



Analysis of the three-dimensional antigenic structure of giant ragweed allergen, Amb t 5

Thorunn Rafnar^{a,*}, Mary E. Brummet^a, Donna Bassolino-Klimas^b, William J. Metzler^b,
David G. Marsh^a

Division of Clinical Immunology, Johns Hopkins Asthma and Allergy Center, Johns Hopkins University School of Medicine, Baltimore, MD 21224, USA

Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543-4000, USA

Received 5 December 1997; received in revised form 27 April 1998; accepted 8 May 1998

Abstract

The ragweed allergens Amb t 5 and Amb a 5 are among the smallest inhaled protein allergens known, containing a single, immunodominant T-cell epitope. In this study we analyzed the B-cell epitope structure of Amb t 5. The three-dimensional structures of Amb t 5 and Amb a 5 have been determined by NMR spectroscopy, providing a rare opportunity to analyze three-dimensional antigenic sites. Amb t 5 residues likely to be important for antigenicity were identified by examining the surface area of Amb t 5 accessible to a probe of the size of an antibody molecule. After changing these residues to the corresponding Amb a 5 residues, recombinant proteins were purified and tested for loss of antigenic activity. Inhibition radio-immunoassays, using sera from 8 individuals who had received immunotherapy with giant ragweed extract, allowed the mutations to be divided into three groups: (1) mutations that had little or no effect on antibody binding, (2) mutations that caused a loss of antigenic activity to a different degree in different sera and (3) mutations that drastically reduced antigenic activity in all sera tested. This last set of mutations clustered in the third loop of Amb t 5, suggesting that antibody recognition of Amb t 5, like T-cell recognition, is primarily directed towards a single, immunodominant site. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Allergens; Epitope structure; Ragweed

1. Introduction

The structural elements of protein allergens are recognized at two levels by the immune system. T-cells recognize a short, linear peptide fragment bound to a major histocompatibility complex class II molecule, whereas B-cells produce IgE and IgG antibodies that can react either with linear epitopes or to antigenic sites that are determined by the tertiary structure of the protein (Benjamin et al., 1984; Germain, 1991). Immunochemical studies of allergens provide information about these immunogenic sites and have resulted in the development of experimental desensitization protocols that aim to provide a safer and more effective treatment for allergic diseases (Briner et al., 1993; Norman, 1993). To date, multiple

allergens have been cloned and their primary structures probed for the presence of B- and T-cell epitopes (Joost van Neerven et al., 1993; Liebers et al., 1993; Seiberler et al., 1994; van Kampen et al., 1994; Zhu et al., 1995). These mapping studies have mostly been performed with synthetic peptides, a method that is sufficient for delineating T-cell epitopes and B-cell epitopes that are comprised of linear amino acid sequences. Synthetic peptides, however, can not be used to identify B-cell epitopes of many important allergens such as the major ragweed, cat and mite allergens, because antibody recognition of these molecules is primarily directed towards conformational determinants (Olson and Klapper, 1986; Nilsen et al., 1991; O'Brien and Thomas, 1994; Seiberler et al., 1994; Vailes et al., 1994). Identification of conformational epitopes on structurally complex allergens can provide insight into which structural elements are important in antigenicity/allergenicity and may help to explain the clinically relevant allergenic crossreactivity that occurs

* Corresponding author. Present address: Icelandic Cancer Society, P.O. Box 5420, IS-125 Reykjavik, Iceland. Tel: +35-4-562-1414; Fax: 35-4-562-1417; E-mail: rafnar@krabb.is

between allergens from distantly related species (Ebner et al., 1995; Frankland, 1995).

To examine how a group of structurally complex allergens is recognized by human B-cells, we chose to study the homologous ragweed allergens, the Amb 5s. Amb t 5 (from giant ragweed, *Ambrosia trifida*) and Amb a 5 (from short ragweed, *Ambrosia artemisiifolia*) are among the smallest protein allergens defined to date, consisting of a single polypeptide chain of 40 and 45 amino acids respectively (Mole et al., 1975; Goodfriend et al., 1985). Specific immune responsiveness to both proteins is strongly associated with DR(α , β 1*1501) (Marsh et al., 1982a, 1982b; Huang et al., 1991) and cellular and molecular evidence suggest that the molecules contain a single, immunodominant T-cell epitope (Rafnar et al., 1993; Zhu et al., 1995). Amb a 5 and Amb t 5 have amino acid sequence identity of 45%, with each protein containing 8 conserved cysteine residues, all of which participate in disulfide-bond pairing. The immunological and physical properties of Amb t 5 and Amb a 5 have been studied extensively; both proteins have been cloned (Ghosh et al., 1991, 1993) and sequenced and their three-dimensional structures have been derived by NMR spectroscopy (Metzler et al., 1992a, 1992b). The Amb 5 allergens thus provide an exceptional opportunity to study the relationship between structure and immunological activity. Topologically, the structures of Amb t 5 and Amb a 5 are very similar; both molecules contain a triple-stranded β -sheet, three loops and a C-terminal α -helix. Despite their overall structural homology, Amb t 5 and Amb a 5 are essentially non-crossreactive antigenically (Goodfriend et al., 1985; Roebber et al., 1985), a feature that was used in the design of this study.

Here, we examined the B-cell epitope structure of Amb t 5, using mutagenesis of recombinant proteins. Amino acids of Amb t 5 predicted to be parts of antigenic sites (Novotny et al., 1986) were mutated into the corresponding Amb a 5 amino acids. The resulting molecules were tested for loss of Amb t 5 activity and gain of Amb a 5 activity. Our results show that while different individuals have distinct profiles of epitope recognition, loop 3 of Amb t 5 contains an immunodominant B-cell epitope that is responsible for a large portion of the IgG antibody response to Amb t 5.

