

Roles of Structure and Structural Dynamics in the Antibody Recognition of the Allergen Proteins: An NMR Study on *Blomia tropicalis* Major Allergen

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SUMMARY

Blo t 5 is the major allergen from *Blomia tropicalis* mites and shows strong IgE reactivity with up to 90% of asthmatic and rhinitis patients' sera. The NMR solution structure of Blo t 5 comprises three long α helices, forming a coiled-coil, triple-helical bundle with a left-handed twist. TROSY-NMR experiments were used to study Blo t 5 interaction with the Fab' of a specific monoclonal antibody, mAb 4A7. The mAb epitope comprises two closely spaced surfaces, I and II, connected by a sharp turn and bearing critical residues Asn46 and Lys47 on one surface, and Lys54 and Arg57 on the other. This discontinuous epitope overlaps with the human IgE epitope(s) of Blo t 5. Epitope surface II is further predicted to undergo conformational exchange in the millisecond to microsecond range. The results presented are critical for the design of a hypoallergenic Blo t 5 for efficacious immunotherapy of allergic diseases.

INTRODUCTION

Worldwide prevalence of allergic diseases has continued to increase in the past two decades, and house dust mite allergy is now recognized as a major risk factor for rhinoconjunctivitis, asthma, and atopic dermatitis (Holgate, 1999; Holt et al., 1999). It is estimated that ~10%–30% of the world population is affected by the house dust mite allergy. Allergens from clinically important mite species have been identified and categorized into groups based on their sequence homology. Groups 1 and 2 are the major allergens of the widely distributed *Dermatophagoides* genus of the dust mites (Thomas and Smith, 1999). Recent studies revealed that *Blomia tropicalis* mite allergens are clinically more important than *Dermatophagoides pteronyssinus* allergens in the densely populated tropical and subtropical regions, where dual sensitization by both mite species is common

(Chew et al., 1999; Puccio et al., 2004). The group 5 allergen Blo t 5 is clinically the most important major allergen from *B. tropicalis* mites. Epidemiological studies revealed that up to 90% of the asthmatic and rhinitis patients, particularly pediatric patients, from tropical and subtropical countries have strong IgE reactivity to Blo t 5 (Yeoh et al., 2003; Manolio et al., 2003). Unlike Blo t 5, its group 5 homolog, Der p 5, shows relatively weak IgE binding and surprisingly low IgE crossreactivity (Kuo et al., 2003). A recent study localizes Blo t 5 to the midgut and hindgut contents of *B. tropicalis* and also to mite fecal particles (Gao et al., 2007).

Three-dimensional structures of only a limited number of important allergens have been solved by NMR or crystallography. The biological functions of these allergens are diverse or unknown, and they lack any particular biological or structural feature that seems to predispose a protein to act as an allergen (Aalberse, 2000; Chapman et al., 2007). This study describes the three-dimensional solution structure of Blo t 5, which is the first, to our knowledge, solved structure of any group 5 dust mite allergen and major allergen identified with a triple-helical bundle fold. Blo t 5 does not have any close sequence homolog except other group 5 mite allergens, which share ~40% identity (Figure 1). Recently, we characterized a monoclonal antibody (mAb), 4A7, recognizing several isoforms of native Blo t 5 (Yi et al., 2005). Here, we also report data from NMR chemical shift perturbation and cross-saturation transfer experiments, and these data conclusively predict the location of the mAb 4A7 epitope. The epitope was found to be discontinuous and subdivided into two regions connected by a sharp turn, an arrangement that forms two closely spaced surfaces that contain 1 lysine residue each. Our data suggest that the two epitope surfaces on Blo t 5 interact with separate complementarity-determining regions (CDRs) of the antibody. Based on the Model-free analysis of the ¹⁵N-NMR relaxation measurements, we predict a slow millisecond to microsecond conformational exchange on one epitope surface and postulate a potential role for the structural dynamics in allergen recognition by antibodies. Our findings collectively shed light on how immunoglobulins recognize both structural and motional features of a protein antigen. The data also provide a basis for the design of hypoallergenic Blo t 5 variants for a safe and effective immunotherapy.

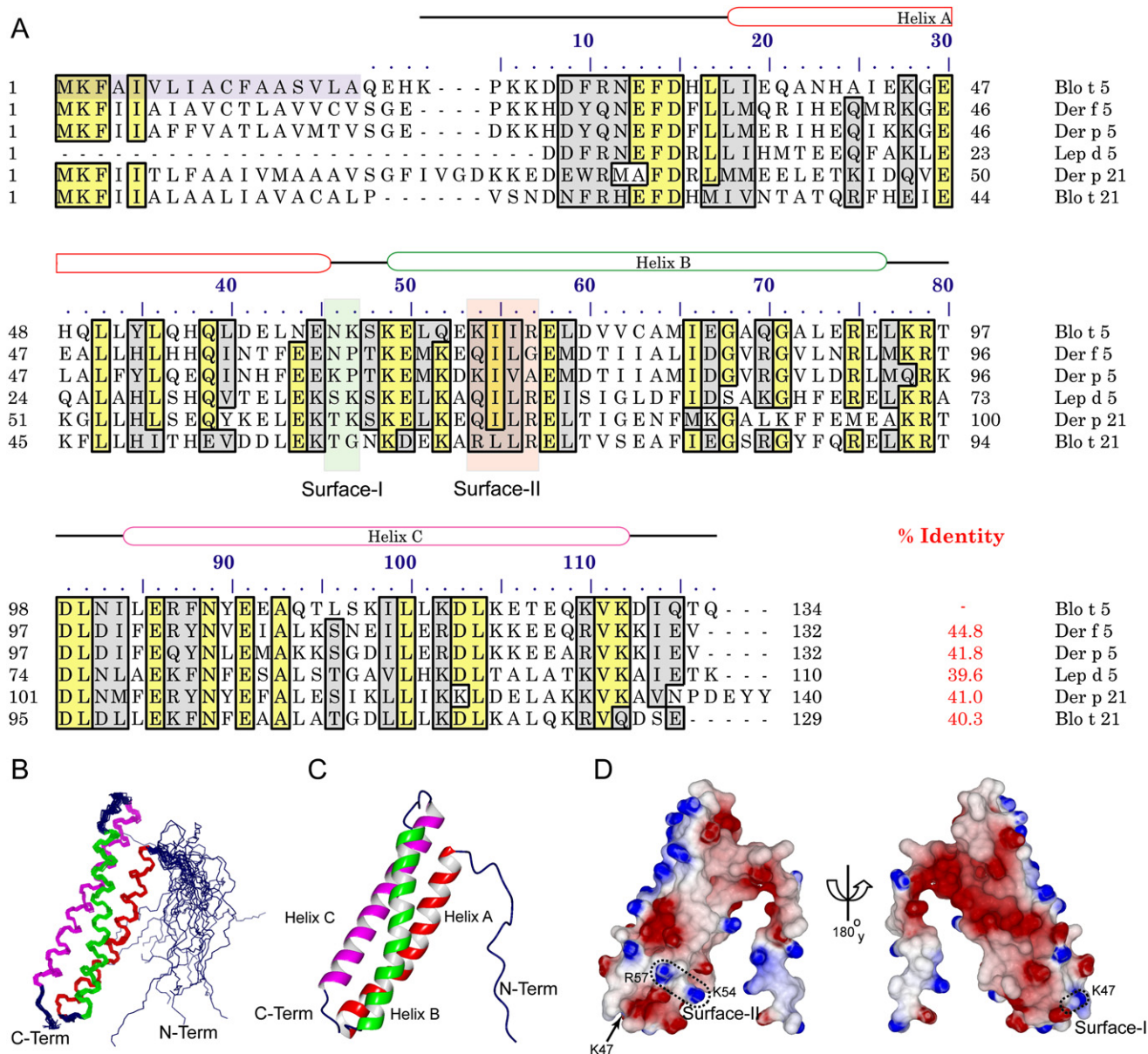


Figure 1. The NMR Solution Structure of the *Blomia tropicalis* Group 5 Allergen, Blo t 5

(A) The primary sequence alignment of the Group 5 and the Group 21 allergens from the different mite species, viz. *Blomia tropicalis*, *Lepidoglyphus destructor*, *Dermatophagoides farina*, and *D. pteronyssinus*. The sequence identities and the conservative substitutions are shaded yellow and gray, respectively, and the percentage identity with respect to Blo t 5 is shown at the end. The numbers on the left and the right sides of the sequences are indicative of the residue numbering for the proteins encoded in the cDNA sequence deposited in the databases with the following accession numbers: Blo t 5, O96870; Der f 5, BAE45865; Der p 5, P14004; Lep d 5, Q9U5P2; Der p 21, ABC73706; and Blo t 21, AY800348. The 17 residue leader peptide of Blo t 5 is boxed, and the residues of mature Blo t 5 are numbered in blue on the top along with the secondary structure. The critical residues of the mAb 4A7 epitope are located on surfaces I and II as indicated.

