

Expression and Analysis of Recombinant *Amb a V* and *Amb t V* Allergens

COMPARISON WITH NATIVE PROTEINS BY IMMUNOLOGICAL ASSAYS AND NMR SPECTROSCOPY*

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Thorunn Rafnar‡, Balaram Ghosh‡, William J. Metzler§, Shau-Ku Huang‡, Michael P. Perry‡, Luciano Mueller§, and David G. Marsh‡

From the ‡Johns Hopkins Asthma and Allergy Center, Baltimore, Maryland 21224-6801 and the §Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543-4000

The *Amb V* allergens are small, highly disulfide-bonded ragweed pollen allergens that serve as useful models for understanding the molecular basis of the human immune response. We have produced recombinant *Amb a V* and *Amb t V* (from short and giant ragweed pollens, respectively) in *Escherichia coli* and have compared their structural and functional characteristics to those of the native proteins. Recombinant *Amb t V* was indistinguishable from native *Amb t V* as determined by NMR spectroscopy and antibody-binding studies. Whereas inhibition analysis showed that recombinant *Amb a V* possessed only ~50% of the antibody-binding activity of native *Amb a V*, the two proteins were similarly effective in stimulating *Amb a V*-specific T-cells. Our results demonstrate that even highly homologous proteins exhibit different abilities to fold into their native three-dimensional conformations and establish the potential and limits of expressing the recombinant *Amb V* allergens intracellularly in *E. coli*.

Type I hypersensitivity to inhaled allergens affects ~15–20% of individuals in the United States (1). Diagnosis and possible therapeutic treatment of allergy rely on the availability of highly purified allergens. Toward this end, the cDNAs of a number of important human allergens have been isolated and sequenced. These include allergens from house dust mites (2–5), insect venom (6), cat (7), and various pollens from ragweeds (8–10), grasses (11–13), and trees (14, 15). Some of the recombinant allergens are able to react with human IgE in solid-phase assays (3, 4, 11, 12, 14–16, 17) and to stimulate allergen-specific T-cells (10, 16). However, none of the recombinant allergens tested so far, with the possible exception of *Der p II* (18), possessed the full antigenic reactivity of their natural counterparts, as assayed in the solution phase under nondenaturing conditions.

We are using a group of homologous, small, well-defined protein allergens from ragweed pollens, the *Amb V* allergens, as models to study the B- and T-cell recognition of allergens. *Amb a V* from short ragweed (*Ambrosia artemisiifolia*) and *Amb t V* from giant ragweed (*Ambrosia trifida*) are small peptides of 45 and 40 amino acids, respectively; each peptide is stabilized by four intrachain disulfide bonds. Specific im-

mune responsiveness to each protein is strongly associated with DR ($\alpha_1\beta_1^*$ 1501) (formerly DR $\alpha\beta_1$ 2.2) (19–22), suggesting that *Amb a V* and *Amb t V* may possess identical MHC¹-binding elements, despite having limited sequence homology (45%). The three-dimensional structures of *Amb a V* and *Amb t V* have been derived by NMR spectroscopy (23)² providing an exceptional opportunity to study the relationship between the structure and function of these proteins.

To resolve the immunological structures of the *Amb V* allergens, we have produced recombinant *Amb t V* (r*Amb t V*) and r*Amb a V* and analyzed these proteins extensively to determine whether they are identical structurally and immunologically to their native *Amb V* (n*Amb V*) counterparts. Despite strong overall three-dimensional homology (23),² the two *Amb V* homologues exhibit very different behaviors when expressed intracellularly in *Escherichia coli*. We conclude that both the r*Amb a V* and r*Amb t V* produced in this system can be used for identification of MHC/T-cell epitopes; however, only r*Amb t V* is suitable for complete B-cell epitope studies. These results emphasize the importance of verifying the configuration of structurally complex recombinant proteins before they are used for immunological characterization.