2. Materials and methods

2.1. Isolation of pollen proteins and generation of Amb t 5 mutants

Amb a 5 and Amb t 5 were purified from short and giant ragweed pollens (Greer Labs, Lenoir, NC) as previously described (Metzler et al., 1992a, 1992b). The cloning and expression of recombinant Amb t 5 (rAmb t 5) in the pGEX-2T vector has been described (Ghosh et al.,

1991; Rafnar et al., 1992). Mutations were introduced into Amb t 5 using oligonucleotide-directed mutagenesis, using a three step mutagenesis protocol (Nelson and Long, 1989). The resultant, full-length, mutated rAmb t V molecules were ligated into the pGEX-2T vector (Smith and Johnson, 1988). Multiple mutations were introduced in the same manner, using rAmb t V molecules with single or double mutations as templates. All mutants were sequenced using the dideoxy sequencing procedure (Sequenase, USB).

The rAmb t 5 mutant proteins were produced in *E. coli* as fusion proteins with the 26 kDa polypeptide from *Schistosoma japonicum* glutathione S-transferase (GST) (Smith and Johnson, 1988). The fusion proteins were isolated from *E. coli* lysates by affinity chromatography on glutathione-agarose (Sigma). The bound proteins were digested with thrombin overnight at 22°C to release the rAmb t 5 from the GST fusion partner; subsequently the thrombin in the eluate was inactivated by passage over benzamidine beads (Promega). The rAmb t V proteins were concentrated by ultrafiltration (Microcon-3, Amicon) and their concentrations were determined by Coomassie staining of SDS acrylamide gels and by the Bradford reagent method (BioRad). SDS-PAGE of the Amb t 5 proteins was performed using 5–20% acrylamide linear gradient gels, followed by fixing and staining with Boston stain (25% methanol, 10% trichloroacetic acid, 3% sulfosalicylic acid, 0.9% Coomassie blue) and destaining in a solution containing 7.5% acetic acid and 10% methanol.

2.2. Double-antibody radioimmunoassay (DARIA)

Inhibition of antibody binding to rAmb t 5 by mutant rAmb t Vs was measured by competition DARIA (Roebber et al., 1982; Roebber et al., 1985). Human antisera were obtained from ragweed-allergic individuals who had previously received immunotherapy with giant ragweed extracts and who had been found to produce substantial IgG antibody responses to Amb t 5. Briefly, 1 ng of ¹²⁵I-labeled rAmb t 5 protein was incubated for 4 h at 23°C with a 1/10 dilution of human serum in the presence of tenfold dilutions (50–0.05 ng) of the mutant rAmb t 5 molecules as a competitor. The same dilutions of unmutated rAmb t 5 were used as control. 100 μ l of 1/2 dilution of goat anti-human IgG antisera were added and the incubation continued at 4°C overnight. The precipitate was pelleted, washed 4 times in borate-buffered saline and counted. Percent inhibition (%I) was calculated using the formula: $\%I = (A - B)/A \times 100$ where *A* is (cpm of test serum without inhibitor – cpm of negative control serum) and *B* is (cpm of test serum with inhibitor – cpm of negative control serum). In addition to the human sera, anti-Amb t 5 antisera produced in rabbits and goats were used for identical inhibition analysis (Roebber et al., 1985). These sera were used at 1:1000 dilution, followed by 1:10

dilution of goat anti-rabbit IgG or 1:5 dilution of rabbit anti-goat IgG second antibodies.

To examine potential gain of Amb a 5 antigenic activity of the rAmb t 5 mutants, radiolabeled Amb a 5 was incubated with 1:10 dilution of serum from short ragweed-allergic individuals. Increasing amounts of mutated rAmb t 5 molecules were added and the percent inhibition was calculated as above.

2.3. Prediction of antigenic sites

Potential antigenic sites were identified using the methodology of Novotny and co-workers, who, for a variety of proteins, including myoglobin, lysozyme, cytochrome c and myohemerythrin, have shown that peaks in the contact surface area profile of a protein correlate well with the location of the experimentally determined antigenic epitopes (Novotny et al., 1986). To estimate the antigenic potential of the various regions of Amb t 5, we calculated their contact surface areas by determining the van der Waals' surfaces contacted by a spherical probe of 10 Å. Conceptually, the contact surface area is a set of patches representing those portions of the surface that are in actual contact with the probe. Residue-contact areas plotted in the profile were obtained by summing the computed atom-contact areas.

3. Results and discussion

3.1. Selection of candidate amino acids and generation of mutant Amb t 5

To determine which amino acids of Amb t 5 are likely to be important in antibody binding, several characteristics of Amb t 5 and Amb a 5 were taken into account. First, the B-cell epitopes are conformational in nature. Thus, overlapping synthetic peptides corresponding to the entire sequence of Amb a 5 show no trace of antigenic activity. Furthermore, both Amb t 5 and Amb a 5 lose all antibody binding upon denaturation and neither protein reacts to human antibodies on Western blots (data not shown). Finally, reduced and alkylated Amb t 5 has no activity in the inhibitory radio-immuno assay. Second, although structurally similar, Amb a 5 and Amb t 5 are non-crossreactive antigenically. This suggests that the major antigenic sites lie in regions where there is relatively little sequence homology between the two molecules, the three loops (Figure 1). These loops are located at the exposed surfaces (Figure 1b) where they would be accessible to antibodies. To identify amino acids in Amb t 5 potentially involved in the antigenic sites, we used the method of Novotny et al. (1986), which predicts antigenic sites by examining the contact surface areas of proteins that are accessible to a probe that is the approximate size of an antibody molecule. Four amino acids in Amb t 5

