Original article

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

Maksymilian Chruszcz, PhD,[a] Martin D. Chapman, PhD,[b] Tomasz Osinski, MSc,[a] Robert Solberg, MSc,[a] Matthew Demas, BSc,[a] Przemyslaw J. Porebski, MSc,[a] Karolina A. Majorek, MSc,[a] Anna Pomés, PhD,[b] and Wladek Minor, PhD[a] *Charlottesville, Va*

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 $Ta_6Br_{12}^{2+}$ 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 Å^2) 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;■■■:■■■-■■■.)

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/m^3) 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

From [a]the Department of Molecular Physiology and Biological Physics, University of Virginia, and [b]Indoor Biotechnologies, Inc.

Supported by National Institutes of Health grant GM53163. The structural results shown in this report are derived from work performed at Argonne National Laboratory at the Structural Biology Center of the Advanced Photon Source. Argonne is operated by University of Chicago Argonne, LLC, for the US Department of Energy, Office of Biological and Environmental Research, under contract DE-AC02-06CH11357. Use of the LS-CAT Sector 21 was supported by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor for the support of this research program (grant 085P1000817). A.P. and M.D.C. were funded in part by grant R01AI077653 from the National Institute of Allergy and Infectious Diseases.

Disclosure of potential conflict of interest: M. D. Chapman is President and CEO of Indoor Biotechnologies and has ownership positions with Indoor Biotechnologies, Inc, and Indoor Biotechnologies Ltd; has received research support from the National Institute for Environmental Health Sciences and the National Institute of Allergy and Infectious Diseases; and is on the Board of Directors for the Virginia Biotechnology Association. A. Pomés is employed by Indoor Biotechnologies Ltd and has received research support from the National Institute of Allergy and Infectious Diseases. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication January 6, 2012; revised February 21, 2012; accepted for publication March 27, 2012.

Corresponding authors: Maksymilian Chruszcz, PhD, or Wladek Minor, PhD, University of Virginia, Department of Molecular Physiology and Biological Physics, 1340 Jefferson Park Ave, Charlottesville, VA 22908. E-mail: maks@iwonka.med.virginia.edu or wladek@iwonka.med.virginia.edu. Or: Martin D. Chapman, PhD, Indoor Biotechnologies, 1216 Harris St, Charlottesville, VA 22903. E-mail: mdc@inbio.com.

0091-6749/$36.00

© 2012 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2012.03.047

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

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 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-μm 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 μL. Crystallization drops were set using 1 μL of protein solution and 1 μL 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 Center[26] 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 $Ta_6Br_{12}^{2+}$ 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 COOT[35] 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] MOLPROBITY[38] and ADIT[39] 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-BLAST[40] 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 Promals3D[41] and manually refined in Jalview[42] and Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Secondary structure was derived using STRIDE.[43] CD-HIT[44] was used to remove the redundancy of sequences at 80% identity threshold.

DALI,[45] FATCAT,[46] iSARST,[47] and PDBeFold[48] 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). PISA[49] 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 APBS[51] 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 $I4_122$ 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 β7a (residues 116-123) and β7b (residues 126-130) were separated by a region containing Pro125 that interrupted the continuity of β7. Strands β8a (residues 138-142) and β8b (residues 146-149) were separated by a β-bulge, whereas strands β8b and β8c (residues 151-153) were separated by Leu150. Strands β8b and β8c 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 β3 and a fragment of the chain in the vicinity of strand β4. This bridge could be described as a clamp holding both barrel-forming β-sheets. The Cys128-Cys140 bridge linked 2 neighboring β-strands, β7b and β8a. 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 $Å^2$. 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.

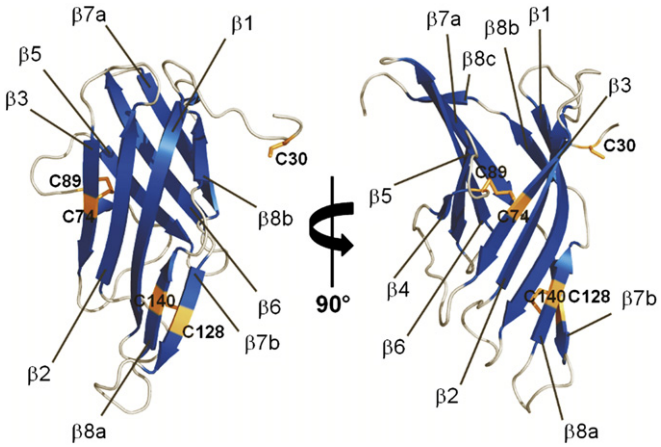

FIG 1. Overall structure of Alt a 1. β-Strands are shown in *blue*, loop regions are shown in *gray*, and cysteine residues are marked in *orange*.

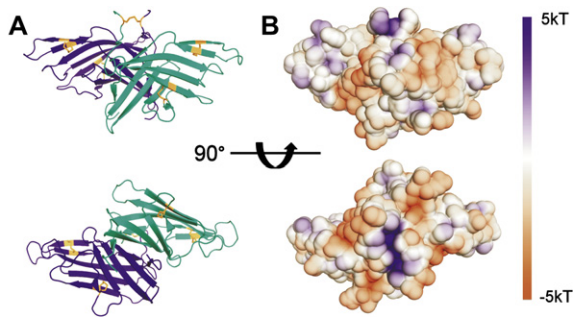

FIG 2. A, Alt a 1 dimer shown as a cartoon representation. Disulfide bridges are shown in *orange*. **B,** Solvent-accessible surface of the Alt a 1 dimer with mapped electrostatic charges (*red*, residues with negative charge; *blue*, residues with positive charge).