MATERIALS AND METHODS

Protein Preparation—*Amb a V* and *Amb t V* were isolated and purified from short and giant ragweed pollens (Greer Laboratories, Lenoir, NC) as previously described (21, 24), except that reverse-phase HPLC with a Waters radially compressed 10 × 25 Delta-Pak C₁₈ column in H₂O, 0.1% trifluoroacetic acid, with an increasing gradient of acetonitrile, 0.1% trifluoroacetic acid, was used as the final purification step (23).

Expression and Purification of Fusion Proteins—In separate experiments, *Amb a V* and *Amb t V* cDNAs (7)³ were amplified by polymerase chain reaction using primers compatible with the *Bam*HI/*Eco*RI cloning site in pGEX-2T (25). The primer sequences were as follows: *Amb a V* 5'-primer: 5'-GAACATATGGGATCCTTAGTGCCGTGTGCGTGGGCGGGG-3'; *Amb a V* 3'-primer: 5'-GAA-CATATGGAATTCATTATTTGCCGCATTTTGGTGCATAT-3'; *Amb t V* 5'-primer: GAACATATGGGATCCGACGATGG-ACTTTGTTATGAGGGG-3'; and *Amb t V* 3'-primer: 5'-GAACA-TATGGAATTCATTAAGTACAATTTTGTTCATATTGC-3'. The amplified products were digested with *Eco*RI and *Bam*HI, purified by agarose gel electrophoresis, and ligated into pGEX-2T using

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¹ The abbreviations used are: MHC, major histocompatibility complex; r*Amb t V*, recombinant *Amb t V*; n*Amb V*, native *Amb V*; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NOESY, nuclear Overhauser effect spectroscopy.

² Metzler, W. J., Valentine, K., Roebber, M., Marsh, D. G., and Mueller, L. M. (1992) *Biochemistry*, in press.

³ B. Ghosh, M. P. Perry, T. Rafnar, and D. G. Marsh, submitted for publication.

standard procedures (26). The recombinant plasmids were used to transform *E. coli* strain JM109 (27). These constructs encode fusion proteins consisting of a 26-kDa polypeptide from *Schistosoma japonicum* glutathione *S*-transferase and 6 additional amino acids spanning a thrombin cleavage site, followed by the Amb *t* V or Amb *a* V peptide.

Growth and Purification of Recombinant Proteins—After confirmation of the DNA sequence by dideoxy sequencing (28), the fusion proteins were produced and purified by affinity chromatography on glutathione-agarose (Sigma) as described (25). The fusion proteins from 1 liter of culture were eluted with 2–3 ml of reduced glutathione (Sigma) (5 mM in 50 mM Tris-HCl, pH 8) and dialyzed overnight against buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM CaCl₂. The Amb V allergens were then cleaved from the glutathione *S*-transferase by the addition of 250 ng of human thrombin (in 25 μ l) and separated by reverse-phase HPLC (Waters 8 $\times$ 10 Delta-Pak C₁₈ column) using a gradient of 0–30% acetonitrile, 0.09% trifluoroacetic acid in H₂O, 0.1% trifluoroacetic acid at the rate of 1% increase in acetonitrile concentration/min. This was followed by a steeper gradient of 2–4%/min to 100% acetonitrile, 0.09% trifluoroacetic acid. The flow rate of the column was 1 ml/min. After lyophilization, the protein fractions were analyzed by electrophoresis on 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 (21).

