

Bet v 1 – a Trojan horse for small ligands boosting allergic sensitization?

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Summary

Background Birch pollen allergy represents the main cause of winter and spring pollinosis in the temperate climate zone of the northern hemisphere and sensitization towards Bet v 1, the major birch pollen allergen, affects over 100 million allergic patients. The major birch pollen allergen Bet v 1 has been described as promiscuous acceptor for a wide variety of hydrophobic ligands.

Objective In search of intrinsic properties of Bet v 1, which account responsible for the high allergenic potential of the protein, we thought to investigate the effects of ligand-binding on immunogenic as well as allergenic properties.

Methods As surrogate ligand of Bet v 1 sodium deoxycholate (DOC) was selected. Recombinant and natural Bet v 1 were characterised physico-chemically as well as immunologically in the presence or absence of DOC, and an animal model of allergic sensitization was established. Moreover, human IgE binding to Bet v 1 was analysed by nuclear magnetic resonance (NMR) spectroscopy.

Results Ligand-binding had an overall stabilizing effect on Bet v 1. This translated in a Th2 skewing of the immune response in a mouse model. Analyses of human IgE binding on Bet v 1 in mediator release assays revealed that ligand-bound allergen-induced degranulation at lower concentrations; however, in basophil activation tests with human basophils ligand-binding did not show this effect. For the first time, human IgE epitopes on Bet v 1 were determined using antibodies isolated from patients' sera. The IgE epitope mapping of Bet v 1 demonstrated the presence of multiple binding regions.

Conclusions and clinical relevance Deoxycholate binding stabilizes conformational IgE epitopes on Bet v 1; however, the epitopes themselves remain unaltered. Therefore, we speculate that humans are exposed to both ligand-bound and free Bet v 1 during sensitization, disclosing the ligand-binding cavity of the allergen as key structural element.

Keywords allergenicity, allergic sensitization, allergic sensitization, Bet v 1, epitope mapping, human IgE epitope, IgE reactivity, NMR

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Introduction

Type I hypersensitivity describes adverse reactions of the immune system towards innocuous environmental antigens mediated by IgE antibodies. Recently, the search for inherent features of allergens underlying their exquisite capacity to induce IgE-mediated immune responses led to the discovery of several mechanisms, which have been postulated as driving forces of the allergic sensitization.

These mechanisms include interactions of allergens with receptors of the innate immune system, proteolytic activity, allergen-associated carbohydrate-structures and other yet undefined structural properties of allergens [1, 2]. Birch pollen allergy represents the main cause of winter and spring pollinosis in the temperate climate zone of the northern hemisphere and sensitization towards Bet v 1, the major birch pollen allergen, affects over 100 million allergic patients [3]. Natural Bet

v 1 represents a complex mixture of isoforms, whereas Bet v 1.0101 is most abundant ranging from 50% to 70% of total Bet v 1 [4]. Bet v 1 was described as highly promiscuous ligand acceptor, and recently, the crystal structure of Bet v 1.0101 has been solved in combination with different bound ligand molecules [5]. The allergen belongs to a large protein family; however, besides the major birch pollen allergen only few family members have the potential to initiate allergic sensitization [6]. This potent allergenicity could be either explained by unique structural properties of Bet v 1 or it could result from Th2-polarizing properties of the allergen source itself. Birch pollen extracts have been shown to have proteolytic activity, which can lead to the degradation of tight junction proteins [7]. Moreover, phytoprostanes and adenosine capable of inducing Th2 polarization have been identified in aqueous birch pollen extracts [8, 9]. We believe that these matrix effects are necessary but not sufficient to elicit full-blown Th2 polarization and that the intrinsic properties of Bet v 1 are mandatory for allergic sensitization. As Bet v 1 is traversed by a Y-shaped amphiphilic cavity where sites for ligand-binding are located, we believe that ligand-binding affects the allergenicity of the protein. Recently, a pollen derived ligand of Bet v 1 has been identified by Seutter *et al.* [10]. However, we believe that this is not the only ligand of Bet v 1; thus, to test our hypothesis that ligand-binding can modulate the immunogenicity of Bet v 1, we used the well-characterized ligand sodium deoxycholate (DOC – at concentrations far below the CMC). DOC has previously been shown in various publications to specifically bind Bet v 1 in a very stable way, whereas two molecules of DOC bind to one molecule Bet v 1 [5, 11, 12]. By using this model, we investigated the effects on physico-chemical, immunological and structural parameters of Bet v 1. To analyse whether Bet v 1-specific IgE was primarily directed against apo or ligand-bound Bet v 1, we isolated Bet v 1-specific IgE antibodies from serum samples of three different birch pollen-allergic donors and performed antibody epitope mapping with or without ligand by nuclear magnetic resonance (NMR) spectroscopy. Human IgE epitopes found on free Bet v 1 were compared with those found on Bet v 1–ligand complexes. Moreover, an animal model of adjuvant-free sensitization was used to investigate the effects of ligand-binding on sensitization *in vivo*.

Methods

Patients and sera

Birch pollen-allergic patients ($n = 7$) were selected based on case history, positive *in vivo* skin prick test and *in vitro* IgE detection (CAP System, Phadia AB, Uppsala, Sweden). Experiments with blood samples

from pollen-allergic patients were approved by the Ethic Committee of the Medical University and General Hospital of Vienna (no. EK028/2006). Informed written consent was obtained from all subjects included in the study.

Recombinant protein expression and purification

Recombinant Bet v 1.0101, termed Bet v 1 in the following, was expressed from a pET28b (Novagen, Merck KGaA, Darmstadt, Germany) construct in *Escherichia coli* BL21 Star™ (DE3) cells (Invitrogen, Carlsbad, CA, USA) in LB medium supplemented with 25 mg/L kanamycin. For isotope labelling, protein expression was performed in *E. coli* OD 2 N or *E. coli* OD 2 CN medium (Silantes, München, Germany) supplemented with 25 mg/L kanamycin. Protein expression was induced by addition of 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at OD600 nm of 0.8, and growth was continued at 37°C for 5 h. Harvested cells were dissolved in 1/50 culture volume of 20 mM sodium phosphate buffer pH 7.4 containing 0.8 M NaCl and 15 mM NaH₂PO₄, and protein purification was performed as previously described [13]. Recombinant proteins were lyophilized and stored at –20°C. The endotoxin content of Bet v 1 used for immunological studies was determined by Limulus amoebocyte lysate (LAL) assay (Associates of Cape Cod, Inc., East Faulmouth, MA, USA). Bet v 1 had shown an endotoxin content of > 0.3 ng/mL.

Purification of natural Bet v 1

Natural Bet v 1, termed nBet v 1 in the following, was extracted from 5 mg *Betula pendula* (Allergen AB, Ängelholm, Sweden) pollen in a volume of 50 mL ddH₂O and 500 mM NaCl. After vigorous shaking for 5 min at room temperature, the supernatant was collected after centrifugation at 12 000 g. The supernatant was filtered through a 0.45- μ m filter, and protein purification was performed using a 10 mL Phenylsepharose column (GE Healthcare Biosciences, Little Chalfont, UK). The protein was found in the flow-through, and a final polishing step was performed by size exclusion chromatography in ddH₂O using a Superdex 75 10/300 GL column (GE Healthcare Biosciences). Pure protein was stored at –20°C.

Purification of human Bet v 1-specific IgE

The purification of Bet v 1-specific IgE antibodies from three allergic donors was performed as reported elsewhere [14] using a NHS-activated Sepharose column (GE Healthcare, Chalfont St. Giles, UK) coupled with rBet v 1. A representative SDS-PAGE is shown in Figure S1.

