

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

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

Objective: We sought to obtain a high-resolution structure of Alt a 1 using 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 by 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 that of 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 (1345 Å²) 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 toward new forms of immunotherapy for *Alternaria* species-sensitive allergic patients. (J Allergy Clin Immunol 2012;130:241-7.)

Key words: Asthma, allergens, molds, Alt a 1, *Alternaria* species, x-ray crystallography, protein structure, oligomeric structure

Alternaria alternata is a quintessentially American allergen. Much of the original research on the clinical significance of *Alternaria* species emanated from the United States.¹ Exposure to high levels of atmospheric *Alternaria* species spores (2000 spores/m³) in the US Midwest 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* species in early childhood was an independent risk factor for asthma at age 22 years in a longitudinal birth cohort study carried out in Tucson, Arizona.³ Outdoor exposure to fungal spores, including *Alternaria* species, in southern California was associated with asthma severity and increased medication use.⁴ Sensitization and exposure to *Alternaria* species was also associated with asthma in the Inner City Asthma Studies and in the latest National Health and Nutrition Examination Survey (2005-2006).⁵⁻⁸

IgE-mediated mechanisms of immediate hypersensitivity to *Alternaria* species correlate with clinical symptoms.⁹⁻¹⁴ Immunotherapy with standardized *Alternaria* species 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, *A alternata* is one of the principal species associated with allergic disease.¹ The major allergen produced by *A alternata*, Alt a 1, elicits IgE antibody responses in approximately 80% of patients with *Alternaria* species allergy. Natural Alt a 1 is a 30-kDa dimer that migrates as 2 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 patients with *Alternaria* species allergy.¹⁷⁻²⁰ A homologous allergen with 90% amino acid sequence identity to Alt a 1 is produced by *Alternaria brassicicola* (an agricultural species), which might 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

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Abbreviations used

4-HTP: 4-Hydroxy-2,5,6-triaminopyrimidine
 8-ACA: 8-Aminocaprylic acid
 DLS: Dynamic light scattering
 gi: GenInfo Identifier
 PDB: Protein Data Bank

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 (PDB).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 (lot #01a; Biomay, Vienna, Austria; for more information, see the Methods section in this article's Online Repository at www.jacionline.org) was dissolved in crystallization buffer (150 mmol/L NaCl and 10 mmol/L Tris HCl, pH 7.5). The solution was filtered by means of centrifugation through a 0.22- μ m filter (Millipore, Billerica, Mass) and concentrated to a final concentration of 5.5 mg/mL by using a 10-kDa cutoff concentrator (Millipore). Crystallization screens were set by 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]- α -lipoic acid, caffeine, 8-aminocaprylic acid [8-ACA], L-threonine, D,L-carnitine, and quinine HCl), and 1% Anameg-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, Ill). Data were collected at 100K and processed with HKL-2000.²⁷ The initial model was obtained from a low-resolution dataset (2.8 Å) collected at 19-BM from a crystal soaked in a solution containing the Ta₆Br₁₂²⁺ cluster. The single-wavelength anomalous diffraction technique was used for structure determination. Calculations were performed with HKL-3000,²⁸ which integrates SHELXC/D/E,²⁹ MLPHARE,³⁰ DM,³¹ PARROT, RESOLVE,³² ARP/wARP,³³ and selected programs from the CCP4 package.³⁴ The partial model was obtained by using a combination of 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 dataset (1.9 Å) collected at 21-ID-G was used. The model was later updated with COOT³⁵ and refined with REFMAC.³⁶ Translation/libration/screw parameterization was used in the final stages of the refinement, and translation/libration/screw parameterization groups were determined by using the TLSMD server.³⁷ MOLPROBITY³⁸ and ADIT³⁹ were used for structure validation. Statistics from data processing and structure determination are reported in Table E1 in this article's Online Repository at www.jacionline.org.

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 nonredundant National Center for Biotechnology Information BLAST database with the Alt a 1 sequence (GenInfo Identifier [gi]: 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 1 additional sequence. Sequences were retrieved from GenBank, and sequences annotated as "incomplete" were removed. The final sequence dataset was aligned with Promals3D⁴¹ and manually refined in Jalview⁴² and Bioedit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Secondary structure was derived using STRIDE.⁴³ CD-HIT⁴⁴ was used to remove the redundancy of sequences at 80% identity threshold.

