



Structural Characterization of the Tetrameric form of the Major Cat Allergen Fel d 1

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Felis domesticus allergen 1 (Fel d 1) is a 35 kDa tetrameric glycoprotein formed by two heterodimers which elicits IgE responses in 95% of patients with allergy to cat. We have previously established *in vitro* conditions for the appropriate folding of recombinant Fel d 1 using a direct linkage of chain 1 to chain 2 (construct Fel d 1 (1+2)) and chain 2 to chain 1 (construct Fel d 1 (2+1)). Although the crystal structure of Fel d 1 (2+1) revealed a striking structural similarity to that of uteroglobin, a steroid-inducible cytokine-like molecule with anti-inflammatory and immunomodulatory properties, no functional tetrameric form of Fel d 1 could be identified. Here we present the crystal structure of the Fel d 1 (1+2) tetramer at 1.6 Å resolution. Interestingly, the crystal structure of tetrameric Fel d 1 reveals two different calcium-binding sites. Symmetrically positioned on each side of the Fel d 1 tetramer, the external Ca²⁺-binding sites correspond to a putative Ca²⁺-binding site previously suggested for uteroglobin. The second Ca²⁺-binding site lies within the dimerization interface, stabilizing the formation of the Fel d 1 tetramer, and inducing important local conformational changes that directly govern the shape of two water-filled cavities. The crystal structure suggests a potential portal for an unknown ligand. Alternatively, the two cavities could be used by the allergen as a conditional inner space allowing for the spatial rearrangement of centrally localized side-chains, such as Asp130, without altering the overall fold of the molecule. The striking structural similarity of the major cat allergen to uteroglobin, coupled to the identification in the present study of a common Ca²⁺-binding site, let us speculate that Fel d 1 could provoke an allergic response through the modulation of phospholipase A2, by sequestering Ca ions in a similar manner as previously suggested for uteroglobin.

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Abbreviations used: CC16, Clara Cell 16; PLA₂, phospholipase A₂; PBMC, peripheral blood mononuclear cells; ELISA, enzyme-linked immunosorbent assay; PPD, tuberculin purified protein derivative; FASCIA, flow-cytometric assay of specific cell-mediated immune response in activated whole blood; PBS, phosphate-buffered saline.

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Introduction

The domestic cat (*Felis domesticus*) is one of the most common household pets as well as a significant source of indoor allergens. IgE-mediated sensitization to allergens from *F. domesticus* affects approximately 10% of the Western world.^{1–3} The severity of symptoms ranges from mild rhinitis to life-threatening asthmatic responses. *F. domesticus* allergen 1 (Fel d 1) is the most prominent and potent allergen in cat dander and was first identified more than three decades ago.⁴ It elicits IgE responses in 95% of patients with allergy to cat.⁵ However, the

structural basis of the powerful allergenicity of Fel d 1 remains enigmatic.

Fel d 1 has been reported as a 35 kDa tetrameric glycoprotein⁶ formed by two heterodimers.⁷ Each heterodimer consists of one 70 residue peptide and one 85, 90, or 92 residue peptide (i.e. chains 1 and 2, respectively) encoded by separate genes. These chains are linked within each heterodimer through three disulfide bonds, formed between cysteine residues Cys3 and Cys73, Cys44 and Cys48, and Cys70 and Cys7, in chains 1 and 2, respectively.^{6,8} Several attempts with only partial success have been made to refold the separate peptide chains into a native-like allergen.^{8–12} We have previously established *in vitro* conditions for the appropriate folding of recombinant Fel d 1 using a direct linkage of chain 1 to chain 2 (construct Fel d 1 (1+2)) and chain 2 to chain 1 (construct Fel d 1 (2+1)).¹³ Both refolded recombinant Fel d 1 protein variants display an identical disulfide-bonding pattern and a comparable secondary structure to the native protein.¹³ Importantly, the *in vitro* immunoreactivity of recombinant Fel d 1 (2+1) protein was previously determined to be indistinguishable from that of the native allergen.¹³

