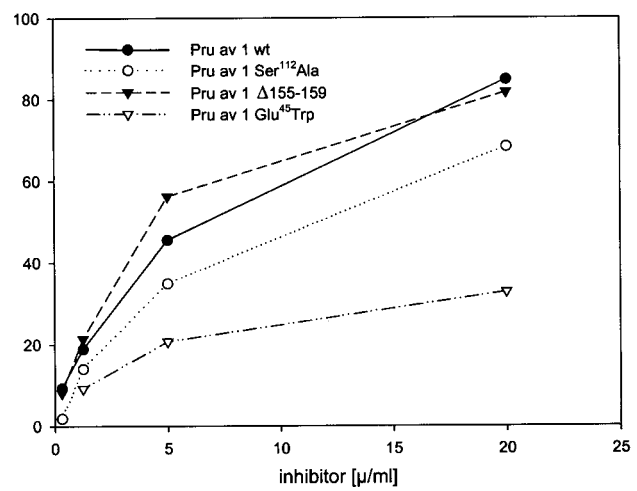

Figure 4 Inhibition of IgE binding to Pru av 1 wt on the solid phase by pre-incubation of a serum pool from cherry allergic patients with Pru av 1 wt (positive control) and its mutants

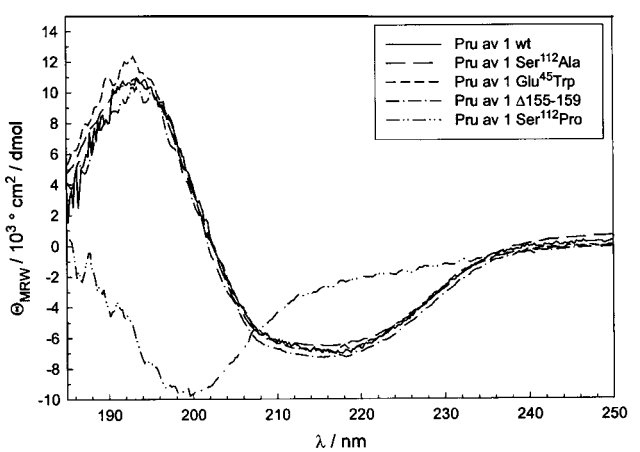

Figure 5 CD spectra of Pru av 1 wt and its mutants

The spectra are largely superimposable and consistent with their mixed α/β secondary structure, except for Pru av 1 Pro^{112} with a spectrum typical for a mostly unstructured protein with a pronounced minimum near 200 nm.

av 1 wt (Figures 3A and 4), demonstrating that the hydroxy group of Ser^{112} does not contribute to the IgE-binding epitopes of Pru av 1. It should be noted that although, in contrast with the carboxy group of Glu^{45}, the hydroxy group of Ser^{112} of both Bet v 1 and Pru av 1 does not protrude into the solvent, it is solvent accessible and therefore a potential interaction site, either directly with a protruding side chain of the IgE antibody or indirectly via any water molecules lining the antibody–antigen interface. Together with our previous observation that the mutation of Thr^{10} to Pro does not affect the IgE-binding capacity of Pru av 1 [14], this suggests that the region on the adjacent first and seventh β-strands around Thr^{10} and Ser^{112}, which was suggested as a potential IgE-binding epitope of Bet v 1 [15], Mal d 1 [16] and Api g 1.0101 [12], does not constitute a clinically relevant cross-reactive IgE-binding epitope of Pru av 1.

The conservation of the tertiary structure of Pru av 1 upon the mutation Glu$^{45}\rightarrow$Trp leaves the altered biophysical properties of