Physicochemical analyses of recombinant proteins

Purity of recombinant and natural allergens was analysed by SDS-PAGE followed by Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA, USA) staining. Protein identity was determined by mass spectrometry using a Quadrupole time-of-flight mass spectrometer with electrospray ionization (ESI-QTOF-MS) (Waters Corp., Milford, Massachusetts, USA). Secondary structure analysis was performed by circular dichroism (CD) spectroscopy using a JASCO J-815 spectropolarimeter fitted with a PTC-423S Peltier-type single position cell holder (Jasco, Tokyo, Japan). Spectra were recorded in 5 mM sodium phosphate buffer pH 7.4. Thermal stability of proteins was determined performing a thermal denaturation from 20 to 95°C with a temperature slope of 1°C/min. Denaturation was monitored at 222 nm.

ANS displacement assay

In a 96-well UV-Star plate (Greiner Bio One, Kremsmünster, Austria), 50 µL of protein solution (final concentration 10 µM) was mixed with DOC in the different molar ratios 1 : 1, 1 : 2 and 1 : 10 and incubated overnight at 4°C. For the measurement, 50 µL of 8-Anilinonaphthalene-1-sulfonic acid (ANS) solution (50 µM final concentration) was added, and after incubation for 5 min at room temperature, measurements were taken from 400 to 600 nm in 2 nm steps with ANS excited at 350 nm. Blanks of DOC with ANS were subtracted.

Mediator release assays

The allergenic potential of Bet v 1 with and without DOC was assessed by rat basophil degranulation assays performed as previously described [15]. In short, RBL-2H3 cells transfected with the human high-affinity IgE receptor (FcεRI) were passively sensitized with serum IgE from birch pollen-allergic donors. Antigen-dependent β-hexosaminidase release into the supernatant was measured by enzymatic cleavage of the fluorogenic substrate 4-methyl umbelliferyl-N-acetyl-β-D-glucosaminide and expressed as percentage of total enzyme content of Triton X-100-treated cells.

Endolysosomal degradation assay

JAWS II cells (American Type Culture Collection, Manassas, VA, USA) were cultured as previously described [16]. For preparation of microsomes, 10⁷ cells were homogenized in 1 mL 10 mM TRIS acetate pH 7, 250 mM sucrose. Cellular debris was precipitated by centrifugation at 6000 g for 10 min. Total microsomes were prepared by ultracentrifugation for 60 min at 100 000 g. Microsomal enzymes were released by

repeated freeze/thaw cycles. Five mg antigen was digested with 7 mg endo-/lysosomal enzymes in a reaction volume of 20 µL in 100 mM citrate buffer pH 4.8, 2 mM dithiothreitol, at 37°C. Digestions were stopped by heat denaturation and analysed by SDS-PAGE.

Basophil activation test (BAT)

Heparinized blood was collected from birch pollen-allergic donors with positive skin prick tests to birch pollen extract (ALK-Abelló, Hørsholm, Denmark), and birch pollen-specific IgE levels > 0.35 kU/L (ImmunoCAP, Thermo Fisher). Whole blood was incubated for 15 min at 37°C with different concentrations of either natural or recombinant Bet v 1 (100, 10, 1, 0.1, 0.01 ng/mL) in RPMI (Hepes Calcium Buffer) supplemented with human IL-3 (5 ng/mL, PeproTech, Rocky Hill, NJ, USA) in the absence or presence of DOC (10 molar excess to the allergen). Medium alone served as negative control. An anti-human IgE Ab (1 µg/mL, Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) served as positive control. After adding PBS containing 20 mM EDTA, cells were stained for 30 min at 4°C with CD63-PE, CD123-FITC and CCR3 (CD193)-APC (all from Biolegend San Diego, CA, USA), or the respective isotype controls (all from BD Biosciences, Franklin Lakes, New Jersey, USA). After lysis of erythrocytes, the percentage of CD63-positive cells in the CCR3⁺CD123⁺ population was assessed by flow cytometry.

Animal experiments

For immunization studies, female BALB/c mice (*n* = 5 per group) (Charles River Laboratories, Wilmington, MA, USA) were used at 8–10 weeks of age. Animals were injected subcutaneously (s.c.) with 10 µg antigen administered as two 50 µL injections in the upper and lower back. Injections were given on day 1, 15 and 29. Sera were collected on day 0 and 1 week after the last booster immunization. Serum IgG1 and IgG2a were analysed by ELISA. Bound antibodies were detected with alkaline phosphatase-conjugated secondary antibodies (SouthernBiotech, Birmingham, AL, USA). All measurements were performed as triplicates; results are presented as mean OD values. Biological activity of serum IgE was determined in mediator release assays using RBL-2H3 cells passively sensitized with murine immune sera. Therefore, the cells were incubated with titrated animal sera and washed to remove serum proteins including IgG. Antigen-dependent β-hexosaminidase release was triggered by addition of 100 µL 0.3 µg/mL antigen solution per well and measured as described [13]. Animal experiments were conducted according to National guidelines approved by the Austrian Ministry of Science (BMWF-66.012/0011-II/10b/2010).

Statistics

Statistics were calculated with student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Nuclear magnetic resonance assignment

Partial Bet v 1 resonance assignment (40%) was obtained by direct comparison with Biological Magnetic Resonance Bank (BMRB) deposit 4417. The backbone assignment of the protein Bet v 1 was further improved (to 88% of assigned resonances) using 3D triple-resonance experiments: HNCACB, HNCA, HNCOC, HNCACO and CBCA(CO)NH. The same experiments were used for Bet v 1 – DOC assignments. The NMR spectra were recorded using ^{15}N - or ^{15}N , ^{13}C labelled samples for Bet v 1 on a Bruker Avance III 800 MHz spectrometer equipped with TXI 5 mm triple-resonance probe. All experiments were performed at 298 K. The sample used for assignment experiment was 400 μM in 10 mM sodium phosphate buffer (pH 7.0), containing 10% D_2O and 0.05% sodium azide. All spectra were processed using TOPSPIN 2.1 and analysed using CARR 1.8.4.

Bet v 1 backbone dynamics

Experiments with ^{15}N -labelled Bet v 1 were performed at 298 K on a Bruker Avance III 800 MHz ^{15}N R_1 , and ^{15}N R_2 relaxation rates were measured from spectra with different relaxation delays (ms): 20, 50, 100, 200, 300, 400, 500, 750, 850, 1000 and 1500 s for R_1 and 16, 32, 48, 80, 64, 96, 112 and 144 ms for R_2 . Errors in the peak intensities were calculated from the triplicate experiments, errors of R_1 and R_2 measurements were obtained for values of three independent experiments (relaxation delay).

Interaction of Bet v 1 with Human IgE

Bet v 1-IgE interactions were monitored by ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) spectra with 1024×360 points. Four micromolar of unlabelled purified human IgE from three patients was used with 40 or 80 μM of ^{15}N -labelled Bet v 1. Chemical shift perturbations (CSPs) were compared to a control sample without IgE. Experiments were conducted in 200 mM sodium phosphate buffer pH 7.0, 30 mM sodium citrate. CSPs from the 2D ^1H - ^{15}N HSQC NMR experiments were used to identify IgE epitopes using the equation $\Delta\delta = (\Delta\delta_{\text{HN}}^2 + (0.1\Delta\delta_{\text{N}})^2)^{1/2}$, where $\Delta\delta_{\text{HN}}$ represents the difference between free and bound ^1H amide chemical shifts (ppm), and $\Delta\delta_{\text{N}}$ the difference between free and bound ^{15}N chemical shifts (ppm).

