

The Crystal Structure of a Major Dust Mite Allergen Der p 2, and its Biological Implications

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The crystal structure of the common house mite (*Dermatophagoides* sp.) Der p 2 allergen was solved at 2.15 Å resolution using the MAD phasing technique, and refined to an *R*-factor of 0.209. The refined atomic model, which reveals an immunoglobulin-like tertiary fold, differs in important ways from the previously described NMR structure, because the two β -sheets are significantly further apart and create an internal cavity, which is occupied by a hydrophobic ligand. This interaction is structurally reminiscent of the binding of a prenyl group by a regulatory protein, the Rho guanine nucleotide exchange inhibitor. The crystal structure suggests that binding of non-polar molecules may be essential to the physiological function of the Der p 2 protein.

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Introduction

Hypersensitivity to house dust mite (*Dermatophagoides* sp.) allergens is one of the most common allergic reactions in the world. Estimates suggest that as many as 10% of the general population and 90% of individuals suffering from allergic asthma are sensitive to house dust mites. The severity of the problem is on the rise, with at least 45% of young people with asthma showing sensitivity. The house dust mite allergen, Der p 2, is a major allergen and 80–90% of patients who react to mite extract, react specifically to this protein or

its homologues.^{1–3} Because of the socioeconomic, emotional, and physical distress that such allergies present, major efforts have been initiated to understand the relation between the structure of allergens and the molecular basis of allergy and asthma. Towards this end, we have undertaken to determine the three-dimensional structure of the Der p 2 allergen, to complement our studies aimed at the determination of the location of epitopes recognized by both murine monoclonal and human patient IgE antibodies. Although the solution structure of the protein by NMR has been reported,⁴ the high-resolution crystal structure solved using experimentally derived phases differs in important ways. These differences may be important in understanding the biological function of the Der p 2 molecule in mites as well as the immunochemistry and immunobiology of human IgE anti-Der p 2 responses in particular, and IgE anti-allergen responses in general. Finally, the crystal structure suggests that Der p 2 is a lipid-binding

Abbreviations used: Der p 2, major group 2 allergen from *Dermatophagoides pteronyssinus*; rDer p 2, recombinant Der p 2; PEG, polyethylene glycol; MAD, multiwavelength anomalous diffraction; NOE, nuclear Overhauser effect.

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Table 1. Crystallographic data

	SeMet-41 PEG inflection point	SeMet-31 PEG peak	SeMet-51 PEG low-energy remote	SeMet-11 PEG high-energy remote	dp2-as AS frozen	hamb-nat AS room temp.	peg-nosp PEG frozen
Beamline	NSLS	NSLS	NSLS	NSLS	NSLS	HAMB	In-house
Resolution (Å)	2.5	2.5	2.5	2.5	2.2	2.15	3.0
Wavelength (Å)	0.9791	0.9784	0.9801	0.9724	1.0715	0.9	1.57
<i>R</i> (<i>I</i>) merge (%) ^a	5.5(32.1)	5.8(24.6)	5.5(34.4)	5.7(22.7)	5.3(30.4)	5.3(38.4)	8.3(46.8)
<i>I</i> / σ (<i>I</i>)	27(4)	30(6)	27(4)	28(7)	27(5)	27(3)	21.3(2)
Completeness (%) ^a	99.8(98.7)	99.6(98.5)	99.8(98.6)	99.8(99.5)	99.3(94.0)	99.2(77.3)	99.3(95.2)
Redundancy (%)	4.8	4.8	4.8	4.8	4.0	6.1	6.9
Multiple anomalous dispersion							
<i>R</i> _{iso} (%)			2.8	3.3	4.3		
<i>R</i> _{cullis} (to 3 Å)			0.94	1.05	0.85		
<i>R</i> _{cullis-ano}			0.75	0.97	0.75		
Phasing power			1.49	0.74	1.48		
Overall figure of merit after Mlphare			0.54(to 3 Å)				
Overall figure of merit after DM			0.76(to 3 Å)				
Refinement							
Resolution in refinement (Å)						20–2.15	
Number of reflections						17,104	
No. reflection in <i>R</i> _{free}						1947	
Protein atoms						1970	
Solvent atoms						169	
Ligand molecules						4	
<i>R</i> -factor (%)						20.9	
<i>R</i> _{free} (%)						27.4	

^a Numbers in parentheses show statistics for the highest resolution shell.

protein, like some of its distant mammalian homologues.

