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Expression and Secondary Structure Determination by NMR Methods of the Major House Dust Mite Allergen Der p 2*

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There exists a strong correlation between asthma and sensitization to indoor allergens. This study reports on the secondary structure of the major house dust mite allergen Der p 2, determined using heteronuclear NMR methods. The DNA was subcloned from the yeast expression vector pSAY1 into the high yield bacterial expression vector pET21a, resulting in yields of 50 mg/liter. The recombinant protein was shown to have immunoreactivity comparable with that of the natural mite protein using competitive inhibition enzyme-linked immunosorbent assay (ELISA) and a modified monoclonal radioallergosorbent test (RAST). The secondary structure was determined by examining chemical shifts, short and long range NOEs, J_H,HN-HA coupling constants, and amide exchange rates. From these data, it is clear that Der p 2 is composed of β-sheets and random coil. Based on long range distance constraints, a number of β-strands were aligned into two three-stranded, anti-parallel β-sheets.

Sensitization to inhalant allergens of the house dust mite Dermatophagoides sp. is commonly associated with asthma, atopic dermatitis, and allergic rhinitis (1, 2). Indeed up to 85% of patients with asthma have IgE Ab 1 to house dust mite Der p 2, determined using heteronuclear NMR methods. The DNA was subcloned from the yeast expression vector pSAY1 into the high yield bacterial expression vector pET21a, resulting in yields of 50 mg/liter. The recombinant protein was shown to have immunoreactivity comparable with that of the natural mite protein using competitive inhibition enzyme-linked immunosorbent assay (ELISA) and a modified monoclonal radioallergosorbent test (RAST). The secondary structure was determined by examining chemical shifts, short and long range NOEs, J_H,HN-HA coupling constants, and amide exchange rates. From these data, it is clear that Der p 2 is composed of β-sheets and random coil. Based on long range distance constraints, a number of β-strands were aligned into two three-stranded, anti-parallel β-sheets.

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The abbreviations used are: Ab, antibody; mAb, monoclonal antibody; NMR, nuclear magnetic resonance; ELISA, enzyme-linked immunosorbent assay; RAST, radioallergosorbent test; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; HSSQC, heteronuclear single quantum coherence.

** Experimental Procedures

Oligonucleotide Mutagenesis and Subcloning of rDer p 2—The coding sequence for Der p 2 was amplified from the pSAY1 yeast vector using standard polymerase chain reaction techniques (19). The 5′ primer contained the appropriate codons to mutate Asp-1 to Ser (designated D1S) to promote efficient cleavage of the N-terminal Met residue after expression in this bacterial system (20, 21). The cloning sequence was ligated into the pET-21a expression vector (Novagen Inc., Madison, WI) and subsequently sequenced to confirm the entire correct sequence including the D1S mutation.

Expression of rDer p 2 (D1S)—The Escherichia coli strain BL21-pLysE (Novagen Inc.) was used to express rDer p 2 (D1S). Protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogly-

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lactopyranoside when the culture \(A_{\text{OD}}\) reached 1.0, and the cells were harvested after 5.5 h.

Isotopically enriched rDer p 2 (DIS) was prepared by growing the bacteria in M9 minimal media with \(^{13}C\)-glucose (5 g/liter) and \(^{15}N\)-ammonium sulfate (1 g/liter) as the sole carbon and nitrogen sources. Selected colonies from plating were labeled as rDer p 2 (DIS) and were isolated using the \(E.\ coli\) trans-aminase-deficient strain DL-39. This strain was transformed with DE3 phage to supply endogenous T7 polymerase for expression of rDer p 2 (DIS). The M9 media was supplemented with 200 mg/liter of \(^{1-2}C\)-glucose in combination with either \(^{15}N\)-valine or \(^{15}N\)-leucine, similar to that described by Pennington and Rule (22).

**Isolation of rDer p 2 (DIS) from Inclusion Bodies—**Extraction of the protein was adapted from a protocol used to isolate Der f 2 from inclusion-like bodies in bacteria (23). The cell pellet was frozen for 3–4 h at \(-20^\circ C\) and subsequently thawed and resuspended to one-twentieth of the original culture volume in TE (100 mM Tris, pH 8.5, 10 mM EDTA) and sonicated for 2 min on ice. The sonicate was centrifuged (GS-54 rotor, 12,000 rpm for 20 min), and the supernatant was discarded. The pellet was dissolved in one-fifth of the original culture volume of 6 M guanidine-HCl and dialyzed against 20 mM Tris, pH 8.5, 1 mM EDTA without stirring overnight at room temperature, followed by a buffer change and an additional 4 h of dialysis. After dialysis, NaCl was added to 100 mM concentration, and the sample was centrifuged (GS-54 rotor, 15,000 rpm for 20 min). Note that at no time did the extraction or refolding buffers contain any disulfide exchange reagents, e.g. N-ethylmethylthiourea, dithiothreitol, and glutathione. Therefore, the disulfide bonds present in the final folded recombinant molecule most probably were formed during synthesis and not during the isolation procedure.

