

The Crystal Structure of the Major Cat Allergen Fel d 1, a Member of the Secretoglobin Family*

Received for publication, May 7, 2003, and in revised form, July 3, 2003
Published, JBC Papers in Press, July 8, 2003, DOI 10.1074/jbc.M304740200

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The domestic cat (*Felis domesticus*) is one of the most important causes of allergic asthma worldwide. The dominating cat allergen, Fel d 1, is composed of two heterodimers. Recently, it has been shown that recombinant Fel d 1, consisting of chain 2 and chain 1 fused together without additional linker, has immunological properties indistinguishable from the natural heterodimeric protein. Herein, we report the crystal structure of recombinant monomeric Fel d 1 at 1.85-Å resolution, determined by multi-wavelength anomalous diffraction using selenomethionine substituted protein. Fel d 1 is an all-helical protein and consists of eight helices. The two halves of the recombinant Fel d 1 molecule, corresponding to the wild-type Fel d 1 chains, are very similar in three-dimensional structure, despite the lack of significant sequence identity. The structure of the Fel d 1 presents a striking similarity to that of uteroglobin, a steroid-inducible cytokine-like molecule with anti-inflammatory and immunomodulatory properties. An internal, asymmetric cavity is formed in the Fel d 1 that could bind an endogenous ligand. The distribution of residues lining this cavity suggests that such a ligand must be amphipathic. The structure of Fel d 1 displays the localization of three previously defined Fel d 1 IgE epitopes on the surface of the protein. The three-dimensional structure provides a framework for rational design of hypoallergenic mutants aimed for treatment of cat allergy.

Proteins from domestic cats (*Felis domesticus*) are some of the most potent elicitors of allergic disease, affecting people worldwide (1). The severity of symptoms ranges from mild rhinitis and conjunctivitis to life-threatening asthmatic responses. The most prominent and potent allergen in cat dan-

der, termed Fel d 1, was identified 3 decades ago (2). Fel d 1 elicits IgE responses in 90–95% of patients with cat allergy (3) and accounts for 60–90% of the total allergenic activity in cat dander (4). The allergen is produced by sebaceous glands and squamous epithelial cells and is transferred to the pelt by licking and grooming (5–7). Fel d 1 is also present in the salivary, perianal, and lachrymal glands (8, 9). The allergen, which is carried by small airborne particles, is abundant in society, including environments in which cats are not present, such as schools and public transportation (10, 11). Subsequent studies have evaluated the clinical importance and biochemical properties of this ubiquitous protein, and clinical vaccination trials have been conducted, using T cell peptides derived from Fel d 1 (12, 13). However, the structural basis for the powerful allergenicity of Fel d 1 has remained enigmatic.

The allergen Fel d 1 belongs to the secretoglobin family of proteins (14). It is a 35-kDa tetrameric glycoprotein (15, 16) formed by two heterodimers (15). Each dimer is composed of two chains derived from two independent genes (17), chain 1, comprising 70 residues, and chain 2, comprising 90 or 92 residues. The two isoforms of the second chain are expressed in the skin and the saliva (17), respectively. Three interchain disulfide bonds linking the two peptide chains have been proposed (16); the distribution of the disulfide bridges (*i.e.* Cys-3 (chain 1)-Cys-73 (chain 2), Cys-44 (chain 1)-Cys-48 (chain 2), and Cys-70 (chain 1)-Cys-7 (chain 2)) suggests an antiparallel orientation of the two chains in each subunit.

In routine allergy praxis, only crude cat allergen extracts have so far been used for diagnosis and treatment of cat allergy. One of the drawbacks with these extracts is that the amount of Fel d 1 may vary markedly. Because the purification and production of native Fel d 1 is a time-consuming and costly process, Fel d 1 obtained by DNA technology may offer means of overcoming these problems. Several attempts have been made to refold the separate peptide chains into a native-like allergen, with only partial success (18–20). Although a mix of the separate chains has proven to be valuable for *in vitro* allergy diagnostics (3, 18), a soluble and correctly folded recombinant molecule would be useful not only for diagnosis and treatment but also for molecular studies of the Fel d 1 protein. Using a direct fusion of the two chains of Fel d 1 (Fig. 1*a*), we have been able to establish *in vitro* conditions for the appropriate refolding of the fused protein. Therefore and hereafter, recombinant Fel d 1 will be denoted as monomer rather than heterodimer, as would be appropriate for native Fel d 1. Refolded recombinant Fel d 1 displays an identical disulfide-bonding pattern and a comparable secondary structure to the native protein as revealed by circular dichroism (21). It forms a dimer of the two fused chains corresponding to the native