Results

Overall structure

Attempts to use the NMR model in the molecular replacement protocol have failed despite numerous efforts. The structure was solved by the multiwavelength anomalous diffraction (MAD) method using a SeMet-substituted recombinant Der p 2. The conventional *R*-factor for the final model is 20.9% (Table 1). The model includes two independent Der p 2 (Figure 1(a)) molecules, with all 129 residues in each, and 169 water molecules. The stereochemical quality of the protein model was assessed with program PROCHECK:⁵ 90% of the residues are in the most favorable region of the Ramachandran plot and no residue is in the disallowed region. The overall error on the atom positions based on the Cruickshank's DPI (diffraction-data precision indicator)⁶ is 0.24 and 0.22 Å based on R_{free} .

The overall tertiary fold of Der p 2 is that of two anti-parallel β -pleated sheets overlying each other at an angle of approximately 30°, in agreement, in general terms, with our earlier report of the NMR structure.⁴ This fold is characteristic of the immunoglobulin superfamily and Der p 2 appears to belong to the E-set domain subfamily. The crystal structure provides further details. The molecule contains ten β -strands and a short (one-turn) 3_{10} helix (Figure 1(b) and (c)). Eight β -strands form a β -sandwich with a $\beta 1' - \beta 6 - \beta 5 - \beta 3 - \beta 3' - \beta 4 - \beta 2 - \beta 1$ topology, related to an s-type (seven-stranded strand-switched) immunoglobulin fold.⁷ Sheet I contains three strands: E (residues 85–93), B (residues 34–42) and A (residues 13–17). Sheet II contains five strands: A' (residues 6–8), C' (residues 61–64), C (residues 51–58), F (residues 104–112) and G (residues 115–122). Although strand A' (residues 6–8) forms a hydrogen bond ladder with the β -sheet II, it is not part of the immunoglobulin fold, and neither are strands B' (29–30) or G' (127–128). Two β -strands (A' and C') seen in the crystal structure are not observed in the NMR structure.⁴

There are three disulfide bonds in Der p 2 forming covalent bonds between residues 21 and 27, 73 and 78, and 8 and 119. None of these is found in the Ig-like molecules, and the disulfide bond that connects strands B and F in many of the Ig-like molecules is absent from the Der p 2 structure.

Putative lipid-binding cavity

In contrast to the NMR model, the X-ray structure shows a greater distance between the two β -sheets than is found in other proteins with related topology. The resulting cavity is ~9 Å wide, 15 Å long and 3.5 Å deep (Figure 2(a) and

(b)), and it narrows along the median, with Ala108 and Ile37 on each side, splitting into two, almost separate, elongated pockets. The initial MAD-phased electron density map contained two distinct, elongated fragments of high electron density within the cavity, each able to accommodate an aliphatic chain of 14–16 carbon atoms in length. Since the crystal used for the MAD experiment was grown from a solution containing 5 mM spermine, our early assumption was that this extra electron density was due to two spermine molecules. However, structures of Der p 2 built using X-ray data collected from crystals grown from either PEG or ammonium sulfate solutions with no additives showed the same cavity and electron density (data not shown). Although we have not established their identity, the ligand molecules must be hydrophobic because the cavity is lined with hydrophobic and aromatic amino acid residues (Figure 2(c)). Sheet II contributes the following: strand G, Ala118, Ala120 and Ala122; strand F, Val104, Val106, Val108, Val110 and strand C, Ile52, Ile54 and Ile58 and Ala56. This conservation of residue types is unique and is not seen in other β -sandwich structures. More variation is observed in Sheet I, which contributes several aromatic residues (Phe41, Tyr90, and Trp92) and several hydrophobic residues (Val16, Val18, Leu37, Ile88, Ile13, and Ala39). This architecture suggests strongly that the Der p 2 molecule has evolved to bind lipid-like molecules. The cavity is connected with the exterior through a short tunnel, about 2.5 Å in diameter, lined with several proline residues: Pro66, Pro95, and Pro99.