The crystal structure of the recombinant Fel d 1 (2+1) molecule revealed a striking similarity to that of uteroglobin, a steroid-inducible cytokine-like molecule with potent anti-inflammatory and immunomodulatory properties.¹⁴ Uteroglobin is a founding member of the secretoglobin superfamily, comprising secreted mammalian-restricted proteins that also includes the human protein Clara Cell 16 (CC16) and the rat-derived androgen-binding protein.^{14–18} In humans the lung is the main site of CC16 production, while several other organs synthesize smaller amounts of mRNA encoding this protein.¹⁹ Murine knock-out studies demonstrated that CC16 provides a protective effect in the lungs against several pathogens, and plays a role in the control of inflammation.²⁰ CC16 levels were down-regulated in patients with allergic rhinitis.²¹ Furthermore, the human secretoglobin gene has been mapped to a region of chromosome 11 previously linked to both atopy and bronchial hyper-responsiveness.²² Uteroglobin, CC16 and ABP may carry ligands such as steroids,^{17,18,23,24} and the expression of both uteroglobin and CC16 in epithelial cells is controlled by steroids.^{18,25–27}

It has been demonstrated that both natural and recombinant uteroglobin are potent inhibitors of extracellular phospholipase A₂ (PLA₂) activity²⁸ and that these anti-inflammatory activities appear to reside in a nonapeptide region (corresponding to residues 39–47) of the third α -helix of uteroglobin.^{29,30} Chowdhury *et al.* thereafter assessed the critical role of residues Lys43 and Asp46 of uteroglobin for its PLA₂-inhibitory activity.³¹ Modulation of PLA₂ may affect the biosynthesis of eicosanoids and platelet-activating factors, potent mediators of inflammatory and allergic responses.³² Uteroglobin only inhibits the function of the enzyme PLA₂ when the concentration of Ca²⁺, an essential

cofactor for PLA₂, becomes limiting.³³ It has also been proposed that uteroglobin may inhibit PLA₂ by sequestering Ca ions.^{33,34} Interestingly a putative calcium-binding site was identified in uteroglobin within a region comprising residues Asp46, Ser47, Leu48 and Pro49.³⁴ However, the potential binding of Ca²⁺ to uteroglobin has been the subject of some controversy, since some groups demonstrated that Ca²⁺ was bound by uteroglobin^{33–35} while others showed the opposite.³¹

Although important information was obtained from the crystal structure of Fel d 1 (2+1), no tetramer could be identified.¹⁴ Here we have solved the crystal structure of the tetrameric form of Fel d 1 at 1.6 Å resolution, using a Fel d 1 (1+2) construct. We demonstrate that the immunological properties of Fel d 1 (1+2) are similar or identical to the previously characterized Fel d 1 (2+1) molecule, as well as to that of the natural Fel d 1. The crystal structure of the Fel d 1 (1+2) tetramer also revealed the presence of a Ca²⁺-binding site within the dimerization interface. Furthermore, the crystal structure of Fel d 1 (1+2) suggests a potential portal for two water-filled cavities of different size, one in each monomer. Two additional Ca²⁺-binding sites are also present on both sides of the tetramer, localized at exactly the same position as suggested for Ca²⁺-binding sites in uteroglobin.³⁴

Results and Discussion

The immunological activity of Fel d 1 (1+2) is equivalent to that of Fel d 1 (2+1) and natural Fel d 1

We have demonstrated that the recombinant molecule Fel d 1 (2+1) displayed similar if not identical structural, immunological and biochemical properties when compared to its natural counterpart.¹³ In order to ascertain the immunological properties of Fel d 1 (1+2) as well as to assess its functional similarity to both the previously characterized Fel d 1 (2+1) and the natural Fel d 1, we made use of a combination of different approaches.