(B) The NMR solution structure of Blo t 5 shown as an ensemble of 20 conformers overlaid with the backbone atoms of the helical residues Leu18–Asp113 on the lowest-energy first conformer.

(C) The ribbon representation of the lowest-energy conformer of Blo t 5. The three helices are numbered A, B, and C and are colored red, green, and purple, respectively.

(D) Two orientations of the Blo t 5 surface charge distribution generated by a 180° flip along the y axis and colored with blue for positive charge and red for negative charge.

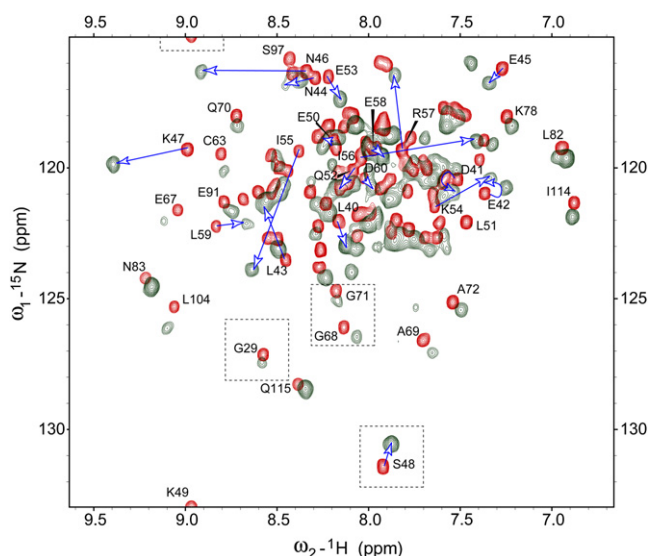


Figure 2. The TROSY-HSQC NMR Spectra of Blo t 5 and Fab'-Bound Blo t 5

An overlay of a 2D ^{15}N 2D-TROSY-HSQC spectrum of Blo t 5 (in red) and the Fab'-Blo t 5 complex (in green) acquired under identical conditions of 37°C and pH 7.5. For simplicity, only isolated peaks are annotated, and the chemical shift perturbation for a stretch of residues between Leu40 and Asp60 is marked by arrows. The peaks boxed with a dotted line are aliased in the indirect ^{15}N dimension. It must be noted that all NMR data used for the Blo t 5 structure and the backbone dynamics calculations were acquired at 22°C.

RESULTS

Three-Dimensional Structure of Blo t 5

We have employed a multifaceted approach, including analytical ultracentrifugation, small-angle X-ray scattering, gel filtration, crosslinking, and NMR, to confirm the monomeric state of Blo t 5 under our experimental conditions (data not shown). Blo t 5 gave a reasonably resolved, monodispersed spectrum (colored red in Figure 2), and practically complete ^1H -, ^{13}C -, and ^{15}N -NMR resonance assignments were attained by using the triple resonance experiments (Naik et al., 2007). The structure of Blo t 5 was determined by using distance and angle restraints derived by using the solution NMR methodology and is represented as a 20 conformer ensemble in Figure 1B. It was calculated by using a total of 1371 or, on average, 12 experimental restraints per residue as described in Table 1. The helical nature of this protein, combined with the high propensity of leucine residues in the hydrophobic core causes severe spectral overlap of aliphatic proton resonances. This is the prime reason for a somewhat lower count of the unambiguously identified NOEs. The first 16 residues from the N terminus of the protein are poorly defined due to the intrinsic disorder, but the remaining structure shows high precision, with a root-mean-square deviation of 0.40 ± 0.06 Å for the backbone atoms. None of the conformers in the ensemble violate experimentally derived distance or torsion angle restraints by more than 0.1 Å and 5° , respectively, and $92.40\% \pm 1.07\%$ of residues fall in the energetically most favored regions of the Ramachandran plot.

A representative conformer of Blo t 5 is shown in a ribbon representation in Figure 1C. Blo t 5 is a triple-stranded, coiled-coil

Table 1. Summary of the Conformational Restraints and the Statistical Analysis

Experimental Restraints	
NOE distances ^a	1105
Intraresidues (i, i)	368
Sequential (i, i ± 1)	315
Medium range ($1 < i - j < 5$)	269
Long range ($ i - j \geq 5$)	153
Hydrogen bonds	156
Dihedral angles	188
ϕ ($^\circ$)	95
ψ ($^\circ$)	93
Rmsd from experimental restraints	
Distances (Å)	0.005 ± 0.001
Dihedral angles ($^\circ$)	0.334 ± 0.064
Rmsd from the Average Structure	
Structured region residues, 17–117	
Backbone (Å)	0.40 ± 0.06
Heavy atoms (Å)	1.01 ± 0.06
Quality Checks	
Combined Z-score (Prosa2003) (Sippl, 1993)	-5.39 ± 0.24
Overall average G factor (PROCHECK) (Laskowski et al., 1993)	0.31 ± 0.02
Ramachandran plot (PROCHECK)	
Most favored regions (%)	92.40 ± 1.07
Additional allowed regions (%)	7.34 ± 1.31
Generously allowed regions (%)	0.27 ± 0.42
Disallowed regions (%)	0
CYANA "Target Function" ($\text{\AA}^2$)	0.84 ± 0.18

^a Derived from 1901 assigned NOESY crosspeaks, including symmetry-related and intraresidue peaks.

bundle in which the first helix (Helix A; residues 18–46) and the third helix (Helix C; residues 84–113) are in parallel arrangement and in which the second helix (Helix B; residues 49–77) runs antiparallel to the other two helices. All three helices of Blo t 5 appear to be slightly tilted, with interhelical angles of $\sim 20^\circ$, and are connected with sharp turns. Each helix in Blo t 5 contains a minimum of 28 residues or 4 heptad repeats—a 7 residue pattern denoted by $(abcdefg)_n$ (Liaw et al., 2001), although the heptad positions for the second helix in the structure are different. A simplistic helical wheel representation of the identified long-range NOE patterns is shown in Figure 3; residues are color coded by using the Wimley and White hydrophobicity scale (Wimley and White, 1996). Most of the 34 hydrophobic residues in Blo t 5 occupy heptad positions *a* and *d*, and they internalize against positions *d* and *a*, respectively, from the neighboring coils. The major exception to this trend exists on Helix B, on which the hydrophobic residues also populate position *e*. The bundle is primarily held together by this alternate hydrophobic burial, which roughly constrains each coil to slightly less than the standard 3.6 residues per α -helical turn and results in substantial curvature in the helical axis. With the exception of Helix B, heptad positions *e* and *g* of Blo t 5 helices are mainly occupied by polar residues, and the water-exposed positions *b*, *c*, and *f* are