As already pointed out, the NMR structure is not consistent with the presence of an internal cavity (Figure 3(a)). It is noteworthy that the original crystals of Der p 2 were obtained from the same protein samples that were used in our NMR experiments. Subsequent samples were obtained following identical expression and purification procedures. Regardless, the crystal structure of Der p 2 is distinctly different from the NMR structure of Der p 2. It is quite possible that the NMR structure represents a distinct form of the protein. For example, there are nuclear Overhauser effects (NOEs) between the β -sheets at the center of the hydrophobic cavity and, specifically, dipolar coupling was observed between residues 37 and 106, 37 and 108, and 52 and 88 (G.S.R. & G.A.M., unpublished results). The existence of these NOEs implies that the two sheets are in contact in at least a significant fraction of molecules, although the exact distance between the sheets is not established.

Comparison with other immunoglobulin-like proteins

Although topologically equivalent, the X-ray structure of the Der p 2 molecule and the IgC (constant) domains do not align well, perhaps due to the lack of the inter-sheet disulfide bond that is

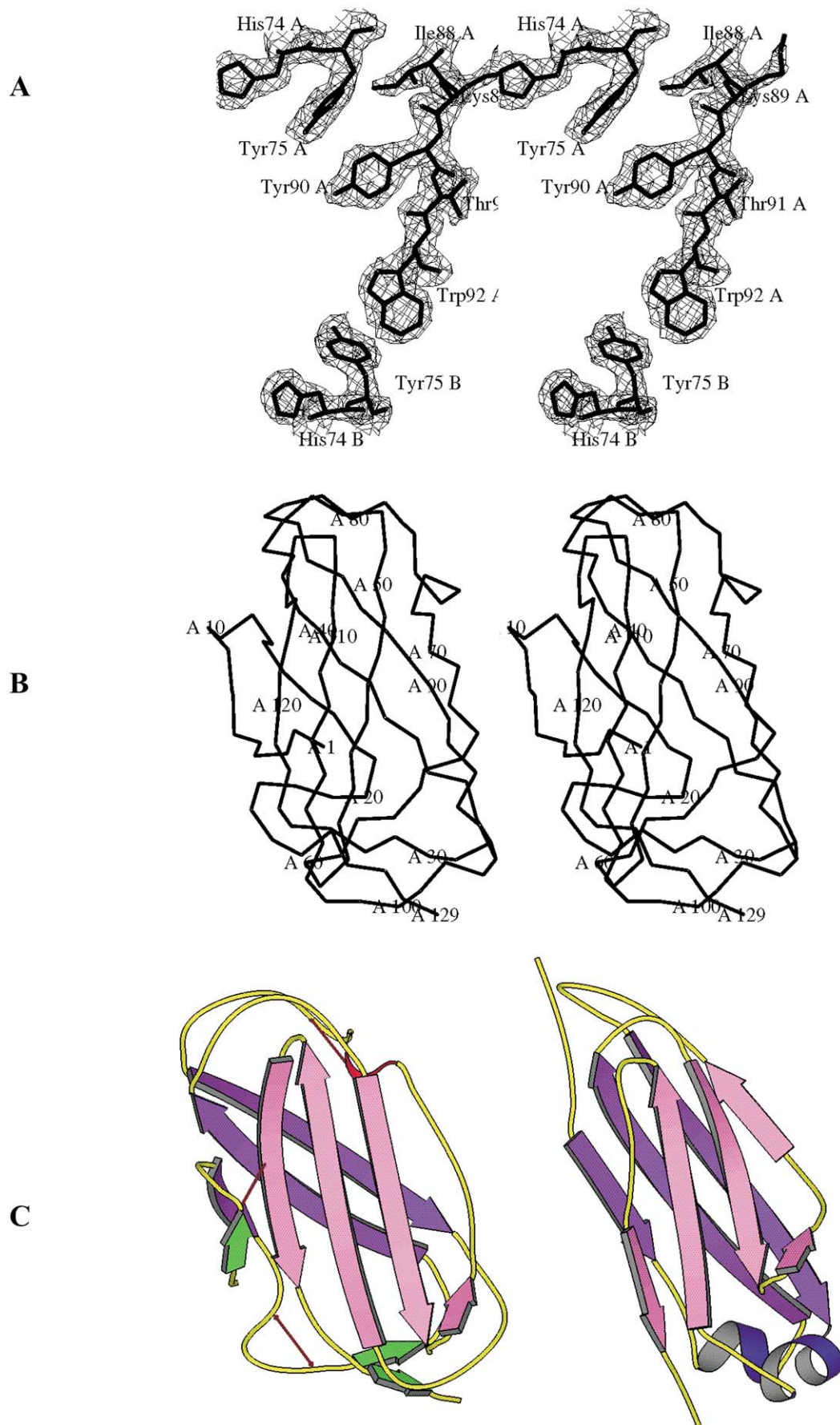


Figure 1 (legend opposite)

found in Ig domains and is responsible for their compactness. In Ig-like structures, the distance between the C α atoms of the B3 and F3 strands increases from 6.1–7.1 Å for structures with the disulfide to 7.2–11.7 Å for those structures without it.⁸ In addition, the cysteine residues are replaced by hydrophobic residues in order to maintain the Ig-fold. In the crystal structure of Der p 2, the distance between the equivalent residues on the two strands, B3 (Ala39) C α and F3 (Val108) C α atoms, is 11.72 Å, similar to the maximum distance listed above. Nevertheless, the crystal structure of Der p 2 does not align well with canonical Ig-like structures, even those lacking the disulfide bond.

The only other molecule with relatively close structural similarity to Der p 2 is the human Rho-specific guanine dissociation inhibitor, RhoGDI (Figure 3(b)).⁹ Interestingly, RhoGDI sequesters the prenylated tail of Rho-family GTPases,^{10,11} and is the only known example of an Ig-like molecule that has an internal ligand-binding cavity. However, the prenyl group occupies less volume than the two ligands in Der p 2, and the remainder of the core of RhoGDI is packed with bulky hydrophobic side-chains. The Der p 2 amino acid sequence shows distant homology to mammalian secretory epididymal protein He1, which is known to bind cholesterol with high affinity.