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***Alternaria alternata* allergen, Alt a 1 - a unique, β -barrel protein dimer exclusively found in fungi**

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Abstract

Background—*Alternaria* is one of the most common molds associated with allergic diseases and 80% of *Alternaria*-sensitive patients produce IgE antibodies to a major protein allergen, Alt a 1. The structure and function of Alt a 1 is unknown.

Objective—To obtain a high resolution structure of Alt a 1 by X-ray crystallography and to investigate structural relationships between Alt a 1 and other allergens and proteins reported in the Protein Data Bank.

Methods—X-ray crystallography was used to determine the structure of Alt a 1 using a custom-designed set of crystallization conditions. An initial Alt a 1 model was determined by the application of a Ta₆Br₁₂²⁺ cluster and Single-wavelength Anomalous Diffraction. Bioinformatic analyses were used to compare the Alt a 1 sequence and structure with other proteins.

Results—Alt a 1 is a unique β -barrel comprising 11 β -strands and forms a ‘butterfly-like’ dimer linked by a single disulfide bond, with a large (1345Å²) dimer interface. Intramolecular disulfide bonds are conserved among Alt a 1 homologs. Currently, the Alt a 1 structure has no equivalent in the Protein Data Bank. Bioinformatics analyses suggest that the structure is found exclusively in fungi. Four previously reported putative IgE binding peptides have been located on the Alt a 1 structure.

Conclusions—Alt a 1 has a unique, dimeric β -barrel structure that appears to define a new protein family with unknown function found exclusively in fungi. The location of IgE antibody binding epitopes is in agreement with the structural analysis of Alt a 1. The Alt a 1 structure will allow mechanistic structure/function studies and immunologic studies directed towards new forms of immunotherapy for *Alternaria*-sensitive allergic patients.

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Keywords

Asthma; Allergens; Molds; Alt a 1; *Alternaria*; X-ray crystallography; Protein structure; Oligomeric structure

Introduction

Alternaria alternata is a quintessentially American allergen. Much of the original research on the clinical significance of *Alternaria* emanated from the United States.¹ Exposure to high levels of atmospheric *Alternaria* spores (2000 spores/m³) in the US Mid-West during the spring and summer months is a risk factor for asthma attacks and has been associated with respiratory arrest among children and young adults.² IgE mediated sensitization to *Alternaria* in early childhood was an independent risk factor for asthma at age 22 in a longitudinal birth cohort study carried out in Tucson, Arizona.³ Outdoor exposure to fungal spores, including *Alternaria*, in southern California was associated with asthma severity and increased medication use.⁴ Sensitization and exposure to *Alternaria* was also associated with asthma in the Inner City Asthma Studies and in the latest National Health and Nutrition Examination Survey (NHANES 2005-6).⁵⁻⁷⁸

IgE-mediated mechanisms of immediate hypersensitivity to *Alternaria* correlate with clinical symptoms.⁹⁻¹⁴ Immunotherapy using standardized *Alternaria* extract significantly reduced combined symptom and medication scores in a double-blind, placebo-controlled clinical trial involving children with rhinitis and asthma.¹⁵

Among fungi, *Alternaria alternata* is one of the principal species associated with allergic disease.¹ The major allergen produced by *Alternaria alternata*, Alt a 1, elicits IgE antibody responses in ~80% of *Alternaria*-allergic patients. Natural Alt a 1 is a 30kDa dimer which migrates as two separate 16.4 and 15.3 kDa bands under reducing conditions on SDS-PAGE, suggesting a disulfide bond linking the monomers.¹⁶ Alt a 1 has been cloned and the expressed recombinant allergen has been used to measure IgE and IgG antibody responses in *Alternaria*-allergic patients.¹⁷⁻²⁰ A homologous allergen with 90% amino acid sequence identity to Alt a 1 is produced by *Alternaria brassicicola* (an agricultural species) that may play a role in fungal pathogenesis.^{21, 22}

Despite its allergenic importance, there are few known Alt a 1 homologs. In addition, little structural data are available on this protein. Here, we present a high resolution X-ray crystal structure of recombinant Alt a 1. The structure reveals that Alt a 1 forms a unique, dimeric, β -barrel structure, unlike any other structure currently reported in the Protein Data Bank.²³ Moreover, proteins with similar sequences to Alt a 1 only occur in a limited number of fungal species. The surface locations of putative IgE binding peptides have also been identified.

