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Evidence for an Alpha Helical T Cell Epitope in the C-Terminus of the Main Birch Pollen Allergen Bet V 1

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Secondary structure prediction of the main birch pollen allergen Bet v 1 was found to be in good agreement with the secondary structural elements found by analysing the Bet v 1 circular dichroism data. According to both experiment and prediction, 32% of 160 amino acids participate in alpha helices, 21% in beta sheets, 24% in turns, and 23% in other structural motifs. The peptide LRAVESYLLAHS which represents one of the major T cell epitopes on Bet v 1 was shown to have a high propensity to form an alpha helix. Time-resolved fluorescence anisotropy measurements of the allergen revealed an overall rotational correlation time of 7.35 ns, which corresponds to a hydrodynamic molecular radius of 19.2 Å. This refers to a monomeric Bet v 1 molecule in solution, which is also reflected in the narrow band width of the ^{1}H-NMR spectrum. The results presented here are in good agreement with the recently solved NMR structure of Amb t 5: both allergens are monomers in solution with an extended C-terminal alpha helix containing a major T cell epitope.

More than 15% of the population of developed countries suffer from type I (IgE-mediated) allergic deseases, the main symptoms of which are allergic rhinitis/conjunctivitis and bronchial asthma. Sources for allergic proteins by which predisposed individuals are sensitized are manifold, ranging from pollen of grasses, trees, weeds, and spores of moulds, to faeces from mites, and dander from pets and other domestic animals (1). The allergic proteins induce IgE synthesis by B cells in a T cell dependent manner (2). Allergen-specific IgE is cross-linked when bound to its high affinity receptor ($Fc_\epsilon RI$) on mast cells and basophils, thus leading to the immediate release of inflammatory mediators like histamine, arachidonic acid and its metabolites. At present it is not completely understood what contributes to the upregulation of the IgE-synthesis which is characteristic for atopic patients.

In the moderate climate zone of the world, pollen from early flowering trees is an important source of Type I allergens, among which birch (*Betula verrucosa*) pollen seems to contain the most potent allergenic molecules. The major allergen with a molecular mass of 17 kDa (3), designated Bet v 1, reacts with the serum IgE of 96% of all tree pollen allergic patients. 60% of patients react exclusively to this allergen (4). Breiteneder et al. (5) have isolated and sequenced a cDNA clone coding for Bet v 1 and found high sequence similarities to the major pollen allergens from alder (6), hornbeam (7) and hazel (8) as well as to pathogenesis related proteins. This is in good

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Abbreviations used: NMR, nuclear magnetic resonance; CD, circular dichroism; HPLC, high pressure liquid chromatography; HFP, hexafluoropropanol; NaP_i, NaH_2PO_4/Na_2HPO_4; MEM, maximum entropy method.

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agreement with the observed strong cross-reactivity between these allergens (9). Obviously, common immunodominant epitopes are recognised at the B cell and T cell levels.

From a structural point of view, these epitopes are of interest for investigating the structure function relationship of allergens. B cell epitopes are primarily surface structures (10) which have been subject to rapid evolutionary change (11). They are therefore often species specific. The nature and the location of immunodominant T cell epitopes in the native allergen molecule are less studied. Margalit et al. (12) proposed amphipathic α-helices to be the preferred conformation of such epitopes. Marsh (13) postulated therefore that these T cell epitopes are relatively conserved regions of the allergenic proteins. Recently, Ebner et al. (14) identified 7 distinct T cell epitopes on the Bet v 1 molecule which are scattered over the entire molecule. The C-terminus of Bet v 1 contains one of the main T cell epitopes. Similarly, the main T cell epitope of the ragweed allergen Amb t 5 is located in the C-terminus. The recently determined solution structure of Amb t 5 by NMR[1] methods revealed this T cell epitope to be located in an α-helix (15).