**Isolation of rDer p 2 (DIS)—**The supernatant containing the refolded rDer p 2 (DIS) was applied to a mAb affinity column consisting of the mAb dDx coupled to Affi-Gel-10 (Sigma) (24). The protein was eluted with 50 mM glycine, 150 mM NaCl, pH 2.3. The pH of the eluate was neutralized by dialysis against phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, 100 mM NaCl), concentrated, and loaded onto a size-exclusion column (Superdex G-75, 500-ml column, 2.5 × 100 cm in phosphate-buffered saline). Der p 2 containing fractions were pooled, and the purity of the sample was assessed by SDS-polyacrylamide gel electrophoresis using the PhastSystem (Pharmacia Biotech Inc.) and stained with Coomassie Brilliant Blue. Protein concentration was determined by a \(A_{\text{OD}}\) using an extinction coefficient (e) of 0.72 l/(g cm).

**Isolation of Natural Mite Der p 2—**D. pteronyssinus-spgent mite culture medium, provided by Dr. Larry Arlian (Wright State University, OH) and performed source of natural mite Der p 2 (15). The extract was passed over the dDx-affinity column, and the Der p 2 was eluted as described above.

**Antibodies—**The murine mAb used in this study were previously described for their specificity and include dDx, 6D6, 7A1, 2B12, 4G7, and 1D8 (25). The mAb 13A4 and 15E11 were raised against Der f 2 and isolated at the Human Investigation Committee of the University of Virginia. Patients were either skin test positive to \(D.\ pteronyssinus\) or RAST positive to Group 2 allergens and presented with asthma (\(n = 24\)), atopic dermatitis (\(n = 19\)), and allergic rhinitis (\(n = 3\)), as well as non-atopic controls (\(n = 5\)).

**Competitive Inhibition ELISA—**The competitive inhibition ELISA was performed as described previously (26). The mAbs were mixed with increasing concentrations of inhibitor, either natural mite Der p 2 or rDer p 2 (DIS). The mixtures were added to natural mite Der p 2-coated wells, and subsequently, bound Ab was detected using horseradish peroxidase-conjugated goat-anti-mouse IgG and substrate. Results are reported as a percent inhibition with respect to the reaction in the absence of inhibitor.

**Monoclonal Antibody-modified RAST Assay—**As described previously, the anti-Der p 2 mAb dDx was coupled to cytochrome c-activated filter paper disks. The mAb-conjugated disks were incubated with either natural mite Der p 2 or rDer p 2 (DIS) at 10 \(\mu\)g/ml. Subsequently, the disks were washed and mixed with dilutions of patient sera. The disks were then incubated with \(^{13}I\)-labelled Der p 2 or \(^{13}I\)-labelled anti-human IgE, washed, and counted for \(^{13}I\) activity (13).

**NMR Spectroscopy—**All spectra of rDer p 2 (DIS) were obtained at 25 °C in a buffer of 10 mM sodium phosphate, pH 6.0, 50 mM NaCl, 100 mM K$_2$SO$_4$, 5 \(\mu\)M EDTA, sodium azide (0.02% w/v), and D$_2$O (5% v/v). The NMR experiments were performed at a proton frequency of 500 MHz using a Varian Unity-Plus spectrometer equipped with a Nalorac 5-mm triple resonance probe with a shielded z-gradient coil. The sweep widths, data processing, and assignment strategy for the heteronuclear experiments were virtually identical to those described by Briercheck et al. (27). The chemical shifts were assigned using the following triple resonance experiments: HNCA (28), CA(CO)NH (29), HN(CA)HA (30), HN(CC)NH (33), HNCO (28), CBACOHA (34), HBBH(CHCA)NH (29), HCCH-TOCSY (35), CH-TOCSY (27), H(HC)NH (36), and C(CO)NH (36). To provide additional sequential information, two double-label preparations (\(^{15}N\)-Leu + \(^{1}C\)-Gly, and \(^{15}N\)-Val + \(^{13}C\)-Gly) provided residue-specific assignments. NMR spectra were referenced as described by Jerala et al. (37).

**High Level Expression of rDer p 2 (DIS)—**The expression and isolation protocol reported here consistently yielded 50 mg or more of purified protein per liter of media. The final protein preparation gave a single band on SDS-polyacrylamide gel electrophoresis. N-terminal amino acid sequencing confirmed the purity of the protein sample and also confirmed the correct processing of the initiator methionine and the serine substitution (data not shown).