METHODS

Structure elucidation

Lyophilized Alt a 1.0101 (Biomay, Vienna, Austria, Lot #01a; see the Online Repository) was dissolved in crystallization buffer (150 mM NaCl, 10 mM tris HCl pH 7.5). The solution was filtered via centrifugation through a 0.22 μ m filter (Millipore, Billerica, MA) and concentrated to a final concentration of 5.5 mg/mL using a 10 kDa cutoff concentrator (Millipore). Crystallization screens were set using hanging drop vapor diffusion. The well solution contained 50% saturated ammonium sulfate, 12.5% of an additive mixture (saturated solution of 4-hydroxy-2,5,6-triaminopyrimidine (4-HTP), (I)-alpha-lipoic acid,

caffeine, 8-aminocaproic acid (8-ACA), L-threonine, D,L-carnitine, and quinine HCl), and 1% Ananias-7 in a total volume of 400 μ L. Crystallization drops were set using 1 μ L of protein solution and 1 μ L of well solution and incubated at 16°C. Crystals typically appeared within 1 week. Crystallization experiments were tracked and analyzed with the XTALDB crystallization system.^{24, 25}

Data collection was performed at the 19-BM beamline of the Structural Biology Center²⁶ and the 21-ID-G beamline of the Life Sciences Collaborative Access Team at the Advanced Photon Source (Argonne, IL, USA). Data were collected at 100K and processed with HKL-2000²⁷. The initial model was obtained from a low resolution data set (2.8Å) collected at 19-BM from a crystal soaked in a solution containing Ta₆Br₁₂²⁺ cluster. SAD technique was used for structure determination. Calculations were performed with HKL-3000²⁸, which integrates SHELXC/D/E²⁹, MLPHARE³⁰, DM³¹, PARROT, RESOLVE³², ARP/wARP³³ and selected programs from the CCP4 package³⁴. The partial model was obtained by a combination of a manual building and building with RESOLVE. This model was used as a starting model for building with ARP/wARP. For the ARP/wARP calculation, a higher resolution, native data set (1.9Å) collected at 21-ID-G was used. The model was later updated with COOT³⁵ and refined with REFMAC³⁶. Translation/Libration/Screw (TLS) parameterization was used in the final stages of the refinement and TLS groups were determined using TLSMD server³⁷. MOLPROBITY³⁸ and ADIT³⁹ were used for structure validation. Statistics from data processing and structure determination are reported in Table E1 in the Online Repository.

The coordinates and structure factor for Alt a 1 were deposited in the PDB with accession code 3v0r.

Bioinformatic analysis

Sequences were obtained by running PSI-BLAST⁴⁰ against the non-redundant NCBI BLAST database with the Alt a 1 sequence (gi number: 14423645) as a query. Searches were performed with an expectation value of 0.001 until convergence, ultimately returning 122 homologous sequences. The homologous sequences identified in the first search were used for PSI-BLAST searches to identify distant homologs. However, these searches resulted in only one additional sequence. Sequences were retrieved from GenBank and sequences annotated as “incomplete” were removed. The final sequence dataset was aligned using Promals3D⁴¹ and manually refined in Jalview⁴² and Bioedit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Secondary structure was derived from STRIDE.⁴³ CD-HIT⁴⁴ was used to reduce the redundancy of sequences to 80 percent identity.

DALI⁴⁵, FATCAT⁴⁶, iSARST⁴⁷ and PDBeFold⁴⁸ were used to identify similar structures. Structures of both the Alt a 1 monomer and dimer were used as search models for homologous structures reported in the PDB (as of October 2011). PISA⁴⁹ was used for analysis of the oligomeric assembly and calculation of the dimer's interface area. Figures were prepared with PYMOL⁵⁰. The electrostatic surface was calculated in APBS⁵¹ using a model prepared by PDB2PQR⁵² as implemented in PYMOL.

PYMOL was also used to show the IgE binding peptides, which were identified by Kurup *et al.*⁵³, on the structure of Alt a 1. Molecular surface areas for the epitopes were calculated with SURFACE RACER.⁵⁴

RESULTS

Structure of Alt a 1

Alt a 1 crystallized in the tetragonal system and in the $I4_122$ space group with one protein chain in the asymmetric unit. Residues 28-157 of Alt a 1 were traced into electron density. The protein chain formed a β -barrel composed of eleven β -strands (Fig. 1). Strands $\beta 7a$ (residues 116-123) and $\beta 7b$ (residues 126-130) were separated by a region containing Pro125 that interrupted the continuity of $\beta 7$. Strands $\beta 8a$ (residues 138-142) and $\beta 8b$ (residues 146-149) were separated by a β -bulge, while strands $\beta 8b$ and $\beta 8c$ (residues 151-153) were separated by Leu150. Strands $\beta 8b$ and $\beta 8c$ were very close to each other but were part of different sheets forming the barrel. The center of the barrel was filled by side chains of hydrophobic residues and there was no inner cavity.