* Financial support for this study was received from the Swedish Research Council, the Swedish Foundation for Strategic Research, the Swedish Foundation for Health Care Sciences and Allergy Research, the Åke Wibergs Foundation, the Magnus Bergwalls Foundation, the Swedish Asthma and Allergy Association, the Hesselman Foundation, the Swedish-Heart Lung Foundation, and the King Gustaf V 80th Birthday Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1PUO) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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TABLE I
Statistics for data collection, phasing, and refinement

Values in parentheses are for the highest resolution shell of each data set. $R_{\text{merge}} = \sum_{\text{hkl}} \sum_{\text{ai}} |I_i(\text{hkl}) - \langle I(\text{hkl}) \rangle| / \sum_{\text{hkl}} \sum_{\text{ai}} I_i(\text{hkl})$. $R_{\text{cryst}} = \sum_{\text{hkl}} \|F_o\| - \|F_c\| / \sum_{\text{hkl}} F_o$, where F_o and F_c are observed and model-derived structure factors, respectively. R_{free} is calculated against 5% of the observations left out throughout the refinement.

	Wild Type	Seleno-Met
X-ray source	I711, MAX-lab	ID29, ESRF
Wavelength (Å)	1.134	0.979
Resolution (Å)	30–1.85	30–2.2
Space group	P2 ₁	P2 ₁
Unit cell parameters		
<i>a</i> (Å)	43.3	43.5
<i>b</i> (Å)	51.5	51.6
<i>c</i> (Å)	67.7	68.2
β (°)	95.3	95.7
Total unique reflections	25113	14922
Number of observed reflections	69889	51253
Completeness (%)	98.4 (99.7)	96.5 (80.2)
<i>R</i> _{sym}	0.089 (0.46)	0.067 (0.16)
<i>I</i> /σ	9.7 (2.5)	14.1 (4.9)
B-factor from Wilson plot (Å ²)	18.0	27.3
Phasing power		1.30
Figure of merit after SOLVE		0.31
<i>R</i> _{cryst} (%)	20.3 (22.6)	
<i>R</i> _{free} (%)	23.9 (24.4)	
Number of protein atoms	2202	
Number of MPD molecules	4	
Number of water molecules	233	
r.m.s.d. bond lengths (Å)	0.01	
r.m.s.d. bond angles (°)	1.27	
B-factors (Å ²)		
Overall	19.4	
Protein atoms	18.1	
Solvent	30.5	
Ramachandran plot: non-glycine residues in		
Favorable regions		96.7%
Additionally allowed regions		2.5%
Generously allowed regions		0.8%

tetramer. Most importantly, the *in vitro* immunoreactivity of the stable recombinant Fel d 1 is indistinguishable from the native allergen (21).

The three-dimensional structures of only a limited number of important animal-, plant-, and dust mite-derived allergens have been revealed (22). The biological functions of these allergens are diverse or unknown, without any particular biological or structural feature that seems to predispose a protein to act as an allergen (23). To increase the understanding of the mechanisms for cat allergy and to obtain knowledge regarding new strategies for treatment, we have solved the crystal structure of the most potent cat allergen, Fel d 1. The fold of the latter presents a striking resemblance to that of uteroglobin, a steroid-inducible, cytokine-like molecule with anti-inflammatory and immunomodulatory properties. This similarity in three-dimensional structure suggests a potential mechanism underlying the potent allergenicity of Fel d 1. The structure also reveals the presence of an internal pocket. The shape of the cavity, as well as the properties of the residues that line it indicates a different kind of ligand to Fel d 1 than those proposed for uteroglobin. Furthermore, the structure of Fel d 1 allows for rational design of hypoallergenic IgE binding epitopes aimed for treatment of cat allergic patients.