DALI,⁴⁵ FATCAT,⁴⁶ iSARST,⁴⁷ 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 PYMOL.⁵⁰ The electrostatic potential on the surface was calculated in APBS⁵¹ by using a model prepared with PDB2PQR,⁵² 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 I₄2₂ space group with 1 protein chain in the asymmetric unit. Residues 28 to 157 of Alt a 1 were traced into electron density. The protein chain formed a β -barrel composed of 11 β -strands (Fig 1). Strands β 7a (residues 116-123) and β 7b (residues 126-130) were separated by a region containing Pro125 that interrupted the continuity of β 7. Strands β 8a (residues 138-142) and β 8b (residues 146-149) were separated by a β -bulge, whereas strands β 8b and β 8c (residues 151-153) were separated by Leu150. Strands β 8b and β 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 5 cysteine residues present in Alt a 1 formed disulfide bridges, 2 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 β 3 and a fragment of the chain in the vicinity of strand β 4. This bridge could be described as a clamp holding both barrel-forming β -sheets. The Cys128-Cys140 bridge linked 2 neighboring β -strands, β 7b and β 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 2 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 Å². However, Cys30 was not conserved in all Alt a 1-homologous sequences (Fig 3).

Dynamic light scattering (DLS) measurements (for details, see the Results section in this article's Online Repository at www.jacionline.org) showed that for both purified nonreduced (see Fig E1 in this article's Online Repository at www.jacionline.org) and reduced Alt a 1, 2 species were observed with average hydrodynamic radii of approximately 8 and 32 Å, each having a polydispersity of 13% or less (see Fig E2 in this article's Online Repository at www.jacionline.org). Peaks were estimated to

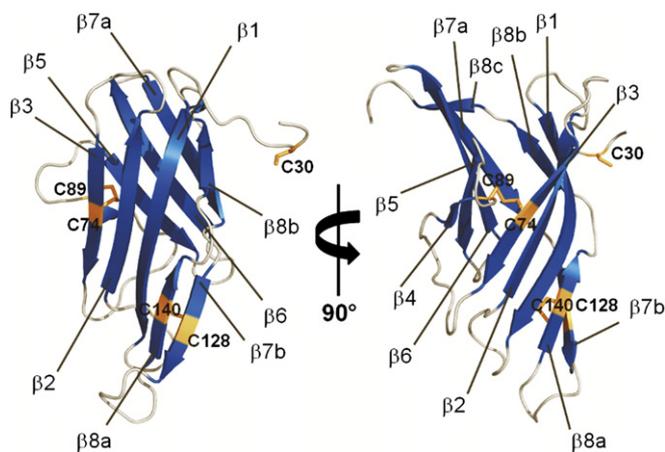


FIG 1. Overall structure of Alt a 1. β -Strands are shown in blue, loop regions are shown in gray, and cysteine residues are marked in orange.

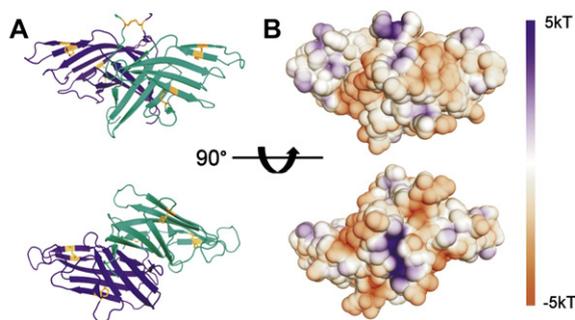


FIG 2. **A**, Alt a 1 dimer shown as a cartoon representation. Disulfide bridges are shown in orange. **B**, Solvent-accessible surface of the Alt a 1 dimer with mapped electrostatic charges (red, residues with negative charge; blue, residues with positive charge).

have molecular weights of approximately 2 kDa (most likely some impurity) and 50 kDa, respectively (ranging between 47 and 56 kDa for nonreduced samples and 40 and 54 kDa for reduced samples). However, these apparent molecular weight differences between nonreduced and reduced samples are unlikely to be statistically significant. During purification of the protein by means of size exclusion chromatography, we also observed 2 species: one corresponding to approximately 2.5 kDa and the other to approximately 40 kDa. Mass spectrometric analysis shows that the dimer of Alt a 1 has a molecular weight of 29.2 kDa (see Fig E3 in this article's Online Repository at www.jacionline.org).