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 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 *Leotiomyceta*.[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 PROFUNC[56] 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 al[53] 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

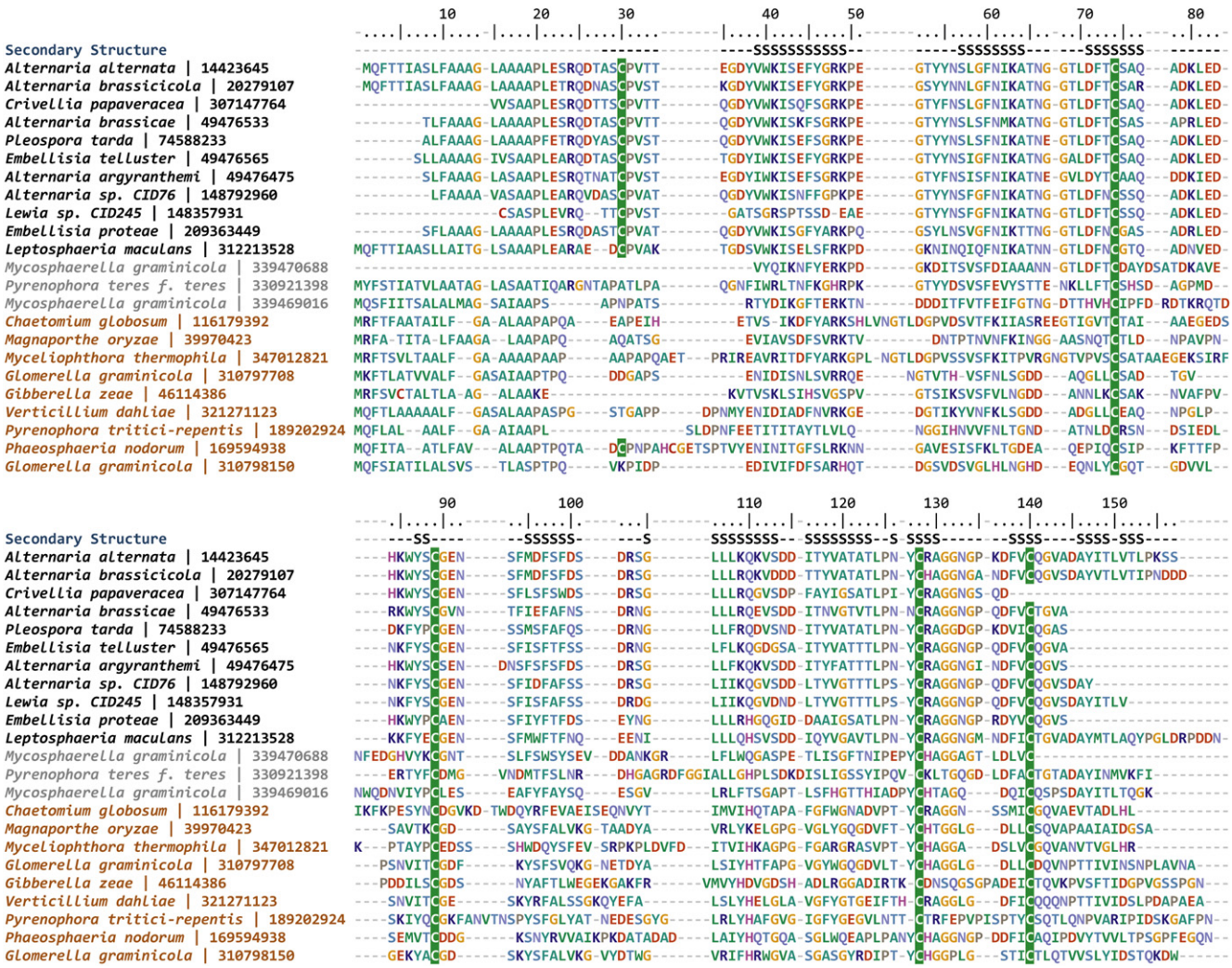

FIG 3. Sequence alignment of selected Alt a 1 homologs. The sequences are colored by using the ClustalX default color scheme. Secondary structure was calculated with STRIDE on the basis of Alt a 1 structure and refined manually (β-strands are marked with the letter *S*). The cysteine residues involved 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*.

as follows: 450 Å^2 (K41-P50), 330 Å^2 (Y54-K63), 500 Å^2 (Y87-D96), and 280 Å^2 (V119-C128).

DISCUSSION

Alt a 1 and homologous proteins are unique and characteristic for the *Dothideomycetes* and *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 intramolecular pattern of the disulfide bonds is present in all known homologs of Alt a 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 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 because the residues forming the dimer interface are not conserved (Fig 6).