¹² Mutations in the gene coding for He1 are known to cause the Niemann-Pick type C2 disease (NP-C2), a fatal hereditary disorder characterized by defective egress of cholesterol from lysosomes.¹³ It is certainly possible that in mites Der p 2 serves a biological function involving binding and/or transport of a lipid-like molecule. In this context, it is interesting to note that there are reports in the literature of lipid-binding proteins eliciting an allergic response.^{14–16}

Implications for understanding and modifying allergic disease

The mapping of epitopes on Der p 2, and in fact on any allergen, is an important and necessary step to the rational design of immunotherapy for allergic reactions to this protein. We have shown that murine monoclonal antibodies to Der p 2 can be placed into three groups on the basis of the antigenic region on the protein surface that they recognize.^{17,18} More recently, we have used NMR and site-directed mutagenesis methods to map the location of three epitopes recognized by three

murine monoclonal antibodies (Figure 2(d)), one from each group.¹⁹ In addition, we have shown that almost all the IgE anti-Der p 2 antibodies from several allergic patients are directed toward two of these antigenic regions suggesting that the Der p 2 allergen in humans is paucivalent rather than polyvalent. This clearly raises the possibility of engineering null Der p 2, which would not react with pre-existing patient IgE, reducing the possibility of systemic anaphylaxis following injection of the null Der p 2 as an immunotherapeutic agent. This therapy could be designed to activate regulatory T-cells to induce suppression of IgE formation by one or more mechanisms including a shift to the Il-4 independent synthesis of IgG4 anti-Der p 2 rather than IgE. In order to produce such a null allergen, its structure must be known in detail. The crystal structure reported here, may allow for a more accurate prediction of which residues lie within the epitope further enhancing the ability to produce a null immunotherapeutic Der p 2 molecule. This approach would, at least theoretically, be of general applicability and thus could be extended to any allergen for which structural information is available.