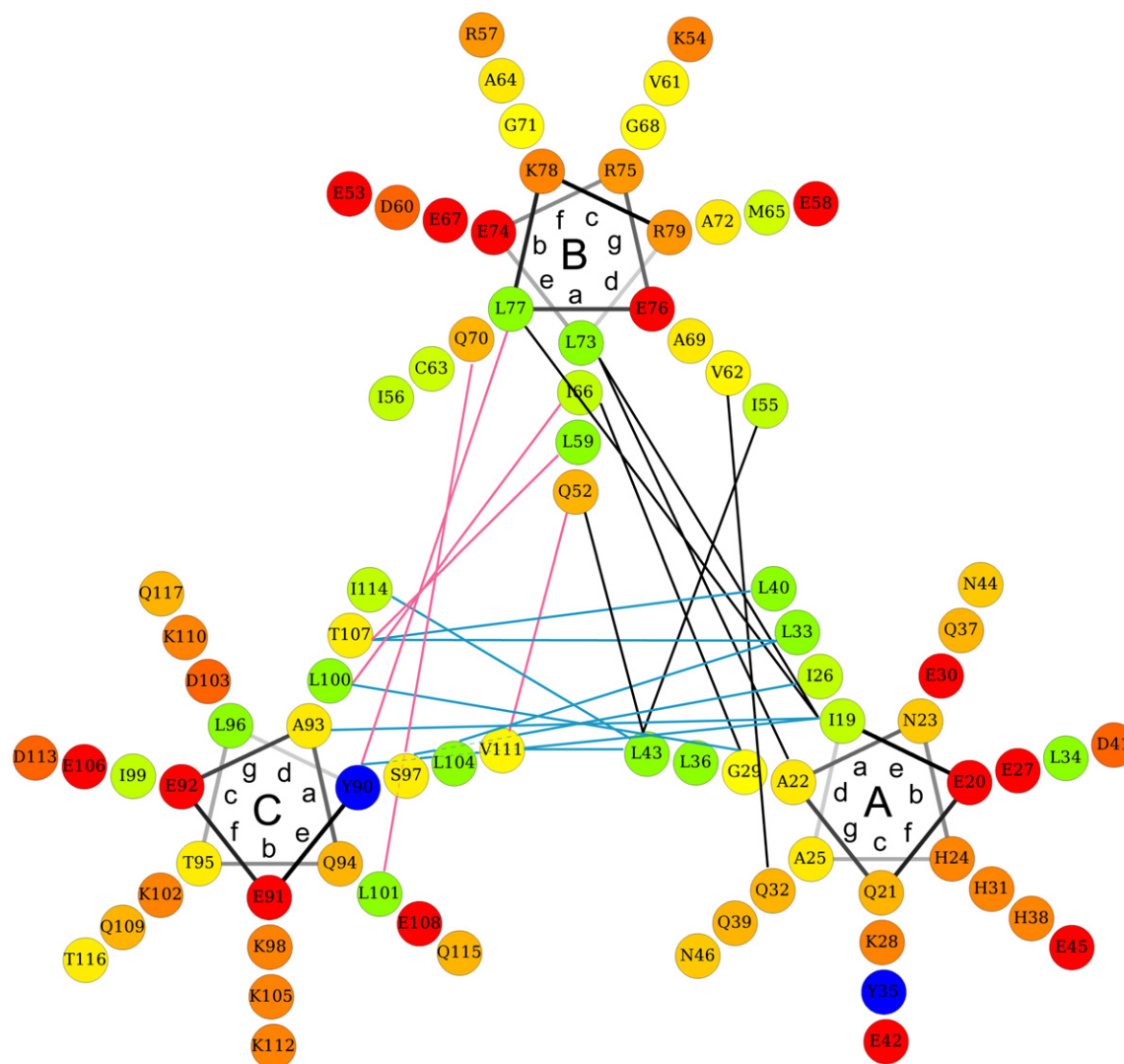


Figure 3. A Helical Wheel Representation

The residues on the helical wheel are color coded in blue-green-yellow-red according to the Wimley and White hydrophobicity scale. The aromatic residues are shown in blue. The hydrophobic aliphatic residues are green, and their shade turns more yellowish with decreasing hydrophobic index; similarly, the polar residues are colored orange, and, finally, the charged residues range from dark orange to red. Identifiable NOEs between Helix A and Helix B are shown with black lines, those between Helix B and Helix C are shown with purple lines, and those between Helix C and Helix A are shown with cyan lines. Helix B is drawn in a reverse direction to indicate its antiparallel orientation with respect to Helix A and Helix C.

predominantly occupied by charged residues. As shown in Figure 1D, the surface charge is localized in large negative or positive patches, but none of these make either of the two epitope surfaces described below. Interestingly, the charge distribution around epitope surface II displays a series of basic-nonpolar patches sandwiched between two large acidic regions. This unique pattern potentially confers the binding specificity to mAb recognition.

¹⁵N Relaxation Study and Backbone Dynamics

Protein structure derived by experimental methods like NMR or X-ray crystallography is generally believed to depict thermodynamic ground state conformation. The conformational exchange and structural mobility potentially play important roles in the pro-

tein function (Boehr et al., 2006). However, there is paucity of information regarding the quantitative relationship between structural coordinates and structural mobility; moreover, there is only limited information available about the motional behavior of the allergenic proteins, and absolutely nothing is known about their epitope regions. To our knowledge, among the allergenic proteins, ¹⁵N relaxation of only minor birch pollen allergen, Bet v 4, has been studied previously (Neudecker et al., 2004), but no information about its epitope is available in the literature. The backbone ¹⁵N atoms of Blo t 5 at 600 MHz have average NMR longitudinal (R_1) and transverse (R_2) relaxation rate constants of $1.03 \pm 0.14 \text{ s}^{-1}$ and $18.23 \pm 3.19 \text{ s}^{-1}$, respectively, as well as a $\{^1\text{H}\}$ -¹⁵N NOE ratio 0.74 ± 0.31 (Figure S1; see the Supplemental Data available with this article online). The uniform

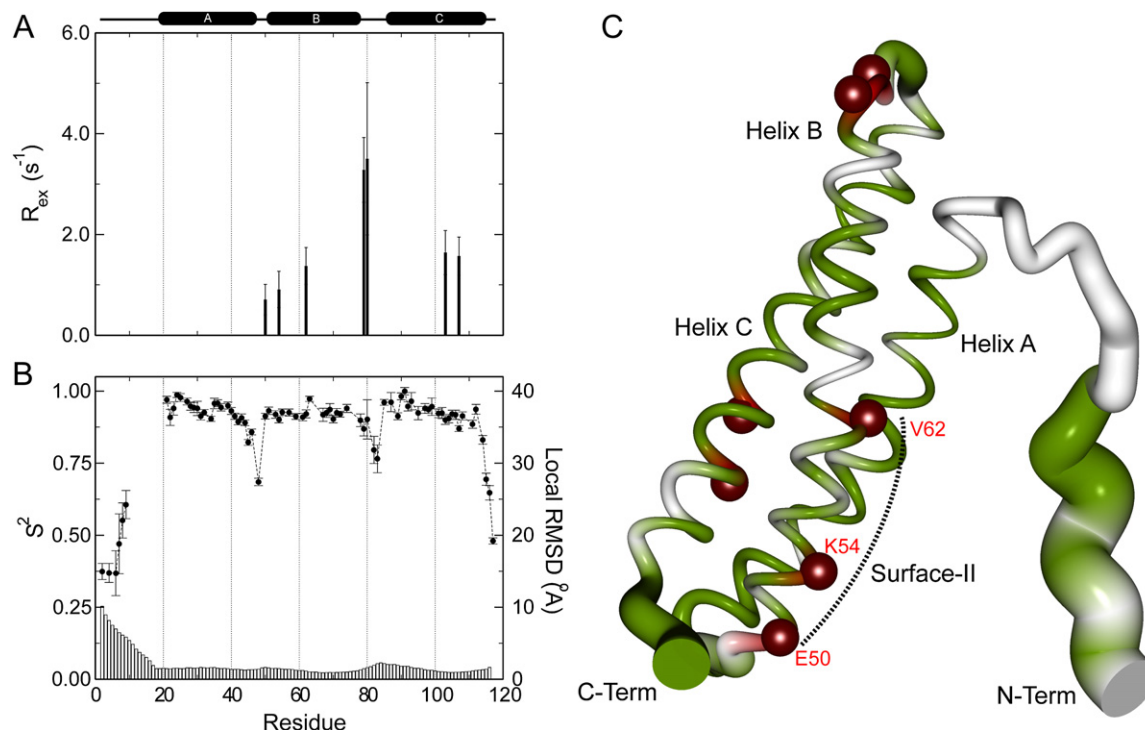


Figure 4. The Backbone Dynamics of Blo t 5

(A) The residues that undergo millisecond-microsecond chemical exchange are shown as a histogram against residue number. The error estimates are as calculated by the program Modelfree.