MATERIALS AND METHODS

Cloning, expression, and purification of recombinant Bet v 1. The construction of a high-yield bacterial expression system and the purification protocol for Bet v 1 were recently described (16). Additionally, a preparative HPLC run was introduced in which the collected Bet v 1-containing fractions were subjected to a linear gradient of acetonitrile (A: 0.1% trifluoroacetic acid, 10% acetonitrile, 89.9% water; B: 0.1% trifluoroacetic acid, 89.9% acetonitrile, 10% water; flow rate 10.0 ml/min) created within 30 min (C4 Delta-Pak (Millipore, Milford, USA) 25 × 100 mm, 15μm). Refolding of the allergen was accomplished in a linear 40 min gradient (A: 50 mM TRIS.HCl, pH 8.3; B: 50 mM TRIS.HCl, pH 8.3, 1 M NaCl) on a Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden). Bet v 1 eluted at 200 mM NaCl. The concentration of Bet v 1 was determined both optically by measuring the absorption at 277 nm (OD(277nm) = 0.623 for 1 mg Bet v 1/ml) and by applying the photometric Pierce assay (Pierce, Illinois, USA).

Circular dichroism. CD spectra of aqueous allergen solutions were recorded in cuvettes with a path length of 0.1 cm on a Jasco J-710 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). Spectra were collected with a response time of 0.25 sec and with a data point resolution of 0.1 nm. Commonly six scans were averaged to yield smooth spectra. The concentration of Bet v 1 was 2.6 μM during all CD measurements. Hexafluoropropanol (HFP) (FLUKA, Buchs, Switzerland) was used to investigate the propensity of a Bet v 1 peptide to adopt a helical conformation. The peptide LRAVESYLLAHS was purchased from Cambridge Research Biochemicals (Cambridge, England). Analysis of the CD spectra with respect to secondary structural elements was accomplished with the program SELCON (17).

Fluorescence spectroscopy. Time-resolved fluorescence anisotropy spectra were obtained on an ultrafast pulsed laser equipment as described earlier (18). Data analysis was performed as detailed by Vos et al. (19) using a reference compound with a few picoseconds fluorescence lifetime for the deconvolution procedure and with the software from Maximum Entropy Data Consultants Inc. (Cambridge, England) (20).

Nuclear magnetic resonance. ^{1}H-NMR spectra were recorded on a Bruker AMX 600 spectrometer (Bruker, Karlsruhe, Germany) as detailed by Lindemann (21). For NMR measurements of Bet v 1, 10 mg of the allergen were dissolved in 540 μl sodium phosphate buffer (50 mM, pH 6.3) containing 5 mM NaCl and 50 μl D_2O.

RESULTS AND DISCUSSION

Time-resolved anisotropy experiments were undertaken to determine the molecular dynamics of the allergen molecule as well as its molecular dimensions. The fluorescence anisotropy decay of Bet v 1 is best described by a bimodal distribution of rotational correlation times (Φ_i). A very fast initial decay with a mean correlation time $\Phi_1 = 50$ ps (Figure 1) is the result of the unrestricted motion of the seven tyrosine residues along their χ_1 and χ_2 dihedral angles (22). From the long rotational correlation time $\Phi_2 = 7.35$ ns, which corresponds to the overall rotation of the entire allergen, the molecular radius of Bet v 1 was computed according to the Einstein-Stokes equation $\Phi = (\eta V)/(RT)$, with η being the viscosity of the solvent, V being the hydrodynamic volume of the molecule, R being the gas constant and T being the temperature. From this equation a hydrodynamic volume of 2.96×10^4 Å^3 can be calculated, which gives a molecular radius of 19.2 Å. This refers to a monomeric allergen under the assumption of a globular protein.

Further proof of the monomeric nature of Bet v 1 was provided by ^{1}H-NMR experiments (Figure 2). The homogeneous and narrow line width of the proton signals result from a non-aggregated,