Interaction of sodium deoxycholate with Bet v 1

Interactions were monitored by CSP in titration experiments. Several ^1H - ^{15}N HSQC (1024×512) points of Bet v 1 (400 μM) were acquired at concentrations of 0.05, 0.15, 0.4, 0.8, 1, 2 and 4 mM DOC (Sigma Chemicals, St. Louis, MO, USA) in sodium phosphate buffer (pH 7.0). CSPs were calculated as described.

Results

DOC binds to natural and recombinant Bet v 1

ANS displacement assays were used to confirm the binding of DOC to the natural, as well as recombinant purified Bet v 1. In both cases, DOC was able to displace ANS binding to the molecules resulting in a decrease of the fluorescent signal (Fig. 1c).

Thermal stability of Bet v 1 is increased in the presence of bound ligand

Circular dichroism spectroscopy was used to determine the effects of ligand-binding on secondary structure elements of Bet v 1, and further to investigate the influence of bound ligands on thermal stability of the allergen. Of note, the secondary structure of Bet v 1 did not change upon binding of sodium DOC (Fig. 1a), whereas ligand-binding had a clear consequence on the stability of the complex. In a denaturation experiment, the melting point of Bet v 1 increased by 3.3°C from $64.19 \pm 0.072^\circ\text{C}$ to $67.49 \pm 0.056^\circ\text{C}$ upon binding of sodium DOC (Fig. 1a).

Increased stability of ligand-bound Bet v 1 translates into proteolytic stability

A direct correlation between thermal and proteolytic stability has been demonstrated for protein antigens [17, 18]. Moreover, there is strong evidence that proteolytic stability has an influence on the quality and quantity of the immune response elicited by an antigen. To simulate proteolysis of antigens in the endo-/lysosomal compartments of dendritic cells, proteases were isolated from microsomes of a murine DC-like cell line. Antigens were subjected to proteolytic degradation at acidic pH, and proteolysis was monitored by SDS-PAGE in a time dependent manner. The control protein BM4 was included in the assay to expulse a direct interference of DOC with the proteases [13]. Binding of sodium DOC had a stabilizing effect on Bet v 1 translating into strong resistance towards endo-/lysosomal degradation, whereas apo Bet v 1 was susceptible to proteolysis (Fig. 1b). In fact, apo Bet v 1 was digested completely after 72 h of proteolysis, while ligand-bound Bet v 1 was not degraded at any time point investigated.

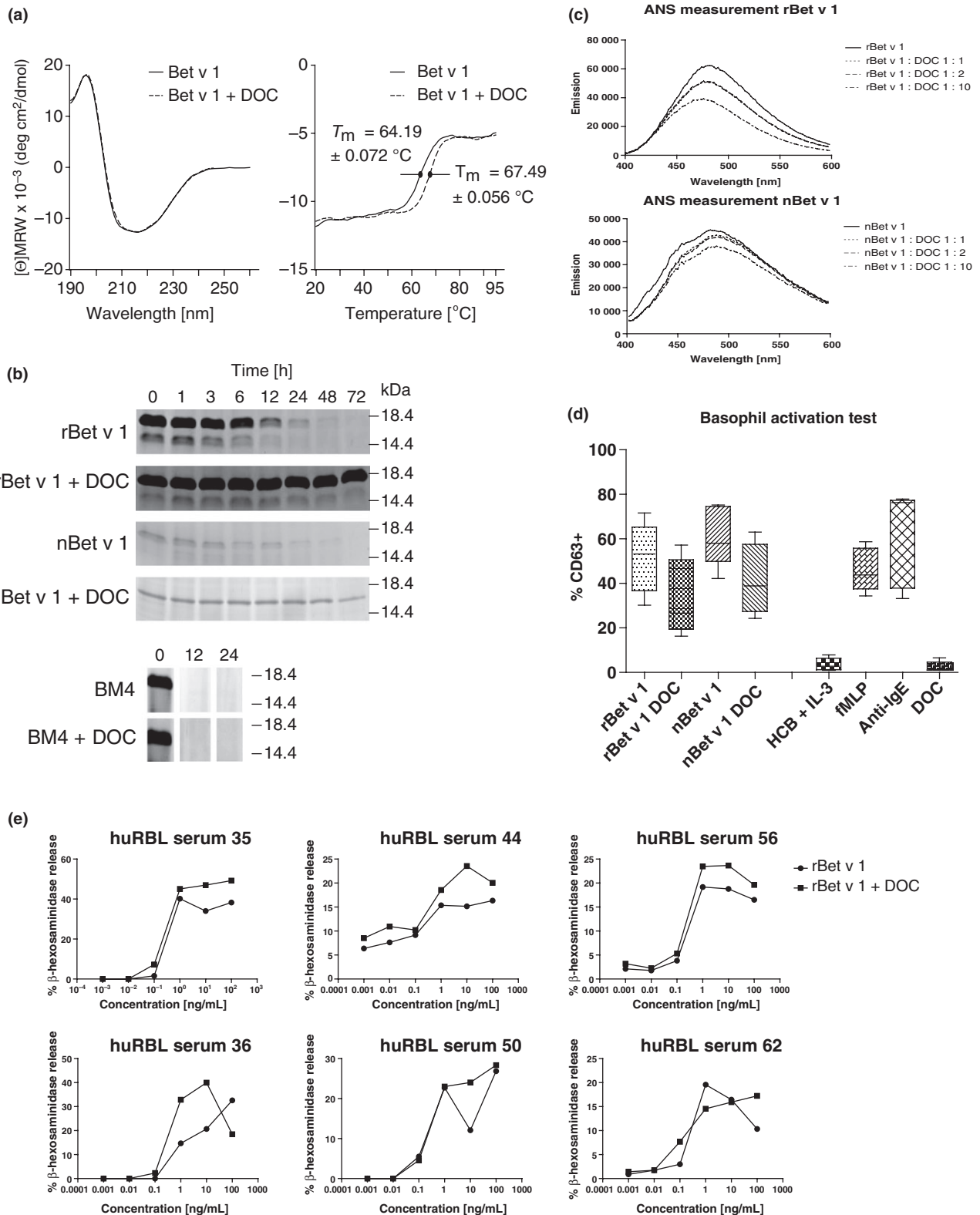


Fig. 1. (a) Circular dichroism (CD) and denaturation spectra of Bet v 1 with and without deoxycholate (DOC). (b) SDS-PAGE analysis of time dependent endo-/lysosomal degradation of natural and recombinant Bet v 1 with and without DOC and the control protein BM4. (c) ANS displacement assay. Fluorescence emission intensities of ANS at 486 nm at different molar ratios nBet v 1/rBet v 1:DOC are shown. (d) Basophil activation tests of atopic donors ($n = 5$). The percentage of CD63⁺ basophils at 100 ng applied allergens with and without ligand is shown. (e) The allergenic activity was determined by mediator release assays using birch pollen allergic patients' sera.

Bet v 1 plus sodium deoxycholate serves as strong stimulus for basophil degranulation

To mimic allergen-induced degranulation of basophils, RBL cells were passively sensitized with serum IgE from allergic donors. Mediator release was triggered by the addition of serial dilutions of Bet v 1 either loaded with DOC or in its apo form. Bet v 1 stabilized by sodium DOC induced basophil degranulation in general at lower concentrations than apo Bet v 1 (Fig. 1e). Although not statistically significant, there was a clear trend that DOC-bound Bet v 1 could activate the RBL cells more efficiently supports the theory that human IgE is at least partially directed towards a Bet v 1–ligand complex during the process of allergic sensitization.