(B) The picosecond timescale plasticity of the Blo t 5 backbone. On the top is a scatter plot of the order parameter, S^2 , for Blo t 5, whereas a histogram of the local root-mean-square deviations seen in the Blo t 5 NMR structure ensemble is shown at the bottom; both are plotted as a function of the residue number. A schematic representation of the secondary structure is shown above the panel. The error estimates are as calculated by the program Modelfree.

(C) The order parameters shown in (B) are mapped on the Blo t 5 backbone, shown as a green sausage. The radius of the sausage is directly proportional to the $(1-S^2)$ value (i.e., wider regions indicate more flexible parts of the protein). White regions indicate the lack of experimental data and are interpolated between the neighboring residues. Red balls indicate residues that, in addition, are identified to undergo millisecond-microsecond chemical exchange and require the R_{ex} term for modeling the relaxation data as shown in (A).

distribution of the R_1 rate constant and heteronuclear NOE ratio, with the exception of the N-terminal and the two interhelical turn regions, suggest highly ordered placement and compact packing of the three helices; whereas the near uniformly elevated R_2 rate constant can be explained by the slower tumbling of Blo t 5 in the solution due to its elongated ellipsoid shape. The relaxation data were then interpreted by using the extended Lipari-Szabo Modelfree formalism to gain insights into the magnitudes and the timescales of the backbone motion (Mandel et al., 1995). The analysis considered five semiempirical forms of the spectral density function, with each form composed of terms describing the motion of the N-H-bond vector, and effectively elucidates both nanosecond-picosecond timescale mobility of the protein backbone (S^2, τ_e) as well as a slow millisecond to microsecond conformational exchange (R_{ex}). It must be emphasized that the motional anisotropy also contributes to the R_2 values and, if wrongly characterized, can cause erroneous interpretation of R_{ex} (Kneller et al., 2002; Tjandra et al., 1995). Our analysis modeled Blo t 5 as an elongated prolate ellipse with the ratio of major to minor axis, $D_{||}/D_{\perp}$, of 1.25 ± 0.03 .

The values of the Modelfree parameters (S^2 , τ_e , and R_{ex}) are listed in Table S1. Out of 80 quantifiable residues in Blo t 5, 77 could be satisfactorily fit by using the Modelfree analysis,

whereas relaxation data of Leu17, Lys47, and Ala64 could not be fit. Model 1 (S^2 -only) was an appropriate fit for 45 residues, 13 residues fit to model 2 (S^2 and τ_e), 1 residue fits to model 3 (S^2 and R_{ex}), 6 residues fit to model 4 (S^2 , τ_e , and R_{ex}), and 12 residues fit to model 5 (S^2 , S^2_{ss} , and τ_e). The sequence variation of the chemical exchange term, R_{ex} , and the generalized order parameter, S^2 , are plotted in Figures 4A and 4B, respectively. The average order parameter for Blo t 5 is 0.88 ± 0.14 ; if only residues located in the regions of regular secondary structure are considered, the average order parameter is 0.943 ± 0.030 . The order parameters are mapped as the radius of a green sausage on the Blo t 5's backbone in Figure 4C. The protein has a long and floppy N terminus, where the order parameter sharply drops below 0.5. Apart from being poorly defined in the structure (Figure 1B and histogram in Figure 4B), these N-terminal residues also have chemical shifts very similar to those in the random coil conformation and very low amide protection factors (Supplemental Data). This information indicates that the N terminus is in a random coil conformation and does not interact with the structured regions of Blo t 5. The order parameter also drops, though to a much lesser extent, around the two interhelical turns and the C terminus. The motional plasticity exhibited by the turns probably is essential to hold the rigid helical assembly.

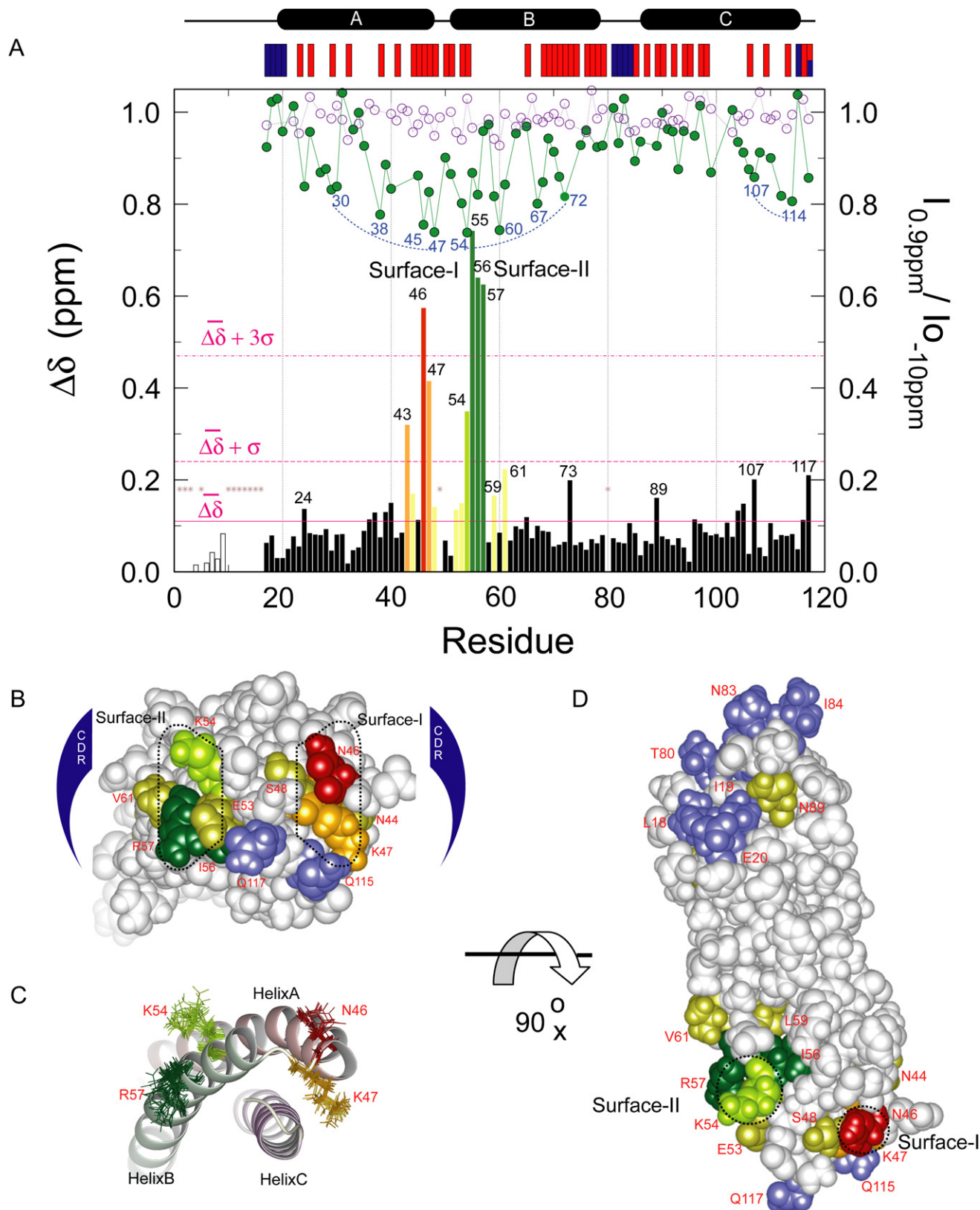


Figure 5. The NMR Localization of the mAb 4A7 Epitope

(A) A histogram showing chemical shift perturbation in Blo t 5 backbone amide resonances upon Fab' complex formation. The statistical significance of the change seen for an individual residue can be judged by the purple horizontal lines. The major changes seen for residues concluded to be the epitope are colored

The presence of slow millisecond to microsecond exchange is also evident in the Blo t 5 molecule (Figure 4A). Residues predicted to undergo slow conformational exchange involve Lys54 from epitope surface II and two other residues, Glu50 and Val62, in the vicinity of Lys54. Two other distal residues, Arg79 and Thr80 from the Helix B-Helix C turn, and residues Asp103 and Thr107 from Helix C are also recognized to undergo slow millisecond-microsecond motion.