were identified as highly accessible: Ile₂₂, Lys₂₄ and Tyr₂₅ in loop 3 and Lys₃₇ in the C-terminal alpha helix (Figure 2a). Tyr₂₅ is conserved between Amb t 5 and Amb a 5, so there was no reason to mutate this residue. However, because Pro₂₁ could be conformationally constraining, we opted to mutate this residue instead. Lys₃₇ is also conserved between Amb t 5 and Amb a 5; therefore, Asn₃₈ was selected as an alternate residue in this region. In addition, Thr₉ and Asn₁₀ in loop 1 and Val₁₄, Gly₁₅ and Lys₁₆ in loop 2 were selected for mutagenesis. Although these residues are less exposed than those in loop 3, they lie on the opposite side of the Amb t 5 structure and thus provide a more extensive probe of the Amb t 5 surface. In total, nine amino acids were selected for mutation.

Each of the candidate Amb t 5 amino acids was changed into the corresponding Amb a 5 residue. This mutational scheme was chosen primarily for two reasons. First, since Amb t 5 and Amb a 5 do not crossreact, amino acid substitutions of this kind would be expected to result in a change in antigenic activity. Second, because Amb a 5 is structurally related to Amb t 5, these substitutions are less likely to cause disruption of the overall structure of Amb t 5. Each of the nine selected mutations was individually introduced into the Amb t 5 sequence using oligonucleotide-directed mutagenesis.

3.2. Effect of single mutations on rAmb t 5 antigenicity

To examine the effect single mutations have on the antigenicity of Amb t 5, each single mutant was used to inhibit binding of human IgG to rAmb t 5. We have previously shown that rAmb t 5, produced and purified by the methods used here, is antigenically and structurally identical to nAmb t 5 (Rafnar et al. 1992); furthermore, these two proteins can be used interchangeably in the inhibition RIA. Sera from 8 allergic individuals who previously received immunotherapy with giant ragweed extract were tested; a set of inhibition curves originating from patient 2 is shown in Fig. 3. In some cases, shifts in inhibition curves indicated a loss of antibody affinity towards a mutated molecule; however, the same molecules could inhibit over 95% of antibody binding at high concentrations. The mutated Amb t 5 molecules varied in their ability to inhibit binding; several mutants inhibited binding as efficiently as unmodified rAmb t 5, while other mutants were able to inhibit only between 45–80% of total binding. The maximum inhibition attained with each mutant is summarized in Table 1. These data show that, while some mutations have little or no effect on epitope structure (T9N, V14K, K16A), other mutations result in partial inhibition in individual sera (N10V, G15R, K24R, N38K). Strikingly, two mutations in loop 3 (P21D, I22P) resulted in significant loss of antigenic activity for all sera tested, suggesting that this region contains an immunodominant antigenic site.

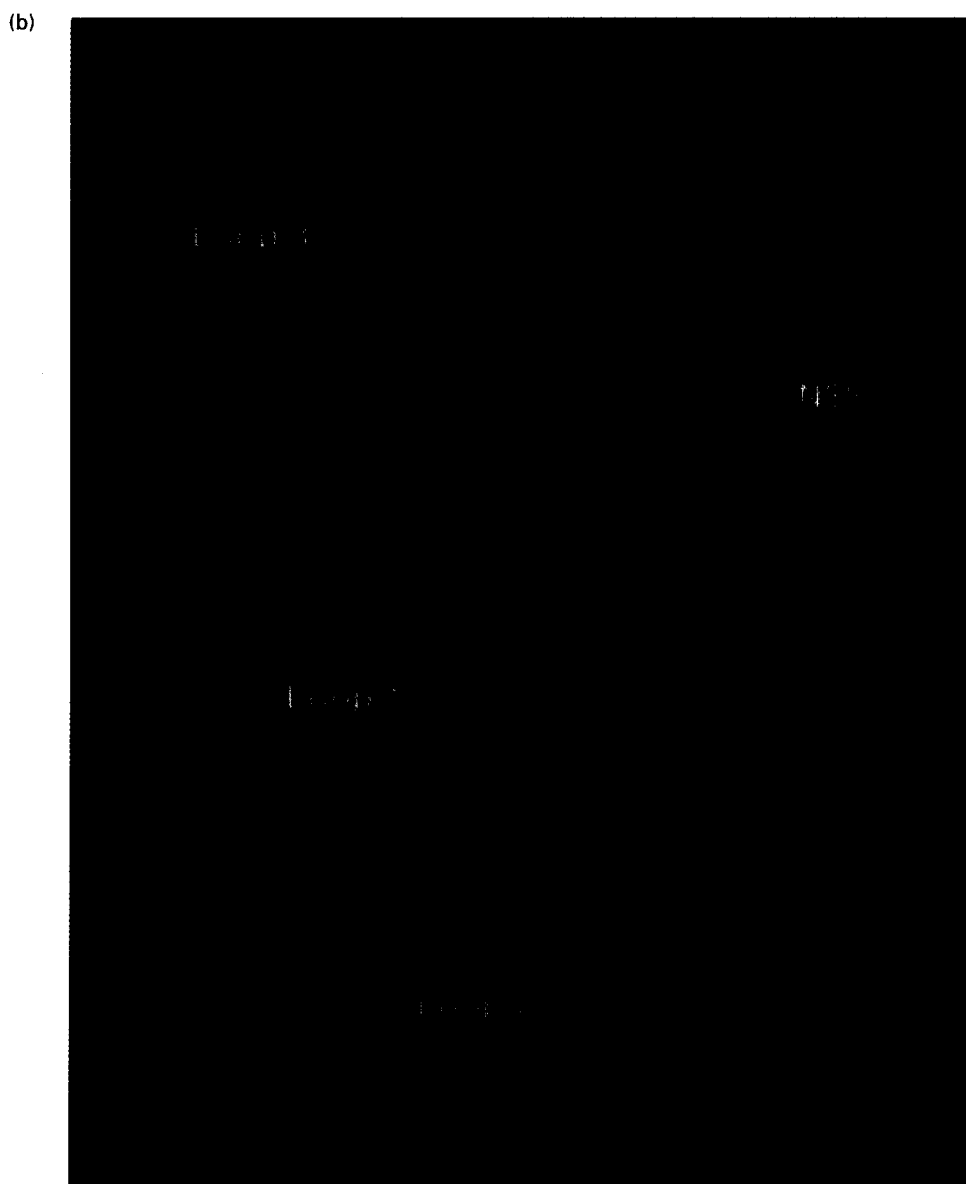
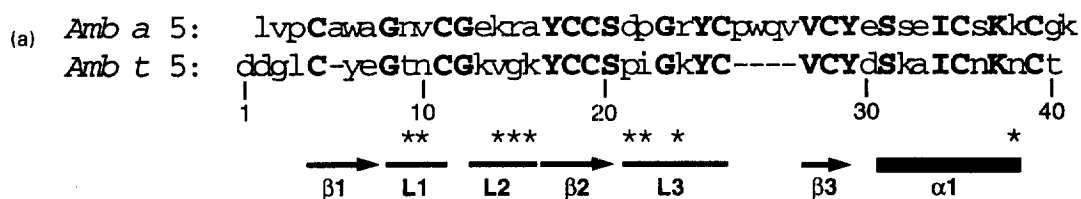