Alternatively, rAmb *a* V was purified from washed inclusion bodies and isolated by the procedure of Lin and Cheng (29), except that proteinase inhibitors were omitted from all solutions. The inclusion bodies were solubilized in 10 ml of denaturing buffer (50 mM Tris-HCl, pH 8, 7 M guanidinium chloride, 5 mM EDTA). The suspension was incubated on ice for 90 min, followed by the addition of up to 800 ml of renaturing buffer (50 mM Tris-HCl, pH 8, 1 mM dithiothreitol, 20% glycerol) and stirred gently at 4 °C overnight. The solution was centrifuged at 13,500 $\times$ *g* for 30 min, and the fusion protein was purified by affinity chromatography on glutathione-agarose beads as described before. After elution with reduced glutathione, CaCl₂ was added to 2.5 mM and NaCl to 150 mM, and the fusion protein was cleaved with thrombin as described above. The majority of the glutathione *S*-transferase was adsorbed by a second passage over glutathione-agarose (1-ml beads in a Pasteur pipette column), and rAmb *a* V was purified by reverse-phase HPLC.

Double-antibody Radioimmunoassay—Antigenic cross-reactivity between recombinant and native Amb *a* V allergens was measured by competition double-antibody radioimmunoassay (21, 24). Human antisera were obtained from ragweed-allergic individuals who had previously received immunotherapy with short ragweed extracts and who had been found to produce substantial IgG antibody responses to Amb *a* V. Briefly, 1 ng of ¹²⁵I-labeled nAmb *a* V protein was incubated for 4 h at 23 °C with a 1:10 dilution of immune human serum in the presence of 10-fold dilutions (50 to 0.05 ng) of either recombinant or native Amb *a* V as a competitor. 100 μ l of a 1:2 dilution of goat anti-human IgG antisera were added, and the incubation was continued at 4 °C overnight. The precipitate was pelleted, washed four times in borate-buffered saline, and counted. Percent inhibition (% I) was calculated using the following formula: % I = ((cpm of test serum without inhibitor – cpm of test serum with inhibitor)/(cpm of test serum without inhibitor – cpm of negative control serum)) $\times$ 100. Similar experiments were performed using ¹²⁵I-labeled nAmb *t* V and human sera from individuals who had received immunotherapy with giant ragweed pollen extract and had high IgG antibody levels to Amb *t* V. In these experiments, nAmb *t* V and rAmb *t* V were used as inhibitors.

T-Cell Assays—The Amb *a* V-specific T-cell clones AP1.2 and AP9 have been described previously (22); the T-cell line AP was generated from the same individual. For T-cell stimulation studies, 2 $\times$ 10⁴ Amb *a* V-specific human T cells were cultured in duplicate, in a volume of 200 μ l, with or without native or recombinant Amb *a* V (2 μ g/ml) in the presence of 2 $\times$ 10⁵ irradiated (5000 rads) autologous peripheral blood mononuclear cells as antigen-presenting cells for 3 days (22, 30). The cell cultures were then pulsed with 1 μ Ci of [³H]deoxythymidine (Du Pont-New England Nuclear), harvested 18 h later, and counted. For T-cell inhibition assays, Amb *a* V-specific T cells (2 $\times$ 10⁴) and autologous antigen-presenting cells (2 $\times$ 10⁵) were incubated in the presence or absence of the rAmb *t* V competitor (1 and 10 μ g/ml) for 1 h. Native Amb *a* V was added to a concentration of 1 μ g/ml, cultured for 3 days, pulsed with [³H]deoxythymidine, harvested, and counted as described before.

NMR Spectroscopy—For the NMR structural determinations of

the Amb *t* V proteins, 9.0 mg of nAmb *t* V and 2.5 mg of rAmb *t* V were dissolved separately in 600 μ l of buffer (50 mM acetate, 0.1 mM EDTA, 0.1 mM NaN₃, pH 5.0, at 23 °C) to yield final concentrations of 3.5 and 1.0 mM, respectively. NMR experiments were performed at a temperature of 30 °C on a Varian Unity 600 spectrometer operating at 599.5 MHz. Details of the data collection and processing, sequence-specific resonance assignment, derivation of structural constraints, and structural calculations are presented elsewhere (23). For the present discussion, analysis of the similarity and differences between the three-dimensional structures of the native and recombinant Amb *t* V proteins was performed by a detailed comparison of the chemical shifts of the proton resonances and the interatomic distances of protons remote in primary structure.