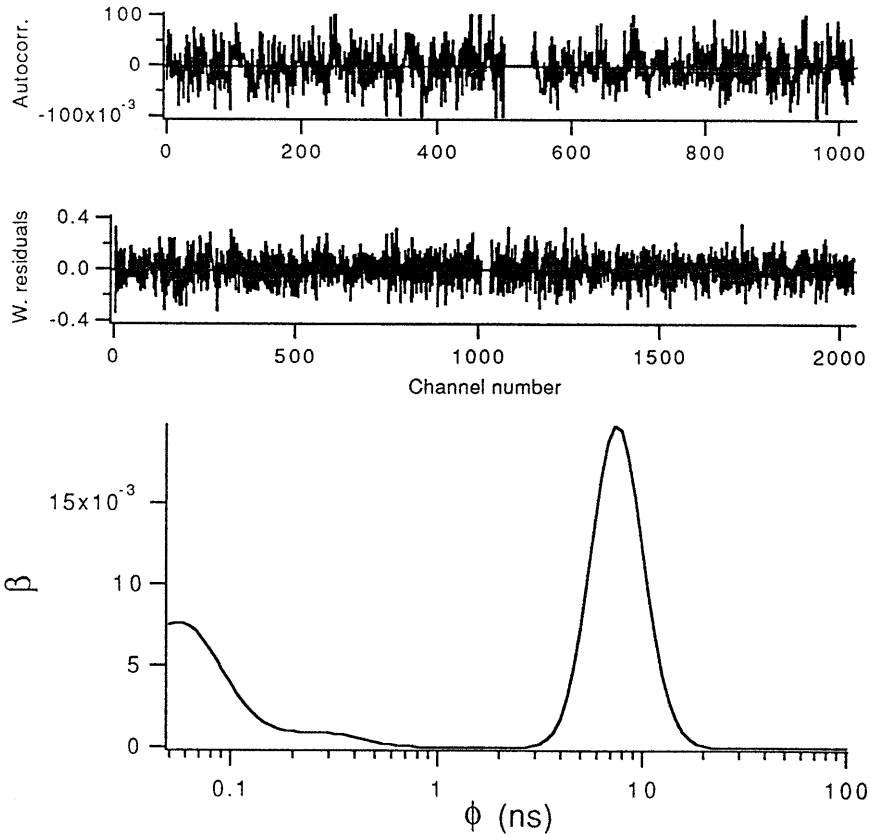

FIG. 1. Maximum entropy (MEM) analysis of the fluorescence anisotropy decay of Bet v 1 (1 μM) in 5 mM NaP_i buffer (pH 7.5) recorded at room temperature with λ_{ex} = 300 nm and λ_{em} = 320 nm. β_i are the weighted amplitudes for the corresponding rotational correlation times ϕ_i.

monomeric protein. Moreover, the NMR spectrum also points to a well defined structure of the allergen because of the high dispersion of the proton signals over the whole spectral range (see for example the clear structure of the amide protons in the range 6.6–9.9 ppm in Figure 2).

Secondary structure prediction of Bet v 1 was performed according to different methods with the aim to compare their results with each other as well as with the analysis of the CD spectrum. With the exception of the method of Burkhard and Sander (23), which predicted 20% of the amino acids to be in α-helical, 38.8% β-sheet and 41.3% loop regions, all methods concluded that the main allergen of birch is a well structured protein, with a high α-helical content ranging from 48% (24) to 35% (25). A long C-terminal α-helix, which extends from D126 to S156 (see Figure 3), was found by all computer prediction methods. No extended flexible regions were predicted, which is in good agreement with the high dispersion and the narrow line width in the ^{1}H-NMR spectrum (Figure 2).

In Figure 4 the CD spectrum of a 2.6 μM Bet v 1 solution in 5mM NaP_i (pH 7.5) is shown. The spectrum is typical for a highly structured protein with transition bands characteristic for α-helices and β-sheets. Fitting the CD raw data by the SELCON algorithm (17) revealed 32% of all amino acids to be in α-helices, 21% to be in β-sheets, 24% to be in turns, and 23% in other structural motifs. Taking also into account parallel and antiparallel β-sheet structures, 17% of all amino acids are found in antiparallel and 5% are found in parallel sheets. This agrees well with the secondary structure prediction (Figure 3).