Alt a 1 is the first structure of an *A alternata* allergen to be determined. It was recently demonstrated that Alt a 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 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

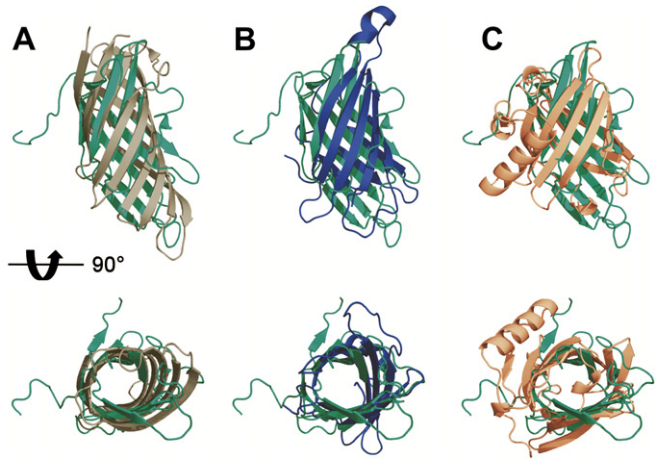

FIG 4. A, Structure of Alt a 1 *(green)* superposed on an uncharacterized protein from *Shewanella denitrificans* (*gray*; PDB code: 2q03). **B,** Structure of Alt a 1 superposed on the structure of streptavidin (*blue*; PDB code: 1nc9). **C,** Superposition of Alt a 1 and Bos d 2 (*pale pink;* PDB code: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

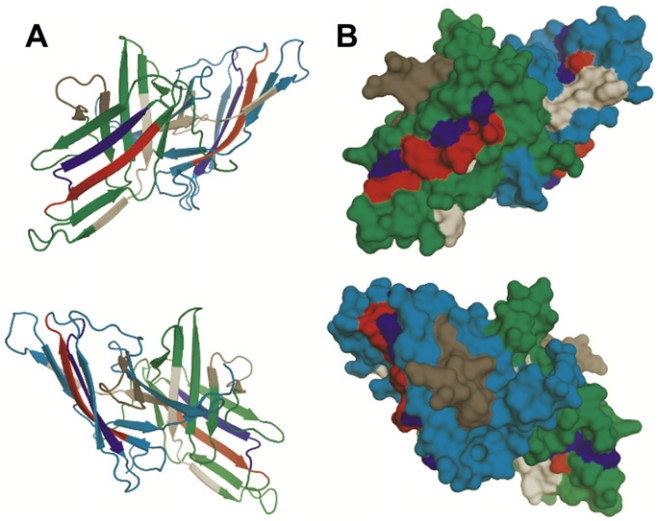

FIG 5. A, Dimer of Alt a 1 shown as a cartoon representation with IgE antibody–binding peptides marked in *red* (residues K41-P50) and *blue* (residues Y54-K63). Peptides Y87-D96 and V119-C128, which showed weak IgE antibody binding, are shown in *dark gray* and *light gray*, respectively. **B,** The 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.

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 physiologic (see Fig E6 in this article's Online Repository at www.jacionline.org). 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 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 β1 and β2 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.

We thank Zbyszek Otwinowski for the idea of using a tantalum cluster for phasing and Dominika Borek for providing the cluster.

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.**

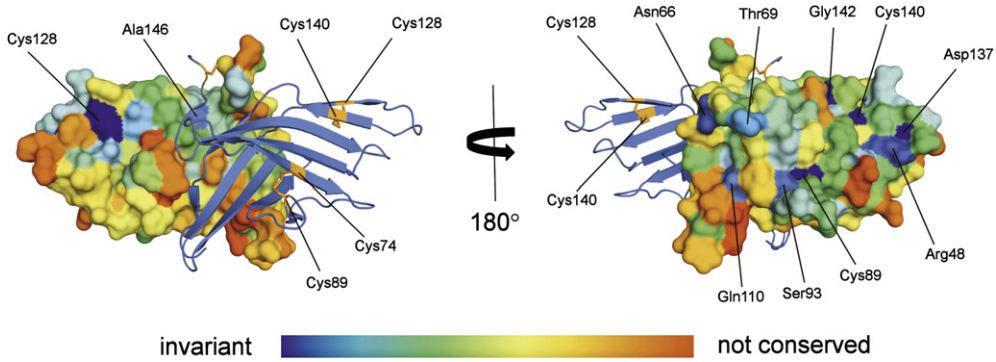

FIG 6. Sequence conservation among Alt a 1–like proteins mapped on the molecular surface of an Alt a 1 molecule. Colors range from *blue* for invariant residues to *red* for residues for no sequence conservation.