RESULTS

Expression of rAmb V Allergens—The cDNAs encoding Amb *a* V and Amb *t* V were amplified by polymerase chain reaction, subcloned into the plasmid vector pGEX-2T, and expressed as fusion proteins with a 26-kDa fragment of glutathione *S*-transferase. The glutathione *S*-transferase-Amb *a* V and -Amb *t* V fusion proteins were purified from bacterial culture lysates by affinity chromatography on glutathione-agarose. This simple procedure yielded highly purified fusion proteins (~31 kDa), which upon digestion with thrombin were cleaved into the expected 26-kDa glutathione *S*-transferase and 5-kDa Amb V (Fig. 1). In the case of rAmb *a* V, proteolytic activity in the cell lysate resulted in the appearance of the 26-kDa glutathione *S*-transferase band, before the addition of thrombin (lane 2). This was not seen for the glutathione *S*-transferase-Amb *t* V fusion protein, suggesting that this product attains a configuration that protects it from this proteolytic degradation. As observed by other investigators (25), the thrombin digestion was only 60–80% effective, despite extended incubation times and increased amounts of enzyme (data not shown). Small amounts of rAmb *a* V were eluted from the HPLC column with the glutathione *S*-transferase fraction (lane 4); however, the majority of rAmb *a* V was easily separated from the glutathione *S*-transferase (lane 5). The glutathione *S*-transferase-Amb *t* V fusion product showed similar results. It is worth noting here that cleavage at the engineered thrombin site in the fusion proteins leaves 2 extra amino acids, Gly-Ser, at the NH₂ termini of the recombinant Amb V allergens. The resultant small difference in size between the recombinant and native Amb V allergens is not detectable by SDS-PAGE (lanes 5 and 7).

The cytoplasmic expression of highly disulfide-bonded proteins in *E. coli* has often proved to be difficult. Because

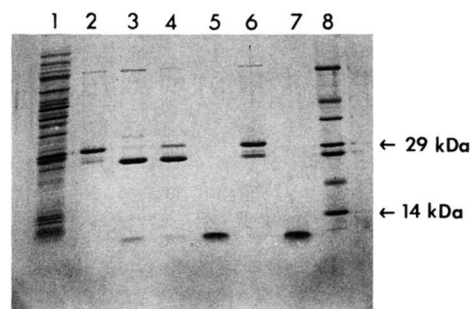


Fig. 1. SDS-PAGE (5–20% linear gradient gel, reducing conditions) of recombinant Amb *a* V protein products. Lane 1, total cell lysate from recombinant Amb *a* V bacteria after single adsorption with glutathione-agarose; lane 2, glutathione *S*-transferase-Amb *a* V fusion protein; lane 3, glutathione *S*-transferase-Amb *a* V fusion protein digested with thrombin; lane 4, glutathione *S*-transferase purified by HPLC; lane 5, rAmb *a* V purified by HPLC; lane 6, glutathione *S*-transferase-Amb *a* V fusion protein after HPLC purification; lane 7, nAmb *a* V; lane 8, size markers; Amb *t* V products showed comparable results.

disulfide bonds do not form in the reducing environment of the cytoplasm, the unfolded peptides tend to aggregate and form inclusion bodies (31). A difference in the folding characteristics of *Amb a V* and *Amb t V* was observed when the recombinant proteins were purified by reverse-phase HPLC. In the case of *rAmb t V* (Fig. 2a), the great majority of the protein eluted as a single sharp peak, which was later found to be identical to *nAmb t V* (see below). This peak was followed by a small diffuse peak, which was shown to possess the same apparent molecular weight as *Amb t V* by SDS-PAGE under reducing conditions (data not shown), indicating that a small fraction of *rAmb t V* is either aggregated or incorrectly folded. Eventually, a large peak, representing glutathione *S*-transferase plus undigested fusion protein, was eluted. Because a mixture of fully and partially folded molecules would compromise the immunological and NMR spectroscopic analyses, this HPLC purification step was essential for obtaining homogeneous *rAmb t V* protein. When the analogous procedure was used to produce *rAmb a V*, the proportion of aggregated molecules was significantly greater than with *rAmb t V* (Fig. 2b), and the yield of soluble protein was very low (10–100 $\mu\text{g/liter}$ for *rAmb a V* as opposed to 200–500 $\mu\text{g/liter}$ for *rAmb t V*). Attempts to reduce the degree of aggregation, namely lowering the culture temperature to 30 $^{\circ}\text{C}$, reducing the amount of the isopropyl-1-thio- β -D-galactopyranoside inducer, or reducing culture time after induction (31), did not increase the amount of soluble protein (data not shown).