NMR Identification of the mAb Epitope

Blo t 5-specific mAb 4A7 used in this study was purified from the mouse ascites fluid (Yi et al., 2005). To facilitate NMR study, its proteolytically cleaved fragment antigen binding Fab' was isolated. A sandwich ELISA assay on the dimer F(ab')₂ fragment and the intact mAb showed identical binding ability for Blo t 5, confirming the integrity of Fab' and ruling out nonspecific damage to the CDR (data not shown). The 2D TROSY-HSQC spectrum (Pervushin et al., 1997) of Fab' bound to the perdeuterated Blo t 5 is shown as the green spectrum in Figure 2. In a systematic NMR titration, the Fab'-bound Blo t 5 population emerges as a distinctly separate set of peaks from those of the free Blo t 5, as expected due to the tight binding. All bound-form resonance assignments were obtained from sequential amide-amide cross-peaks in an acquired 3D-¹⁵N-edited NOESY spectrum, taking advantage of the TROSY (Horst et al., 2006), at a 100 ms mixing time on the purified Fab'-Blo t 5 complex sample (Figure S2). Backbone amides of all but Gln1-His16, Lys49, and Thr80 were assigned for both free and Fab'-bound Blo t 5 at 37°C. Additional tentative assignments for N-terminal residues Lys4, Lys6, Lys7, Asp8, and Asp9 in the Fab'-Blo t 5 complex were achieved at 22°C by sheer comparison to the free form. All five of these resonances can only be observed at a lower temperature, belong to the randomized N terminus, and do not show any significant perturbation from the free form. The compounded magnitude ($\Delta\delta$) of the ¹H chemical shift change (δ_{HN}) and the ¹⁵N chemical shift change (δ_{N}) on Fab' binding was calculated by using $\Delta\delta = ([\delta_{\text{HN}}]^2 + [\delta_{\text{N}}/6.49]^2)^{1/2}$ (Ayed et al., 2001), and the results are shown in Figure 5A. Most of the amides, except those belonging to residues concluded as being the epitope regions, show uniform chemical shift perturbation with a 10% trimmed average of 0.09 ppm, and this finding indicates that Blo t 5 retains its triple-helical fold in the Fab'-bound state.

The chemical shift perturbations are most pronounced on two closely spaced surfaces on Blo t 5. Epitope surface I comprises residues Leu43–Lys47 of the C-terminal end of Helix A, and epitope surface II comprises residues Lys54–Arg57, located in Helix B.

These two epitope surfaces are separated by 6 residues in the sequence. The most perturbed residues, i.e., residues with $\Delta\delta > 0.24$ ppm or one standard deviation from the average $\Delta\delta$, are Leu43, Asn46, Lys47, Lys54, Ile55, Ile56, and Arg57. Among these residues, Leu43 and Ile55 are buried next to each other in the free form, and NOEs can be seen between their side chain protons (Figure 3); thus, both of these residues appear to be an integral part of the hydrophobic core and are unlikely to have direct interaction with Fab'. Similarly, Ile56 is a partially buried residue. Few other distal, buried residues, like Leu73, Asn89, and Thr107, show perturbation higher than the average. The large chemical shift perturbation of these buried residues suggests that, upon binding to the Fab' fragment, the protein core undergoes certain degree of structural rearrangement, particularly around the epitope surfaces. An interesting feature of epitope surface I perturbation is that only the proton chemical shifts vary, with minimal change in the ¹⁵N chemical shifts, and that the peaks move in an opposite downfield direction compared to the upfield movement of epitope surface II peaks (Figure 2). We do not have an explanation for this observation, although ring current shift due to adjacent aromatic ring(s) on the antibody CDR can potentially be the cause.

Figures 5B–5D show spatial localization of the perturbed residues with different color code for the extent of chemical shift perturbation. The two epitope surfaces are located at the C-terminal end of the helical bundle, and surfaces I and II are positioned on the opposite faces of the molecule (Figures 5B and 5D). In the NMR ensemble, the shortest distance between backbone amides of the two surfaces is from Lys47 to Lys54 (11.6 ± 0.4 Å), and if the side chains of these lysines are taken into account, the distance between the two N^ε atoms is 19.9 ± 1.1 Å. Strikingly, residues between surface I and surface II, namely, Ser48, Glu50, Leu51, Gln52, and Glu53, show very insignificant chemical shift changes. Thus, epitope surface I perturbation does not appear to be a secondary effect of binding on epitope surface II, and these residues probably are approached by different CDRs on the Fab' fragment. Epitope surface II is perturbed more strongly and is probably the site of major and broader interaction.

We performed the cross-saturation transfer experiment to further confirm epitope localization (Shimada, 2005). The experiment was designed to identify interfacial residues on uniformly ²H, ¹⁵N-labeled Blo t 5 by selectively irradiating the protonated methyl resonances in the larger Fab' fragment. Any reduction in the peak intensity of Blo t 5 amide resonances compared to the control experiment can mainly be attributed to the saturation

yellow to red and yellow to green for surfaces I and II, respectively. The data collected at 37°C are shown with filled bars, whereas those collected at 22°C are shown with open bars. Unassigned residues are shown by asterisks. Also shown on top is a scatter plot of the change in peak intensity against the residue number for the cross-saturation transfer experiment performed on ²H, ¹⁵N-Blo t 5 bound to nonlabeled Fab' (green, filled circles) and the control experiment performed on the free ²H, ¹⁵N-Blo t 5 (violet, open circles). The connecting line and the dotted curve are shown to highlight the trend. Shown on top is a strictly qualitative assessment of the change in water accessibility upon Fab' binding at 37°C by using a CLEANEX-PM experiment at a fairly long 100 ms mixing time. The residues coded blue can be seen in both the free and Fab'-bound Blo t 5 spectra, whereas the residues coded red are seen only in the free Blo t 5 spectrum. Gln117 is coded with both red and blue, as only its side chain amide is visible in the bound-form spectrum.

(B and C) (B) The space-filled and (C) the ribbon representation of the spatial distribution of the two epitope regions; epitope surface I is colored yellow to red, and epitope surface II is colored yellow to green. Each individual residue is color coded yellow for a $0.11 \text{ ppm} < \Delta\delta < 0.24 \text{ ppm}$ chemical shift change, orange or pale green for a $0.24 \text{ ppm} < \Delta\delta < 0.47 \text{ ppm}$ chemical shift change, and red or olive green for a $\Delta\delta > 0.47 \text{ ppm}$ chemical shift change. Residues that are water exposed in both the free and Fab'-bound form are colored blue. (C) shows how the side chains of Asp46, Lys47, Lys54, and Arg57 are defined in the different conformers of the NMR ensemble.

(D) An orthogonal view of (B). The N-terminal residues, Gln1–His16, which obstruct the epitope visualization, are not shown in the figure.

transfer from the nearby Fab' fragment. The results are shown as a scatter plot in Figure 5A. It must be noted that spin diffusion by the NH-NH pathway, particularly in the helical proteins, reduces the efficiency of this experiment to pinpoint the exact surface. Therefore, the contact surface predicted by this experiment is broader than that seen by the chemical shift perturbation analysis. Nevertheless, up to a 20% reduction in the intensity was observed around the putative epitope surfaces I and II, confirming the finding of the chemical shift perturbation data. Residues Asn46, Lys47, and Lys54 appear to be the three key residues, which strongly interact with the mAb CDRs. The intensity ratio can also be seen dropping away from these residues with a periodicity of 6–8 residues, which appears to be consistent with the heptad arrangement.