Ligand-binding influences the immunological behaviour of Bet v 1 in vivo

To determine the effects of ligand-binding on immunogenicity and immune polarization of Bet v 1, BALB/c mice were immunized with Bet v 1 with or without bound DOC in an adjuvant-free model, and the humoral immune response was investigated. This allows to determine allergenicity and to investigate immune polarization of the allergen without any influence of an external immune stimulator. The IgG1 immune response elicited by Bet v 1 in the presence of DOC was significantly lower as compared to apo Bet v 1, and also IgG2a antibody levels were reduced (Fig. 2a). However, the IgE response against Bet v 1 plus DOC was not reduced as investigated in mediator release assays using basophils passively sensitized with murine immune sera (Fig. 2b).

Bet v 1 in complex with DOC influences the activation of human basophils

To demonstrate the allergenic activity of apo- vs. ligand-bound natural as well as recombinant Bet v 1

on human basophils, BAT assays with the respective allergens were performed. After the purification of human basophils from the peripheral blood of five allergic donors, the cells were stimulated with the allergens, and their activation level was measured by the surface-expression of CD63. The assay revealed that the apo states of Bet v 1 induced a stronger activation of human basophils compared to their ligand-bound state (Fig. 1d). These findings indicate that the higher stability of the allergens upon binding of DOC directly influences the activation of human basophils.

Human IgE Epitope Mapping of Bet v 1

The NMR assignments of Bet v 1.0101 were obtained by triple-resonance experiments and data comparison with the BMRB deposit 4417 (M139L). Bet v 1-specific antibodies were purified by affinity chromatography using a Bet v 1-coated sepharose column, and allergen-specific IgE was separated from IgG by protein G sepharose. Homogeneity of the fractions was verified by SDS-PAGE. Antibodies were purified from three different Bet v 1 allergic donors (Fig. 3). Epitope mapping on Bet v 1 was performed by analysing CSP of $^1\text{H} - ^{15}\text{N}$ coherence (HSQC) experiments, where each residue will give one cross peak related to its amide NH. For these experiments, we used ^{15}N labelled Bet v 1 in the presence of IgE at a ratio of 10 : 1. IgE epitopes were mapped by comparing the spectrum of labelled Bet v 1 with and without IgE. Control experiment performed in the absence of IgE was used to analyse any interference of changes in pH, temperature and salt concentration. The observed CSPs among control experiments were always smaller than the ones observed in the presence of IgE.

Figure 3 shows that upon antibody binding, we observed CSP and also changes in line width, which is related to R_2 , the transverse relaxation rate. For small

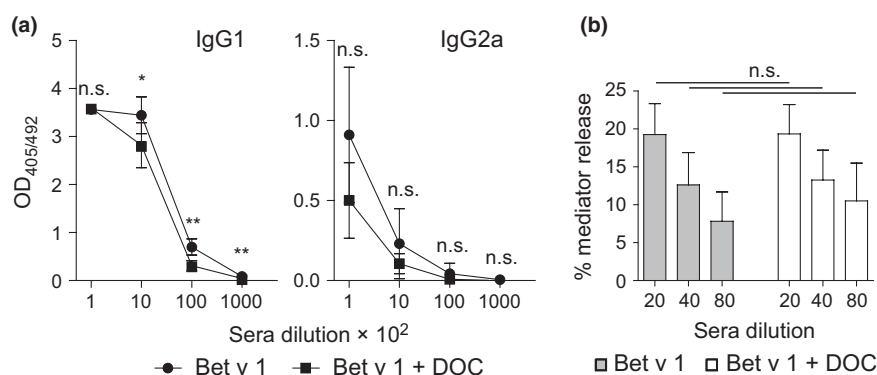


Fig. 2. (a) Bet v 1-specific murine antibody levels of animals ($n = 5$ per group) immunized with Bet v 1 with or without deoxycholate (DOC) were determined by ELISA, (b) IgE activity by mediator release assays. ELISA data are presented as mean $\pm$ SD, release data as mean $\pm$ SEM. P -values were calculated with t -tests (n.s. not significant, * $P < 0.05$, ** $P < 0.01$).

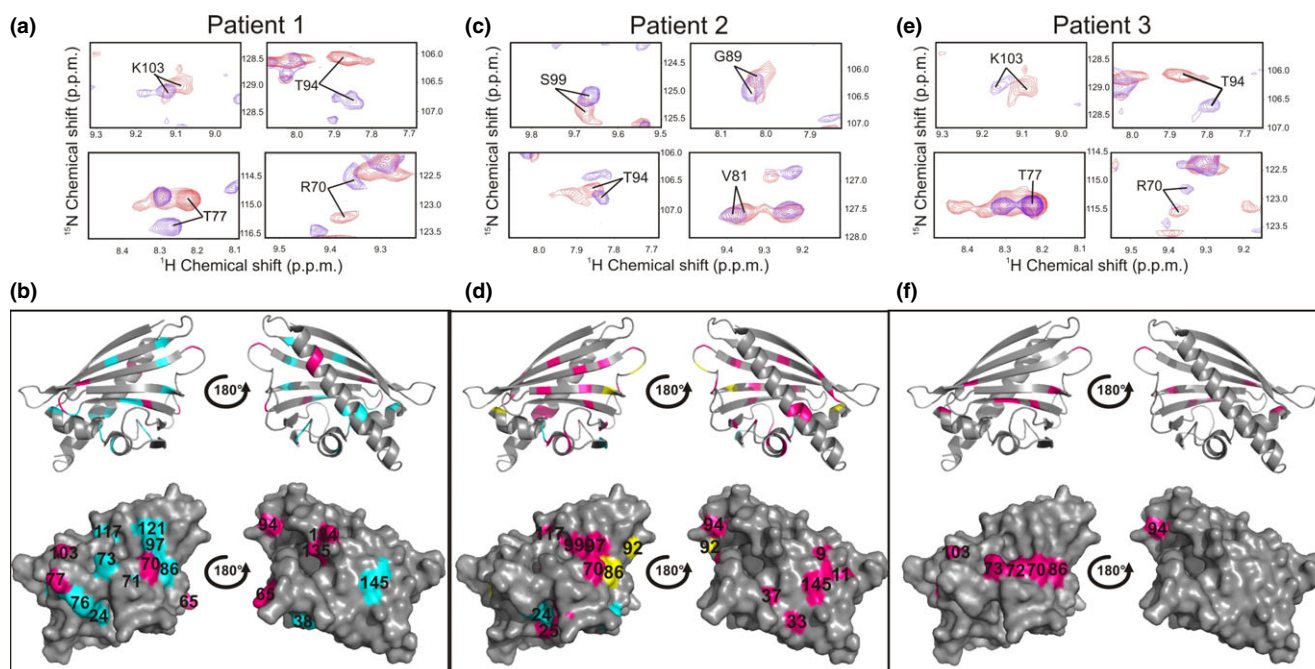


Fig. 3. IgE epitope mapping using IgE from three allergic patients; (a, c and e) show selected peaks for Bet v 1 free (red), and in the presence of IgE (blue); (b, d and f) show Bet v 1 structure (4A88) in which the residues with chemical shift perturbation bigger than 0.034, for patient 1 and 3, and bigger than 0.017 for patient 2 were coloured in pink, residues that disappeared in cyan and that appeared in yellow.