EXPERIMENTAL PROCEDURES

Production and Crystallization of Fel d 1—Recombinant Fel d 1 was produced with chain 2 directly linked to chain 1 followed by a His₆ tag as in *Escherichia coli* strain BL21 (DE3) pLysS and isolated from inclusion bodies. The protein was further purified to homogeneity using a Ni²⁺ chelate column, size exclusion, and ion exchange chromatography (24). After concentration to ~7.5 mg/ml, the protein was stored at –80 °C. The SeMet-substituted Fel d 1 was produced in *E. coli* strain BL21 (DE3) pLysS (25) and purified in the same way as the wild-type

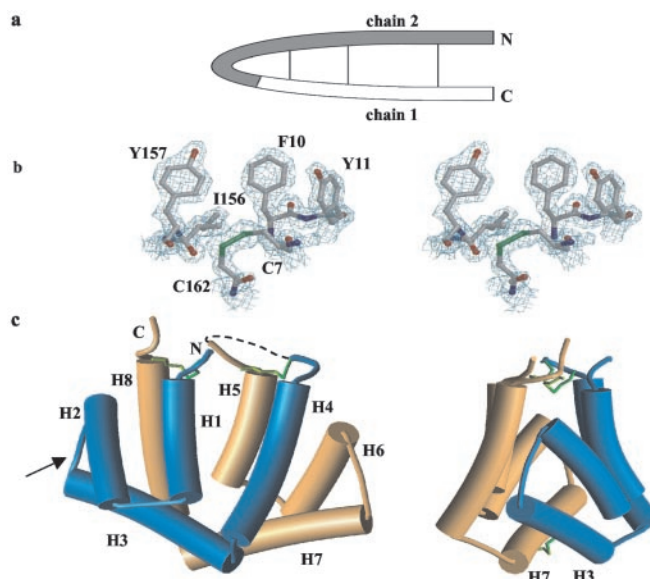


FIG. 1. **Overall structure of Fel d 1.** *a*, arrangement of chain 2 and 1 in recombinant Fel d 1. *b*, stereo view of a representative portion of the refined $2F_o - F_c$ electron density map of Fel d 1, contoured at 1.5 σ . The disulfide bond between residues Cys-7 and Cys-162 is colored in green. *c*, schematic diagram of the Fel d 1 monomer. The monomer is displayed from two different orientations, with a rotation of about 90° around the vertical axis. The helices corresponding to chains 2 and 1 are colored in blue and gold, respectively. The dotted line indicates the disordered loop (residues 75 to 92). The three disulfide bridges that link chains 1 and 2 are displayed in green. An arrow indicates the unique glycosylation site at residue N33.

protein. Fel d 1 crystals were obtained by hanging drop vapor diffusion. Typically, 2 μ l of protein in 20 mM Tris-HCl, pH 7.5, were mixed in a 1:1 ratio with the crystallization reservoir solution, and allowed to equilibrate at 4 °C. The best Fel d 1 crystals appeared after seeding in 13% 2-methyl-2,4-pentanediol (MPD)¹ and 0.1 M sodium acetate, pH 4.8, using a protein concentration of 2.5 mg/ml. The SeMet-Fel d 1 crystals were produced by seeding from wild-type crystals in 16% MPD and 0.1 M sodium acetate, pH 4.8, using a protein concentration of 2 mg/ml.

Data Collection and Processing—The crystals were soaked in cryoprotectant solution (20% MPD) before flash-freezing in a cold nitrogen stream. A data set for native Fel d 1 was collected at beamline I711 at MaxLab (Lund, Sweden). The crystals belong to space group P2₁ with unit cell parameters $a = 43.3$ Å, $b = 51.5$ Å, $c = 67.7$ Å, and $\beta = 95.3^\circ$. The asymmetric unit contains two molecules of Fel d 1 with a molecular mass of 19,177 Da, giving a solvent content of 37.4%. The data for the SeMet-Fel d 1 protein were collected at the ID29 beam line (ESRF, Grenoble, France). The cell parameters of the crystal from SeMet protein were very similar to the native one (Table I). A fluorescence spectrum was measured, and the wavelength was set at the absorption peak. The crystals of SeMet substituted Fel d 1 decayed rather rapidly in the beam of ID29 despite attenuation of the beam. The structure was solved by single wavelength anomalous diffraction experiment from the peak data set. The data were processed with the program MOSFLM (26) and scaled and reduced using SCALA from the CCP4 suite of programs (27).