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* (in which *A alternata* belongs) and *Sordariomycetes* classes, which belong to the *Pezizomycotina* subphylum (Fig 3). Both *Dothideomycetes* and *Sordariomycetes* are classified as *Leotiomyces*.⁵⁵ Cysteines corresponding to Cys30 of Alt a 1 were only conserved among the closest homologs of Alt a 1, whereas 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 2 groups, and each group could be divided further into 2 subgroups (see Fig E4 in this article's Online

Repository at www.jacionline.org). The first subgroup contained *A alternata* and was characterized by the presence of conserved Cys30 residues. Proteins from this subgroup had 89% to 63% sequence identity and 98% to 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% to 31% sequence identity and 71% to 58% sequence similarity to Alt a 1). Sequences from the third and fourth subgroups were less similar to Alt a 1 (32% to 25% sequence identity and 61% to 55% sequence similarity, Fig 3). See Table E2 in this article's Online Repository at www.jacionline.org 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 root-mean-square deviation values of approximately 3 Å, and the aligned residues were mainly those forming the β -barrel. Sequence identity between fragments was very low and ranged between 5% and 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, and lipid-binding proteins. The only allergens identified as having a structural similarity to Alt a 1 were lipocalins (eg, Bos d 2; Fig 4, C), 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 2 domains, and each of the domains was similar to the Alt a 1 monomer (see Fig E5 in this article's Online Repository at www.jacionline.org). FATCAT flexible alignment, comparing structures of synaptotagmin-3 (PDB code 3hn8, chain C) with the Alt a 1 dimer, showed that these structures were similar. The structure alignment had 161 equivalent positions with a root-mean-square deviation value of 3.5 Å ($P = 4.9E-10$ and 3 twists).

A search for 3-dimensional functional fragments with PRO-FUNC⁵⁶ did not show any functional relatedness to enzyme-active sites, ligand-binding sites, or DNA-binding templates. Moreover, a reverse template search (with 260 autogenerated 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 al⁵³ identified 4 IgE-binding linear epitopes. Two of these epitopes, which showed consistent reactivity with sera of patients with *Alternaria* species allergy, 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 3-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 Å² (K41-P50), 330 Å² (Y54-K63), 500 Å² (Y87-D96), and 280 Å² (V119-C128).

DISCUSSION

Alt a 1 and homologous proteins are unique and characteristic for the *Dothideomycetes* and *Sordariomycetes* classes of fungi. In



FIG 3. Sequence alignment of selected Alt 1 homologs. The sequences are colored by using the ClustalX default color scheme. Secondary structure was calculated with STRIDE on the basis of Alt 1 structure and refined manually (β -strands are marked with the 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 names of the least similar sequences are shown in *brown*.

addition, Alt 1 forms a structure that has no equivalent in the current version of the PDB. Although the β -barrel in Alt 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 1 and its homologs among the fungi define a different structural family of proteins. It is likely that the intramolecular pattern of the disulfide bonds is present in all known homologs of Alt 1 because the cysteine residues involved are conserved. The intramolecular disulfide bridges increase the stability of these proteins and appear to explain the extreme stability of Alt 1 (having IgE epitopes that can resist heat treatment of 95°C).⁵⁷ Conservation of Cys30 among the closest Alt 1 homologs suggests that they also form covalently linked dimers. Alt 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 1 dimers. The possibility that some of the Alt 1-like proteins exists in a monomeric form cannot be entirely excluded because the residues forming the dimer interface are not conserved (Fig 6).

Alt 1 is the first structure of an *A alternata* allergen to be determined. It was recently demonstrated that Alt 1 is localized exclusively in the cell wall of *Alternaria* species spores, which access the respiratory tract and mediate allergic reactions.⁵⁷ This observation is consistent with a previous report speculating that Alt 1 was involved in spore germination.⁵⁸ However, it is not known how Alt 1 is attached to the cell wall. Despite some similarity of Alt 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 1, originated from the crystallization solution, and it is unlikely that their binding is physiologic (see Fig E6 in this article's Online Repository at www.jacionline.org). Moreover, the Alt 1 crystal structure does not have a binding cavity characteristic of lipocalins, streptavidin, and other proteins with

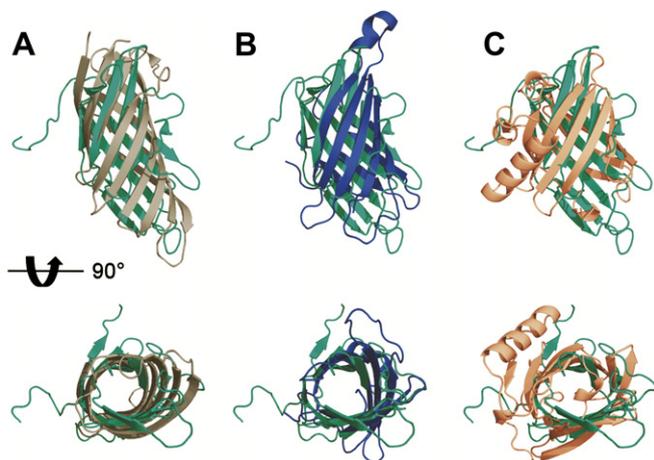


FIG 4. **A**, Structure of Alt a 1 (green) superposed on an uncharacterized protein from *Shewanella denitrificans* (gray; PDB code: 2q03). **B**, Structure of Alt a 1 superposed on the structure of streptavidin (blue; PDB code: 1nc9). **C**, Superposition of Alt a 1 and Bos d 2 (pale pink; PDB code: 1bj7) structures.