Fig. 1. (a) Sequence alignment of *Amb t 5* and *Amb a 5*. Amino acids identical in both proteins are in bold. Amino acids that were substituted are indicated with an asterisk (*). Also included are the assignments of the three beta sheets ($\beta 1$ – $\beta 3$), the alpha helix ($\alpha 1$) and the three loops (L1–3). (b) A ribbon representation of the three-dimensional structure of *Amb t 5* is colored to highlight residues identical in *Amb t 5* and *Amb a 5* (green) and residues mutated in this work (red). Disulfide bridges are shown in yellow.

3.3. Multiple mutations within loops of *rAmb t 5*

To analyze further the immunogenic structures within the loops of *Amb t 5*, multiple mutations were introduced into each loop. Thus, molecules with mutations in loop 1 (T9N and N10V), loop 2 (V14K and K16A) or loop 3

(P21D, I22P, and K24R) were generated and assayed (Table 1). In accordance with the single mutants, multiple mutations in loop 3 resulted in significant loss of antigenic activity in all the sera tested, again suggesting the presence of a major antigenic site in the mutated region. The rationale behind mutating only amino acids 14 and 16 in

Table 1
Maximum inhibition (%) of binding of rAmb t 5 to human and animal sera

Mutant	Patient 1 ^a	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Rabbit	Goat
rAmb t 5	100	99	97	92	95	95	99	99	99	99
T9N	100	98	93	96	99	94	99	99	99	99
N10V	95	78	97	58	94	94	71	95	95	76
V14K	100	97	93	81	89	91	99	99	96	99
G15R	99	98	72	96	81	61	82	78	96	97
K16A	96	98	96	97	95	94	99	85	96	98
P21D	57	48	69	55	65	86	85	84	66	96
I22P	52	44	45	52	66	52	80	85	60	99
K24R	75	77	90	73	96	80	99	99	84	100
N38K	100	97	91	84	87	95	99	78	72	94
Loop 1 ^b	99	97	99	59	99	97	96	95	98	82
Loop 2 ^b	58	98	80	70	74	78	74	75	89	96
Loop 3 ^b	42	ND ^c	55	65	46	53	82	68	63	99

^a Patients 1–8 denote sera from 8 Amb t 5-allergic individuals. ^b Multiple mutants are defined by loop 1 (T9N, N10V), loop 2 (V14K, K16A) and loop 3 (P21D, I22P, K24R). ^c ND: not determined.

loop 2 was to see if the two mutations together added up to more antigenic change than the sum of the two individual mutations. This was indeed the case in 7 of the 8 human sera tested. Mutations in loop 1 resulted in loss of activity in only one patient (patient 4). It is notable that in some sera, the effect of multiple mutations was less than that of single mutations alone. For example, in patient 3 a single mutation at site 22 resulted in only 45% of normal antigenic activity while the molecule with all three mutations in loop 3 was able to inhibit 55% of antibody binding. These results were highly reproducible for each individual (less than 2% variation between multiple readings of the same sera). A possible explanation for this observation can be offered when the nature of

the mutations is examined. Mutation of amino acid 22 results in the substitution of a proline for an isoleucine, while mutation of amino acid 21 leads to the substitution of an aspartic acid for a proline. In the I22P substitution, the resultant molecule contains two consecutive proline residues, both of which will cause the loop structure to be conformationally restricted. Conversely, the P21D substitution leaves the loop without a proline residue which may be needed for proper orientation of this loop. After all three mutations have been inserted, loop 3 is again left with one proline, potentially allowing for more natural bending of the whole loop.