REFERENCES

1. Bush RK, Portnoy JM. The role and abatement of fungal allergens in allergic diseases. J Allergy Clin Immunol 2001;107(suppl):S430-40.
2. O'Hollaren MT, Yunginger JW, Offord KP, Somers MJ, O'Connell EJ, Ballard DJ, et al. Exposure to an aeroallergen as a possible precipitating factor in respiratory arrest in young patients with asthma. N Engl J Med 1991;324:359-63.
3. Stern DA, Morgan WJ, Halonen M, Wright AL, Martinez FD. Wheezing and bronchial hyper-responsiveness in early childhood as predictors of newly diagnosed asthma in early adulthood: a longitudinal birth-cohort study. Lancet 2008;372:1058-64.
4. Delfino RJ, Zeiger RS, Seltzer JM, Street DH, Matteucci RM, Anderson PR, et al. The effect of outdoor fungal spore concentrations on daily asthma severity. Environ Health Perspect 1997;105:622-35.
5. Arbes SJ, Sever M, Mehta J, Collette N, Thomas B, Zeldin DC. Exposure to indoor allergens in day-care facilities: results from 2 North Carolina counties. J Allergy Clin Immunol 2005;116:133-9.
6. O'Connor GT, Walter M, Mitchell H, Kattan M, Morgan WJ, Gruchalla RS, et al. Airborne fungi in the homes of children with asthma in low-income urban communities: the Inner-City Asthma Study. J Allergy Clin Immunol 2004;114:599-606.
7. Wang J, Visness CM, Calatroni A, Gergen PJ, Mitchell HE, Sampson HA. Effect of environmental allergen sensitization on asthma morbidity in inner-city asthmatic children. Clin Exp Allergy 2009;39:1381-9.
8. Salo PM, Calatroni A, Gergen PJ, Hoppin JA, Sever ML, Jaramillo R, et al. Allergy-related outcomes in relation to serum IgE: results from the National Health and Nutrition Examination Survey 2005-2006. J Allergy Clin Immunol 2011;127:1226-35.
9. Chan-Yeung M, Anthonisen NR, Becklake MR, Bowie D, Sonia BA, Dimich-Ward H, et al. Geographical variations in the prevalence of atopic sensitization in six study sites across Canada. Allergy 2010;65:1404-13.
10. Downs SH, Mitakakis TZ, Marks GB, Car NG, Belousova EG, Leuppi JD, et al. Clinical importance of *Alternaria* exposure in children. Am J Respir Crit Care Med 2001;164:455-9.
11. Fernandez C, Bevilacqua E, Fernandez N, Gajate P, de la Camara AG, Garcimartin M, et al. Asthma related to Alternaria sensitization: an analysis of skin-test and serum-specific IgE efficiency based on the bronchial provocation test. Clin Exp Allergy 2011;41:649-56.
12. Marks GB, Bush RK. It's blowing in the wind: new insights into thunderstorm-related asthma. J Allergy Clin Immunol 2007;120:530-2.
13. Perzanowski MS, Sporik R, Squillace SP, Gelber LE, Call R, Carter M, et al. Association of sensitization to *Alternaria* allergens with asthma among school-age children. J Allergy Clin Immunol 1998;101:626-32.
14. Randriamanantany ZA, Annesi-Maesano I, Moreau D, Raherison C, Charpin D, Kopferschmitt C, et al. *Alternaria* sensitization and allergic rhinitis with or without asthma in the French Six Cities study. Allergy 2010;65:368-75.
15. Kuna P, Kaczmarek J, Kupczyk M. Efficacy and safety of immunotherapy for allergies to *Alternaria alternata* in children. J Allergy Clin Immunol 2011;127:502-8, e1-6.
16. Deards MJ, Montague AE. Purification and characterisation of a major allergen of *Alternaria alternata*. Mol Immunol 1991;28:409-15.
17. Achatz G, Oberkofler H, Lechenauer E, Simon B, Unger A, Kandler D, et al. Molecular cloning of major and minor allergens of *Alternaria alternata* and *Cladosporium herbarum*. Mol Immunol 1995;32:213-27.
18. De Vouge MW, Thaker AJ, Curran IH, Zhang L, Muradia G, Rode H, et al. Isolation and expression of a cDNA clone encoding an *Alternaria alternata* Alt a 1 subunit. Int Arch Allergy Immunol 1996;111:385-95.
19. Unger A, Stoger P, Simon-Nobbe B, Susani M, Crameri R, Ebner C, et al. Clinical testing of recombinant allergens of the mold *Alternaria alternata*. Int Arch Allergy Immunol 1999;118:220-1.
20. Vailes LD, Perzanowski MS, Wheatley LM, Platts-Mills TA, Chapman MD. IgE and IgG antibody responses to recombinant Alt a 1 as a marker of sensitization to *Alternaria* in asthma and atopic dermatitis. Clin Exp Allergy 2001;31:1891-5.
21. Cramer RA, Lawrence CB. Cloning of a gene encoding an Alt a 1 isoallergen differentially expressed by the necrotrophic fungus *Alternaria brassicicola* during *Arabidopsis* infection. Appl Environ Microbiol 2003;69:2361-4.
22. Hong SG, Cramer RA, Lawrence CB, Pryor BM. Alt a 1 allergen homologs from *Alternaria* and related taxa: analysis of phylogenetic content and secondary structure. Fungal Genet Biol 2005;42:119-29.
23. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, et al. The Protein Data Bank. Nucleic Acids Res 2000;28:235-42.
24. Cymborowski M, Klimecka M, Chruszcz M, Zimmerman MD, Shumilin IA, Borek D, et al. To automate or not to automate: this is the question. J Struct Funct Genomics 2010;11:211-21.
25. Zimmerman MD, Chruszcz M, Koclega KD, Otwinowski Z, Minor W. The Xtaldb system for project salvaging in high-throughput crystallization. Acta Crystallogr A 2005;61:c178-9.
26. Rosenbaum G, Alkire RW, Evans G, Rotella FJ, Lazarski K, Zhang RG, et al. The Structural Biology Center 19ID undulator beamline: facility specifications and protein crystallographic results. J Synchrotron Radiat 2006;13:30-45.
27. Otwinowski Z, Minor W. Processing of x-ray diffraction data collected in oscillation mode. In: Carter CW Jr , Sweet RM, editors. Macromolecular Crystallography Pt A. New York: Academic Press; 1997. p. 307-26.
28. Minor W, Cymborowski M, Otwinowski Z, Chruszcz M. HKL-3000: the integration of data reduction and structure solution—from diffraction images to an initial model in minutes. Acta Crystallogr D Biol Crystallogr 2006;62:859-66.
29. Sheldrick GM. A short history of SHELX. Acta Crystallogr A 2008;64:112-22.
30. Otwinowski Z, editor. Isomorphous replacement and anomalous scattering. Warrington (United Kingdom): Daresbury Laboratory; 1991.
31. Cowtan KD, Main P. Improvement of macromolecular electron-density maps by the simultaneous application of real and reciprocal space constraints. Acta Crystallogr D Biol Crystallogr 1993;49:148-57.
32. Terwilliger T. SOLVE and RESOLVE: automated structure solution, density modification and model building. J Synchrotron Radiat 2004;11:49-52.
33. Perrakis A, Morris R, Lamzin VS. Automated protein model building combined with iterative structure refinement. Nat Struct Biol 1999;6:458-63.
34. The CCP4 suite: programs for protein crystallography. Acta Crystallogr D Biol Crystallogr 1994;50:760-3.
35. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol Crystallogr 2004;60:2126-32.
36. Murshudov GN, Skubak P, Lebedev AA, Pannu NS, Steiner RA, Nicholls RA, et al. REFMAC5 for the refinement of macromolecular crystal structures. Acta Crystallogr D Biol Crystallogr 2011;67:355-67.
37. Painter J, Merritt EA. TLSMD web server for the generation of multi-group TLS models. J Appl Crystallogr 2006;39:109-11.
38. Chen VB, Arendall WB 3rd, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, et al. MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr 2010;D66:12-21.