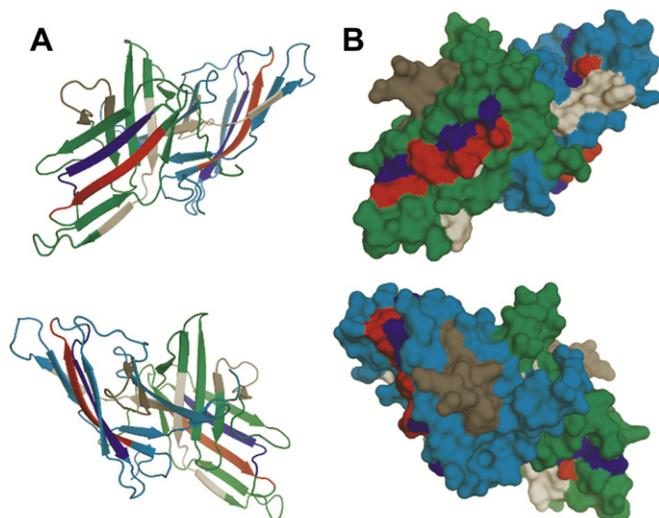


FIG 5. **A**, Dimer of Alt a 1 shown as a cartoon representation with IgE antibody-binding peptides marked in red (residues K41-P50) and blue (residues Y54-K63). Peptides Y87-D96 and V119-C128, which showed weak IgE antibody binding, are shown in dark gray and light gray, respectively. **B**, The same IgE-binding peptides shown on the molecular surface of the Alt a 1 dimer. The top and bottom parts of the figure correspond to 2 views of the allergen dimer rotated by 180° about the y-axis.

a β -barrel fold. One might speculate that the binding of a physiologic Alt a 1 ligand might 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 the 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 2 antiparallel β -sheets. Structures of Der p 2, determined by using either nuclear magnetic resonance or x-ray crystallography, revealed differences regarding the presence of an internal cavity.⁶⁰ The molecular flexibility of Alt a 1 would be consistent with the larger molecular

weight observed for Alt a 1 by using DLS and size exclusion chromatography. The DLS data showed that the Alt a 1 dimer, in the solution, might have a less compact structure than in the crystal or that the allergen could form higher-order oligomeric assemblies, such as tetramers, in solution.

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 mAb 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 patients with *A alternata* allergy, are surface exposed in strands β 1 and β 2 of Alt a 1 and easily accessible for interaction with antibodies. Similarly, the 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 after inhalation of *Alternaria* species 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 might 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- 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* species-sensitive patients. The data provide 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.

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Key messages

- The structure of Alt a 1, as solved by using 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.

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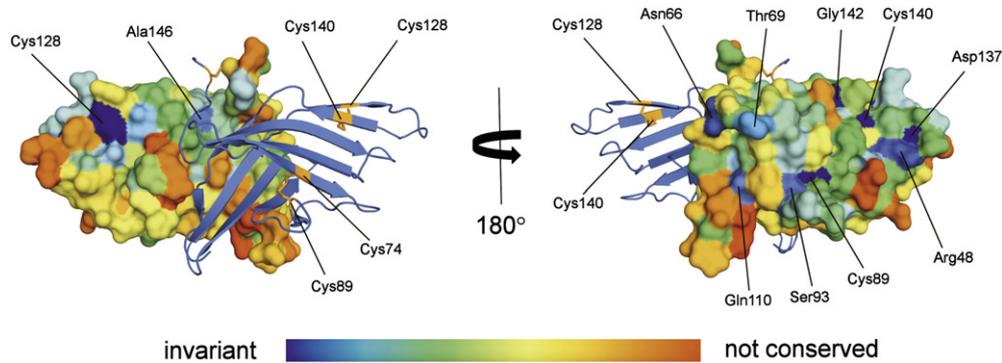


FIG 6. Sequence conservation among Alt a 1-like proteins mapped on the molecular surface of an Alt a 1 molecule. Colors range from *blue* for invariant residues to *red* for residues for no sequence conservation.

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METHODS

Protein sample

According to the protein's manufacturer, recombinant Alta 1.0101 has a molecular weight of 14,593 Da and corresponds to a methionine (replacing the leader sequence) followed by residues 26 (Asp) to 157.