The epitope structure of Amb t 5 was also analyzed using antisera raised in rabbit and goat (Table 1). The

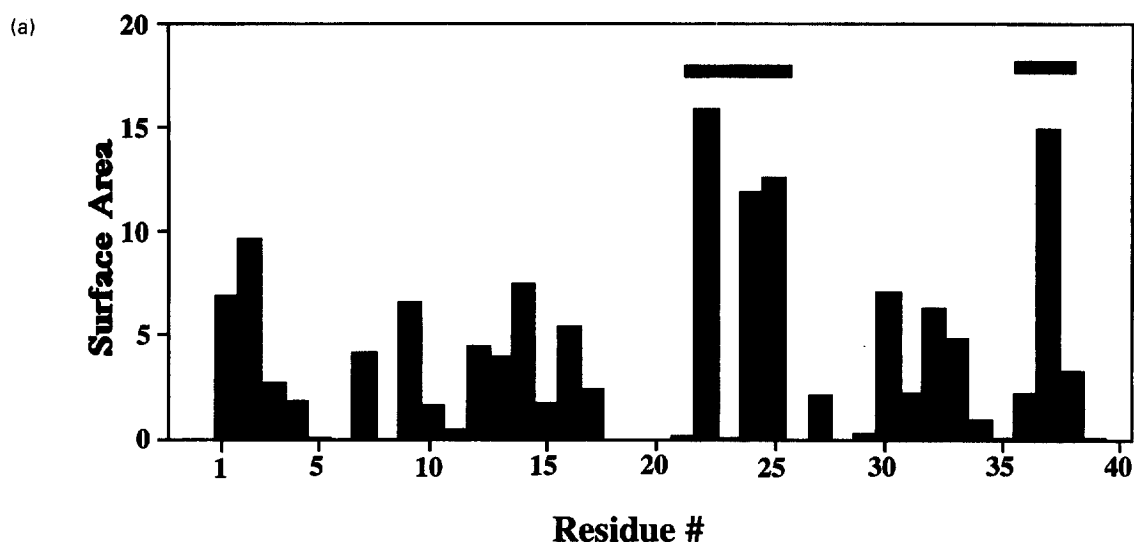


Fig. 2—caption overleaf.

(b)