**Immunoreactivity of rDer p 2 (DIS)—**The immunoreactivity of rDer p 2 (DIS) and the natural allergen was compared using competitive inhibition ELISA and a modified monoclonal RAST assay. The results for the competitive inhibition ELISA are shown in Fig. 1, arranged in clusters based on previous studies which showed that the panel of Group 2 specific monoclonal antibodies recognized four distinct and non-overlapping antigenic regions on the surface of Der p 2 (25). Monoclonal antibodies recognizing the three antigenic regions defined by 15E11, dDx, 7A1, 13A4, and 6D6 did not distinguish between natural Der p 2 and rDer p 2 (DIS) in that they gave overlap.
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Fig. 2. $^{15}\text{N}/^{1}\text{H}$-HSQC spectra of rDer p 2 (D1S). The highlighted regions, A and B, show examples of residues with multiple $^{15}\text{N}/^{1}\text{H}$ peaks in comparison with other peaks.

Fig. 3. Secondary structure of rDer p 2 (D1S). a, Chemical Shift Index. The bars indicate the predicted secondary structure for each residue based on the chemical shift of the $\delta_{\text{C}_t}$, $\delta_{\text{C}_z}$, CO, and H$_{\text{N}}$, b, $d_{\text{NN}}$-NOEs. The bar height indicates the intensity, on an arbitrary scale, of the H$_{\text{N}}(i) \rightarrow$ H$_{\text{N}}(i+1)$ NOESY cross-peak in the $^{15}\text{N}$-NOESY-HSQC. Blanks indicate the absence of a cross-peak or a residue that was too degenerate to determine unambiguously. c, $d_{\text{NN}}$-NOEs. The bar height indicates the intensity, on an arbitrary scale, of the H$_{\text{N}}(i) \rightarrow$ H$_{\text{N}}(i+1)$ NOESY cross-peak in the $^{15}\text{N}$-NOESY-HSQC. CH$_{\text{N}}$-NOESY, and CN-NOESY. Blanks indicate the absence of a cross-peak or a residue that was too degenerate to determine unambiguously. d, $J_{\text{HN-HA}}$. The bar height indicates to $J_{\text{HN-HA}}$ coupling constant for that residue. β-sheet residues typically have coupling constants of 8–10 Hz, and α-helices typically have coupling constants of 4–7 Hz. These ranges are indicated by dashed lines. Residues that could not be found in the HNHA or were too degenerate to measure accurately are indicated with a zero bar height. e, amide exchange. A value of 1 indicates that the amide proton of that residue remained protonated for greater than 1 week after exchange into D$_2$O buffer.

The secondary structure of Der p 2 (D1S) was determined by analysis of chemical shift, short and long range NOE-SYs, and $J_{\text{HN-HA}}$ coupling constants (Fig. 3). Using the Chemical Shift Index, the deviations of the chemical shift for $\delta_{\text{C}_t}$, $\delta_{\text{C}_z}$, CO, and H$_{\text{N}}$ were analyzed for each residue, and the type of secondary structure was predicted (43). The protein is predicted to be composed solely of β-sheet and random coil (Fig. 3a).

The intensity of certain short range NOE-SYs are indicative of local secondary structure. For example, β-sheet residues show weak $d_{\text{NN}}$-NOEs and strong $d_{\text{NN}}$-NOEs while α-helical residues show the opposite trend. Regions predicted by the chemical shift index to have a β-sheet conformation tend to have weak $d_{\text{NN}}$-NOEs and strong $d_{\text{NN}}$-NOEs (compare Figs. 3, a–c). Regions with strong $d_{\text{NN}}$-NOEs correlate well with the chemical shift index prediction of random coil.

The $J_{\text{HN-HA}}$ coupling constant for each residue is shown in Fig. 3d. Coupling constants of 8–10 Hz generally correspond to β-sheet residues and 4–7 Hz generally correspond to α-helical residues. In general, regions predicted to be in sheet by the Chemical Shift Index show high coupling constants. Fig. 3e shows which amides were classified as strongly protected. Strongly protected amides are indicative of hydrogen bonding and/or residues involved in a form of stable secondary structure. The protected residues appear to be involved in the formation of β-strands (Fig. 4).

Alignment of β-strands—The alignment of β-strands as shown in Fig. 4 was based on the analysis of long range NOE-
SYs including HN-HN', HN-Hs', and Hs'-Hs' NOEs that are displayed as dotted lines. Ovals encircle strongly protected amides and their potential hydrogen bonding partner. A majority of the residues predicted to be involved in β-strands are shown to have long range interactions with another strand. The amide protection data correlate well with the long range NOESYs to define the β-strand interactions.