R_2 , sharp lines are recorded whereas for bigger R_2 the lines broaden reflecting conformation exchange in the μ s–ms timescale. For example, residues 24, 38, 71, 73, 76, 86, 97, 117, 121 and 145 disappeared in the experiments with patient 1 IgE, those peaks increased the line width beyond detection. In contrast, interaction with IgE from patient 2 lead to the appearance of the peaks 15, 86 and 92 (details are presented in Supporting Information). All these changes are indicative that these residues are directly involved in antibody binding. Three major IgE binding regions on Bet v 1 could be mapped by NMR spectroscopy. These regions include the N-terminal part, a region around residues 70–100, and the C-terminal α -helix of the allergen. For patients 1 and 2 (Figs 3a, b, c and d), regions were equally mapped while for patient 3 (Figs 3e and f) the region around residues 70–100 presented CSP values three times bigger, whereas the C-terminal residues showed only slight changes. Of note, all patients exhibited largest CSPs around residues 70–100.

The conformational dynamic of Bet v 1 is drastically reduced upon ligand-binding

Triple-resonance experiments were acquired to assign Bet v 1 bound to DOC. Upon ligand-binding the ^1H - ^{15}N HSQC of Bet v 1 changed profoundly (Fig. 4) and DOC binding induced a generalized change in the chemical shift. Titration experiments revealed that, as reported,

two DOC molecules can bind Bet v 1 simultaneously [5, 11]. Further, the exchange between free and bound state occurred in two different time scale modes: one slow exchange, which corresponds for the vast majority of peaks and the other in fast exchange, which corresponded to two peaks (residue K65 and Q36). To describe the complex binding behaviour of Bet v 1, the backbone dynamics were analysed by investigating the relaxation parameters R_1 and R_2 for free and ligand-bound allergen. In the apo state, residues I56, R70 and Y83 and showed high R_2 values, K65 and G140 displayed such high R_2 values that they could not be measured by the methodology applied. In complex with DOC, these R_2 values decreased (Fig. 5). This suggests a decrease in conformational dynamic, in μ s–ms time scale, upon binding of DOC to the hydrophobic cavity – an indicator that free Bet v 1 exists in solution as a family of conformational states, whereas one is selected upon ligand binding.

IgE epitope analysis of ligand-bound Bet v 1

To investigate effects of ligand-binding on antibody epitopes, DOC-bound Bet v 1 was incubated with allergen-specific serum IgE of a birch pollen-allergic donor (Fig. 6). ^1H - ^{15}N HSQC spectra were collected for Bet v 1 complexed with DOC both in the presence or absence of IgE (Fig. 6). Analyses of the mapped regions showed that the same antibody binding regions were mapped in

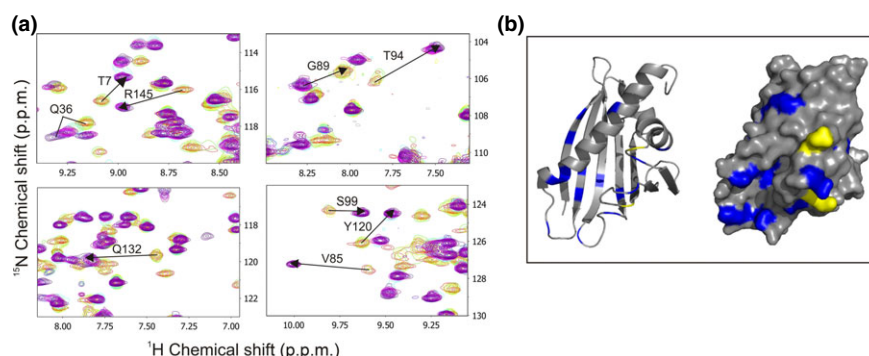


Fig. 4. Structural changes upon deoxycholate (DOC) interaction. (a) Show the superposition of HSQCs of apo Bet v 1 (red), and with different DOC concentrations: 50 μ M (yellow); 150 μ M (green); 400 μ M (cyan); 800 μ M (pink); 1 mM (light pink); 2 mM (blue); 4 mM (Purple); (b) residues in slow exchange are coloured in blue and residues in fast exchange in yellow.

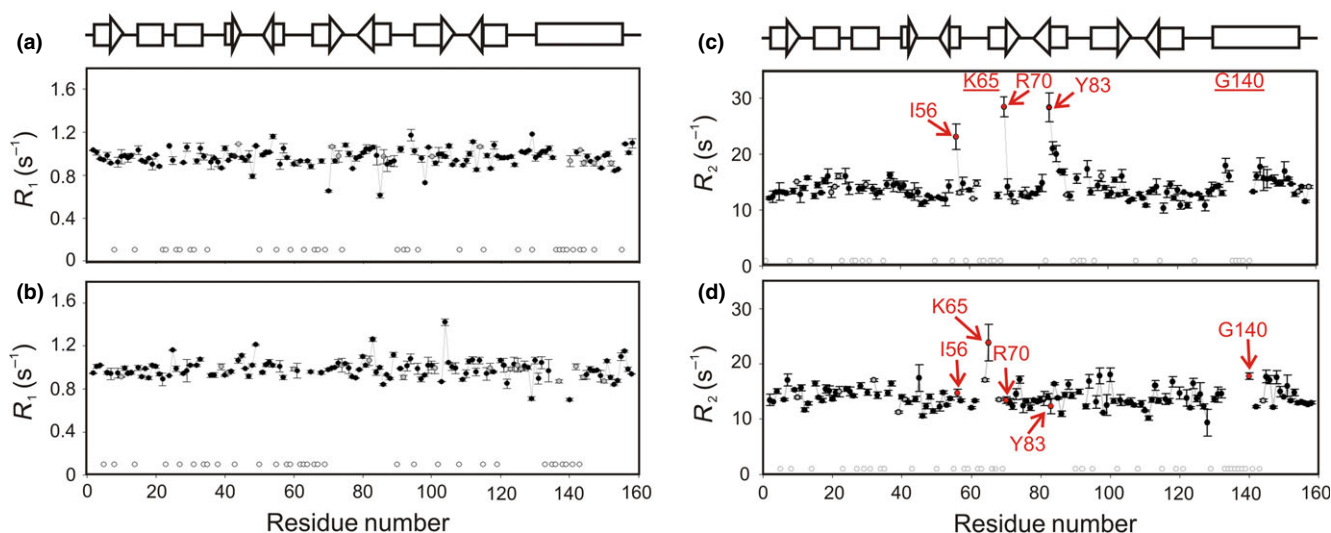


Fig. 5. Conformation stabilization upon deoxycholate (DOC) binding. (a and c) Shows the ^{15}N relaxation parameters of Bet v 1 apo and bound to DOC (b and d). Residues with big changes in dynamic behaviour were highlighted in red. Residues for which R_2 values could not be measured accurately because of high relaxation rates (fast decaying curves) are indicated in red and underlined.

Bet v 1 – DOC complex, however, residues 126 and 132 were only perturbed when DOC was bound to Bet v 1. Of note, these residues are located at the opening of the hydrophobic cavity, which indicates that in the presence of DOC the residues are more exposed. Moreover, line width was decreased pointing towards a reduced conformational exchange (in the μ s–ms time scale) of IgE antibodies with ligand-bound Bet v 1. Thus, DOC binding stabilizes conformational IgE epitopes on Bet v 1.