39. Yang H, Guranovic V, Dutta S, Feng Z, Berman HM, Westbrook JD. Automated and accurate deposition of structures solved by X-ray diffraction to the Protein Data Bank. Acta Crystallogr D Biol Crystallogr 2004;60:1833-9.
40. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389-402.
41. Pei J, Kim BH, Grishin NV. PROMALS3D: a tool for multiple protein sequence and structure alignments. Nucleic Acids Res 2008;36:2295-300.
42. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics 2009;25:1189-91.
43. Frishman D, Argos P. Knowledge-based protein secondary structure assignment. Proteins 1995;23:566-79.
44. Li W, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 2006;22:1658-9.
45. Holm L, Rosenstrom P. Dali server: conservation mapping in 3D. Nucleic Acids Res 2010;38:W545-9.
46. Ye Y, Godzik A. FATCAT: a web server for flexible structure comparison and structure similarity searching. Nucleic Acids Res 2004;32:W582-5.
47. Lo WC, Lee CY, Lee CC, Lyu PC. iSARST: an integrated SARST web server for rapid protein structural similarity searches. Nucleic Acids Res 2009;37:W545-51.
48. Krissinel E, Henrick K. Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. Acta Crystallogr D Biol Crystallogr 2004;60:2256-68.
49. Krissinel E, Henrick K. Inference of macromolecular assemblies from crystalline state. J Mol Biol 2007;372:774-97.
50. DeLano WL. The PyMOL molecular graphics system. 2002.
51. Holst M, Baker N, Wang F. Adaptive multilevel finite element solution of the Poisson-Boltzmann equation I. Algorithms and examples. J Comput Chem 2000;21:1319-42.
52. Dolinsky TJ, Czodrowski P, Li H, Nielsen JE, Jensen JH, Klebe G, et al. PDB2PQR: expanding and upgrading automated preparation of biomolecular structures for molecular simulations. Nucleic Acids Res 2007;35:W522-5.
53. Kurup VP, Vijay HM, Kumar V, Castillo L, Elms N. IgE binding synthetic peptides of Alt a 1, a major allergen of *Alternaria alternata*. Peptides 2003;24:179-85.
54. Tsodikov OV, Record MT Jr, Sergeev YV. Novel computer program for fast exact calculation of accessible and molecular surface areas and average surface curvature. J Comput Chem 2002;23:600-9.
55. Schoch CL, Wang Z, Townsend JP, Spatafora JW. Geoglossomycetes cl. nov., Geoglossales ord. nov. and taxa above class rank in the Ascomycota tree of life. Persoonia 2009;22:129-38.
56. Laskowski RA, Watson JD, Thornton JM. ProFunc: a server for predicting protein function from 3D structure. Nucleic Acids Res 2005;33:W89-93.
57. Twaroch TE, Arcalis E, Sterflinger K, Stoger E, Swoboda I, Valenta R. Predominant localization of the major *Alternaria* allergen Alt a 1 in the cell wall of airborne spores. J Allergy Clin Immunol 2012;129:1148-9.
58. Mitakakis TZ, Barnes C, Tovey ER. Spore germination increases allergen release from *Alternaria*. J Allergy Clin Immunol 2001;107:388-90.
59. Flower DR. The lipocalin protein family: structure and function. Biochem J 1996;318:1-14.
60. Derewenda U, Li J, Derewenda Z, Dauter Z, Mueller GA, Rule GS, et al. The crystal structure of a major dust mite allergen Der p 2, and its biological implications. J Mol Biol 2002;318:189-97.
61. Vailes L, Sridhara S, Cromwell O, Weber B, Breitenbach M, Chapman M. Quantification of the major fungal allergens, Alt a 1 and Asp f 1, in commercial allergenic products. J Allergy Clin Immunol 2000;107:641-6.
62. Barnes C, Portnoy J, Sever M, Arbes S Jr, Vaughn B, Zeldin DC. Comparison of enzyme immunoassay-based assays for environmental *Alternaria alternata*. Ann Allergy Asthma Immunol 2006;97:350-6.
63. Rouvinen J, Janis J, Laukkanen ML, Jylha S, Niemi M, Paivinen T, et al. Transient dimers of allergens. PLoS One 2010;5:e9037.
64. Li M, Gustchina A, Alexandratos J, Wlodawer A, Wunschmann S, Kepley CL, et al. Crystal structure of a dimerized cockroach allergen Bla g 2 complexed with a monoclonal antibody. J Biol Chem 2008;283:22806-14.
65. Radauer C, Bublin M, Wagner S, Mari A, Breiteneder H. Allergens are distributed into few protein families and possess a restricted number of biochemical functions. J Allergy Clin Immunol 2008;121:847-52.e7.