DLS

Approximately 1 mg of lyophilized Alt a 1 powder was dissolved in 1 mL of crystallization buffer and filtered through a 0.22- μ m filter. After taking a 50 μ L prepurification sample, the Alt a 1 was purified in crystallization buffer on a Sephadex G-200 column using an ÄKTA purification system (GE Healthcare, Piscataway, NJ). Eluted fractions were concentrated to 0.8 mg/mL using a 10-kDa cutoff concentrator. Purified protein was analyzed under nonreducing conditions in crystallization buffer or under reducing conditions in 20 mmol/L 2-mercaptoethanol.

The 3 samples were analyzed with a Dynapro Titan DLS instrument (Wyatt Technology, Santa Barbara, Calif). Five trials of Alt a 1 in the nonreduced state and 4 trials in the reduced state were performed. Each trial consisted of 20 acquisitions lasting between 20 and 60 seconds. A regularization algorithm (Wyatt DYNAMICS software) was used to plot the percentage of intensity scattered versus the hydrodynamic radius for each trial. The instrument is sensitive to samples in the range of approximately 5 to approximately 5000 Å. Molecular weight estimates were obtained based on the hydrodynamic radius.

RESULTS

Structural analysis revealed that molecules of 4-HTP and 8-ACA, which were used for crystallization, mediate crystal contacts formed by Alt a 1 molecules. Although 4-HTP is located on symmetry elements, 8-ACA is located close to one of the 4-HTP molecules but not on a symmetry element (see Fig E4). The 8-ACA is oriented such that the amino group is pointing toward solution and the carboxylic group forms 2 hydrogen bonds with an amide group of Gln110. Asp37 and Tyr38 residues provide additional nonspecific interactions for 8-aminocaprylate binding. Biotin would not be expected to bind to Alt a 1 because of the presence of W40 in the equivalent position in which biotin binds to streptavidin superimposed with Alt a 1.

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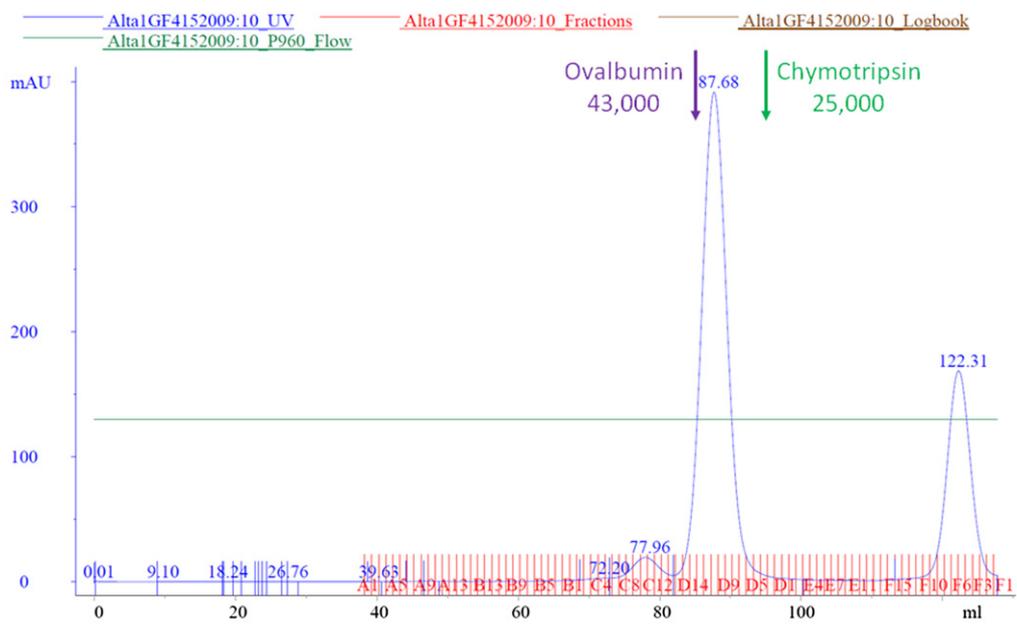


FIG E1. Results of gel filtration on Superdex 200. Protein was dissolved in 10 mmol/L Tris-HCl and 150 mmol/L NaCl, pH 7.5, and the same buffer was used for purification.