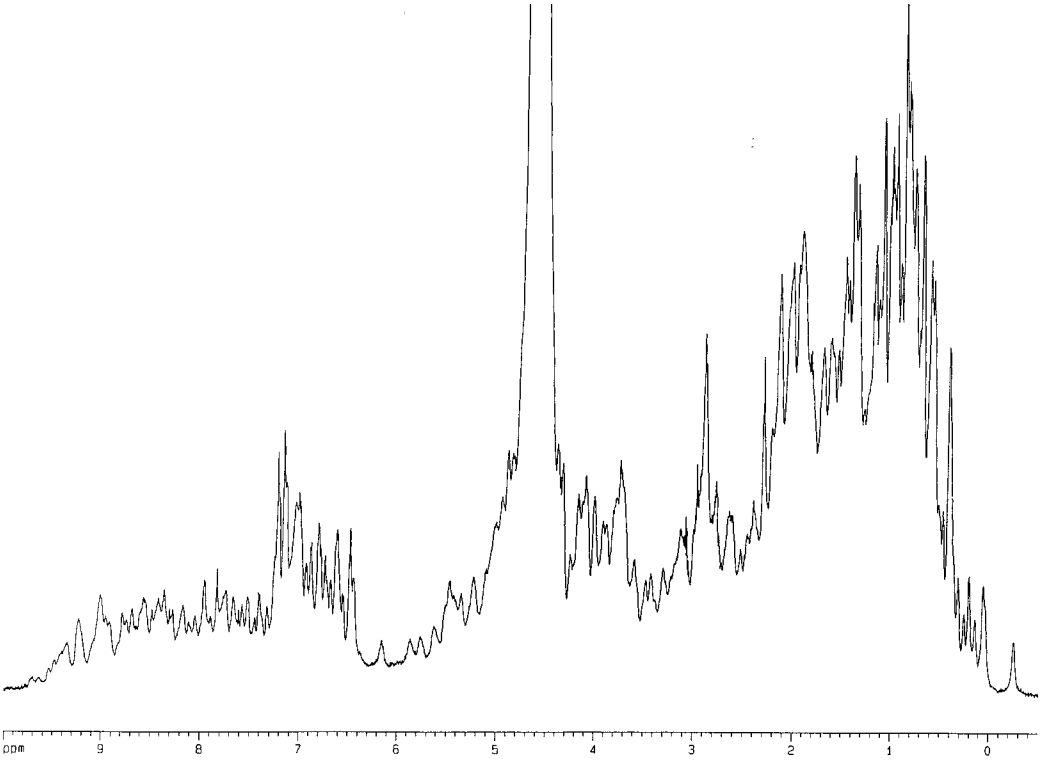

FIG. 2. ^{1}H-NMR spectrum of Bet v 1 (2 mM) in 5 mM NaP_i buffer (pH 6.3) containing 10% (v/v) D_2O and 0.02% (w/v) NaN_3. The spectrum was recorded at 35°C and 600Mhz (80 scans). The chemical shift is given in ppm relative to 5,5-dimethylsilapentanesulfonate.

Of particular interest, due to the location of one of the multiple T cell epitopes therein, is the predicted 32 amino acid C-terminal α-helix which extends from D126 to S156 (Figure 3). The CD spectra as a function of HFP of the major T cell epitope (145)LRAVESYLLAHS(156) are shown in Figure 5. In phosphate buffer no structure could be detected (Figure 5). NaCl was shown to have no secondary structure inducing effect on the peptide (data not shown). However, increasing amounts of hexafluoropropanol induced a well defined α-helical signal in this peptide (Figure 5). Hexafluoropropanol and trifluoroethanol are well documented as inducing alpha helical formation in a linear peptide sequence only when there is a significant preference for helical formation (26–28). Assuming that a mean residue ellipticity of -28900 deg.cm^2.dmol^{-1} at 222 nm corresponds to the maximum α-helical structure which this 12 amino acid peptide could adopt (29), 20% HFP induced approximately 32% α-helix in the peptide (Figure 5).

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1       MGVFNYETET TSVIPAARLF KAFILDGDNL FPKVAPQAIS SVENIEGNGG
              ttt t aaaaaaaa abbbbttt    tt  bbbbb bbbbtttt

51      PGTIKKISFP EGFPFKYVKD RVDEVDHTNF KYNYSVIEGG PIGDTLEKIS
          bbbbbbb  tt   ttt   aaaaaaattt tttbbbbb       aaaaaa

101     NEIKIVATPD GGSILKISNK YHTKGDHEVK AEQVKASKEM GETLLRAVES
        aaabbbbb t ttbbbbb tt t    aaaaaaaaaaaaaaa aaaaaaaaaa

151     YLLAHSDAYN
        aaaaaatttt
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FIG. 3. Secondary structure prediction of Bet v 1 according to Garnier *et al.* (25): a, alpha helix; b, beta sheet; t, turn. The unlabeled regions are sequences with a low probability for secondary structure formation.