METHODS

Protein sample

According to the protein's manufacturer, recombinant Alta a 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-μm filter. After taking a 50 μL 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.

REFERENCES

E1. Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. Proc Natl Acad Sci U S A 1988;85:2444-8.
E2. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731-9.
E3. Whelan S, Goldman N. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Mol Biol Evol 2001;18:691-9.

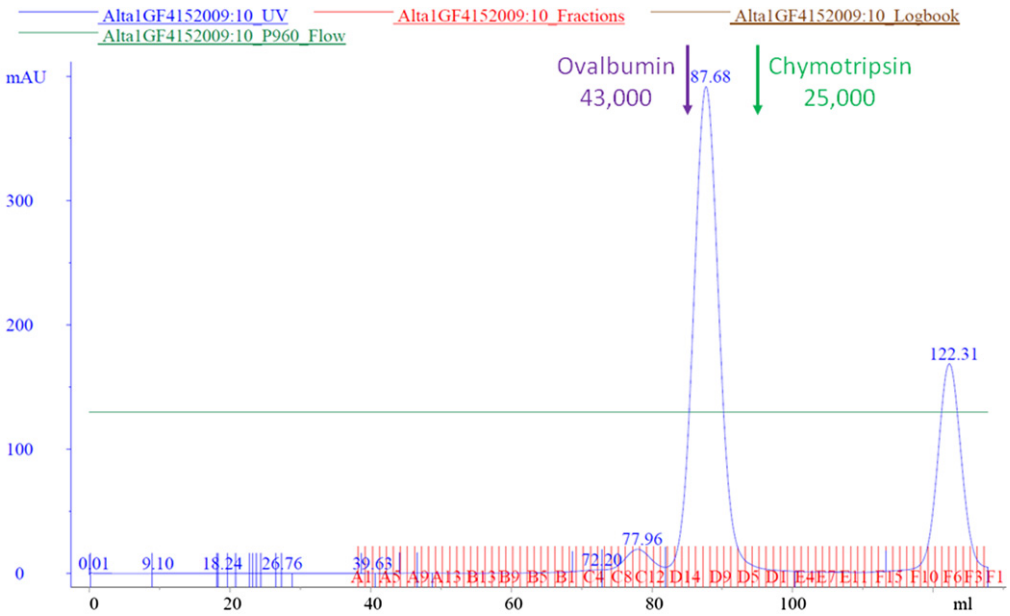

FIG E1. Results of gel filtration on Superdex 200. Protein was dissolved in 10 mmol/L Tris-HCl and 150 mmol/L NaCl, pH 7.5, and the same buffer was used for purification.

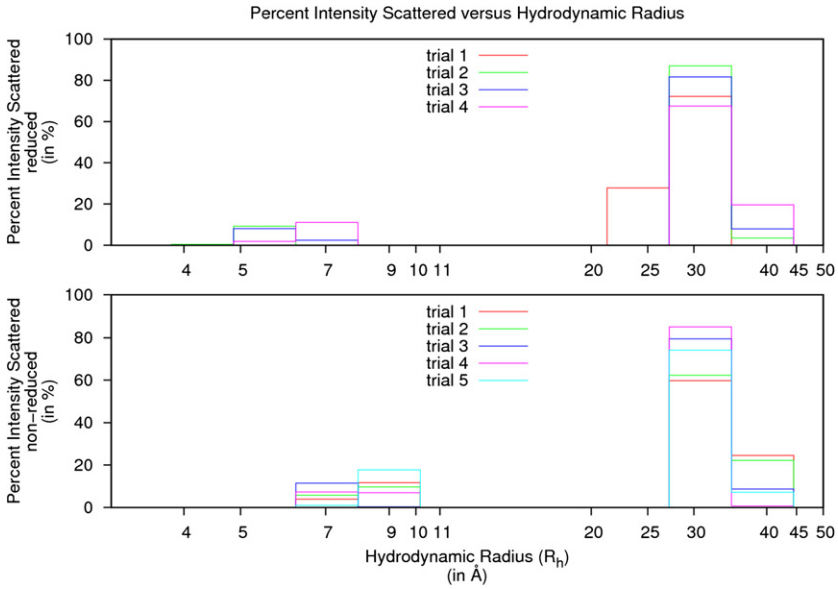

FIG E2. Results of the DLS measurements. The percentage of light intensity scattered versus hydrodynamic radius computed by using a regularization algorithm is shown for both reduced *(top)* and nonreduced *(bottom)* conditions.