Water Accessibility of the Epitope

Hydrogen/Deuterium (H/D) exchange rates provide information about the water accessibility of different backbone amides and have previously been used for epitope mapping of the major allergen Der p 2 (Mueller et al., 2001). We calculated H/D protection factors for Blo t 5 at 22°C, and the results are shown in Figure S3. Unfortunately, quantitative analysis of the Fab' fragment-bound Blo t 5 at 37°C was hindered due to ~3 hr of acquisition time required to collect a meaningful two-dimensional spectrum on the 0.2 mM sample used in our study. A qualitative estimate of the change in water accessibility when using the CLEANEX-PM experiment (Hwang et al., 1998; Supplemental Data) is shown as color-coded bars on top of Figure 5A. Residues from both epitope surfaces appeared to be water accessible only in the free Blo t 5 (red bars on top of Figure 5A), whereas those located far from the epitope, toward the N-terminal end of the helical bundle, i.e. residues Leu17–Glu20 and Asp81–Ile84, were found to be water accessible in free as well as bound Blo t 5 (blue bars on top of Figure 5A). Thus, the bulky Fab' fragment appears to hinder the free approach of water molecules to the epitope region and is in general agreement with our epitope localization. Interestingly, Gln115 backbone and side chain amides as well as Gln117 side chain amides can still be seen in the CLEANEX-PM spectrum. Both of these residues are situated at the tip of the helical bundle flanked by epitope surfaces I and II, a region that is likely to be buried inside the bulky Fab' fragment (Figure 5B). This observation is in general agreement with a model that shows that Blo t 5, like Hyaluronidase (Hyal), is engulfed by two or more CDRs and that the exchanging water is trapped inside the cavity. Water molecules are commonly seen at the Ag–Ab interface and are proposed to improve the fit by neutralizing unpaired hydrogen-bonding groups (Padavattan et al., 2007).

IgE Reactivity of Blo t 5

The localization of human IgE epitopes in Blo t 5 was performed by using two overlapping Blo t 5-derived peptides, Blo t 5_{1–80} and Blo t 5_{46–117}. The peptides were designed to keep the helical elements intact; thus, as they either begin or terminate at the interhelical turn positions of the full-length protein, they retain the key residues comprising the two epitope surfaces for mAb 4A7 determined by NMR. Except for the first three N-terminal residues, Blo t 5_{46–117} resembles an extensively studied truncated form of Blo t 5 designated as BtM (Caraballo et al., 1998). The

IgE reactivity of these peptides was tested against 28 Blo t 5-specific IgE-positive human sera and was compared to the full-length protein by direct and crossinhibition IgE ELISA. In the direct ELISA, the Blo t 5_{1–80} peptide reacted poorly with the Blo t 5-specific IgE ($p = 0.005$), whereas the Blo t 5_{46–117} bound a substantial but smaller amount of Blo t 5-specific IgE as compared to that of the full-length Blo t 5 ($p = 0.05$) (Figure 6A). The antigen preabsorption study showed significantly lower inhibitory capability of Blo t 5_{46–117} to the plate-bound Blo t 5 as compared to that of the full-length Blo t 5 ($p < 10^{-5}$), with a maximum inhibition $< 50\%$, with two exceptions. The inhibition capability of Blo t 5_{1–80} to Blo t 5 was even lower than that of Blo t 5_{46–117}, with a maximum inhibition of less than 20% ($p < 10^{-5}$) (Figure 6B). Both peptides were further studied by circular dichroism and NMR. Peptide Blo t 5_{1–80} was found to be unfolded at room temperature, which explains the major role played by Helix C in the structural assembly of Blo t 5. Analytical centrifugation and gel filtration results show that peptide Blo t 5_{46–117} exists as a dimer and has a heavily perturbed HSQC spectrum compared to that of Blo t 5 (Figure S4). It appears that the lack of Helix A in Blo t 5_{46–117} is compensated by an alternate packing, leading to a probable homodimeric, antiparallel coiled-coil arrangement. These data reveal that the two large overlapping Blo t 5-derived peptides fail to retain the essential epitope conformation of the full-length Blo t 5, which results in significant reduction in IgE binding.

Since the key residues Asn46, Lys47, Lys54, and Arg57 comprising the discontinuous mAb 4A7 epitope are located in peptide Blo t 5_{46–117}, we further examined whether mAb 4A7 binding could block Blo t 5-specific human IgE binding to full-length Blo t 5 and Blo t 5_{46–117}. The preabsorption of mAb 4A7 inhibited human IgE binding to the full-length Blo t 5, but not to Blo t 5_{46–117}. mAb 4A7 could inhibit up to 45% of the total binding of Blo t 5-specific human IgE to the full-length Blo t 5 (Figure 6C). The mAb can inhibit IgE binding by several potential mechanisms, including (a) complete or partially overlapped epitopes, (b) separate, but closely spaced, epitopes in which the steric hindrance limits co-occupancy, and (c) strong perturbation of the distal IgE epitopes due to IgG binding. Low inhibition ($< 30\%$) is generally attributed to steric hindrance (Padavattan et al., 2007). Blo t 5 is an elongated helical protein that shows localized perturbation upon Fab' binding (Figure 5B), which strongly indicates that there is a substantial overlap of mAb 4A7 and dominant human IgE epitopes located in this region. The truncation of Helix A in Blo t 5_{46–117} likely altered both, or at least epitope surface I, epitope surfaces beyond recognition by mAb 4A7, which probably accounts for the lack of inhibition.

DISCUSSION

Structural Features of Blo t 5

Analysis of the protein hydrophobic core with the Socket program (Walshaw and Woolfson, 2001) with a distance cutoff of 7 Å showed that the hydrophobic core of Blo t 5 is mainly made up of Crick's knob-in-hole type of packing; a hydrophobic side chain or knob is buried against the hole formed by four or more side chains on the neighboring helices. Such packing can be further categorized depending on how the buried knob approaches the hole, and if it's an isolated one-way (Type-2)