In an effort to increase the yield of *rAmb a V*, it was isolated from washed inclusion bodies, followed by solubilization in 7 M guanidium chloride and renaturation by dilution (29). Using this method, the yield of *rAmb a V* was comparable to that of *rAmb t V*. Fig. 3 shows SDS-PAGE analysis of the products of various steps in this isolation procedure. A considerable amount of the glutathione *S*-transferase-*Amb a V* fusion protein was present in the cell lysate (lane 4), and repeated washings of the inclusion bodies eliminated some of the contaminating proteins (lane 5). After solubilization and renaturation, the fusion protein bound efficiently to glutathione-agarose (lane 6). The glutathione *S*-transferase and *rAmb a V* were subsequently separated by digestion with thrombin (lane 7) and purified by HPLC. It was evident from the HPLC profiles that the majority of *rAmb a V* eluted from the column in an aggregated form (data not shown). It was concluded that these standard methods, commonly used to restore native configuration to denatured proteins, are insufficient to allow

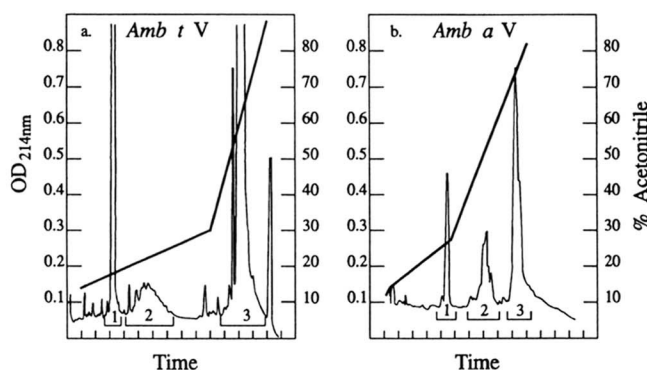


FIG. 2. HPLC profile of *rAmb V* protein products. a, *rAmb t V* (peak 1), aggregated or incorrectly folded *rAmb t V* (peak 2), and glutathione *S*-transferase plus undigested fusion protein (peak 3). The profile was proportionally enlarged to demonstrate the presence of the minor peak 2. b, *rAmb a V* (peak 1), aggregated *rAmb a V* (peak 2), and glutathione *S*-transferase plus undigested fusion protein (peak 3). *rAmb a V* was isolated from bacterial lysate (not from inclusion bodies).