Discussion

Over 50% of major allergens have the ability to bind ligand molecules [19]; amongst them are group 2 mite allergens, Bet v 1-like proteins, non-specific lipid-transfer proteins, 2S albumins, lipocalins or secretoglobins, to name a few. For Der p 2, LPS-binding directly

influenced the TLR-4 mediated development of experimental allergic asthma in mice [20], thus demonstrating remarkable effects of ligand-binding on allergenicity. Bet v 1 itself is a promiscuous acceptor of a wide variety of small, hydrophobic ligands [5]. Here, we investigated the effects of ligand-binding on the structure of Bet v 1, on the formation of IgE epitopes, and on the allergenicity of the complex in a mouse model. Ligand-binding stabilizes the conformation of Bet v 1 resulting in an increased melting point as well as drastically increased resistance towards endo-/lysosomal proteolysis. NMR analyses of Bet v 1 in complex with DOC confirmed the data. Despite increasing the stability of the complex, ligand-binding does not affect the overall structure of the protein. However, the flexibility of Bet v 1 is substantially reduced. This has a direct influence on the subsequent immune response as demonstrated in

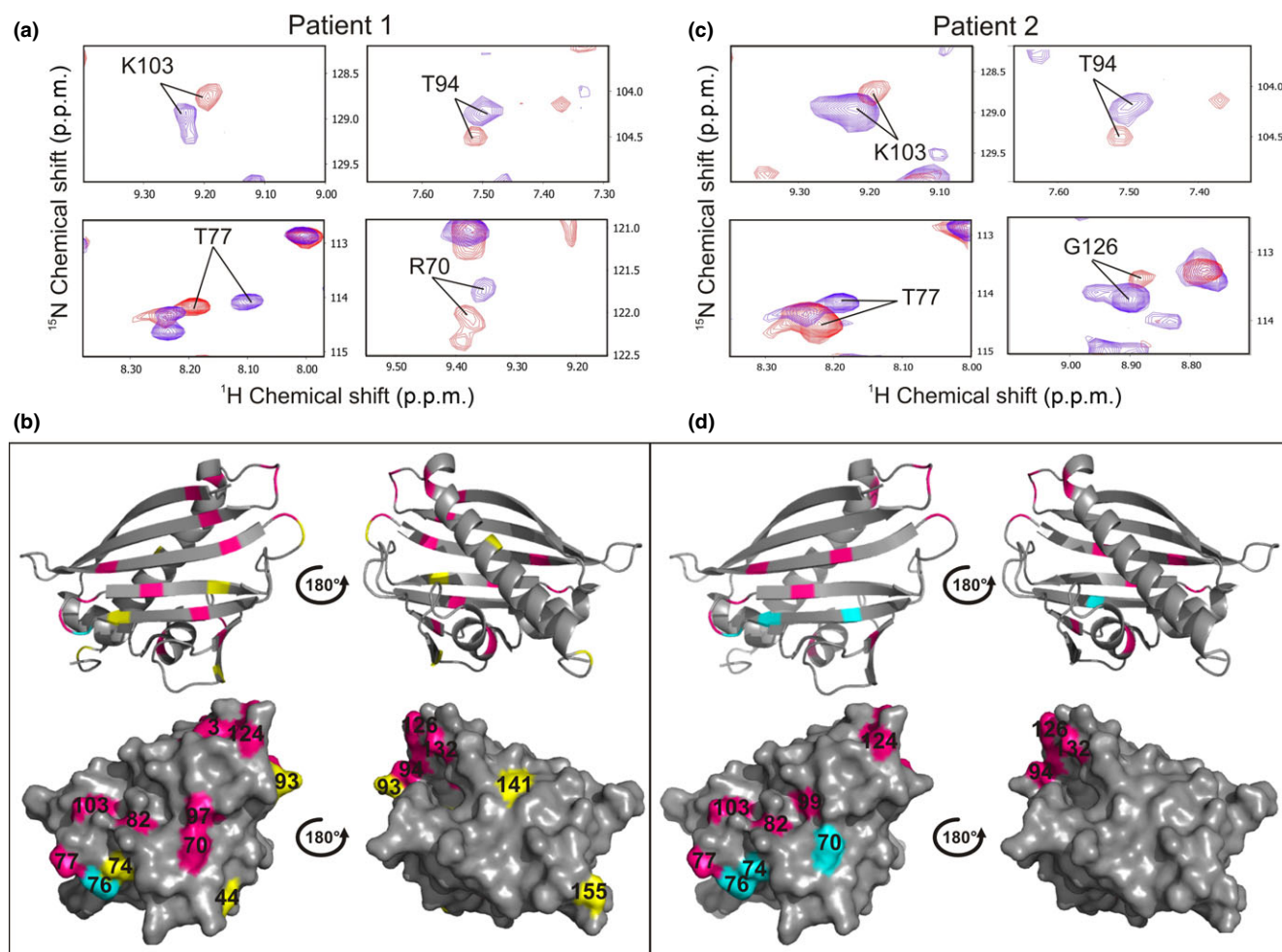


Fig. 6. IgE epitope mapping in the presence of deoxycholate (DOC). (a and c) Show selected peaks for Bet v 1–DOC complex (red), and this complex in the presence of IgE (blue). (b and d) Show Bet v 1–DOC X-ray structure (4A83) where high chemical shift perturbation (CSP) residues were coloured in pink, residues that disappeared in cyan and that appeared in yellow.

an adjuvant-free mouse model. Comparing the immunological properties of Bet v 1 vs. ligand-bound Bet v 1, we found that Bet v 1 in complex with DOC elicited a significantly decreased IgG1 immune response compared to the apo allergen, whereas the IgE response was similar. Thus, ligand-binding increased the allergenicity of the protein but decreased the protein's immunogenicity. The findings are remarkable because published data suggest that a decreased rather than an increased protein stability is accompanied by low immunogenicity and a Th2-biased immune response [16, 18]. However, this highly increased stability could hinder optimal proteolytic processing of the allergen, which would again favour the development of a Th2 immune response in a similar manner as observed for proteolytically susceptible antigens. Obviously, ligand-binding affects the molecule in a complex way, which requires further investigation and should be considered as a key parameter of allergenicity [5]. Humans most likely are

exposed to a mixture of ligand-bound and free Bet v 1, which then would initiate allergic sensitization. Using birch pollen-allergic patients' sera, we performed mediator release assays of passively sensitized RBL cells. In those assays, we found a trend that DOC-bound Bet v 1 was the more potent trigger of cell activation compared to the apo allergen. However in BAT assays we did not see this trend, there DOC binding had a rather restraining effect on cell activation. Apparently, the higher stability and lower dynamic of the protein directly influences IgE binding. The effects of DOC binding obviously also depend on the assay format, thus it will be of highest interest to perform deeper research on this topic. Nevertheless, the findings support the view that during sensitization humans could be exposed to both forms of Bet v 1. However, at present it remains elusive whether ligand-bound or free Bet v 1 would initiate the process of sensitization. To define the IgE epitopes on Bet v 1 in more detail, NMR analyses were performed.