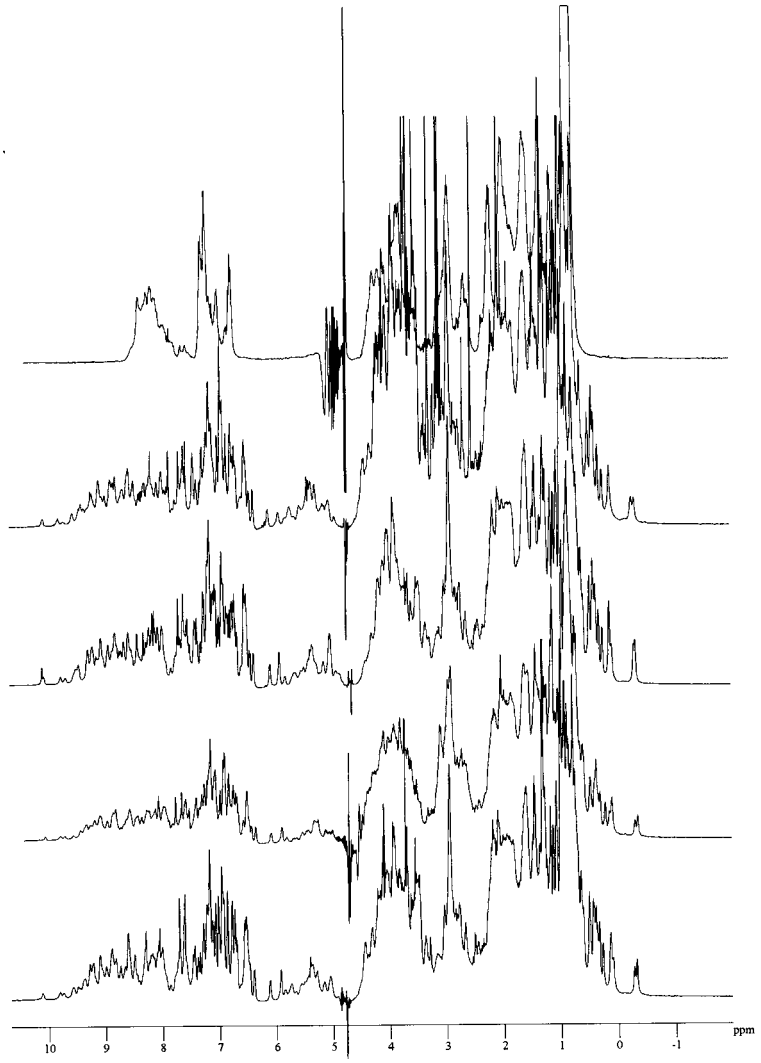

Figure 6 1D ^{1}H NMR spectra of (from bottom to top) Pru av 1 wt, Ala^{112}, Trp^{45}, Δ155–159 and Pro^{112}

The spectra are largely superimposable with excellent chemical shift dispersion due to their high content of β-strands, except for Pru av 1 Pro^{112} with a spectrum typical for a mostly unstructured protein with little chemical shift dispersion and no methyl resonances shifted to high field. The additional sharp resonance at 10.13 ppm of Pru av 1 Trp^{45} stems from Trp^{45} $H_{\varepsilon}1$ (see Figure 8).

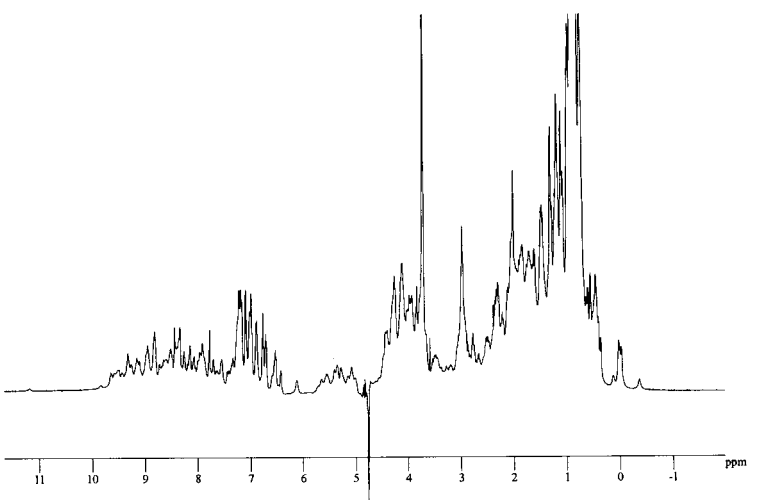

Figure 7 1D ^{1}H NMR spectrum of Api g 1.0101

The excellent chemical shift dispersion indicates a high content of β-strands, such as Bet v 1 and Pru av 1. The intensive sharp resonance at 3.71 ppm stems from a Tris impurity.