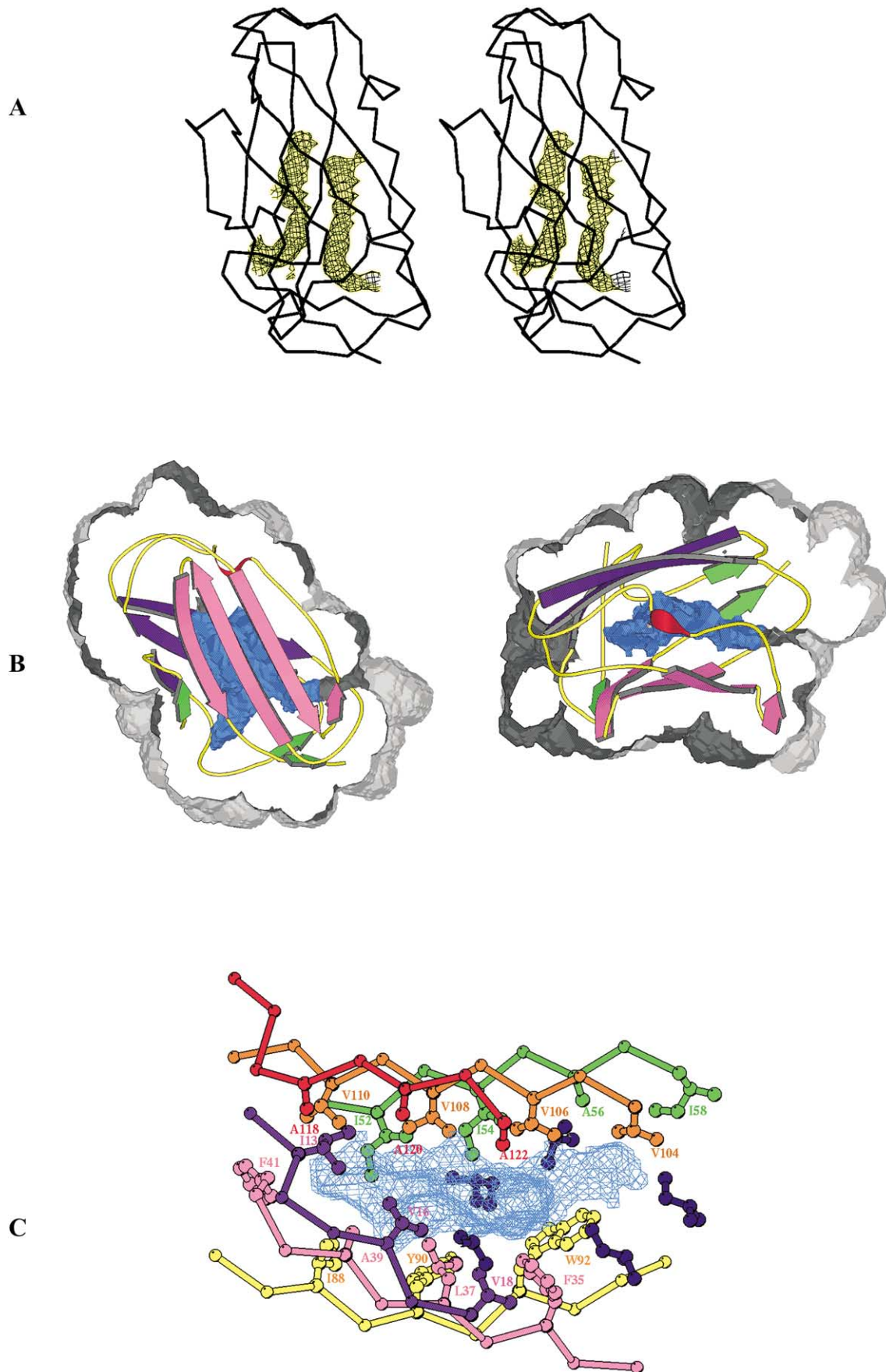
Materials and Methods

Production and crystallization of Der p 2

Recombinant Der p 2 was prepared as described.¹⁸ The plasmid used in this study has the N-terminal Asp replaced with Ser. Recombinant Der p 2 was purified by monoclonal antibody affinity chromatography followed by size-exclusion on Sephacryl-200 to remove trace amounts of a single 30 kDa contaminant. The protein was concentrated to approximately 5 mg/ml, dialyzed against distilled water, and concentrated to 20 mg/ml. Crystallization conditions were evaluated using Crystal Screen reagents (Hampton Research Inc.) using the hanging drop method. Der p 2 crystallizes from either ammonium sulfate or from PEG 4000 with a sodium citrate or a sodium acetate buffer in pH from 4.6 to 5.6. The best conditions with ammonium sulfate as the precipitating agent are 1.8 M ammonium sulfate, 100 mM sodium citrate, pH 4.6. The best PEG conditions are 35% (w/v) PEG 4000, 5 mM spermine (or 0.02% (v/v) β -octylglucoside), 100 mM sodium citrate, pH 4.6. In general, the PEG crystals are easier to grow and handle.

The PEG and ammonium sulfate crystals belong to the same orthorhombic space group, C22₁. The unit cell dimensions of the crystals grown from PEG are $a = 68.8$ Å, $b = 105.7$ Å, and $c = 89.7$ Å. The ammonium

Figure 1. (a) Stereo view of representative electron density for Der p 2 molecule. The map was calculated in SIG-MAA mode with $2mF_o - dF_c$ coefficients, at the 1.5σ level. (b) Stereo view of the C α trace of the Der p 2 molecule. (c) Comparison of Der p 2 (left) with a constant domain of N10 Fab fragment (1NSN.pdb, right). The Der p 2 molecule displays a Greek-key motif. It is composed of two β -sheets. Sheet II (pink) contains strand A' (residues 6–7), strand G (residues 115–122), strand F (residues 104–112), strand C (residues 51–58) and strand C' (residues 61–63). Sheet I (purple) consists of strand A (residues 13–17), strand B (residues 34–42) and strand E (residues 85–93). Strands that do not partake in the Ig-like fold are colored green. The single turn 3_{10} helix (residues 72–74) is colored red. Brown sticks represent disulfide bonds.