The surface area of Bet v 1 accessible for antibody binding covers 7203 Å [2, 20], whereas binding of a murine Fab to the allergen covered an area of 931 Å [2, 21]. Using NMR spectroscopy simultaneous binding of multiple antibodies to a protein antigen can be determined by investigating CSPs of single residues on the molecule. The shifts in CSPs occur with a high frequency at the contact site of the antibody. Bet v 1-specific IgE antibodies were isolated from sera of three birch pollen-allergic donors by affinity chromatography, and IgE epitopes were determined. Three regions of high CSPs indicative for antibody epitopes were identified for each patient's serum, which comprise the N-terminal part of the protein, an area covering residues 70–100, and the C-terminal alpha-helix. Using amino acids showing CSPs after IgE binding as anchor points, we calculated the surface areas of several epitopes (i.e. the epitope including residues 24, 73, 76, 77 and 103; the epitope including residues 70, 86, 97, 117 and 121) and determined area sizes of approximately 1000 Å [2]. Recently, conformational IgE epitopes of Bet v 1 were indirectly mapped using peptide specific murine monoclonal antibodies. Monoclonals reactive for the minimal peptides 49–58, 73–88 and 88–103 were found to inhibit human serum IgE binding [3]. The regions 73–88 and 88–103 overlap with IgE epitopes identified by NMR spectroscopy, whereas region 49–58 was not identified to be directly involved in IgE binding in the present study. Interestingly, the IgE epitopes identified by NMR only partially overlap with regions containing amino acid positions described as crucial for IgE recognition by site-directed mutagenesis of Bet v 1 isoforms [22,23]. Mutagenesis of distinct residues might cause not only local but also global perturbations in the protein fold, thus amino acids are

not necessarily directly involved in IgE binding. In this case, we can clearly conclude that none of the amino acid positions that were mutated is directly involved in IgE binding to Bet v 1. The determination of the correct IgE epitopes is, however, crucial for rational protein design thus the results exemplify the differences between the two approaches for epitope mapping. In total, we mapped the IgE profile of three patients and were able to identify several different IgE epitopes. Our data confirm that the IgE response towards Bet v 1 is heterogeneous. However, we could identify hot spots on the allergen, which seem to be preferred for antibody binding. Binding of DOC did not change the IgE epitopes, still the exchange of the ligand-bound state was reduced. This is indicative for a more stable interaction of DOC-Bet v 1 with the antibodies. Based on these findings in combination with the immunological data, we conclude that humans are exposed to both forms of Bet v 1, ligand bound and free. Nevertheless, the ligand-binding property of Bet v 1 seems to be one of the mechanisms responsible for the high allergenicity of the protein.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. IgE used in the NMR experiments. The purification of Bet v 1-specific IgE antibodies from three allergic donors was performed as reported elsewhere [14] using a NHS-activated Sepharose column (GE Healthcare, Chalfont) coupled with rBet v 1. This gel is a representative example of the IgE samples used in this study.

Figure S2. Bet v 1 - IgE epitope mapping. HSQC of ^{15}N labeled rBet v 1 were acquired in the absence and presence of specific human IgE purified from the peripheral blood of three allergic patients: Patient 1 – a, b and c; Patient 2 – d, e and f; and Patient 3 – g, h and i. Panel (a, d and g) show the chemical shift perturbation (CSP) as a function of residue number obtained for 40 μM Bet v 1 and 4 μM IgE. Yellow bars and red stars represent peaks that appeared and disappeared in the presence of IgE, respectively; Panels (b, e and h) show the superposition of ^1H - ^{15}N HSQCs obtained for Bet v 1 free (red), and in the presence of IgE (blue) showing

specific resonances that change in the presence of IgE; Panels (c, f and i) show Bet v 1 structure (4A88) in which the residues with chemical shift perturbation bigger than 0.034, for patient 1 and 3, and bigger than 0.017 for patient 2 were colored in pink, residues that disappeared in cyan and that appeared in yellow.

Figure S3. Bet v 1 and IgE epitope mapping at different ratios of Bet v 1 : IgE. Results were obtained with IgE of patient 3 and two different ratios 1 : 30 Bet v 1 (a, b, c) or 1 : 10 (d, e, f). (a and d) Shows the CSP as a function of residue number. (b and e) Shows representative regions of the ^1H - ^{15}N HSQC in the absence and presence of IgE. The results were obtained as described in Figure S1. Panels (c and f) show Bet v 1 structure (4A88) in which the residues with chemical shift perturbation bigger than 0.034 were colored in pink. Note that similar regions are mapped in these two conditions but the results obtained with smaller ratio showed bigger CSP.

Figure S4. Bet v 1 titration with deoxycholate. CSP was calculated from ^1H - ^{15}N HSQC spectra of the Bet v 1 in the free state and in the presence of different concentration of deoxycholate.

Supporting Information

Bet v 1 – a Trojan horse for small ligands boosting allergic sensitization?

Condensed title: Bet v 1 dynamics and IgE epitopes

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Supporting Information

Figure Legends:

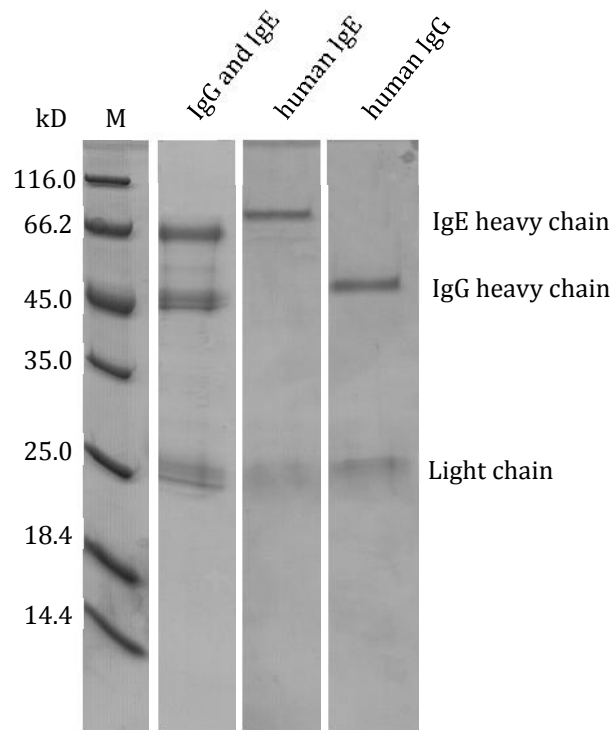


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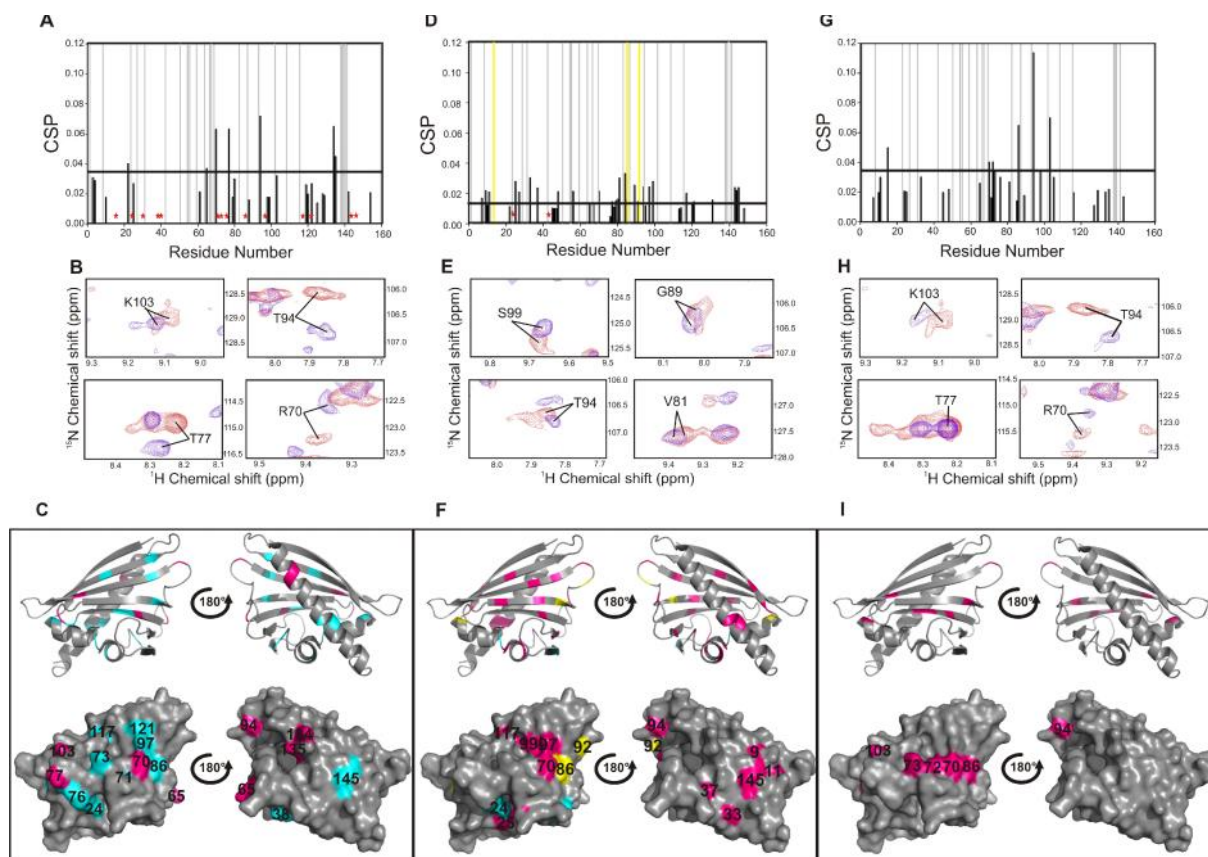