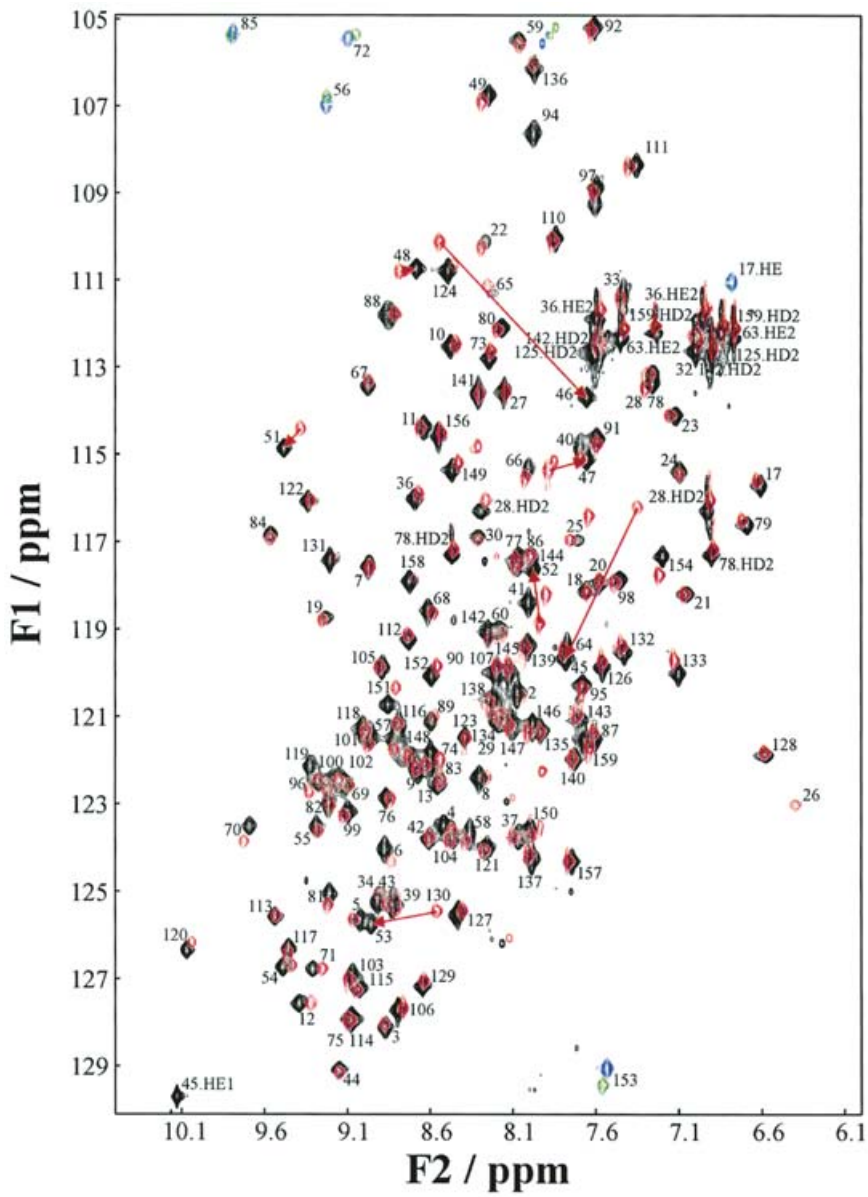

Figure 8 Overlay of the [^{1}H, ^{15}N] HSQC spectra of uniformly ^{15}N-labelled Pru av 1 wt (positive signals in red, negative signals in green) and Trp^{45} (positive signals in black, negative signals in blue)

Amide proton resonances are labelled according to their residue numbers. Negative resonances are aliased in the indirect ^{15}N dimension F1. Significant deviations are indicated by red arrows.

the mutated side chain as the only major structural difference between wt and mutant. The patient-specific modulation of the IgE-binding capacity to Pru av 1 Trp^{45} compared with Pru av 1 wt (Figures 3A and 4) therefore has to be attributed to the side chain of Trp^{45}, which provides strong evidence that Glu^{45} is indeed a key residue of one of the cross-reactive IgE-binding epitopes of Pru av 1. We had observed similar effects in a previous study [14] and the patients PEI82 and Bo111, whose sera did not any longer react with Pru av 1 Pro^{46} and ΔThr^{52}, were also among the patients with the highest decrease in IgE reactivity upon the mutation Trp^{45} in this study.