Phasing and Model Building—The localization of the positions of the anomalous scatterers and the single anomalous diffraction phasing were carried out using the program SOLVE (28). Six of 10 possible selenium sites in the asymmetric unit were found. The four other methionine residues were later found in flexible regions, not visible in the electron density map. The phasing power was 1.3 for acentric reflections for the resolution range 29.8–2.7 Å. The phases were extended to 2.2 Å with the use of non-crystallographic symmetry averaging and solvent flattening with the program RESOLVE (29), which resulted in an interpretable electron density map.

Automated chain tracing by the program RESOLVE gave an initial model comprising 67% of main chain atoms, and the remaining parts were built with the program O (30). The structural similarity with

¹ The abbreviations used are: MPD, 2-methyl-2,4-pentanediol; r.m.s.d., root mean square deviation.

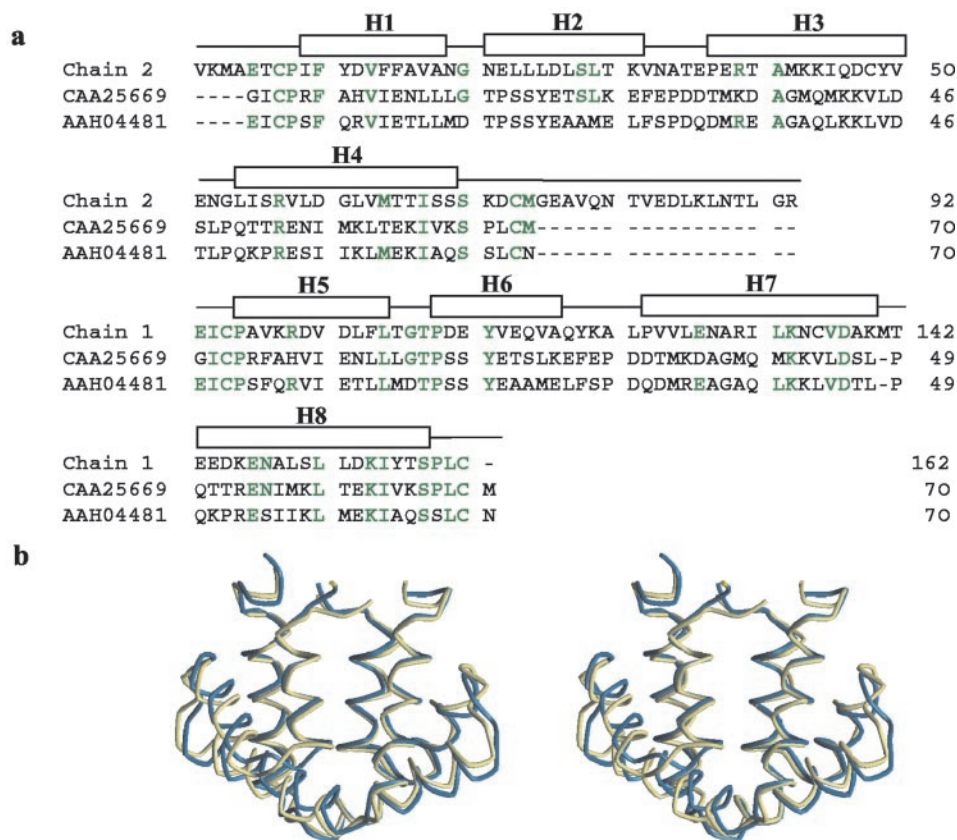


FIG. 2. **Comparison of Fel d 1 and uteroglobin.** *a*, structure-based alignment of Fel d 1 chains 2 and 1 and of uteroglobin from rabbit (GenBank accession number CAA25669) and human (GenBank accession number AAH04481). Identical residues are indicated in green. The secondary structure elements are indicated above the sequence. *b*, stereo view of a ribbon diagram of the main chain of Fel d 1 (blue) superimposed on the oxidized form of rabbit uteroglobin (Protein Data Bank code 2UTG) (yellow).

uteroglobin was recognized at an early stage of model building, and the superposition of the crystal structure of uteroglobin (Protein Data Bank code 2UTG) with the electron density map of Fel d 1 accelerated the chain-tracing process. The three disulfide bonds and the selenium positions facilitated fitting of the biological sequence to the electron density map.