All five cysteine residues present in Alt a 1 formed disulfide bridges, two of which were intramolecular and stabilized the β -barrel. A third disulfide bridge contributed to the formation of the Alt a 1 dimer. The Cys74-Cys89 bridge connected strand $\beta 3$ and a fragment of the chain in the vicinity of strand $\beta 4$. This bridge could be described as a clamp holding both barrel-forming β -sheets. The Cys128-Cys140 bridge linked two neighboring β -strands, $\beta 7b$ and $\beta 8a$. Cys30 was covalently linked to the equivalent residue from the second Alt a 1 chain (symmetry related in the crystal) and this bridge held two dimer forming β -barrels in a 'butterfly-like' configuration (Fig. 2). The Alt a 1 dimer was stabilized not only by the disulfide bridge but also by a mixture of hydrophobic and polar interactions. Both N- and C-terminal regions of the protein participated in dimer formation (Fig. 2). Several residues (Asp100, Arg103, Thr121, Thr123, Thr149, Leu150 and Thr152) formed hydrogen bonds between the protein chains. The dimer interface was large, having a surface area of $1345 \text{ \AA}^2$. However, Cys30 was not conserved in all Alt a 1-homologous sequences (Fig. 3).

Dynamic Light Scattering (DLS) measurements (for details see Online Repository) showed that for both purified non-reduced (Fig. E1, Online Repository) and reduced Alt a 1, two species were observed with average hydrodynamic radii of approximately $8 \text{ \AA}$ and $32 \text{ \AA}$, each having a polydispersity of 13% or less (Fig. E2, Online Repository). Peaks were estimated to have molecular weights of approximately 2 kDa (most likely some impurity) and 50 kDa respectively (ranging between 47 and 56 kDa for non-reduced samples and 40 and 54 kDa for reduced samples). However, these apparent MW differences between non-reduced and reduced samples are unlikely to be statistically significant. During purification of the protein by size exclusion chromatography, we also observed two species: one corresponding to $\sim 2.5 \text{ kDa}$ and other to $\sim 40 \text{ kDa}$. Mass spectrometry analysis shown that the dimer of Alt a 1 has molecular weight of 29.2 kDa (Fig. E3, Online Repository).

Alt a 1 has a unique β -barrel structure

Results obtained from the exhaustive sequence database searches contained only 123 fungal proteins from the *Dothideomycetes* (where *Alternaria alternata* belongs) and from the *Sordariomycetes* classes which belong to *Pezizomycotina* subphylum (Fig. 3). Both *Dothideomycetes* and *Sordariomycetes* are classified as *Leotiomyceta*.⁵⁵ Cysteines corresponding to Cys30 of Alt a 1 were only conserved among the closest homologs of Alt a 1, while cysteine residues 74, 89, 128 and 140 of Alt a 1 were conserved among all sequences except one (gi: 307147764), which was shorter than the other sequences. Phylogenetic analysis allowed all Alt a 1-related sequences to be divided into two groups, and each group could be divided further into two subgroups (Fig. E4, Online Repository). The first subgroup contained *Alternaria alternata* and was characterized by the presence of conserved Cys30 residues. Proteins from this subgroup had 89-63% sequence identity and 98-74% sequence similarity to Alt a 1. The second subgroup, which was closely related to

Alt a 1, contained only sequences from *Mycosphaerella graminicola* (41-31% sequence identity and 71-58% sequence similarity to Alt a 1). Sequences from the third and fourth subgroups were less similar to Alt a 1 (32-25% sequence identity and 61-55% sequence similarity) (Fig. 3). See the Online Repository (Table E2) for results of pairwise sequence comparisons.

Searches with DALI, FATCAT, iSARST and PDBeFold did not reveal any structures with significant similarity to Alt a 1. The best matching structures superimposed with RMSD values $\sim 3\text{\AA}$ and the aligned residues were mainly those forming the β -barrel. Sequence identity between fragments was very low and ranged between 5-10% in most cases. DALI and FATCAT returned the structure of an uncharacterized protein (YP_563039.1) from *Shewanella denitrificans* as the most similar to Alt a 1 (Fig. 4). Bioinformatics searches listed proteins containing β -barrels as the structures most similar to Alt a 1. Those proteins included membrane proteins, streptavidin (and related proteins), odorant binding proteins, plant transcriptional regulators, RNA binding proteins, lipid binding proteins. The only allergens identified as having a structural similarity to Alt a 1 were lipocalins (e.g. Bos d 2, Figure 4C), which have an α -helix in addition to a β -barrel.