In contrast with Bet v 1, Pru av 1 and other homologous allergens of the Rosaceae family, the P-loop is not conserved in Api g 1.0101 (Figure 1) and Dau c 1 (accession number O04298), the homologous carrot allergen (results not shown). A homology model of Api g 1.0101 based on crystal structures of Bet v 1 (Protein Data Bank accession numbers 1QMR, 1FSK and 1BV1) created with SwissModel [46], followed by 100 steps of energy minimization in Sybyl 6.5 (Tripos Inc., St. Louis, MO, U.S.A.), predicts that Lys^{44} simply extends the second β-strand to bridge the little bulge formed by Leu^{44} and Glu^{45} that initiates the P-loop in Bet v 1 and Pru av 1, with the solvent-exposed side chain of Lys^{44} located halfway between the side chains of Leu^{44} and Glu^{45} in Bet v 1 and Pru av 1 (results not shown). This structural difference appears to be a major cause for the lack of IgE cross-reactivity between Pru av 1 and Api g 1.0101 and the lower overall IgE reactivity of Api g 1.0101 [7,12], and the mutation Glu^{44} accordingly had a marked effect on the IgE-binding capacity of Api g 1.0101 (Figure 3B). Interestingly, a lack of IgE cross-reactivity with Api g 1.0101 has been reported for the

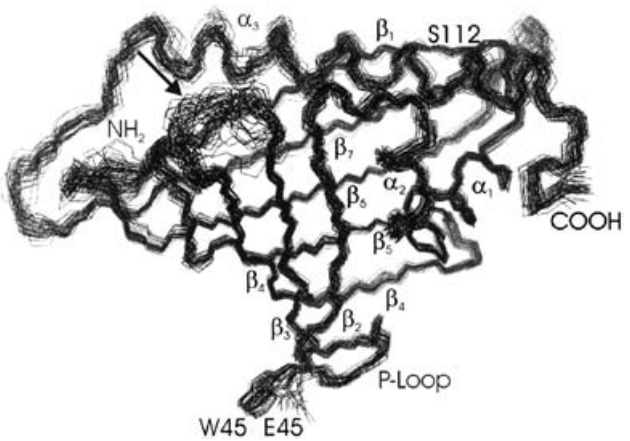

Figure 9 Backbone overlay of the 24 accepted structures of Pru av 1 Trp^{45} with the 22 accepted structures of Pru av 1 wt [7]

The NH_2-terminus on the left-hand side is hidden by the loop from Ile^{86} to Glu^{96}, the C-terminus can be seen on the right-hand side. The loop from Glu^{60} to Tyr^{64} indicated by an arrow shows increased flexibility [7]. The side chains of Trp^{45} and Glu^{45} are shown at the bottom, Ser^{112} is hidden by the C-terminal helix. The overlay was performed using Sybyl 6.5.

isoform Api g 1.0201 [13], which comprises a P-loop homologous with Bet v 1 and Pru av 1. Furthermore, Ballmer-Weber et al. [47] recently described a lack of IgE cross-reactivity between Dau c 1 and Bet v 1 in a subset of carrot allergic patients with sensitization to Dau c 1 and Bet v 1. We could show that the truncated C-terminus of Api g 1.0101 and Dau c 1 does not contribute significantly to the lack of cross-reactivity between Pru av 1 and Api g 1.0101 and the lower overall IgE reactivity of Api g 1.0101, since deletion of the C-terminal residues of Pru av 1 is not associated with a reduced IgE reactivity (Figures 3A and 4).

Similar mAb reactivity to Pru av 1 and its mutants revealed that the conformational IgG epitopes on the genetically engineered proteins are not affected by the mutation Trp^{45}. The heterogeneity between the epitopes on the surface of the IgE cross-reactive allergens among the Bet v 1 family is supported by the observation that mAbs raised against Bet v 1 showed different reactivity to Pru av 1 and Api g 1.0101. Two mAbs recognizing an immunodominant epitope formed by the P-loop region were selected by their enhanced reactivity to Api g 1.0101 Glu^{44} compared with Api g 1.0101 wt. These results showed that a negatively charged side chain is essential for these mAbs, but the lack of cross-reactivity with Pru av 1 indicates additional immunologically relevant differences in the P-loop region of the food allergens compared with Bet v 1.