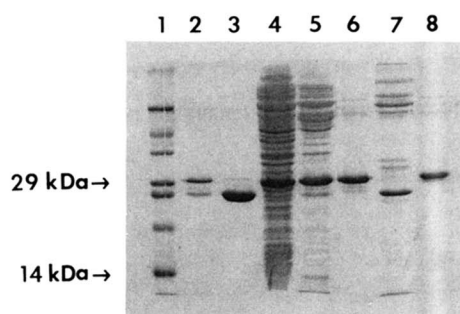


FIG. 3. SDS-PAGE (12% gel, reducing conditions) of various fractions from *rAmb a V* inclusion bodies. Lane 1, size markers; lane 2, glutathione *S*-transferase-*Amb a V* fusion protein from soluble fraction; lane 3, glutathione *S*-transferase isolated from bacteria transfected with pGEX-2T without the *Amb V* insert; lane 4, total cell lysate of recombinant *Amb a V* bacteria; lane 5, washed glutathione *S*-transferase-*Amb a V* inclusion bodies; lane 6, glutathione *S*-transferase-*Amb a V* fusion protein purified on glutathione-agarose after renaturation; lane 7, sample in lane 6 digested with thrombin (*rAmb a V* was not clearly identified because, due to its small size, it is lost during electrophoresis on a 12% acrylamide gel); lane 8, glutathione *S*-transferase-*Amb t V* fusion protein.

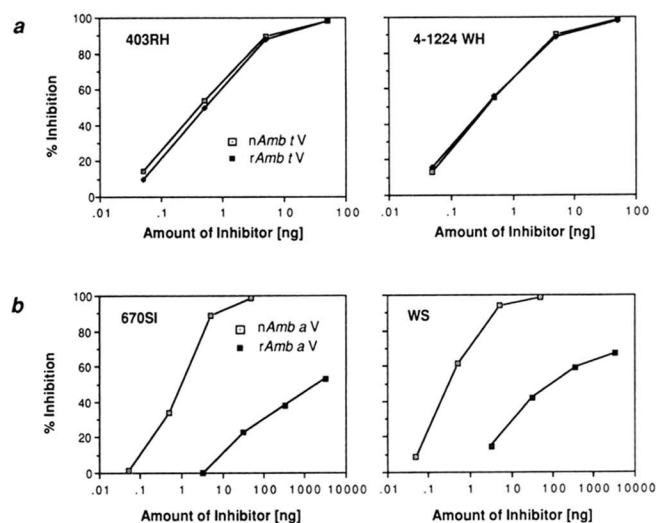


FIG. 4. Representative experiments showing competitive inhibition of ^{125}I -*nAmb V* binding to human anti-*Amb V* IgG antibodies using *nAmb V* or *rAmb V* allergens as inhibitors. a, radiolabeled *nAmb t V* was incubated with human immune serum in the presence of increasing amounts of either recombinant or native *Amb t V* as inhibitor; b, radiolabeled *nAmb a V* was incubated with human immune serum in the presence of increasing amounts of either recombinant or native *Amb a V* as inhibitor.

for the correct folding of *rAmb a V*. Therefore, soluble *rAmb a V* used in subsequent experiments was derived from the bacterial culture lysate procedure discussed above.

Antigenic Structure of *rAmb V* Allergens—We have shown previously that *Amb a V* and *Amb t V* are essentially noncross-reactive antigenically (21). The antigenic reactivities of the HPLC-purified *rAmb V* and *nAmb V* allergens were compared using inhibition double-antibody radioimmunoassays. For each of the eight individual human immune sera tested, *nAmb t V* and *rAmb t V* were able to inhibit IgG antibody binding to radiolabeled *nAmb t V* equally well, indicating that the two proteins are indistinguishable antigenically (two representative samples are shown in Fig. 4a). These data indicate that the presence of the Gly-Ser dipeptide at the NH_2 terminus of *rAmb t V* does not interfere with antibody binding. In contrast, *rAmb a V* was able to inhibit binding of ^{125}I -*nAmb a V* to human IgG antibodies only up to ~50–60% of the inhibition

produced by nAmb a V and only when a large excess of rAmb a V was used (Fig. 4b). This demonstrates that the renaturation of rAmb a V does not restore all of its antigenic epitopes. Thus, contrary to Amb t V, the pGEX expression system does not lead to the biosynthesis of rAmb a V that is suitable for a full investigation of B-cell epitopes. This could result from incorrect folding of rAmb a V; or, less likely, the dipeptide at the NH₂ terminus of the molecule might interfere with antibody binding.