DISCUSSION

The recombinant expression system reported here provides ample quantities of rDer p 2 (D1S) for labeling with heavy isotopes preparatory to NMR analysis. In addition, immunological analyses showed the recombinant protein to have the same conformation as the natural mite allergen.

In multiple competitive inhibition assays, mAb recognizing three of four antigenic regions did not distinguish between the natural and recombinant Der p 2 forms. This strongly suggests that rDer p 2 (D1S) has an overall native conformation. In contrast, mAb that recognize epitopes of the fourth region did see differences. However, this is likely due to the isoform specificity of these three antibodies. For example, when the same isoform is expressed in yeast, without the N-terminal mutation, there was similar lack of reactivity with the 1D8 and 4G7 mAb. Therefore, the lack of reactivity of these two mAb with the rDer p 2 (D1S) protein used in the present study was not due to the D1S change. Furthermore, site-directed mutants that mimicked substitutions found in a Der p 2 isoform were made, and the mAb 2B12 was found to react better with the Asp-114 isoform than it did with the Asn-114 isoform. Our study used the Asp-114 isoform, whereas the natural allergen stock used as a competitive inhibitor presumably contains all possible isoforms since it was isolated with the mAb aDpX, which has not been shown to be isoform-specific. Consequently, the results showing that mAb 2B12 reacts more strongly with the recombinant allergen are likely due to the isoform specificity of mAb 2B12 and not to any overt conformational difference in this region.

Similarly, the results of the RAST assay show a good correlation between the reactivity of human IgE antibody with the

3 G. A. J. Hakkaart, unpublished observations.
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FIG. 5. Potential tertiary homologues. The global fold of Der p 2 as determined by the alignment of the strands indicated in Fig. 4 is shown. The global fold of a member of the immunoglobulin superfamily (Ig) and alpha amylase inhibitor (AA) are shown for comparison.

recombinant and natural forms of Der p 2. These results strongly suggest the rDer p 2 (D1S) is in the same conformation as the natural mite Der p 2 allergen.

The heteronuclear resonances of the rDer p 2 (D1S) protein were assigned, and the secondary structure was characterized. The majority of resonance assignments of each residue fall in the normal range of resonances associated with that particular residue type.

The NMR data strongly suggest that the protein is composed predominantly of $\beta$-sheet and random coil. The secondary structure results, long range NOEYs, and strongly protected amides show an excellent correlation. These data show that Der p 2 is composed of two three-stranded $\beta$-sheets involving residues 15–17, 34–48, 51–57, 79–94, 105–111, and 118–123 as shown in Fig. 4. The secondary structure of these strands can be described as regular $\beta$-sheet, except in the region of Gly-83. In this region, the interstrand interactions appear to be broken, suggesting the presence of a $\beta$-bulge.

Since there appear to be two three-stranded anti-parallel $\beta$-pleated sheets, a search of several structural data bases was initiated. Only a single potential structural homologue, the $\alpha$-amylase inhibitor (1hoe.pdb), was found. The alignment of the sheets relative to the preliminary model of Der p 2 is shown in Fig. 5. The arrangement of secondary structural elements in $\alpha$-amylase inhibitor is clearly unlike that of Der p 2. An examination of other strand-loop-strand motifs in the data base revealed that the two-dimensional model of Der p 2 appears similar to that of the immunoglobulin superfamily, also shown in Fig. 5. Strand 4 of the immunoglobulin superfamily has been replaced with an extended loop that comprises residues 59–78 of Der p 2. However, a crucial element of the immunoglobulin superfamily is the Greek-key motif, and Der p 2 is lacking the strand that would define a Greek-key. Whether the Der p 2 fold is a new fold or a distant relative of the $\alpha$-amylase inhibitor or the immunoglobulin superfamily remains to be determined. What is clear is that the structure is not similar to that of the avian lysozymes as was previously suggested (44). Hen egg white lysozyme contains several $\alpha$-helices that span residues clearly defined as $\beta$-sheet in Der p 2.

In conclusion, we report here the expression, purification, and characterization of the secondary structure of the major mite allergen Der p 2. The data from the competitive inhibition ELISA and the serum IgE RAST indicate that the recombinant is markedly indistinguishable from the natural mite protein, thus meeting an important criteria for using the recombinant allergen in structural studies. The secondary structure analysis using chemical shift, short and long range NOEYs, $J_{\text{NN-HA}}$ coupling constants, and amide protection corroborate that the protein is composed of $\beta$-sheet and random coil. Efforts now are being directed at determining a well refined solution structure of the protein.

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