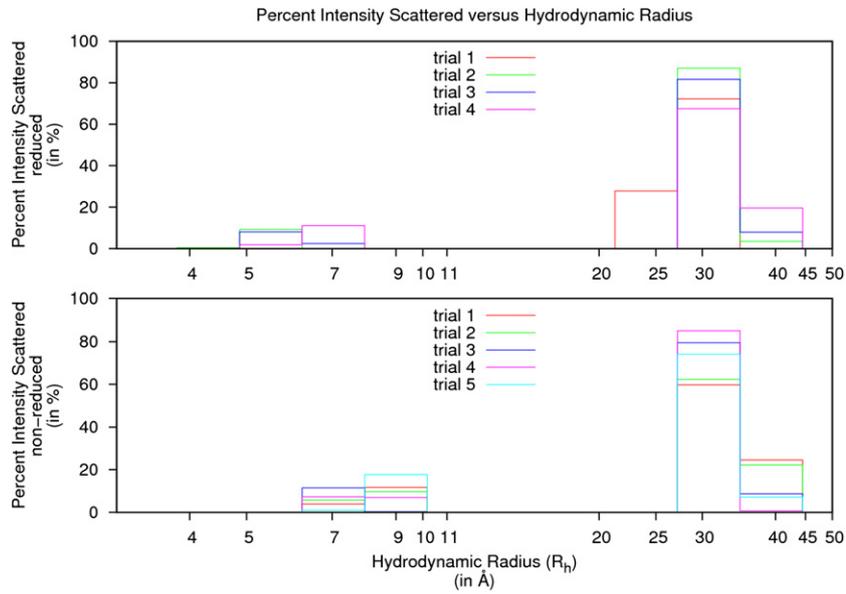


FIG E2. Results of the DLS measurements. The percentage of light intensity scattered versus hydrodynamic radius computed by using a regularization algorithm is shown for both reduced (*top*) and nonreduced (*bottom*) conditions.

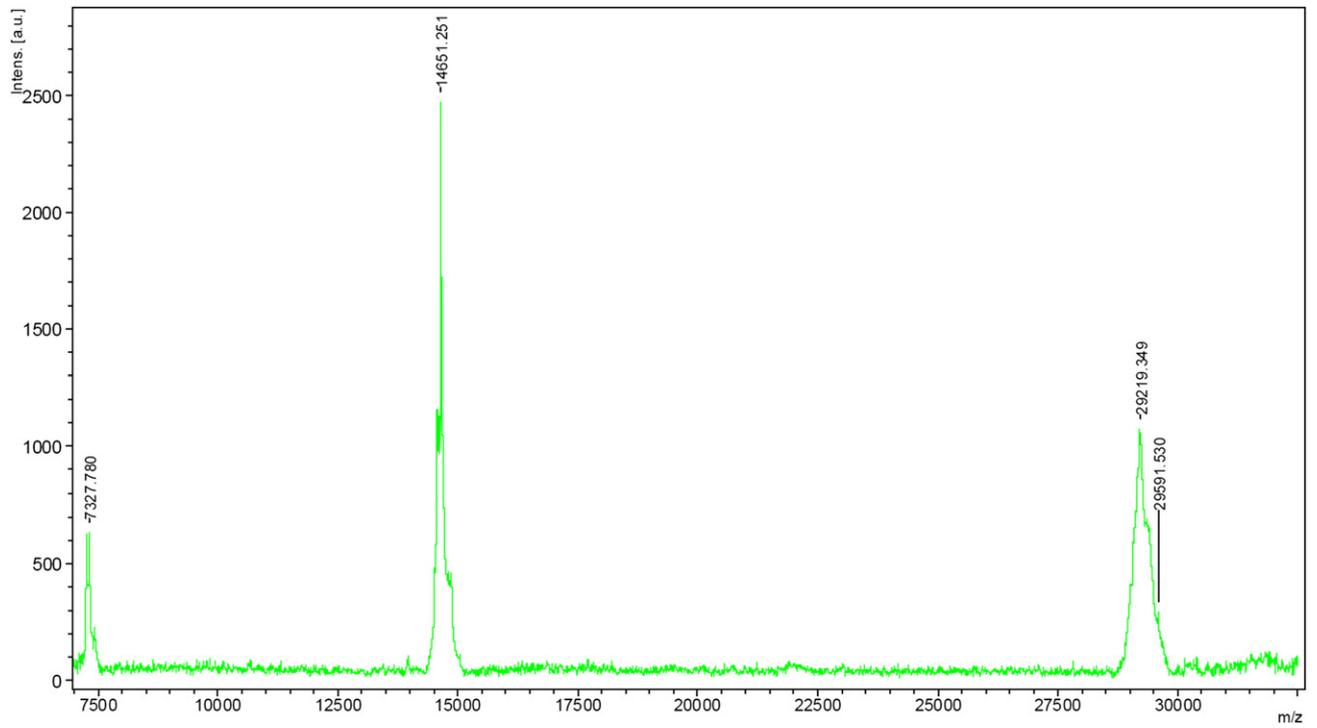


FIG E3. Results of mass spectrometric analysis of the Alt a 1 sample that was used for crystallization.