We first analyzed the lymphoproliferative responses of peripheral blood mononuclear cells (PBMC) derived from five cat-allergic patients. Fel d 1 (1+2) was able to stimulate the isolated PBMC to proliferate at all tested concentrations (Figure 1(a)). We thereafter compared the T cell stimulatory effect of Fel d 1 (1+2) and Fel d 1 (2+1) antigens by measuring the proliferation of CD3⁺/CD4⁺ T helper cells in allergen-stimulated whole blood (Table 1). Both proteins were similarly active in whole blood and induced equivalent CD3⁺/CD4⁺ cell proliferative responses. Conversely, CD3⁺/CD4⁺ T cells derived from a healthy control did not proliferate when exposed to both allergens, while still proliferating to a positive control antigen (PPD). It should be noted that the endotoxin contents in both protein preparations were very low (2 EU/mg protein for Fel d 1(2+1) and 4.3 EU/mg protein for Fel d 1 (1+2)),

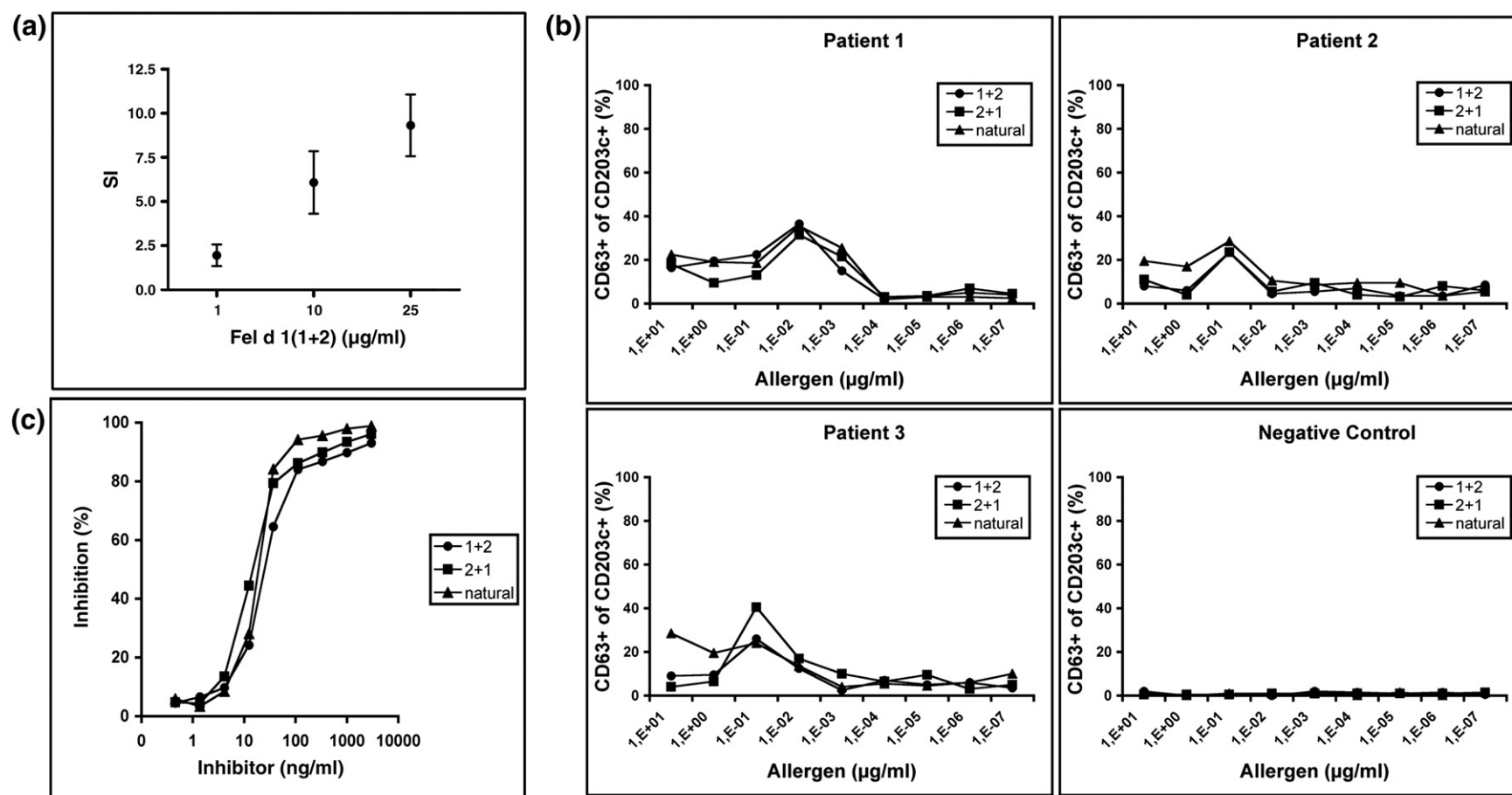


Figure 1. The immunological activity of Fel d 1 (1+2) is equivalent to that of Fel d 1 (2+1) and natural Fel d 1. (a) Fel d 1 (1+2) stimulates lymphoproliferative responses. The Fel d 1 (1+2) protein was able to stimulate lymphoproliferative responses of PBMC isolated from five allergic donors at all tested concentrations. (b) Similar capacity of Fel d 1 (1+2), Fel d 1 (2+1) and natural Fel d 1 to induce basophil degranulation. Natural Fel d 1, Fel d 1 (1+2) and Fel d 1 (2+1) induced a nearly identical dose-dependent degranulation of basophils, measured through the evaluation of CD63/CD203c expression up-regulation in three patients, but