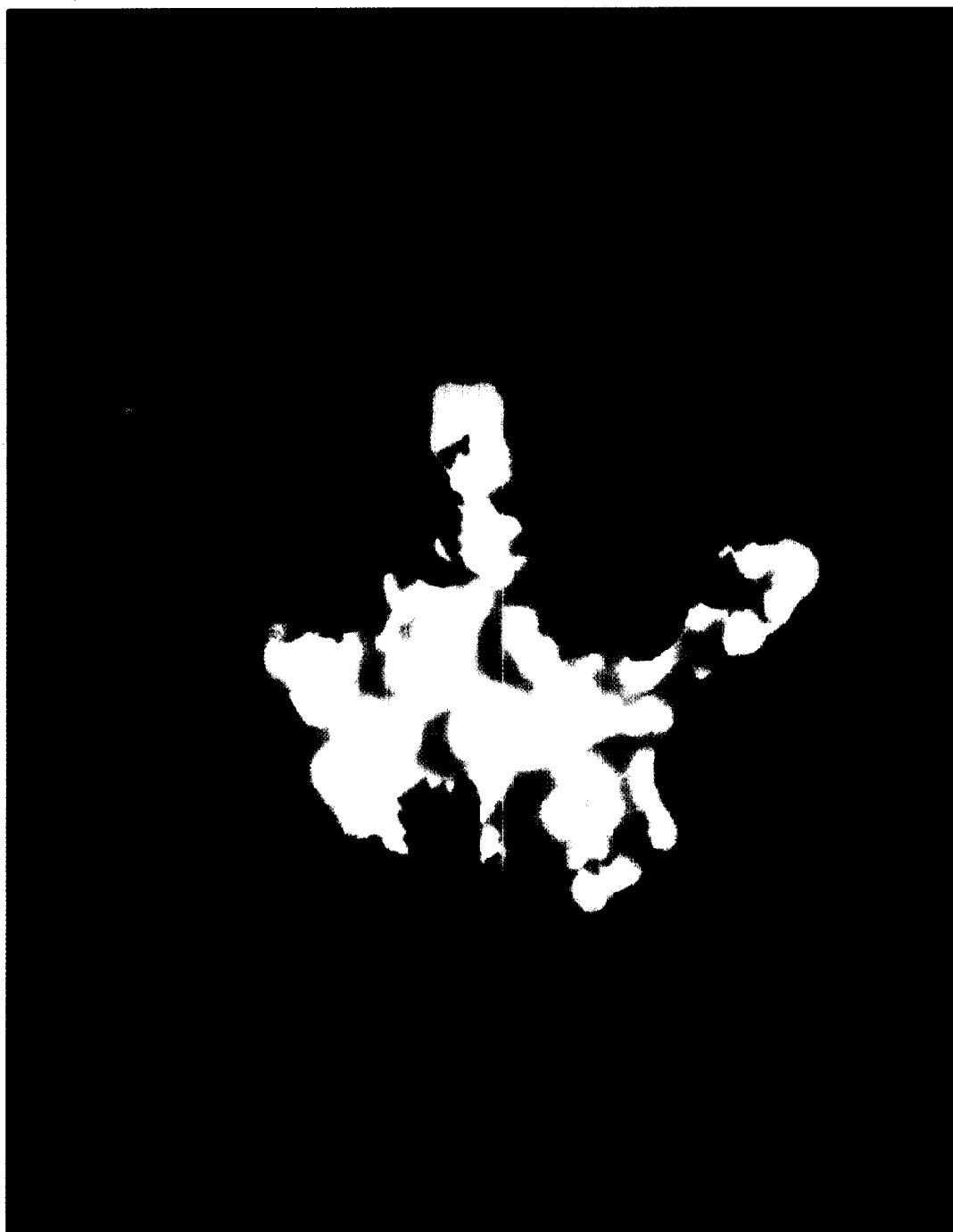


Fig. 2. (a) Contact surface area profile for Amb t 5. Peaks in the profile (horizontal bars) indicate regions of high accessibility to antibody binding: I₂₂, K₂₄, Y₂₅ and K₃₇. (b) Molecular surface plot of Amb t 5 colored to highlight regions probed by mutagenesis: P21–K₂₄ and K₃₇–N₃₈ (red); T₉–N₁₀ and V₁₄–K₁₆ (cyan). The orientation in this figure was obtained by rotating $\sim 180^\circ$ about the z-axis relative to the orientation shown in Fig. 1(b).

rabbit antiserum showed similar results to the human sera in that mutations in loop 3 caused a drastic loss of antigenic activity. Smaller drops in activity were observed for N38K in the C-terminal alpha helix and combined mutations in loop 2. Substitution of N10V resulted in loss of antibody binding as determined by shift in inhibition

curves, however, this loss was overcome by high doses of inhibitor. Interestingly, the goat antiserum reacted only to a single amino acid substitution. Thus, the N10V mutation resulted in the loss of approximately 20% of antibody binding, while none of the other mutations had notable effect. Mutations of loop 2 and loop 3 resulted

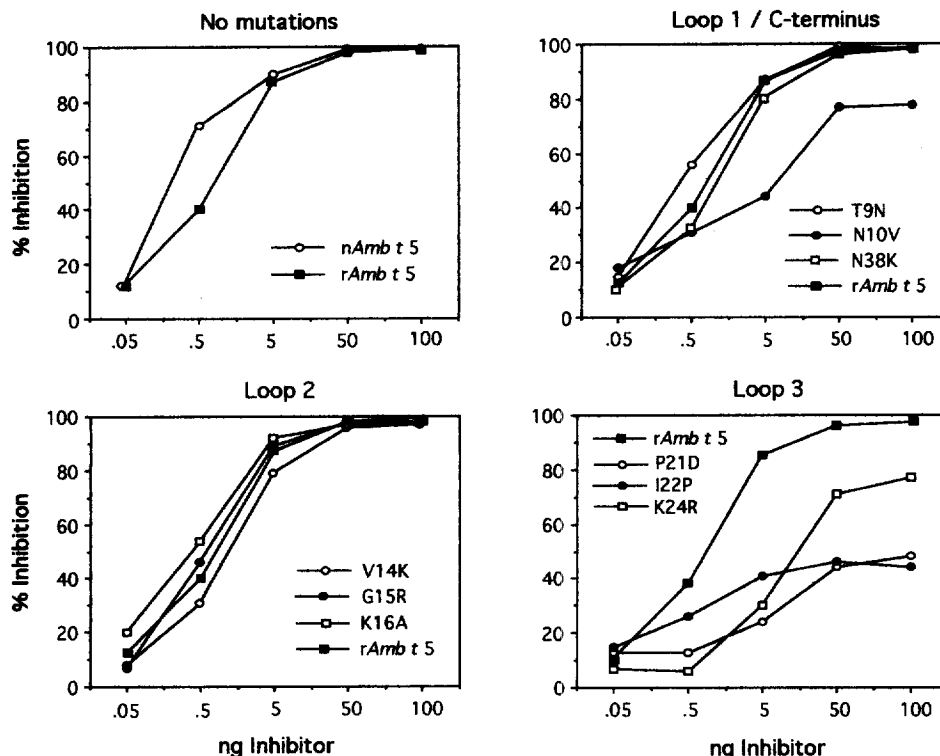


Fig. 3. Antigenic activity of mutant Amb t 5 proteins. Antigenic properties of mutant rAmb t V molecules were assayed by their ability to inhibit binding of human serum to ¹²⁵I-labeled rAmb t V. The figure demonstrates an example (from patient 2) of the set of inhibition curves obtained for each serum. The difference between native and recombinant Amb t 5 in the top left hand panel (0.5 ng inhibitor) was not seen in several other experiments with the same serum (Rafnar et al., 1992) and is probably due to an inaccurate dilution of native Amb t 5 in this one experiment.

in loss of antibody binding but this change in affinity was again not detectable at high doses of inhibitor. The majority of antibody reactivity in the goat antiserum, therefore, is directed towards sites other than those mutated here. These sites are conformational in nature as the goat antiserum showed no reactivity towards denatured Amb t 5 on Western blots and reduced, alkylated and boiled Amb t 5 exhibited no activity in the inhibition RIA (data not shown).

Comparison of the structures determined for Amb t 5 and Amb a 5 revealed that most of the conserved residues are located in the interior of the molecules, being involved in disulfide bonds or hydrophobic interactions (Metzler et al., 1992a, 1992b). This 'core' of the ragweed proteins is quite rigid and perturbation of the core residues would be expected to have dramatic impact on structure. In contrast, the surfaces of the two proteins display significant conformational and chemical variability, consistent with the observation that Amb a 5 and Amb t 5 show no antibody cross-reactivity. The mutations made here were expected to have little effect on protein folding, as they involved substitution of surface amino acids between two homologous proteins. As evidence for this, for every mutant, there was at least one serum that did not lose activity for that mutant. Thus, even Amb t 5 with a triple mutation in loop 3 retained full antigenic

activity when tested against the goat antiserum. As we had previously shown that the goat antibodies do not recognize denatured Amb t 5, these data strongly argue against a major loss of structural integrity.