Crystallographic Refinement—The native data set to 1.85 Å resolution was used for the refinement, which was carried out using the program REFMAC5 (31). Five percent of the reflections were set aside for validation. Several rounds of rebuilding with O combined with five to seven cycles of maximum likelihood refinement were performed, resulting in the final model, consisting of 283 residues. Isotropic, individual B-factor refinement was used throughout the procedure. Finally, 233 water molecules were added by ARP-WARP (32), and their positions were checked in O. The final validation included calculation of a composite omit map (33) and analysis using PROCHECK (34). Structural comparisons were carried out with O using default parameters. Figures were prepared with the programs WebLab ViewerLight, SwissPDB Viewer (35), BobScript (36), and GRASP (37).

RESULTS AND DISCUSSION

Overall Structure—The three-dimensional structure of the allergen Fel d 1 was determined to a resolution of 1.85 Å using anomalous diffraction from a selenomethionine-substituted protein crystal (Table I). The model displays good stereochemistry, and the R_{cryst} and R_{free} values for the refined structure are 20.3 and 23.9%, respectively (Table I). The asymmetric unit includes two Fel d 1 monomers, with 233 water molecules as well as four MPD molecules. The two monomers present in asymmetric unit are very similar in structure, with a root-mean-square deviation (r.m.s.d.) of 0.3 Å for all $C\alpha$ -atoms.

The final electron density map is of high quality with well-defined polypeptide chains. An example of the refined σ_a weighted $2F_o - F_c$ map is displayed in Fig. 1b. The final model of one molecule consists of residues 5 to 74 (chain 2) and 93 to

164 (chain 1). The four N-terminal and the six C-terminal histidine residues are not defined by electron density. In addition, electron density is missing for residues 75 to 92, which correspond to the C-terminal part of chain 2 (Fig. 1c), indicating disorder of this region of the molecule. Except for these residues the model is well ordered. The average B-factor of the final model is very close to the B-factor, calculated from the Wilson plot (see Table I).

The fold of recombinant Fel d 1 is different from those of previously structurally characterized allergens (22). It consists of eight helices, H1–H4 and H5–H8, that correspond to chains 2 and 1, respectively, in native Fel d 1 (Fig. 1c). The N-terminal part comprising H1–H4 packs against the C-terminal part of the chain, H5–H8, forming a globular molecule of dimensions $40 \times 30 \times 40$ Å. Three disulfide bonds, linking the N-terminal with the C-terminal half of the monomer, are found at positions Cys7–Cys162, Cys48–Cys136, and Cys73–Cys95. Using mass spectrometry, we have recently demonstrated that the disulfide bonds are identical to those described for native Fel d 1 (21). The parts corresponding to chain 1 and 2 in natural Fel d 1 are similar in structure, with an overall r.m.s.d. of 1.7 Å for 67 equivalent $C\alpha$ atoms, despite a sequence identity of only 9%. Among the few conserved amino acids between the two chains are the six cysteines that participate in the formation of the disulfide bridges. The conservation of the three-dimensional structure in chains 1 and 2 and of the critical cysteine residues suggests a common evolutionary origin.

An internal cavity is formed at the packing interface between the two halves of the molecule, and residues lining the cavity originate from practically all of the helices in the monomer. Strong residual electron density, which cannot be attributed to side chains or ordered water molecules, indicates a bound li-