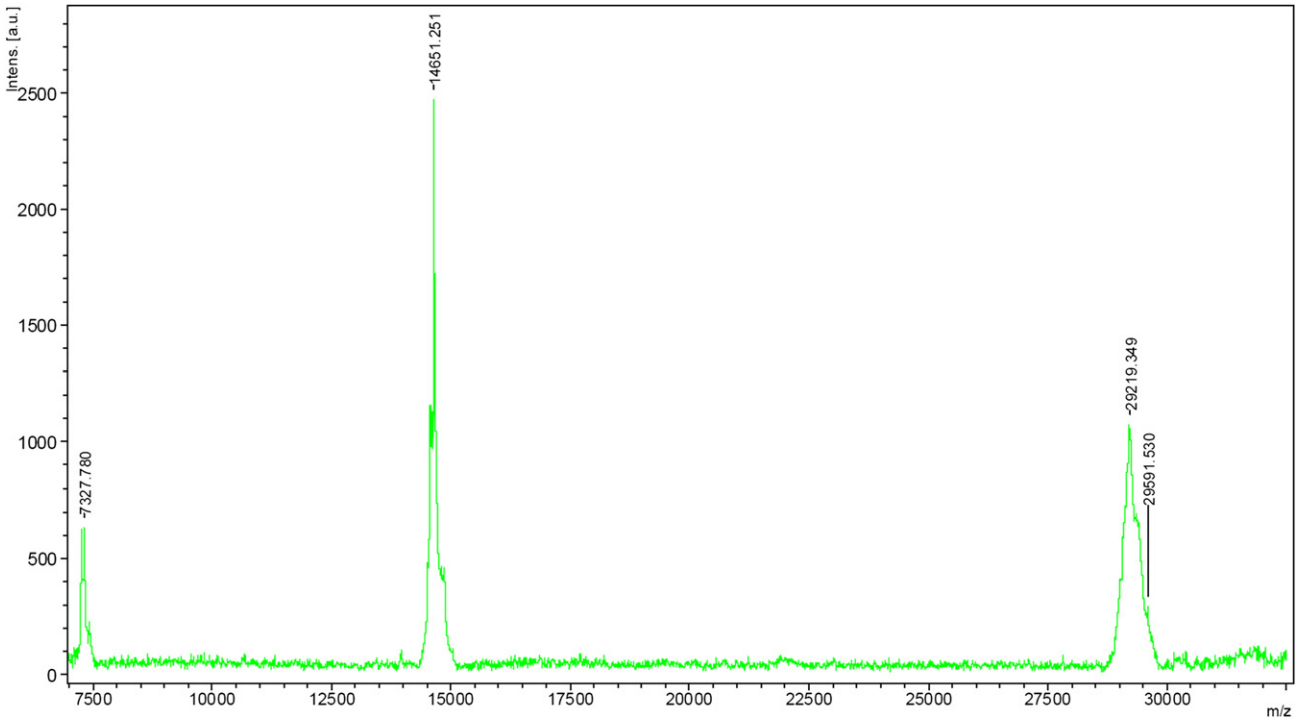

FIG E3. Results of mass spectrometric analysis of the Alt a 1 sample that was used for crystallization.