not in a non-allergic negative control. (c) Similar dose-dependent inhibition of binding to IgE in a pool of sera from individuals sensitized to cat. Pooled sera from three individuals were pre-incubated with threefold dilutions of natural Fel d 1 (▲), Fel d 1 (1+2) (●), Fel d 1 (2+1) (■), and subsequently added to ELISA plate-bound Fel d 1 (1+2).

Table 1. Both Fel d 1 (1+2) and (2+1) have similar immunological activity, resulting in equivalent proliferative cell responses in cultures with whole blood

	Fel d 1 (1+2) CD3 ⁺ /CD4 ⁺ /ml	Fel d 1 (2+1) CD3 ⁺ /CD4 ⁺ /ml	PPD CD3 ⁺ /CD4 ⁺ /ml	Medium CD3 ⁺ /CD4 ⁺ /ml
Patient 1	33.6 × 10 ³	44.8 × 10 ³	41.4 × 10 ³	16.4 × 10 ³
Patient 2	28 × 10 ³	36.2 × 10 ³	1348.2 × 10 ³	17.2 × 10 ³
Negative control	11 × 10 ³	3.8 × 10 ³	14,951.6 × 10 ³	9.4 × 10 ³

Number of CD3⁺/CD4⁺ cells/ml of whole blood in response to seven days stimulation with Fel d 1 (1+2) (20 µg/ml), Fel d 1 (2+1) (20 µg/ml), PPD (1 µg/ml) and control medium, as measured in the FASCIA proliferation assay.

well below the values in vaccine preparations for human use.⁴⁸

We also compared the ability of the two recombinant Fel d 1 proteins and natural Fel d 1 to induce basophil degranulation through the evaluation of CD63 expression on CD203c⁺ cells in three cat allergic patients. All allergic patients displayed a nearly identical dose-dependent degranulation following the addition of Fel d 1 (1+2), Fel d 1 (2+1) or natural Fel d 1. In contrast, no degranulation was observed in basophils from a healthy control at any Fel d 1 (1+2) or Fel d 1 (2+1) concentration (Figure 1(b)).