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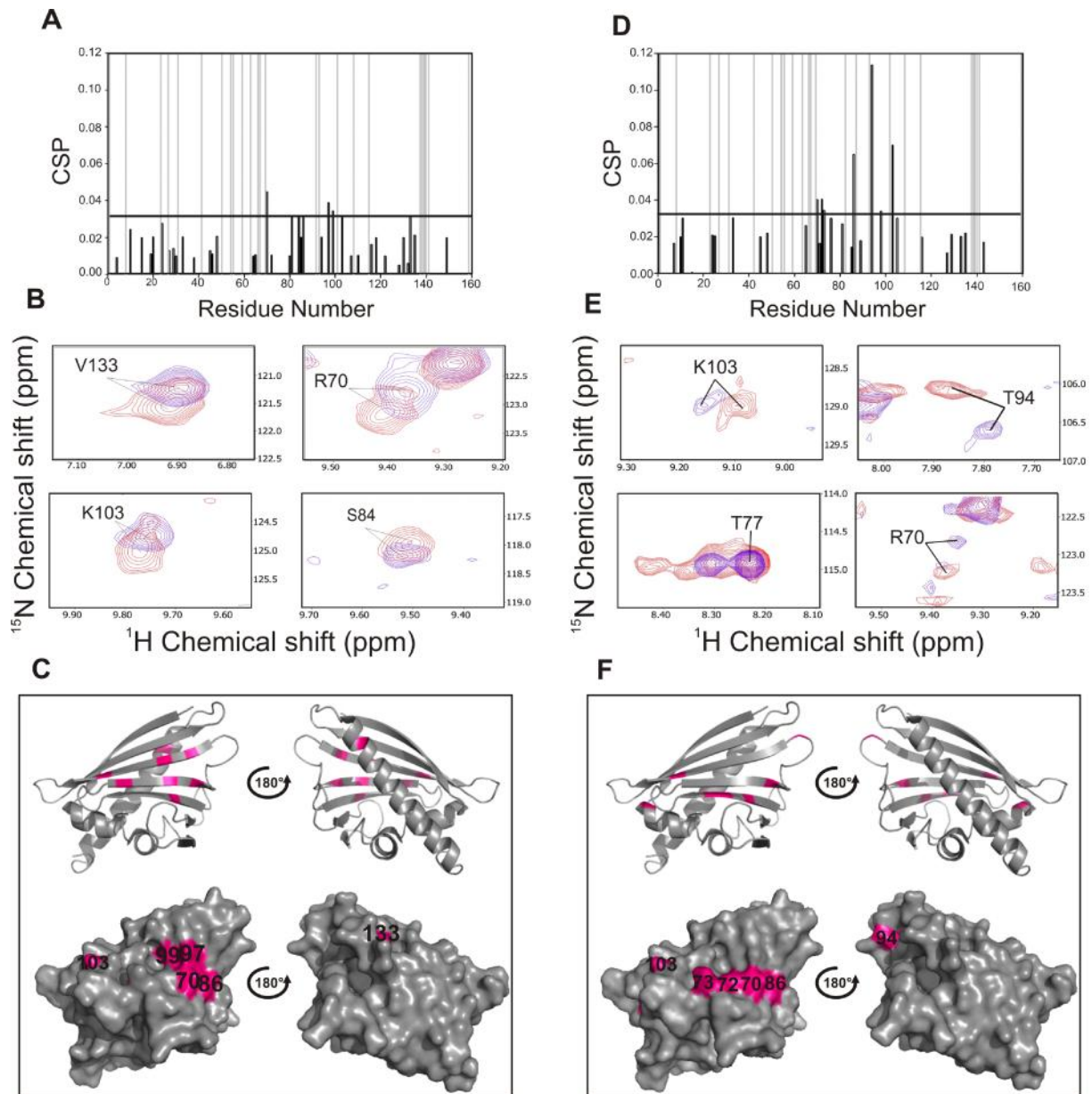


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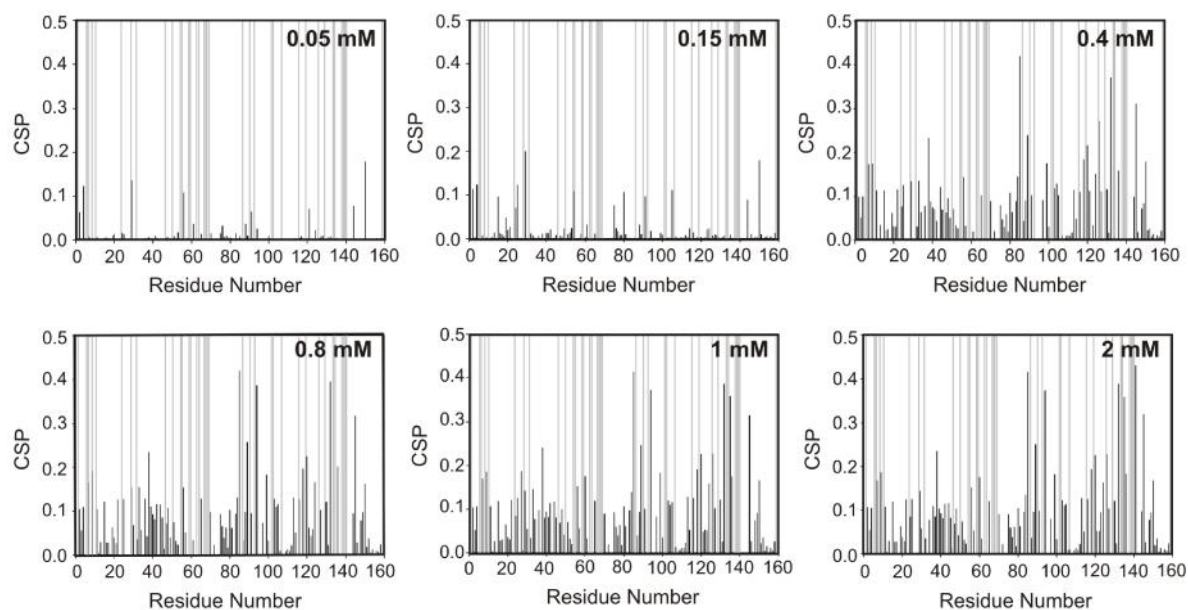


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