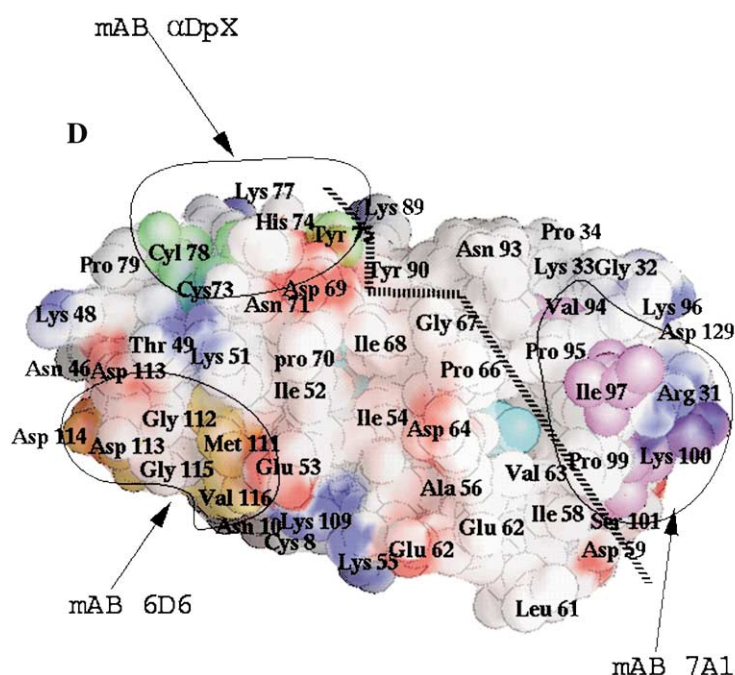


Figure 2. (a) Chicken-wire representation of the $F_o - F_c$ electron density map displayed at the 1.5σ level. The map is phased using protein atoms only. The program Bobscrip³¹ was used to generate the Figure. (b) Representation of the inner cavity inside the Der p 2 molecule. The secondary structure elements are colored as in Figure 1(c). The accessible surface of the Der p 2 molecule is shown in gray. The cavity surface (calculated with a 1.37 \AA probe radius) is colored in blue. Left, top view of the molecule; right, a view perpendicular to Figure 1(c), looking from the C strand side. The surfaces were calculated using the program VOIDOO³⁰ and displayed in Bobscrip.³¹ (c) A close-up of the inner cavity (chicken-wire) and of the amino acid residues lining the cavity (ball-and-stick). Strand C residues are in green, strand F in orange, strand G in red. Purple represents A strand; pink, B strand and yellow, E strand. Dark blue residues belong to the inter-strand connecting loops. (d) GRASP³² representation of the Der p 2 crystal structure. Circled are three known immunogenic epitopes on the surface of the molecule, based on amide exchange measurements.¹⁹ The broken line marks the groove between two β -sheets. A fragment of one of the two unknown molecules filling the inner cavity, colored turquoise, reveals the entrance to the cavity.

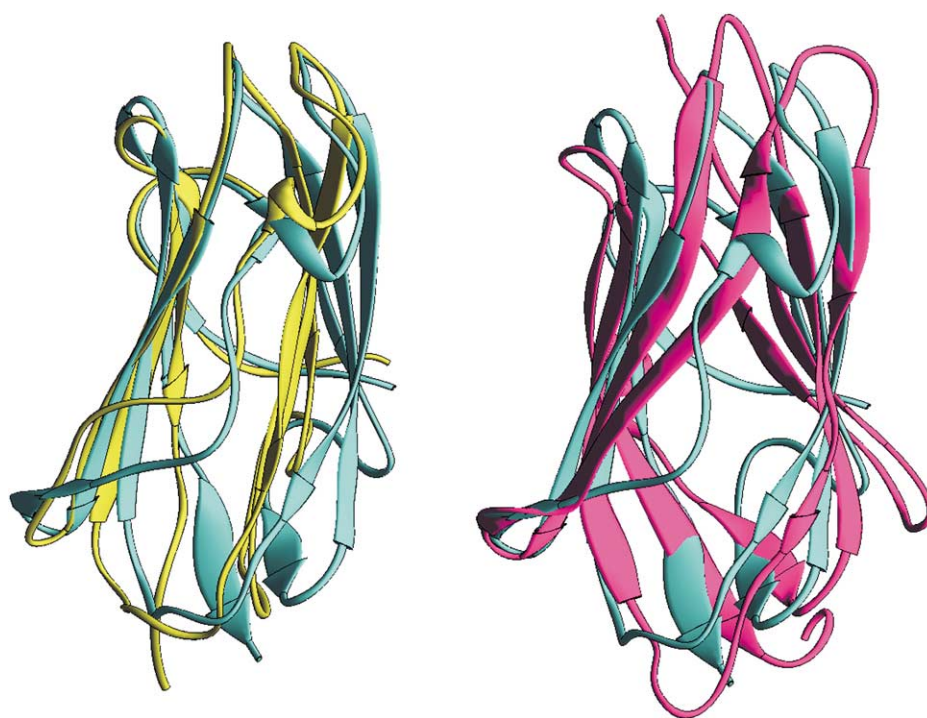


Figure 3. Left, comparison of the X-ray structure (turquoise) of the Der p 2 molecule (molecule A) with the NMR structure (yellow, 1A9V.pdb). Right, comparison of the X-ray structure (turquoise) of the Der p 2 molecule (molecule A) with the RhoGDI molecule (pink, 1DOA.pdb). The molecules were overlapped on residues in sheet II.

sulfate crystals have a slightly larger unit cell with $a = 71.1 \text{ \AA}$, $b = 107.1 \text{ \AA}$, and $c = 91.4 \text{ \AA}$.