The closest human protein with structural similarity to Alt a 1 was synaptotagmin-3. This protein contained two domains and each of the domains was similar to the Alt a 1 monomer (Fig. E5, Online Repository). FATCAT flexible alignment, comparing structures of synaptotagmin-3 (PDB code: 3hn8, chain C) with dimer of Alt a 1, showed that these structures were similar. The structure alignment had 161 equivalent positions with RMSD value of $3.5\text{\AA}$ (P -value of $4.9\text{e-}10$ and three twists).

A search for three-dimensional functional fragments with PROFUNC⁵⁶ did not show any functional relatedness to enzyme active sites, ligand-binding sites, or DNA-binding templates. Moreover, a reverse template search (with 260 auto-generated templates from the Alt a 1 structure) against representative PDB structures did not return any significant results.

Localization of IgE binding peptides

Studies on synthetic peptides of Alt a 1 conducted by Kurup *et al.*⁵³ identified four IgE binding linear epitopes. Two of these epitopes, which showed consistent reactivity with sera of *Alternaria*-allergic individuals, were associated with the K41-P50 and Y54-K63 peptides. Two other peptides (Y87-D96 and V119-C128) showed weak IgE binding. Mapping of these peptides on the three-dimensional structure of the Alt a 1 dimer revealed that all of them were partially localized on the surface of the allergen (Fig. 5). Surface exposed molecular areas of the peptides were as follows: $450\text{ \AA}^2$ (K41-P50), $330\text{ \AA}^2$ (Y54-K63), $500\text{ \AA}^2$ (Y87-D96) and $280\text{ \AA}^2$ (V119-C128).

DISCUSSION

Alt a 1 and homologous proteins are unique and characteristic for the *Dothideomycetes* and for the *Sordariomycetes* classes of fungi. In addition, Alt a 1 forms a structure that has no equivalent in the current version of the PDB. Although the β -barrel in Alt a 1 is similar to those in lipocalins, this similarity could be explained by assuming an evolutionary convergence leading to the same structural scaffold. Therefore, Alt a 1 and its homologs among the fungi define a different structural family of proteins. It is likely that the intra-molecular pattern of the disulfide bonds is present in all known homologs of Alt a 1, since the cysteine residues involved are conserved. The intra-molecular disulfide bridges increase stability of these proteins and appear to explain the extreme stability of Alt a 1 (having IgE epitopes that can resist heat treatment of 95°C).⁵⁷ Conservation of Cys30 among the closest Alt a 1 homologs suggests that they also form covalently-linked dimers. Alt a 1 homologs in

which Cys30 is not conserved could form dimeric structures stabilized by the hydrophobic and hydrogen bonding interactions, which also contribute to the formation of Alt a 1 dimers. The possibility that some of the Alt a 1-like proteins exists in a monomeric form cannot be entirely excluded as the residues forming the dimer interface are not conserved (Fig. 6).

Alt a 1 is the first structure of an *Alternaria alternata* allergen to be determined. It was recently demonstrated that Alt a 1 is localized exclusively in the cell wall of *Alternaria* spores, which access the respiratory tract and mediate allergic reactions.⁵⁷ This observation is consistent with a previous report speculating that Alt a 1 was involved in spore germination.⁵⁸ However, it is not known how Alt a 1 is attached to the cell wall. Despite some similarity of Alt a 1 to other proteins with β -barrel cores, structural comparison does not provide a definitive function of the allergen, other than as a possible transporter of small ligands. For example, β -barrels from lipocalins are well known to act as proteins binding small hydrophobic ligands such as odorant pheromones, retinoids, steroids and arachidonic acid.⁵⁹ However, small molecular compounds, which were present in the crystal structure of Alt a 1, originated from the crystallization solution and it is unlikely that their binding is physiological (see Fig. E6, Online Repository). Moreover, the Alt a 1 crystal structure does not have a binding cavity characteristic of lipocalins, streptavidin and other proteins with a β -barrel fold. One may speculate that the binding of a physiological Alt a 1 ligand may induce conformational changes, resulting in the opening of the hydrophobic β -barrel core, or that the molecules with closed conformation were captured by the crystallization process. It is also possible that Alt a 1 in solution has an internal cavity. A similar molecular flexibility leading to changes in internal cavity was observed for the mite allergen Der p 2. Although no β -barrel is present in Der p 2, the allergen has an immunoglobulin-like fold comprised of two anti-parallel β -sheets. Structures of Der p 2, determined by either NMR or X-ray crystallography, revealed differences regarding the presence of an internal cavity.⁶⁰ Molecular flexibility of Alt a 1 would be consistent with the larger molecular weight observed for Alt a 1 by DLS and by size exclusion chromatography. The DLS data showed that, in solution, the Alt a 1 dimer may have a less compact structure than in the crystal, or that the allergen could form higher order oligomeric assemblies, such as tetramers.