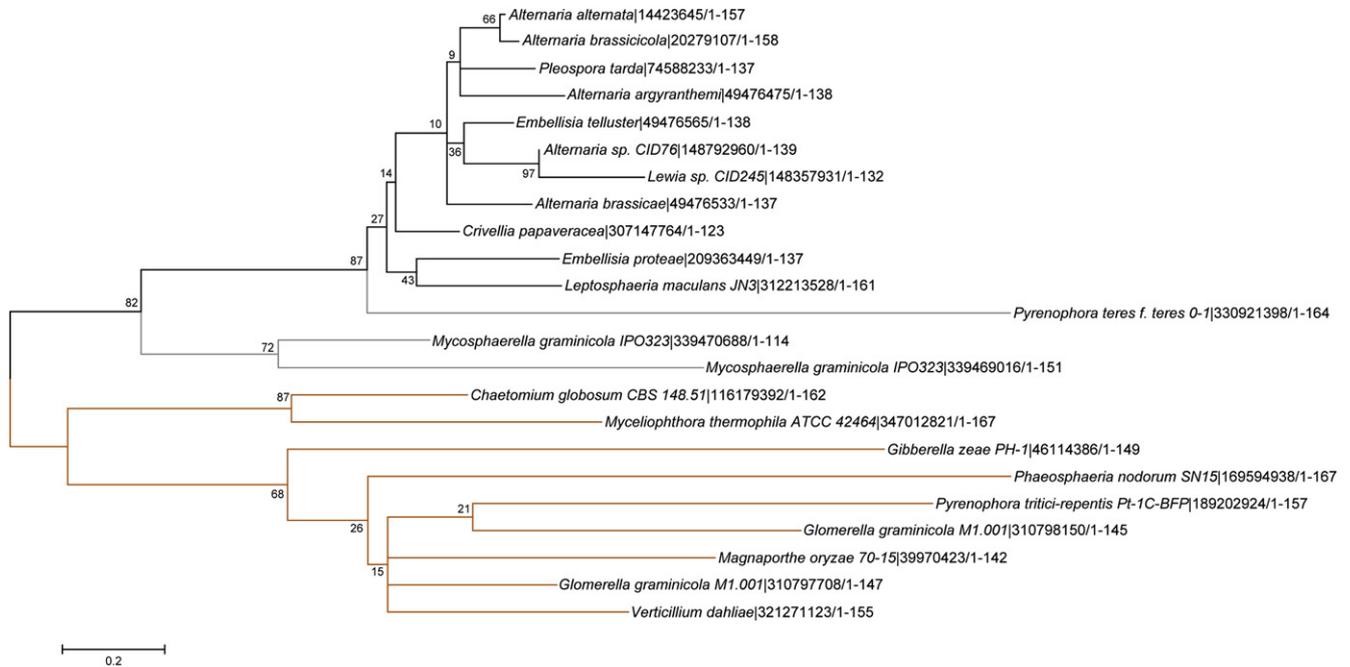


FIG E4. Results of sequence phylogenetic analysis. Sequences are labeled with organism name, gi number, and sequence length used for analysis, respectively. The MEGA package^{E2} was used for phylogenetic analysis. The tree was computed by using the Maximum Likelihood statistical method with the WAG amino acid substitution model.^{E3} The number of discrete gamma categories (G) was set to 5. Gaps and missing data were treated as complete deletion. The Nearest-Neighbor-Interchange was used as a maximum likelihood heuristic method. The initial tree was created automatically. Phylogeny was tested with the bootstrap method by using 1000 replications. Numbers near nodes show bootstrap values for that particular node. Branches are colored according to alignment shown in Fig 3.

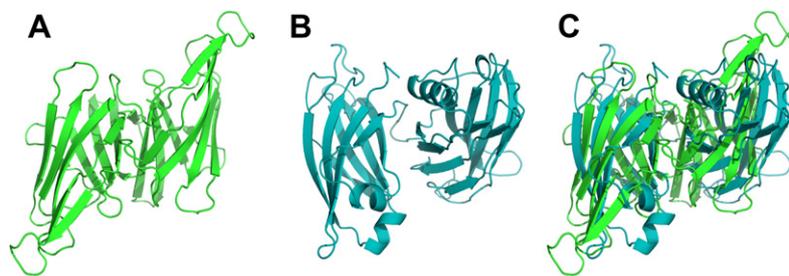


FIG E5. **A**, Alt a 1 dimer. **B**, Structure of synaptotagmin-3 (PDB code: 3hn8). **C**, Superposition of the structures of Alt a 1 and synaptotagmin-3, which is a human protein with the biggest structural similarity to the allergen. Superposition is done with secondary-structure matching, as implemented in COOT.