Overexpression and crystallization of SeMet Der p 2

For Se-labeled recombinant Der p 2, the pET21a expression vector was transformed into *Escherichia coli* strain B834, a methionine auxotroph. The bacteria were grown in LeMaster medium^{20,21} enriched with a vitamin and nutrient mix. Protein expression was induced with 1 mM IPTG and the cells were harvested 18 hours later. The solubilization and isolation of Se-rDer p 2 was carried out as described above, except that 5 mM methionine was added to all buffers to prevent oxidation. Following purification, the protein was concentrated to 20 mg/ml in water with addition of 2 mM DDT. The crystallization conditions for Se-Met Der p 2 were the same as for native Der p 2. No X-ray quality Se-labeled crystals were obtained without the addition of spermine or β -octylglucoside, or using ammonium sulfate as the precipitating agent.

Data collection

Multiwavelength anomalous diffraction (MAD) data were collected at the National Synchrotron Light Source, beamline X9B, at Brookhaven National Laboratory using ADSC CCD. Four data sets were collected: at the inflection point of the rising edge of the selenium absorption spectrum (0.9791 \AA wavelength), at the peak of the absorption (0.978 \AA), and at two remote points at the ends of the selenium absorption spectrum (0.980 and 0.972 \AA). The crystals for the MAD experiment were grown from PEG solution containing 5 mM spermine. All data sets were collected using a single crystal flash-frozen in mother liquor containing 28% (v/v) glycerol.

The native data set from ammonium sulfate grown crystals was collected at the EMBL outstation, Hamburg, Germany. This data set was collected at room temperature from several crystals at 0.9 \AA wavelength. Another data set was collected from PEG-grown crystals without the addition of spermine or β -octylglucoside using a rotating anode source and a Raxis IV scanner. See Table 1 for the statistics on all data sets.

Phasing and refinement

The X-ray structure of Der p 2 allergen was solved using phases from Se-MAD experiment. The positions of the four selenium atoms were found using either Patterson function or direct methods algorithm in SHELX.²² The phasing of the data using MLPHARE^{23,24} was adequate for calculation of the initial electron density maps (see Table 1 for phasing summary). Calculation of electron density provided a visual difference between the protein and solvent region for one of the enantiomers. Density modification with solvent flattening and histogram matching using programs DM²⁵ improved the phases and raised the figure of merit to 0.7. Symmetry averaging using RAVE²⁶ improved the electron density maps to the point where most of the residues could easily be modeled into the electron density. The model of one molecule of Der p 2 was built at this point using the program O.²⁷ The other molecule in the asymmetric unit was generated by applying non-crystallographic symmetry. The model of those two molecules was then subjected to refinement using the slow-cooling protocol

in X-PLOR.²⁸ The non-crystallographic symmetry restraints were used at this point.

The high-resolution 2.15 \AA data (collected from an ammonium sulfate grown crystal) was used for the final refinements. Due to the slight discrepancy in the unit cell dimensions between the PEG and ammonium sulfate-grown crystals (see above), rigid body refinement was required prior to the crystallographic refinement. The final model was refined with maximum likelihood method using the program REFMAC.^{24,29} The final model contains all residues in both molecules, and 169 ordered water molecules. Two molecules of spermine per molecule of Der p 2 were added during the refinement. Due to the lower resolution of diffraction data from PEG-grown crystals, the models of the Der p 2 molecule in those crystals were refined only partially, just enough to satisfactorily evaluate the extra electron density inside the molecule.

Protein Data Bank accession code

The coordinates and structure factors have been deposited with the RCSB Protein Data Bank under accession code 1KTJ.

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