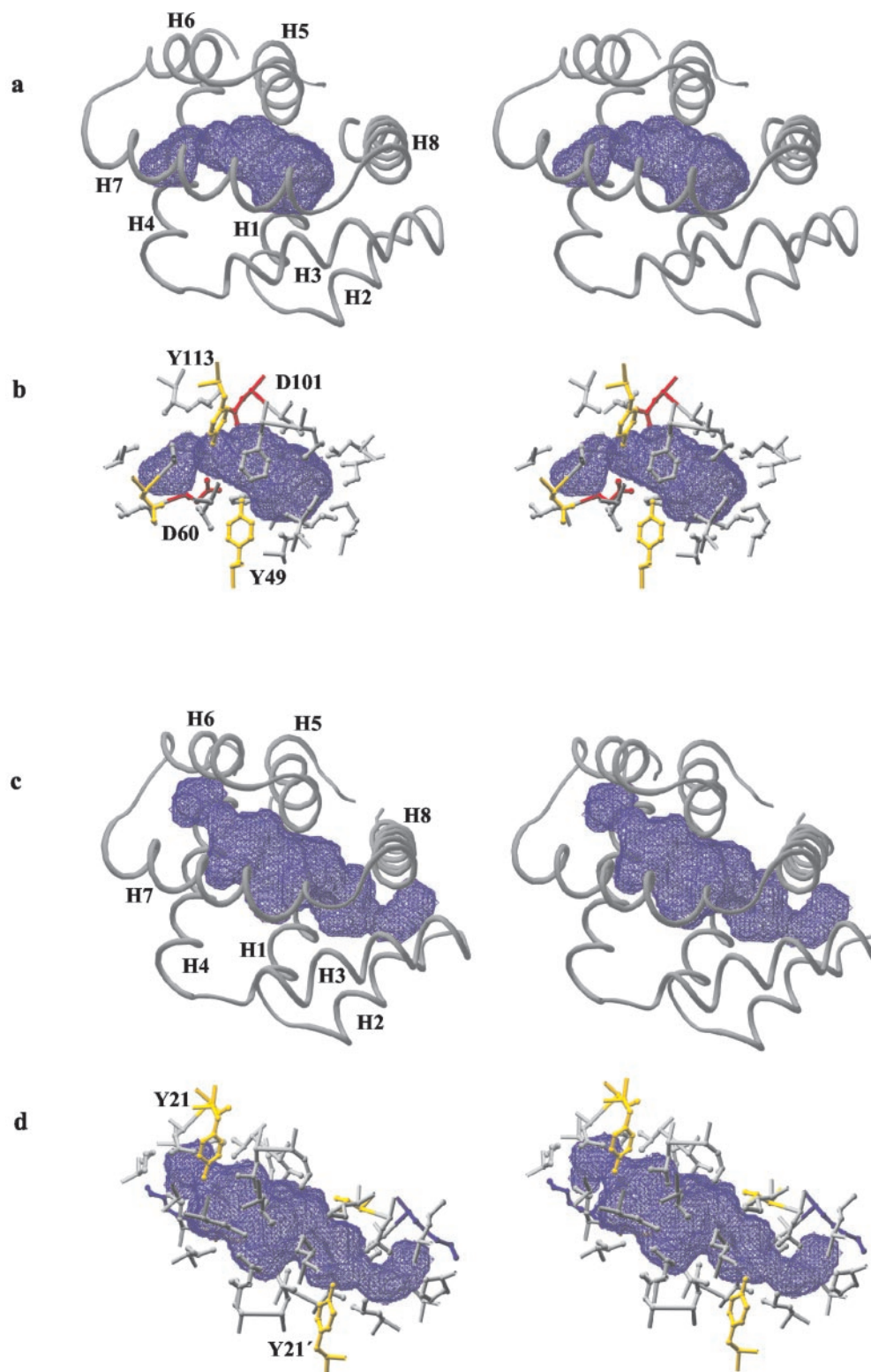


FIG. 3. **Comparison of the cavities in Fel d 1 and uteroglobin.** *a*, representation of the cavity inside the Fel d 1 allergen. *b*, residues lining the Fel d 1 cavity. *c*, representation of the cavity within uteroglobin (Protein Data Bank code 2UTG). *d*, residues lining the uteroglobin cavity. The molecular surface of the cavities was calculated with a 1.4-Å probe radius and is colored in *blue*. Hydrophobic, polar, acidic, and basic residues lining the cavities within both molecules are colored in *gray*, *yellow*, *red*, and *blue*, respectively.

gand within this cavity. The nature of this ligand in the recombinant protein is unknown; it has been modeled as an MPD molecule, which is present at a high concentration in the crystallization solution. In addition, six well ordered water molecules were found buried at identical positions in the cavity of each molecule.