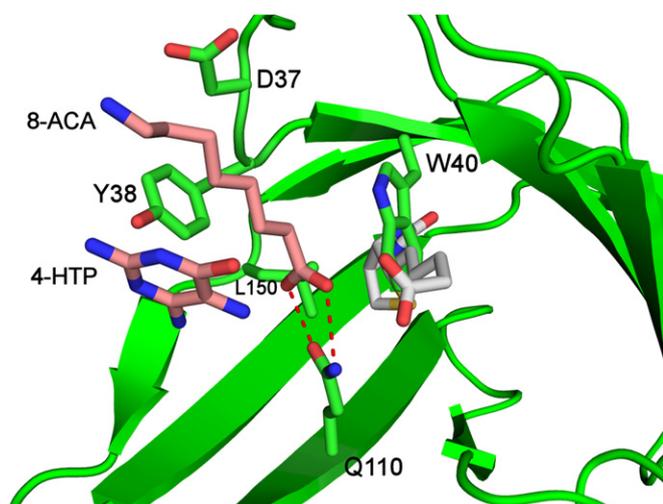


FIG E6. Binding of 4-HTP and 8-ACA, components of the crystallization solution. The position of biotin (*white sticks*) observed in a streptavidin structure (PDB code: 3ry2) superimposed with Alt a 1 would overlap with W40 from Alt a 1 (shown in *green*).

TABLE E1. Data collection and refinement statistics

PDB code	3v0r
Data collection	
Space group	I4 ₁ 22
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	<i>a</i> = <i>b</i> = 70.0, <i>c</i> = 179.3
α , β , γ (°)	90, 90, 90
Resolution (Å)	50.00-1.90 (1.93-1.90)
<i>R</i> _{sym}	0.068 (0.777)
<i>I</i> / σ <i>I</i>	59.4 (4.2)
Completeness (%)	99.9 (100.0)
Redundancy	13.8 (13.9)
Refinement	
Resolution (Å)	1.90
No. of reflections	18,063
<i>R</i> _{work} / <i>R</i> _{free}	16.7/19.0
No. of atoms	
Protein	1,014
Ligand/ion	61
Water	161
<i>B</i> factors (Å ²)	
Protein	42.7
Ligand/ion	57.4
Water	54.0
RMSD values	
Bond lengths (Å)	0.020
Bond angles (°)	1.9

Numbers in parentheses refer to the highest-resolution shell.
RMSD, Root-mean-square deviation.

TABLE E2. Sequence identity and sequence similarity to Alt a 1 (gi: 14423645)

Organism	Identity (%)	Similarity (%)
<i>Alternaria brassicicola</i> (gi: 20279107)***	89.6	98.7
<i>Crivellia papaveracea</i> (gi: 307147764)***	82.1	95.9
<i>Alternaria brassicae</i> (gi: 49476533)***	81.8	95.6
<i>Embellisia telluster</i> (gi: 49476565)***	81.6	96.3
<i>Pleospora tarda</i> (gi: 74588233)***	81	94.2
<i>Alternaria argyranthemis</i> (gi: 49476475)***	80.6	93.5
<i>Alternaria</i> species CID76 (gi: 148792960)***	79.9	97.1
<i>Embellisia proteae</i> (gi: 209363449)***	70.1	92.7
<i>Lewia</i> species CID245 (gi: 148357931)***	66.9	84.5
<i>Leptosphaeria maculans</i> (gi: 312213528)***	63.2	85.8
<i>Mycosphaerella graminicola</i> (gi: 339470688)**	40.9	71.3
<i>Pyrenophora teres f. teres</i> (gi: 330921398)**	39.3	74.2
<i>Myceliophthora thermophila</i> (gi: 347012821)*	32.4	55.3
<i>Verticillium dahliae</i> (gi: 321271123)*	31.9	60.1
<i>Phaeosphaeria nodorum</i> (gi: 169594938)*	31.7	56.1
<i>Mycosphaerella graminicola</i> (gi: 339469016)**	31.2	58.1
<i>Chaetomium globosum</i> (gi: 116179392)*	31.1	60.5
<i>Pyrenophora tritici-repentis</i> (gi: 189202924)*	30.1	56.9
<i>Magnaporthe oryzae</i> (gi: 39970423)*	29	60
<i>Glomerella graminicola</i> (gi: 310797708)*	27	58.3
<i>Glomerella graminicola</i> (gi: 310798150)*	25.6	56.9
<i>Gibberella zeae</i> (gi: 46114386)*	24.7	55.1

Sequences are sorted by identity. Names of the most similar sequences are marked with *** (they are shown in black on Fig 3 and Fig E4), sequences with lower degree of similarity are marked with ** (they are shown in gray on Fig 3 and Fig E4), and names of the least similar sequences are marked with * and are shown in brown on Fig 3 and Fig E4. Values were calculated with *lalign* program from the FASTA package.^{E1}