Presentation of rAmb V Allergens to T-Cells—Amb a V and Amb t V are presented to T-cells in conjunction with DR(α , β 1*1501), as demonstrated by experiments in which proliferative responses of an Amb a V-specific T-cell line and clones were abolished by anti-DR(α , β 1*1501), but not by anti-DQ or anti-DP, antibodies (22). Despite its incomplete antibody-binding activity, rAmb a V was as efficient as nAmb a V in stimulating the proliferation of two Amb a V-specific T-cell clones and a T-cell line (Fig. 5). Currently, Amb t V-specific T-cell lines and clones have not been established. Therefore, an indirect method for assaying the T-cell activity of the Amb t V proteins was used. We had shown that although Amb t V does not stimulate Amb a V-specific T-cells, Amb t V can inhibit presentation of Amb a V to Amb a V-specific T-cells in the presence of DR(α , β 1*1501)-positive antigen-presenting cells (22). Thus, although the two proteins have an overall amino acid identity of only 45%, the sequence similarity in the DR-binding regions (agropopes) of the proteins is sufficient to lead to competition for binding to DR. The inhibitory effect of rAmb t V on an Amb a V-specific T-cell line was examined (Fig. 6) and showed identical results to those previously obtained for nAmb t V (30), i.e. rAmb t V was not able to stimulate proliferation of Amb a V-specific T-

cells, but it blocked proliferation of the T-cells by nAmb a V in a dose-dependent manner.

Comparison of nAmb t V and rAmb t V Structures—We compared the structures of the recombinant and native Amb t V allergens by NMR spectroscopy. Because of the low yields and incomplete antibody binding of rAmb a V obtained in this system, we did not pursue studies of its structure by NMR. The chemical shift of a proton resonance provides a sensitive probe of a protein's three-dimensional structure because it is highly dependent upon the proton's local electronic environment. For perspective, differences in chemical shift of >0.1 ppm are often found for small changes in the three-dimensional structure. The chemical shifts for nearly all the protons in nAmb t V have been determined by two-dimensional NMR experiments (23). To compare the structures of rAmb t V and nAmb t V, we determined the proton chemical shifts for rAmb t V and found them to be essentially identical to those for nAmb t V. The chemical shifts of identical protons in the two Amb t V proteins never deviated by >0.03 ppm, strongly suggesting that the three-dimensional conformations are identical. We also analyzed NMR data from two-dimensional NOESY experiments, which provide direct structural information; the intensity of a NOESY cross-peak correlating two protons is inversely proportional to the sixth power of the distance between the protons. Comparison of the NOESY cross-peak intensities of nAmb t V and rAmb t V indicated that all measurable interproton distances (~400 distances) arising from identical protons are indistinguishable. The similarity in the proton chemical shifts and interproton distances of nAmb t V and rAmb t V provides strong evidence that the two proteins adopt identical conformations in solution.

DISCUSSION

Recently, the three-dimensional structures of both Amb a V and Amb t V have been derived by NMR analysis (23),² providing a unique opportunity for structure-function studies of these highly disulfide-bonded allergens. As a first step in the process of identifying their T-cell epitope(s) and antigenic sites, we have expressed rAmb V allergens, analyzed their immunological properties, and compared the three-dimensional structures of rAmb t V and nAmb t V.

Isolation of Amb V allergens from their respective ragweed pollens is a tedious process, resulting in low yield of the allergen protein (21, 24). To circumvent this problem, various approaches have been used to obtain the proteins in quantities sufficient for analysis of their antigenic and allergenic properties. One approach has been the direct synthesis of the entire Amb V molecule by Choudhury and Goodfriend (32). This synthesis was particularly challenging due to the 8 cysteines contained in the Amb a V sequence and the difficulties encountered when trying to entice the correct disulfide linkages to form. The Amb a V obtained was shown to be closely similar to the native protein as judged by circular dichroism and antigenic and allergenic activities. Although an overall yield of ~6% was obtained, the method is not feasible for producing the large quantities of Amb a V and Amb t V proteins and variants needed for detailed B- and T-cell epitope mapping. In an approach to mapping T-cell epitopes, Huang and Marsh (30) used synthetic peptide fragments to examine the T-cell epitope structure of Amb a V. To overcome the problems of cystine oxidation, the troublesome cysteines were replaced with alanines. A prospective DR(α , β 1*1501)-binding site was placed in a peptide spanning amino acids 31–44 in Amb a V. This peptide was not able to stimulate the Amb a V-specific T-cells, but was able to block