3.4. Gain of Amb a 5 activity

None of the single mutants exhibited significant binding to IgG from Amb a 5 sensitized individuals, indicating that these mutations do not create an Amb a 5 epitope (data not shown). We then tested whether Amb t 5 with multiple mutations within each loop showed any gain of Amb a 5 activity, by examining whether the mutated Amb t 5 molecules could inhibit binding of Amb a 5 to IgG from Amb a 5-allergic individuals. None of the multiple mutants showed any gain of Amb a 5 activity, indicating that the introduced mutations are not sufficient to create a significant Amb a 5 B-cell epitope. It is possible that the primary epitope(s) of Amb a 5 may involve residues of more than one loop and it would seem to be a logical next step to make recombinant Amb t 5 including such mutations. However, we have previously shown that recombinant Amb a 5 does not fold properly when expressed in *E. coli* (Rafnar et al., 1992) and we anticipated that the more the recombinant hybrid molecule looked like Amb a 5, the less likely it was to obtain

a three-dimensional structure that was appropriate for epitope analysis.

In summary, we have probed various surface residues of Amb t 5 for the presence of human antibody binding sites. While all three loops and the C-terminal α -helix of Amb t 5 contribute to antigenic activity in one or more individuals, loop 3 contains a dominant human B-cell epitope that is recognized by IgG antibodies from all patients tested. This epitope was not recognized by goat anti-Amb t 5 antiserum, demonstrating a potential species-specific difference in the recognition of this antigen. Furthermore, antibodies to Amb a 5 do not react with rAmb t 5 in which substantial Amb a 5 substitutions have been made on the surface of the molecule. This suggests that although Amb t 5 and Amb a 5 are topologically very similar, Amb a 5 antibodies discriminate strongly between the relatively small differences in the tertiary structure of the two molecules.

Acknowledgements

We thank Dr. David G. Klapper for providing reduced and alkylated rAmb t 5 and for technical advice. We thank Dr. Amina Woods for assistance in the purification of rAmb t 5. This work was supported by National Institutes of Health Grant AI19727.