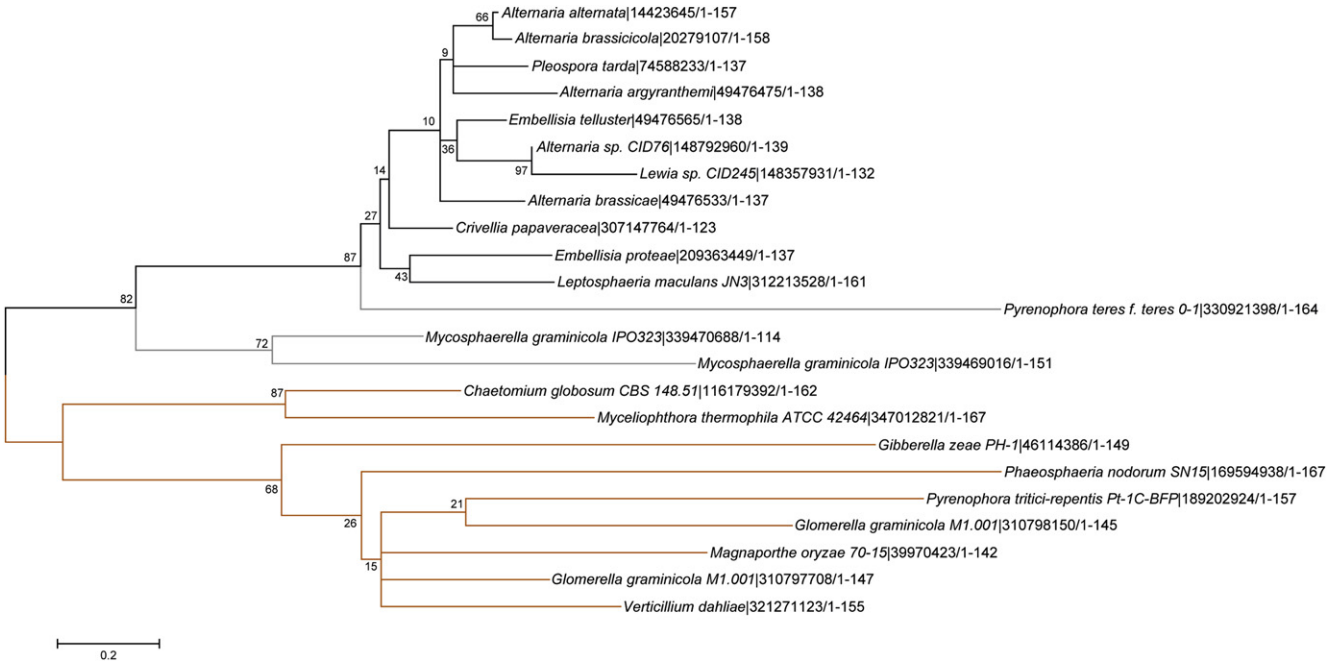

FIG E4. Results of sequence phylogenetic analysis. Sequences are labeled with organism name, gi number, and sequence length used for analysis, respectively. The MEGA package[E2] was used for phylogenetic analysis. The tree was computed by using the Maximum Likelihood statistical method with the WAG amino 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.

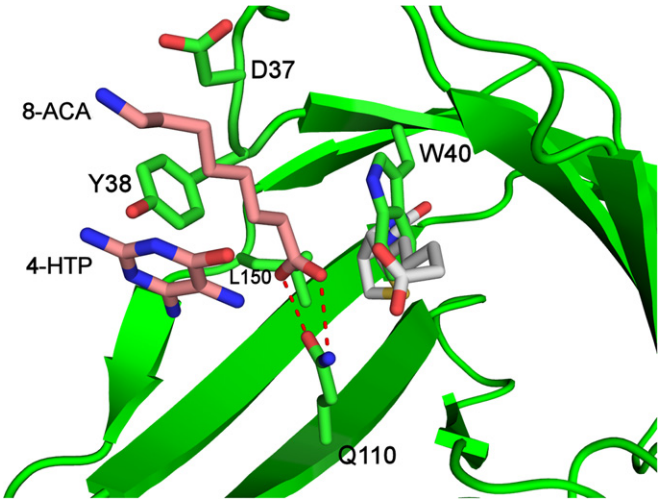

FIG E6. Binding of 4-HTP and 8-ACA, components of the crystallization solution. The position of biotin *(white sticks)* observed in a streptavidin structure (PDB code: 3ry2) superimposed with Alt a 1 would overlap with W40 from Alt a 1 (shown in *green*).

TABLE E1. Data collection and refinement statistics

PDB code	3v0r
Data collection	
Space group	$I4_122$
Cell dimensions	
a, *b*, *c* (Å)	a = b = 70.0, c = 179.3
α, β, γ (°)	90, 90, 90
Resolution (Å)	50.00-1.90 (1.93-1.90)
R_{sym}	0.068 (0.777)
$I/\sigma I$	59.4 (4.2)
Completeness (%)	99.9 (100.0)
Redundancy	13.8 (13.9)
Refinement	
Resolution (Å)	1.90
No. of reflections	18,063
R_{work}/R_{free}	16.7/19.0
No. of atoms	
Protein	1,014
Ligand/ion	61
Water	161
B 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 (%)
Alternaria brassicicola (gi: 20279107)***	89.6	98.7
Crivellia papaveracea (gi: 307147764)***	82.1	95.9
Alternaria brassicae (gi: 49476533)***	81.8	95.6
Embellisia telluster (gi: 49476565)***	81.6	96.3
Pleospora tarda (gi: 74588233)***	81	94.2
Alternaria argyranthemi (gi: 49476475)***	80.6	93.5
Alternaria species *CID76* (gi: 148792960)***	79.9	97.1
Embellisia proteae (gi: 209363449)***	70.1	92.7
Lewia species *CID245* (gi: 148357931)***	66.9	84.5
Leptosphaeria maculans (gi: 312213528)***	63.2	85.8
Mycosphaerella graminicola (gi: 339470688)**	40.9	71.3
Pyrenophora teres f. teres (gi: 330921398)**	39.3	74.2
Myceliophthora thermophila (gi: 347012821)*	32.4	55.3
Verticillium dahliae (gi: 321271123)*	31.9	60.1
Phaeosphaeria nodorum (gi: 169594938)*	31.7	56.1
Mycosphaerella graminicola (gi: 339469016)**	31.2	58.1
Chaetomium globosum (gi: 116179392)*	31.1	60.5
Pyrenophora tritici-repentis (gi: 189202924)*	30.1	56.9
Magnaporthe oryzae (gi: 39970423)*	29	60
Glomerella graminicola (gi: 310797708)*	27	58.3
Glomerella graminicola (gi: 310798150)*	25.6	56.9
Gibberella zeae (gi: 46114386)*	24.7	55.1

Sequences are sorted by identity. Names of the most similar sequences are marked with *** (they are shown 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]