It has previously been determined that a glycosylation site is located at residue N33 in chain 2 (16). Analysis by mass spectrometry identified the glycan as a heterogeneous tri-antennary complex structure (16). The structure of recombinant Fel d 1 reveals that residue N33 is located in the loop connecting the H2 and H3 helices and that the side chain is exposed to the

solvent (Fig. 1c). Fel d 1 occurs naturally as a tetramer composed of two identical heterodimers (15), but it is difficult to define the dimer of recombinant Fel d 1 corresponding to the native tetramer from the crystal structure. The sizes of the contact areas between either adjacent monomers in the asymmetric unit or symmetry-related molecules are very similar, ranging from 420 to 470 Å², typical of crystal contacts rather than oligomer interfaces. Therefore, assignment of the natural tetramer is not possible.

Fel d 1 Is a Member of the Secretoglobin Family—Despite an overall sequence identity with human uteroglobin of only 20%, the structure of Fel d 1 presented here reveals a striking similarity to the three-dimensional structure of the uteroglobin (38–47) (Fig. 2). Sequence comparisons of the first chain of Fel d 1 with members of the uteroglobin family indicate that chain 1 is related to this protein family (14). However, the sequence homology between chain 2 and human uteroglobin (13% identity based on three-dimensional structural alignment) is barely detectable and has not been noted until recently (40). It was therefore unclear how similar the structure of Fel d 1 would be to that of uteroglobin.

The overall r.m.s.d. for 124 equivalent Cα atoms after superposition of Fel d 1 and the uteroglobin dimer was 1.7 Å, whereas the r.m.s.d. of chain 1 (involving helices H1–H4) and of chain 2 (helices H5–H8) of Fel d 1 to uteroglobin are 1.2 and 1.1 Å, respectively. It should be noted that the structural similarity of chain 1 and chain 2 of Fel d 1 to uteroglobin is more pronounced than that between the two chains of Fel d 1, reflecting the higher degree of sequence identity in the primary structures. Two of the disulfide bridges are conserved between uteroglobin and Fel d 1, Cys7–Cys162, and Cys73–Cys95 (Fig. 1c). Although the third disulfide bridge at position Cys48–Cys136 is not present in rabbit uteroglobin (38), the structures are very similar in the vicinity of these amino acids. In many cases residue V44 in uteroglobin (corresponding to Cys48 in Fel d 1) is exchanged to a cysteine (40), indicating the possibility of a third disulfide bridge in these uteroglobin species.

Native Fel d 1 exclusively forms heterodimers consisting of chains 1 and 2; a mixture of both chains results only in the formation of the heterodimer, and no homodimers of either chain 1 or chain 2 are found (19). There is no detectable sequence conservation between the two chains of Fel d 1, and there is therefore no *a priori* indication that the interface between the two chains could be preserved in such homodimers. Indeed, models of the corresponding chain1-chain 1 and chain 2-chain 2 homodimers show that in both cases, severe sterical clashes and charge repulsion would occur, which prevent homodimer formation.

The allergen Fel d 1 and the cytokine-like uteroglobin share more than a similarity in three-dimensional structure (Fig. 2b). The expression of both proteins in epithelial cells (6, 48) is controlled and induced by steroids (49–51). The structural similarities of the two proteins suggest that anti-Fel d 1 antibodies might cross-react with uteroglobin, hypothetically decreasing the anti-inflammatory properties of the latter (49), and thereby aggravating the allergic disease. Despite the low sequence identity between Fel d 1 and uteroglobin, three clusters composed of identical residues are present on the surface of the two molecules: cluster 1 (Thr-109, Pro-110, Asp-138) cluster 2 (Glu-5, Pro-8, Lys-155, Leu-161), and cluster 3 (Cys-73, Cys-95, Pro-96, and Glu-93). These clusters may form the basis for cross-reactive epitopes in Fel d 1 and uteroglobin. The structural similarity between Fel d 1 and uteroglobin may also suggest that the allergen harbors cytokine-like properties, rendering it capable of modulating the immune response.