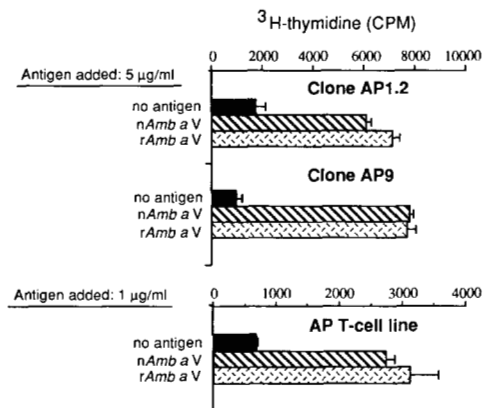


FIG. 5. Stimulation of two Amb a V-specific T-cell clones and T-cell line with rAmb a V and nAmb a V. The Amb V allergens show comparable activities in all cases.

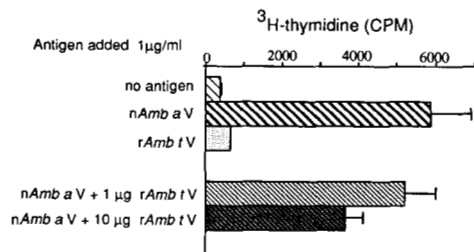


FIG. 6. Inhibition of Amb a V-specific T-cell line response by rAmb t V. rAmb t V does not stimulate Amb a V-specific T-cells, but does inhibit proliferation by nAmb a V in a dose-dependent fashion.

FIG. 7. Amino acid sequences of *Amb a V* and *Amb t V*. Amino acids that are conserved between the two proteins are shown in **boldface type**. Sequences were originally published by Mole *et al.* (34) and Goodfriend *et al.* (35).

rAmb a V and *rAmb t V* exhibit marked differences in their abilities to fold into their native conformations, which seems surprising given the 45% homology. It has been shown that at least two factors play a role in the ability of proteins to fold correctly in *E. coli* (reviewed in Ref. 33): the fraction of cysteine and the number of turns, particularly those created by proline. Disulfide bonds do not form in the cytoplasm, and a cysteine-rich recombinant protein may form insoluble aggregates upon lysis of the *E. coli* cells. We do not believe that this was the problem encountered here because *rAmb t V* was structurally and immunologically indistinguishable from *nAmb t V*, despite the fact that 8 of its 40 amino acids are cysteines. A majority of the *rAmb t V* molecules formed the correct disulfide bonds after lysis of *E. coli*. It is notable that *Amb a V* contains 3 prolines, compared with the single proline in *Amb t V*, which may explain the extensive aggregation of *Amb a V* during the expression/purification procedure. Pro²⁷ of *Amb a V* is especially noteworthy; this residue is the first amino acid in a tetrapeptide sequence (residues 27–30) that has no homologue in *Amb t V* (Fig. 7). The tetrapeptide creates an extended loop in the backbone of *Amb a V* and leads to the largest observed structural difference between *Amb a V* and *Amb t V* (23).²

is crucial to establish the structural integrity of the antigen when investigating antibody-antigen binding. We have produced a recombinant *Amb t V* molecule that is both structurally and antigenically indistinguishable from native *Amb t V*, and we are using *in vitro* mutagenesis and structural modeling to identify the binding sites of human IgE and IgG. Thus, these r*Amb V* allergens will enable us to study in considerable detail the relationships between the structure of an allergen and its function in the allergic response.

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