IgE binding to Pru av 1, Api g 1 and the mutants was investigated by EAST analysis, an enzyme immunoassay in which, in contrast with immunoblotting, the allergen is kept under native conditions. The results of the EAST analysis are highly significant in respect to the presence of allergen-specific IgE in sera, but do not necessarily correlate with biological activity of allergens determined by, for example, skin tests or basophil activation tests. Our IgE-binding assays were restricted to EAST, which is the appropriate test to study a single conformational IgE epitope. Additional assays such as basophil activation tests which determine the presence of two epitopes for cross-linking of receptor-bound IgE were not performed. However, such tests would be required for safety assessment before investigating a hypo-allergenic mutant *in vivo* by skin tests.

SIT (specific immunotherapy) of type I allergy has been succesfully established on the basis of allergen extracts for several respiratory allergies and bee venom allergy, but not for food allergy. Although the immunological mechanisms of SIT are not fully understood, successful SIT requires preserved T-cell epitopes of the allergens. Therefore, low IgE-binding proteins, such as allergen mutants or fragments with retained T-cell epitopes, are interesting candidates for vaccines for a new strategy of specific immunotherapy with reduced anaphylactic side effects [15,48], and residues involved in IgE binding are obviously promising targets for mutation to generate such hypo-allergenic variants. However, results of tests *in vivo* with hypo-allergenic variants of food allergens have not been published so far. Furthermore, the results of this and previous studies [14,16] remind us that the IgE-binding epitopes are highly patient specific and site-directed mutagenesis can also enhance the IgE reactivity for at least a subgroup of patients, as observed for Pru av 1 Trp^{45} (Figure 3A) and Api g 1 Glu^{44} (Figure 3B). Hence, with allergens containing exclusively conformational IgE-binding epitopes, hypo-allergenic variants may be produced more easily by irreversibly preventing

Table 2 Summary of the structure calculations

Except for the experimental restraints, all values are average values over the 24 accepted structures as average value ± S.D. 1 Kcal ≡ 4.184 kJ.

Parameter	Value	
Experimental restraints used for the structure calculation		
Intraresidual NOEs	661	
Inter-residual NOEs		
Sequential	733	
Medium-range	332	
Long-range	593	
Dihedral angle restraints	71	
Hydrogen bonds	34	
Molecular dynamics simulation statistics		
Energies (kcal per mol)		
Total	242 ± 6	
Bond lengths	7.4 ± 0.4	
Bond angles	178.5 ± 2.0	
Improper angles	21.6 ± 0.6	
van-der-Waals repulsion	14.0 ± 1.3	
Distance restraints	21 ± 3	
Dihedral angle restraints	0.06 ± 0.03	
RMSDs from ideal distances (Å)		
Bond lengths	0.00171 ± 0.00005	
Distance restraints	0.0132 ± 0.0009	
RMSDs from ideal angles(°)		
Bond angles	0.507 ± 0.003	
Improper angles	0.431 ± 0.004	
Dihedral angle restraints	0.035 ± 0.010	
Atomic RMSDs from the average structure (Å)		
	Backbone	Heavy atoms
Overall*	0.60 ± 0.10	0.91 ± 0.12
Regular secondary structure†	0.43 ± 0.07	0.70 ± 0.08
β-Strands‡	0.27 ± 0.04	0.56 ± 0.07
C-terminal α-helix§	0.44 ± 0.10	0.82 ± 0.14
Comparison with Pru av 1 wt‖		
Overall*	0.73 ± 0.12	1.07 ± 0.13
P-loop¶	0.70 ± 0.18	0.93 ± 0.13

* Residues 1–159.
† Residues 2–58, 65–85, 97–104, 112–122 and 130–153.
‡ Residues 2–11, 41–58, 65–85, 97–104 and 112–122.
§ Residues 130–153.
‖ From reference [7]; average structure.
¶ Residues 45 (side chain only, up to Cβ), 46, 47, 48, 51, 52 and 53.

the folding process, as in the case of Pru av 1 Pro^{112}, than by mutation of all assumed IgE-binding epitopes while maintaining the native tertiary structure. Such products would then be used in a similar way as chemically modified allergoids, which are currently produced from natural allergen extracts, but would contain much less vigorous and structurally better defined alterations than the natural product.

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