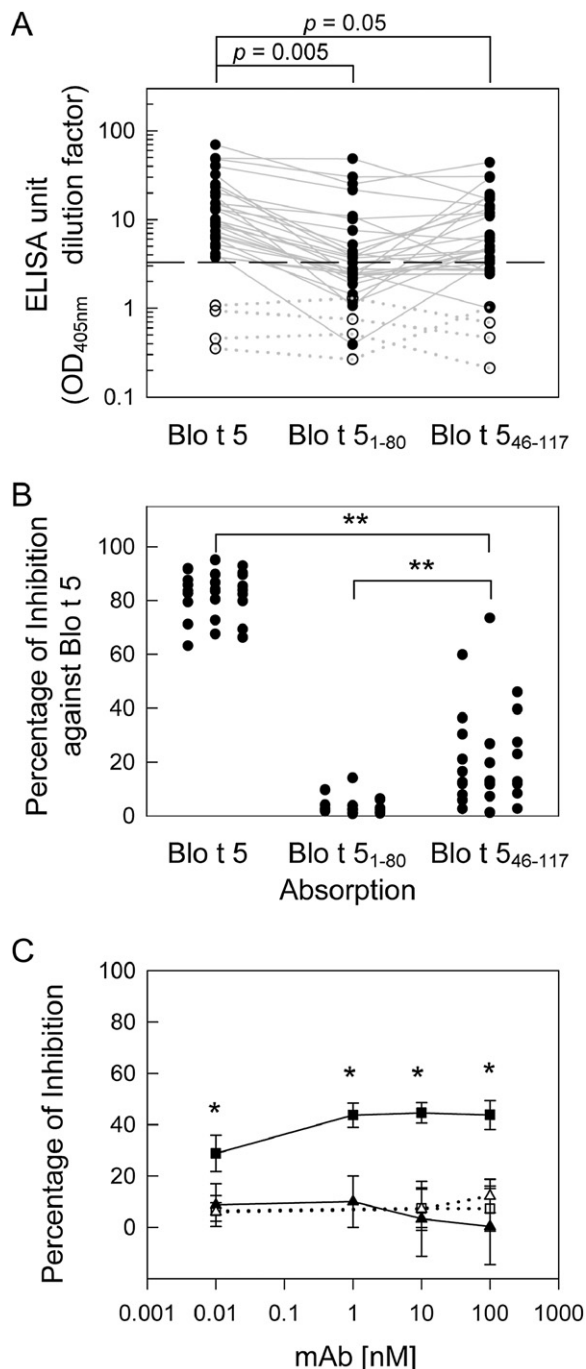


Figure 6. The Human IgE Reactivity of Blo t 5 and Blo t 5 Fragments

(A) The human IgE epitopes of the full-length Blo t 5, Blo t 5₁₋₈₀, and Blo t 5₄₆₋₁₁₇ were evaluated with 28 Blo t 5-positive sera (solid circles) from asthmatic children by direct ELISA. Four sera from nonatopic subjects (open circles) were used as controls. The cutoff value (long-dashed line) was determined by the mean plus 3-fold of the standard deviation of the control sera.

(B) Diluted sera were preabsorbed with 1 μ M full-length Blo t 5, Blo t 5₁₋₈₀, or Blo t 5₄₆₋₁₁₇ at 4°C for 16 hr and reacted with the plate-bound Blo t 5. The percentage of inhibition was calculated as $(1 - [\text{OD}_{405 \text{ nm}} \text{ with inhibitor} / \text{OD}_{405 \text{ nm}} \text{ without inhibitor}]) \times 100$. **, $p < 10^{-5}$ with Student's *t* test.

(C) Inhibition of the human IgE reactivity with mAb 4A7. The plate-bound full-length Blo t 5 (1–117, square) or the Blo t 5₄₆₋₁₁₇ (46–117, triangle) fragment

or a complementary burial with the knob itself acting as hole (Type-4). In triple- and higher-order helical bundles, a cyclically complementary Type-4 daisy chain arrangement can arise out of the hydrophobic burial in an interleaved fashion (Walshaw and Woolfson, 2001). The Blo t 5 structure has one such daisy chain arrangement formed by Leu36, Leu59, and Thr107 and their neighbors, from Helices A, B, and C, respectively. The rest of the core is mainly made up of isolated or Type-2 packing. Helix C appears to be the best packed among the three, utilizing either one-way or complementary burial of its side chain knobs against the side chain holes in either of the two neighboring helices. Unfavorable burial of polar or charged residues like Thr107 and Glu108 in the hydrophobic core is seen in 20% of the instances and is proposed to play an important role in determining structural uniqueness and specificity (Akey et al., 2001). In the context of Blo t 5, Glu108 can be seen to be buried against a hole formed by the side chain of the key epitope residue Ile56 and its neighbors. One of the main reasons for the left-handed supercoiling of the entire bundle is that Helix C preferentially partners Helix A in the beginning, with side chains of Ala93, Leu100, and Thr107 buried deeply in Helix A holes, and with Helix B toward its end, with Leu104, Glu108, and Val111 buried in Helix B. Truncation of an entire helix, as in peptide Blo t 5₁₋₈₀ and Blo t 5₄₆₋₁₁₇, compromises the structure and/or the active epitope conformation. Thus, all three helices are necessary for the structural integrity of Blo t 5 and demonstrate the limitation of mapping the conformational B epitopes with overlapping peptides (Figure 6).

Motional Features of Blo t 5

There are increasing evidences suggesting that conformational exchange plays an important role in many biological processes, like enzyme catalysis (Boehr et al., 2006). Modelfree analysis on Blo t 5 predicted conformational exchange along straight-stretch residues on Helix B. These residues involve Lys54 from epitope surface II and two other residues, Glu50 and Val62, in vicinity of Lys54. Of note, Glu58 amide resonance overlapped with Ile56 and hence no reliable relaxation data could be estimated for either of these residues. The residues showing slow millisecond-microsecond motion on epitope surface II are spaced 4 residues apart from each other, and H bonds typical of a helical backbone exist between them. Thus, the Modelfree prediction for this continuous stretch of water-exposed residues appears to be valid, and the carboxyl terminus end of Helix B, including the entire epitope surface II, is proposed to undergo slow conformational exchange. Interestingly, the amide of residue Thr107 and its H-bond partner, Asp103, were also recognized to undergo millisecond-microsecond exchange motion in the Modelfree analysis. This conformational exchange for epitope surface II thus appears to be a result of unfavorable burial of Glu108 and is potentially a prime feature recognized by mAb 4A7. However, Modelfree analysis involves assumptions that need further verification with more stringent experiments.

was incubated with serial diluted mAb 4A7 (closed symbol) or the mouse IgG1 (anti-TNP) as an isotype control (open symbol). Diluted human sera were reacted with the mAb-blocked antigens at 4°C for 16 hr. The results are presented as mean $\pm$ SD ($n = 5$); *, $p < 0.001$ by comparison with any of the other groups with the Student's *t* test.

Thus, we have predicted an interesting feature of allergen epitope surfaces, but our current data are insufficient to confirm if such motions are predominantly present on other allergen molecule epitopes.

Discontinuous B Cell Epitopes on Blo t 5

Though structure determination of the allergen-Ab complex will be the most conclusive method by which to elucidate B cell epitopes, disregarding most of the other antigen-Ab complexes found in literature, only two such structures have been published before now (Mirza et al., 2000; Padavattan et al., 2007). A discontinuous B cell epitope was observed on birch pollen major allergen, Bet v 1, for mAb BV16. This conformational epitope is mainly made up of residues between Glu42 and Thr52 (Mirza et al., 2000). Glu45Ser mutation was shown to completely abolish mAb binding and to reduce the polyclonal human IgE reactivity by 50% (Spangfort et al., 2003). In a recent study on the major bee venom allergen, Hyal, a 9 residue continuous epitope was identified against mAb 21E11. The conformational epitope is made up of Arg138 and a linear array of residues between His141 and Arg148. It is located at the tip of a helix-turn-helix motif that protrudes from the protein core and fits into a pocket formed by the six CDRs of Fab (Padavattan et al., 2007). mAb BV16 could inhibit ~40% of the binding to Bet v 1-specific human IgE (Mirza et al., 2000), whereas mAb 21E11 could inhibit up to 57% of IgE binding to Hyal (Padavattan et al., 2007). In comparison, mAb 4A7 inhibited ~45% of the Blo t 5-specific human IgE binding (Figure 6C), and its epitope was mapped on two surfaces connected by a turn, with the critical residues Asn46 and Lys47 on surface I and Lys54 and Arg57 on surface II. Interestingly, the group 5 allergen from *D. pteronyssinus*, Der p 5, showed relatively low IgE binding with only 40%–50% of sera from the mite allergic asthmatics. Despite the 42% sequence identity between these two group 5 allergens, the IgE cross-reactivity of Blo t 5 and Der p 5 is also surprisingly low (Kuo et al., 2003). The sequence alignment (Figure 1) reveals that the two epitope regions of Blo t 5 are not conserved in its homologs from other mite species. The critical Lys47 is replaced with proline residue in Der f 5 and Der p 5, whereas Arg57 is replaced with a glycine and an alanine in Der f 5 and Der p 5, respectively. Indeed, one may speculate that replacing the charged residues like lysine and arginine with a more rigid residue like proline could probably account for the lower allergenicity of these two allergens.