The structural features of Alt a 1 have several implications. Dimerization of Alt a 1 provides an explanation for the ability to use a single monoclonal antibody binding for capture and detection of the allergen in “sandwich” ELISA.⁶¹ Different molecular forms of Alt a 1 could also explain the variability of immunoassays that have been developed for Alt a 1.⁶² Structural data are consistent with the results of IgE binding to the synthetic peptides of Alt a 1.⁵³ Two peptides (K41-P50 and Y54-K63), which were shown to strongly bind IgE from sera of *Alternaria alternata* allergic individuals, are surface exposed in strands β 1 and β 2 of Alt a 1 and easily accessible for interaction with antibodies. Similarly, weak IgE binding peptides Y87-D96 and V119-C128 are also partially accessible. These results suggest that IgE antibodies are produced against intact, dimeric Alt a 1 in the respiratory tract following inhalation of *Alternaria* fungal spores or their fragmented cell walls. Although Alt a 1 has a classic, dimeric structure, the data do not necessarily support the hypothesis that dimerization is an important prerequisite for allergenicity.⁶³ Many important allergens are protein monomers and, to that extent, we believe that dimerization of Alt a 1 may contribute to, but is not essential, for its allergenicity, as reported for the cockroach allergen Bla g 2.⁶⁴ The structure will allow the location of B cell and T cell epitopes on Alt a 1 to be determined and mechanisms for modulation of immune responses to the allergen to be investigated. Ultimately, this could result in improved strategies for recombinant allergen or peptide-based immunotherapy for *Alternaria* sensitive patients. The data provides clear evidence that Alt a 1 belongs to a new family of dimeric fungal proteins, which adds to the complement of protein families in the Pfam database that induce IgE responses.⁶⁵

Determination of the function of this unique protein family will provide insights into protein function, fungal biology, allergenicity and the immune response to fungi.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

4-HTP	4-hydroxy-2,5,6-triaminopyrimidine
8-ACA	8-aminocaprylic acid
DLS	Dynamic Light Scattering
NHANES	National Health and Nutrition Examination Survey
SAD	Single-wavelength Anomalous Diffraction
TLS	Translation/Libration/Screw parameterization

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Key messages

- The structure of Alt a 1, solved by X-ray crystallography, is unique and defines a new protein family of homologous proteins exclusively found in molds.
- The Alt a 1 structure consists of a β -barrel that dimerizes through a disulfide bond and hydrophobic and polar interactions, exposing residues that were reported to be IgE antibody binding epitopes.

FIG 1.

Overall structure of Alt a 1. β -strands are shown in blue, loop regions are shown in gray, and cysteine residues are marked in orange.

FIG 2.

A Alt a 1 dimer shown in cartoon representation. Disulfide bridges are shown in orange. **B** Solvent accessible surface of the Alt a 1 dimer with mapped electrostatic charges (red - residues with negative charge, blue - residues with positive charge).

FIG 3.

Sequence alignment of selected Alt a 1 homologs. The sequences are colored using the ClustalX default color scheme. Secondary structure was calculated with STRIDE on the basis of Alt a 1 structure and refined manually (β -strands are marked using letter S). The cysteine residues involved in disulfide bonds formation are highlighted in green. Names of the most similar sequences are shown in black, sequences with lower degree of similarity are shown in gray and the names of the least similar sequences are shown in brown.

FIG 4.

A Structure of Alt a 1 (green) superposed on an uncharacterized protein from *Shewanella denitrificans* (gray; PDB code: 2q03). **B** structure of Alt a 1 superposed on structure of streptavidin (blue; PDB code: 1nc9). **C** Superposition of Alt a 1 and Bos d 2 (pale pink; PDB code: 1bj7) structures.

FIG 5.

A Dimer of Alt a 1 in cartoon representation with IgE antibody binding peptides marked in red (residues K41-P50) and blue (residues Y54-K63). Peptides Y87-D96 and V119-C128 that showed weak IgE antibody binding are shown in dark gray and light gray, respectively. **B** The same IgE binding peptides shown on the molecular surface of the Alt a 1 dimer. Top and bottom parts of the figure correspond to two views of the allergen dimer rotated by 180° about y-axis.

FIG 6.

Sequence conservation among Alt a 1-like proteins mapped on a molecular surface of an Alt a 1 molecule. Colors range from blue for invariant residues to red for residues for no sequence conservation.