Finally, enzyme-linked immunosorbent assay (ELISA) assays were performed in order to identify any differences in IgE-binding properties between natural Fel d 1, Fel d 1 (1+2) and Fel d 1 (2+1). Direct ELISA revealed that the IgE-reactivity to the two recombinant proteins was identical in five subjects tested (four cat allergic and one negative control) (data not included). A competition ELISA was also performed in order to evaluate the ability of natural Fel d 1, Fel d 1 (1+2) and Fel d 1 (2+1) to compete with solid phase bound Fel d 1 (1+2) for binding to IgE. Inhibition through addition of natural Fel d 1, Fel d 1 (1+2), or Fel d 1 (2+1) was dose-dependent and comparable (Figure 1(c)).

Determination of the crystal structure of Fel d 1 (1+2)

The structure was solved by molecular replacement and refined to a crystallographic *R*-factor of 19% (free *R*-factor 22%). The final model consists of two Fel d 1 (1+2) molecules (subunit A, Met0 to Gly145; and subunit B, Met0 to Met144), four Ca ions and 270 water molecules. The electron density map is of high quality with well-defined polypeptide chains. A total of 98.9% of the non-glycine residues have main-chain dihedral angles within the additionally allowed regions of a Ramachandran plot as determined by the program PROCHECK.⁴³ For the residues that fall into the generously allowed region (Lys129 in subunit A and Cys77 in subunits A and B), clear electron density is visible (data not included). The average *B*-factor of the final model is very close to the *B*-factor calculated from the Wilson plot (see Table 2). It should also be noted that the 17 C-terminal residues in chain 2 and the histidine tags are not defined by electron density in both subunits, indicating disorder in these regions. The biological role of this latter section of the

molecule may be of potential interest, since the last 18 C-terminal residues of chain 2 define the major difference in primary structure between the CC16/uteroglobin chain and the Abpb (androgen binding protein chain b)/Fel d 1 chain 2.¹⁷ The C-terminal residues of Abpb/Fel d 1 chain 2 have been predicted to form a helix,^{14,17} but it has also been demonstrated that chain 2 in Fel d 1 is naturally truncated from position 85, removing the last eight residues.⁶

Overall structure of the Fel d 1 tetramer

In contrast to the previously solved crystal structure of Fel d 1 (2+1), which did not show any evidence for tetramerization,¹⁴ we present here the

Table 2. Statistics for data-collection, phasing and refinement

X-ray source	I711, MAX-lab
Wavelength (Å)	1.087
Resolution (Å)	37.8–1.6
Space group	P1
Unit cell parameters:	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	38.5 42.9 49.0
α, β, γ (°)	70.7 80.5 81.5
Total no. of unique reflections	33, 386
Number of observed reflections	135, 810
Completeness ^a (%)	92.1 (87.5)
<i>R</i> _{sym}	0.06 (0.26)
<i>I</i> / <i>σ</i> ^a	17.4 (4.2)
<i>B</i> -factor from Wilson plot (Å ²)	21
<i>R</i> _{cryst} (%)	19.9
<i>R</i> _{free} (%)	22.2
Number of protein atoms	2274
Number of water molecules	270
rmsd bond lengths (Å)	0.01
rmsd bond angles (°)	1.36
<i>B</i> -factors (Å ²)	
Overall	12.0
Protein atoms	10.1
Ca ²⁺ atoms	25.6
Solvent	27.4
Ramachandran plot, percentage of non-glycine	
Residues in favorable regions	94.0
Additionally allowed regions	4.5
Generously allowed regions	1.5

$R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.
 $R_{\text{cryst}} = \sum_{hkl} ||F_o| - |F_c|| / \sum_{hkl} F_o$, 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.

^a Values in parentheses are for the highest resolution shell of each data set.