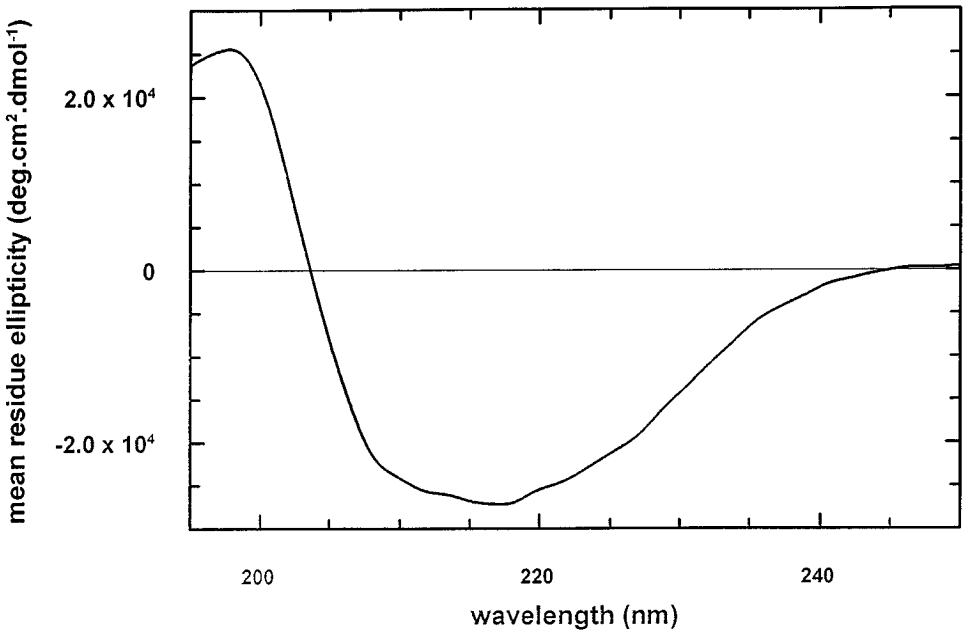

FIG. 4. CD spectrum of Bet v 1 (2.6 μM) in 5 mM NaP_i buffer (pH 7.5) at room temperature. Six scans in a cuvette with 0.1 cm path length were run to obtain smooth data.

A C-terminal α-helix was shown by Metzler et al. (15) to be a main structural feature of the ragweed allergen Amb t 5, the only allergen for which the 3-D structure has been solved by NMR methods. In Amb t 5, as in Bet v 1, the C-terminal helix contains a T cell epitope in agreement with the model of Margalit et al. (12). We thus hypothesise that the presence of this structural domain, as well as its location in the C-terminus, are general characteristics of allergenic proteins. Recent

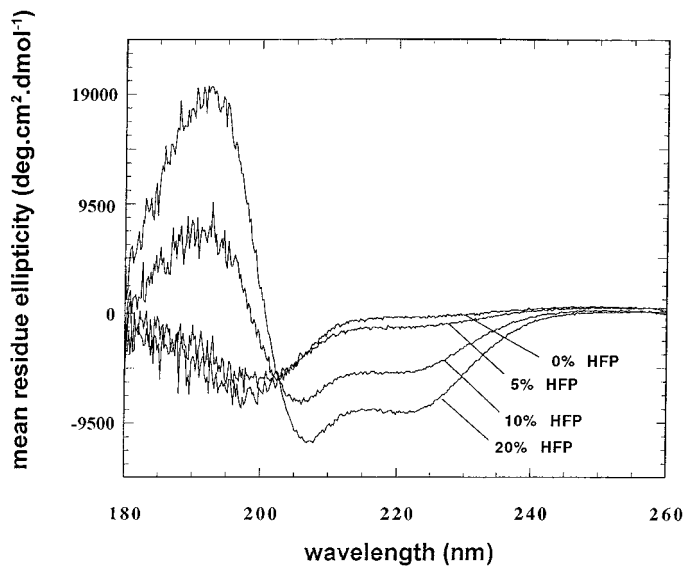

FIG. 5. CD spectra of the peptide L145-S156 of Bet v 1 (1 mg/ml) in 5 mM NaP_i buffer (pH 7.5) containing different concentrations (% v/v) of hexafluoropropanol (HFP).

crystallographic data have shown, moreover, that a peptide which represents a T cell epitope is bound to the antigen-presenting histocompatibility protein class II in a conformation characteristic of the type II polyproline helix (30). The alpha helical structure of a T cell epitope might therefore be a prerequisite for or at least facilitate MHC II antigen presentation.

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