The Ligand-binding Cavity of Fel d 1 Is Different from That

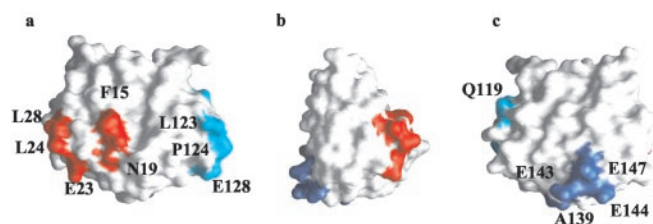


FIG. 4. **Predicted IgE epitopes.** The relative localization of three IgE epitopes is indicated on the molecular surface of Fel d 1. The surface accessible residues of the epitopes 15–28 (chain 2), 117–130, and 138–151 (chain 1) are colored in red, cyan, and blue, respectively. a, same orientation as in Fig. 1c, left. b, perpendicular view to a. c, perpendicular view to b.

of Uteroglobin—The shape of the cavity within Fel d 1 differs from the corresponding cavity within uteroglobin (Fig. 3, a and b). The size of the pocket is smaller (480 Å³) than in uteroglobin (750 Å³). Furthermore, the Fel d 1 cavity is asymmetric. One part of the cavity is lined by only hydrophobic residues, whereas polar and charged residues dominate on the opposite site. The hydrophilic end is created by two buried aspartic acids, Asp-60 and Asp-101 and two polar tyrosines Tyr-49 and Tyr-113 (Fig. 3b). In contrast, the cavity in homodimeric uteroglobin is symmetrical, mainly composed of hydrophobic residues, with one tyrosine from each chain (Tyr-21 and Tyr-21') at opposite ends of the cavity (Fig. 3, c and d). It has been demonstrated that uteroglobin is a carrier of small ligands such as polychlorinated biphenyl derivatives, phosphatidyl inositol, phosphatidyl choline, retinol, and progesterone (41, 47, 49). The tyrosine residue Tyr-21 is involved in the binding of ligands to uteroglobin (41), and the mutation of this position to alanine or phenylalanine results in a dramatic decrease of progesterone binding to uteroglobin (52). The particular distribution of residues in the cavity of Fel d 1 provides an amphipathic character to the cavity, which may be related to the physicochemical properties of the endogenous ligand.

Structural Localization of IgE Epitopes—The only curative treatment for allergy today is immunotherapy, based on repeated subcutaneous injections of allergen (53). One major disadvantage of current immunotherapy is that it can cause severe side effects, such as asthma attacks and anaphylactic shock. To overcome these problems, new strategies have been developed to reduce the allergenic potential of allergens (54). The identification of IgE epitopes on allergens allows the modification of important allergens such that they display strongly reduced allergenic activity by disrupting the conformational IgE epitopes. Such hypoallergenic allergen derivatives can be used as candidates for vaccines in allergen-specific immunotherapy, with a reduced risk of immediate side effects (54).

Three important IgE epitopes have been defined on the Fel d 1 allergen using 14-residue overlapping peptides spanning both chains, two in chain 1 (residues 25–38 and 46–59) and one in chain 2 (residues 15–28) (55). Epitopes 25–38 (corresponding to 117–130 in the construct), 46–59 (138–151 in the construct), and 15–28 (15–28 in the construct) are located on the neighboring helices H6–H7, H7–H8, and H1–H2, respectively, and connecting loops (Fig. 4). Interestingly, epitope 117–130 was recognized by 46% of the analyzed patient sera (55). The crystal structure of Fel d 1 reveals that the side chains of residues Gln-119, Leu-123, Pro-124, Glu-128, Ala-139, Glu-143, Glu-144, and Glu-147 in chain 1 and residues Phe-15, Asn-19, Glu-22, Leu-23, and Leu-27 in chain 2 are exposed to the solvent and are thereby available for IgE binding (Fig. 4).

CONCLUSIONS

The purpose of this study was to determine the structure of the major cat allergen Fel d 1. Herein, we report that the

crystal structure of Fel d 1, resolved at 1.85 Å resolution, displays a striking structural similarity to that of uteroglobin, a steroid-inducible, cytokine-like molecule with anti-inflammatory and immunomodulatory properties. The three-dimensional structure of Fel d 1 provides a framework for further studies aimed at understanding the pathogenesis of and creating new tools for diagnosis and therapy of cat allergy.

Acknowledgments—We acknowledge access to synchrotron beam lines I711 (MAX II laboratory, Lund, Sweden) and ID29 (ESRF, Grenoble, France). We also thank Dr. Robert A. Harris for assistance in manuscript preparation.

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