References

- Benjamin, D.C., Berzofsky, J.A., East, I.J., Gurd, F.R.N., Hannum, C., Leach, S.J., Margoliash, E., Michael, J.G., Miller, A., Prager, E.M., Reichlin, M., Sercarz, E.E., Smith-Gill, S.J., Todd, P.E., Wilson, A.C., 1984. The antigenic structure of proteins: a reappraisal. *Ann. Rev. Immunol.* 2, 67–101.
- Briner, T.J., Kuo, M.C., Keating, K.M., Rogers, B.L., Greenstein, J.L., 1993. Peripheral T-cell tolerance induced in naive and primed mice by subcutaneous injection of peptides from the major cat allergen *Fel d1*. *Proc. Natl. Acad. Sci. U.S.A.* 90, 7608–7612.
- Ebner, C., Hirschwehr, R., Bauer, L., Breitender, H., Valenta, R., Ebner, H., Kraft, D., Scheiner, O., 1995. Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch allergens Bet v 1 and Bet v 2 (birch profilin). *J. Allergy Clin. Immunol.* 95, 962–969.
- Frankland, A.W., 1995. Food reactions in pollen and latex allergic patients. *Clin. Exp. Allergy* 25, 580–581.
- Germain, R.N., 1991. Antigen presentation. The second class story. *Nature* 353, 605–607.
- Ghosh, B., Perry, M.P., Marsh, D.G., 1991. Cloning the cDNA encoding the Amb t V allergen from giant ragweed (*Ambrosia trifida*) pollen. *Gene* 101, 231–238.
- Ghosh, B., Perry, M.P., Rafnar, T., Marsh, D.G., 1993. Cloning and expression of immunologically active recombinant Amb a V allergen of short ragweed (*Ambrosia artemisiifolia*) pollen. *J. Immunol.* 150, 5391–5399.
- Goodfriend, L., Choudhury, A.M., Klapper, D.G., Coulter, K.M., Dorval, G., DelCarpio, J., Osterland, C.K., 1985. Ra5G, a homologue of Ra5 in giant ragweed pollen: isolation, HLA-DR-associated activity and amino acid sequence. *Mol. Immunol.* 22, 899–906.
- Huang, S.-K., Zwollo, P., Marsh, D.G., 1991. Class II MHC restriction of human T-cell responses to short ragweed allergen, Amb a V. *Eur. J. Immunol.* 21, 1469–1473.
- Joost van Neerven, R., van t'Hof, W., Ringrose, J.H., Jansen, H.M., Aalberse, R.C., Wierenga, E.A., Kaspenberg, M.L., 1993. T-cell epitopes of house dust mite major allergen *Der p II*. *J. Immunol.* 151, 2326–2335.
- Liebers, V., Raulf, M., Mazur, G., Modrow, S., Baur, X., 1993. Epitope mapping with peptides of *Chi t 1* component III and immunomodulation of the *Chi t 1* immune response. *J. Allergy Clin. Immunol.* 92, 334–339.
- Marsh, D.G., Hsu, S.H., Roebber, M., Ehrlich-Kautzky, E., Freidhoff, L.R., Meyers, D.A., Pollard, M.K., Bias, W.B., 1982. HLA-Dw2: A genetic marker for human immune response to short ragweed allergen Ra5. I. Response resulting primarily from natural antigenic exposure. *J. Exp. Med.* 155, 1439–1451.
- Marsh, D.G., Meyers, D.A., Freidhoff, L.R., Ehrlich-Kautzky, E., Roebber, M., Norman, P.S., Hsu, S.H., Bias, W.B., 1982. HLA-Dw2: A genetic marker for human immune response to short ragweed allergen Ra5. II. Response after ragweed immunotherapy. *J. Exp. Med.* 155, 1452–1463.
- Metzler, W.J., Valentine, K., Roebber, M., Friedrichs, M.S., Marsh, D.G., Mueller, L., 1992. Determination of the three-dimensional solution structure of ragweed allergen Amb t V by nuclear magnetic resonance spectroscopy. *Biochemistry* 31, 5117–5127.
- Metzler, W.J., Valentine, K., Roebber, M., Marsh, D.G., Mueller, L., 1992. Proton resonance assignments and three-dimensional solution structure of the ragweed allergen Amb a V by nuclear magnetic resonance spectroscopy. *Biochemistry* 31, 8697–8705.
- Mole, L.E., Goodfriend, L., Lapkoff, C.B., Kehoe, J.M., Capra, J.D., 1975. The amino acid sequence of allergen Ra5. *Biochemistry* 14, 1216–1220.
- Nelson, R.M., Long, G.L., 1989. A general method of site-specific mutagenesis using a modification of the *Thermus aquaticus* polymerase chain reaction. *Anal. Biochem.* 180, 147–151.
- Nilsen, B.M., Sletten, K., Paulsen, B.S., O'Neill, M., van Halbeek, H., 1991. Structural analysis of the glycoprotein allergen *Art v II* from the pollen of mugwort (*Artemisia vulgaris* L.). *J. Biol. Chem.* 266, 2660–2668.
- Norman, P.S., 1993. Modern concepts of immunotherapy. *Curr. Opin. Immunol.* 5, 968–973.
- Novotny, J., Handschumacher, M., Haber, E., Bruccoleri, R.E., Carlson, W.B., Fanning, D.W., Smith, J.A., Rose, G.D., 1986. Antigenic determinants in proteins coincide with surface regions accessible to large probes (antibody domains). *Proc. Natl. Acad. Sci. U.S.A.* 83, 226–230.
- O'Brien, R.M., Thomas, W.R., 1994. Immune reactivity to *Der p I* and *Der p II* in house dust mite sensitive patients attending paediatric and adult allergy clinics. *Clin. Exp. Allergy* 24, 737–742.
- Olson, J.R., Klapper, D.G., 1986. The major allergenic sites on ragweed pollen allergen antigen E identified by using monoclonal antibodies. *J. Immunol.* 136, 2109–2115.
- Rafnar, T., Ghosh, B., Metzler, W.J., Huang, S.-K., Perry, M.P., Mueller, L., Marsh, D.G., 1992. Expression and analysis of recombinant Amb a V and Amb t V allergens. Comparison with native proteins by immunological assays and NMR spectroscopy. *J. Biol. Chem.* 267, 21119–21123.
- Rafnar, T., Huang, S.-K., Ghosh, B., Nag, B., Sharma, S.D., Marsh, D.G., 1993. Dominant T-cell epitope on allergen, Amb a V. *J. Immunol.* 150, 175A.
- Roebber, M., Klapper, D.G., Marsh, D.G., 1982. Two isoallergens of short ragweed component Ra5. *J. Immunol.* 129, 120–125.
- Roebber, M., Klapper, D.G., Goodfriend, L., Bias, W.B., Hsu, S.H., Marsh, D.G., 1985. Immunochemical and genetic studies of Amb t V (Ra5G), an Ra5 homologue from giant ragweed pollen. *J. Immunol.* 134, 3062–3069.
- Seiberler, S., Scheiner, O., Kraft, D., Lonsdale, D., Valenta, R., 1994.

- Characterization of a birch pollen allergen, *Bet v* III, representing a novel class of Ca^{2+} binding proteins: specific expression in mature pollen and dependence of patients' IgE binding on protein-bound Ca^{2+} . *EMBO J.* 13, 3481–3486.
- Smith, D.B., Johnson, K.S., 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene* 67, 31–40.
- Vailes, L.D., Li, Y., Bao, Y., DeGroot, H., Aalberse, R.C., Chapman, M.D., 1994. Fine specificity of B-cell epitopes on *Felis domesticus* allergen I (*Fel d* I): effect of reduction and alkylation or deglycosylation on *Fel d* I structure and antibody binding. *J. Allergy Clin. Immunol.* 93, 22–33.
- van Kampen, V., Becker, W.M., Chen, Z., Rihs, H.P., Mazur, G., Raulf, M., Liebers, V., Isringhausen-Bley, S., Baur, X., 1994. Analysis of B-cell epitopes in the N-terminal region of *Chi t* I component III using monoclonal antibodies. *Mol. Immunol.* 31, 1133–1140.
- Zhu, X., Greenstein, J.L., Rogers, B.L., Kuo, M.C., 1995. T-cell epitope mapping of ragweed pollen allergen *Ambrosia artemisiifolia* (Amb a 5) and *Ambrosia trifida* (Amb t 5) and the role of free sulfhydryl groups in T-cell recognition. *J. Immunol.* 155, 5064–5073.