B Cell Epitopes of Other Mite Allergens

Only limited structural information is available regarding the conformational antibody epitopes of the dust mite allergens. The mAb epitopes of Der p 2, a major allergen from *D. pteronyssinus* mites, were predicted by NMR by using the H/D exchange experiments and were confirmed by mutagenesis (Mueller et al., 2001). The extensive study suggested that the probable epitope residues include Arg31, Lys33, Asn93, Lys97, and Ile96 along with other residues. Another study on Der f 2 used the NMR differential exchange broadening experiments in the presence of a detergent and identified residues Asp69, Asn71, and His74 as key residues of the epitope of mAb 15E11, and Arg128 and Asp129 as key residues of the epitope of mAb 13A4 (Ichikawa et al., 2005). The mutagenesis study published with the NMR structure

of a minor allergen, Der f 13, suggested that the major IgE epitope comprises residues Asp41, Lys63, Lys91, and Lys103 (Chan et al., 2006). In all three studies, the epitopes determined are discontinuous and invariably contain charged residues, with a notable proportion of lysine and arginine. Our experimental data are more stringent in mapping the epitope as compared to the published reports, partially due to the ellipsoid shape of Blo t 5, but it is very likely that the proposed experiments will work equally well for the epitope mapping of other more globular allergens.

Implication for Immunotherapy

Immunotherapy remains the only truly disease-modifying treatment for asthma and allergic rhinitis (Nelson, 2007). Traditional allergen extracts used in immunotherapy are prepared from natural sources and have numerous disadvantages, such as the presence of undefined material, huge variability in sample composition, and contamination of allergens from other sources (Valenta and Niederberger, 2007). These problems can be easily overcome with recombinant allergen and modified hypoallergen proteins (Linhart and Valenta, 2005). Here, we have solved the three-dimensional structure of the major allergen Blo t 5 and have mapped critical residues of the mAb 4A7 epitope by NMR. This discontinuous epitope is also shown to overlap with the human IgE epitope(s) of Blo t 5. Our current research focuses on the design of a hypoallergenic Blo t 5 protein. We have generated a panel of point and deletion Blo t 5 mutants, primarily targeting the various charged residues identified in the current study. These mutants are currently being evaluated by NMR analysis and human IgE binding assays.

In summary, we have solved the three-dimensional structure of the major allergen Blo t 5, which, incidentally, is the first major allergen identified, to our knowledge, with a triple-helical bundle fold. The critical residues of the mAb 4A7 epitope were conclusively mapped by NMR strategies that, to our knowledge, had previously never been implemented for the allergen epitope mapping. We have also studied backbone dynamics of Blo t 5 and predicted the presence of interesting slow conformational exchange on one epitope surface. Taken together, ours as well as previously published studies (Mirza et al., 2000; Spangfort et al., 2003) demonstrated that the mapping of conformational epitopes of monoclonal antibodies is an effective approach for identifying critical residues potentially recognized by the polyclonal human IgEs. Such valuable information greatly facilitates the design and production of the hypoallergenic allergens for safe and efficacious immunotherapy for allergic diseases.

EXPERIMENTAL PROCEDURES

Structure and Backbone Dynamics

NMR data were acquired in Shigemi tubes on 1 mM Blo t 5 buffered at pH 7.5 in 50 mM potassium phosphate, 100 mM NaCl, 2 mM EDTA, 0.001% (w/v) NaN₃, and minimum 10% (v/v) D₂O. All NMR experiments used for the structure calculation were carried out at 22°C on the Bruker Avance 800 MHz, 600 MHz, or 500 MHz NMR spectrometers equipped with a 5 mm triple-resonance cryoprobe and a single-axis pulsed field gradient by using standard pulse programs made available by the manufacturer. The data were acquired and processed by using the software XwinNMR, version 3.5 (Bruker, Germany), and were further analyzed in Sparky, version 3.112 (Goddard and Kneller, 2007).

^1H , ^{13}C , and ^{15}N resonance assignments for Blo t 5 were achieved by using standard triple-resonance experiments as described in the [Supplemental Data](#). The NOE distance restraints were based on a 3D simultaneous ^{15}N - and ^{13}C -edited NOESY-HSQC experiment (Sattler et al., 1995) and a 2D-NOESY, both acquired with a 100 ms mixing time. Semiautomated NOE cross-peak assignments were performed by using the CANDID module (Herrmann et al., 2002) in the CYANA software package and were manually checked for correctness in an iterative manner. Additional dihedral angle and hydrogen-bond restraints were derived as described in the [Supplemental Data](#). CYANA, version 2.1, was used for the structure calculation by using a simulated annealing protocol with torsion angle molecular dynamics (Güntert et al., 1997). A total of 100 random conformers were annealed in 10,000 steps for 7 cycles, and 20 structures with the lowest target function after the final cycle were selected to represent Blo t 5.

^{15}N relaxation experiments were performed on ^{15}N -labeled Blo t 5 samples at 22°C on a 600 MHz spectrometer. Details of the ^{15}N relaxation data acquisition and analysis, the diffusion tensor optimization, and the Model-free analysis are provided in the [Supplemental Data](#).

Fab' Preparation and NMR Epitope Mapping

The preparation and characterization of a monoclonal antibody (mAb), 4A7, recognizing several isoforms of Blo t 5 has been reported (Yi et al., 2005). A dimer, F(ab')_2 , was obtained by digesting the mAb with 20:1 pepsin for 2 hr at pH 4.0 and 37°C. The undigested intact mAb was separated from the F(ab')_2 by using a protein A affinity column and was redigested. The F(ab')_2 , either free or as a complex with Blo t 5, was subjected to mild reduction by using 0.1 mM β -mercaptoethanol (pH 8.5). This preferentially broke the interdomain disulfide bridge, resulting in Fab' or the Fab'-Blo t 5 complex, which was further purified with FPLC by using a Sephadex-200 column. The NMR data were acquired at 37°C on a 0.2 mM Fab'-Blo t 5 complex.

Fab' complexed with ^2H , ^{15}N -Blo t 5 was used for the TROSY cross-saturation transfer experiment (Shimada, 2005). The cross-saturation transfer was performed by using a 68.2 Hz band-selective inversion pulse centered at 0.9 ppm for a total saturation period of 1.5 s. The reference spectrum was acquired by centering the inversion pulse far upfield at -10 ppm. A control set of experiments was performed on the free ^2H , ^{15}N -Blo t 5 to check the marginal effect of the residual protonated aliphatic side chains. Slight variations due to the temperature change in the free Blo t 5 chemical shifts were crosschecked by using an HNCA experiment.

ELISA for Blo t 5-Specific Human IgE

A total of 28 asthmatic children's (age: 7–15 years) sera tested for the high IgE against Blo t 5 were used in this study. Four control sera from healthy volunteers (age: 20–40 years) without a history of allergy that tested negative to *D. pteronyssinus* and *B. tropicalis* extracts, native Der p 1, recombinant Der p 2, Der p 5, and Blo t 5 were used as the control sera. The collection of human blood was done according to the guidelines and regulations set by the National University of Singapore, Singapore.

The human IgE ELISA were carried out on 0.5 μM Blo t 5 or its truncated mutants with 50 μl per well as described previously (Kuo et al., 2003). Blo t 5-positive sera were diluted from 1:10 to 1:40 and were incubated with the allergen-coated wells at 4°C for 16 hr. For the absorption assay, final concentrations of 1 μM inhibitors (Blo t 5 or its truncated mutants) were preincubated with the individual serum at 4°C for 16 hr. The preabsorbed sera were then reacted with the plate-bound full-length Blo t 5. For the mAb inhibition assay, the plate-bound full-length Blo t 5 or Blo t 5_{46–117} was incubated with the serial-diluted mAb 4A7 or the isotype control for 1 hr and was washed before it was incubated with sera. The plate-bound IgE were developed as the direct ELISA described above.

Supplemental Data

Supplemental Data include additional Supplemental Experimental Procedures, one table, four figures, and Supplemental References and are available at <http://www.structure.org/cgi/content/full/16/1/125/DC1/>.

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Accession Numbers

The coordinates and the experimental restraints for the Blo t 5 structure are available from the Protein Data Bank under accession code 2JMH. The NMR chemical shift assignments were deposited in the Biological Magnetic Resonance Bank with accession code 7276.