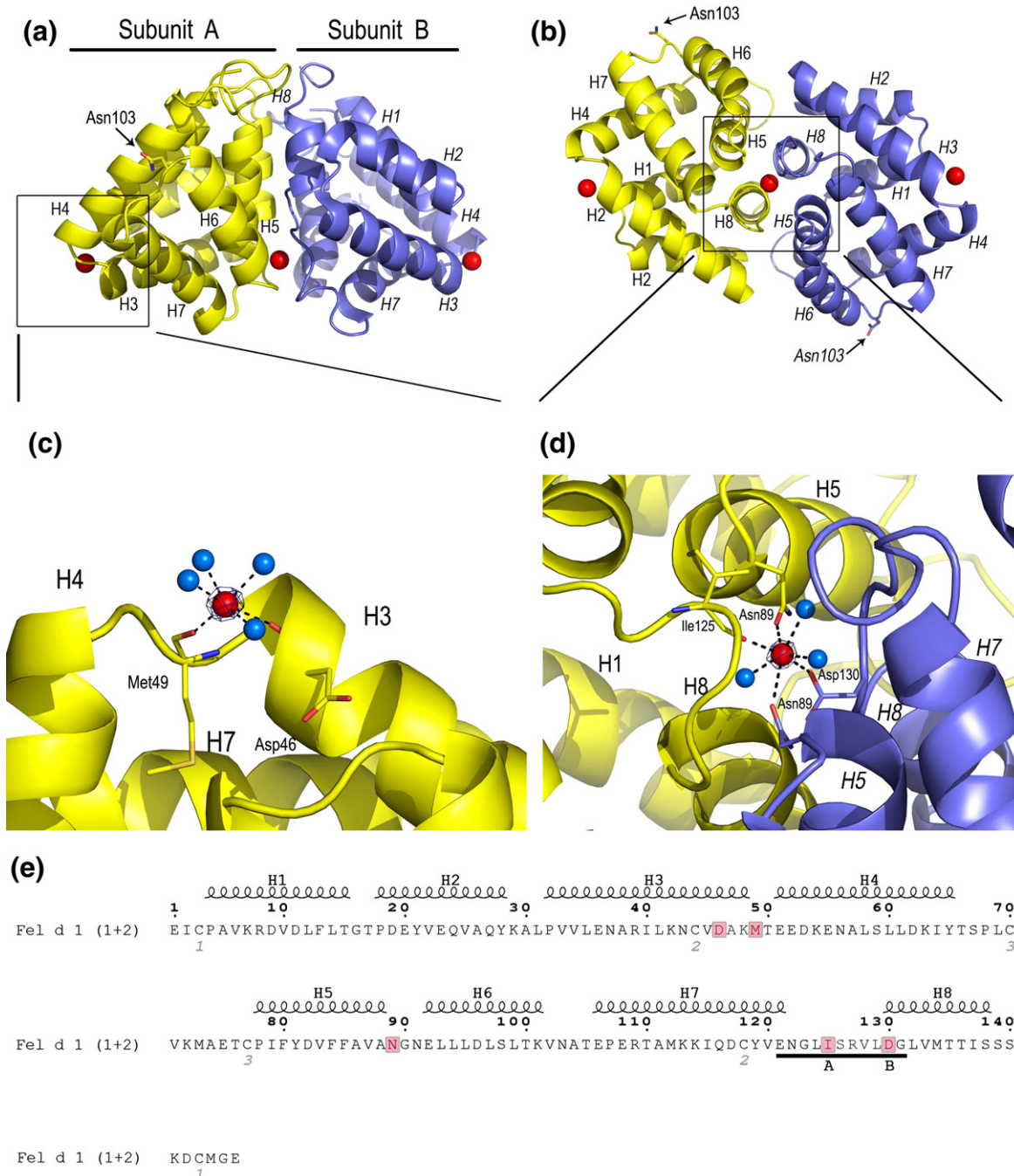


Figure 2. Overall structure of the Fel d 1 (1+2) tetramer. (a) Schematic view of the Fel d 1 (1+2) tetramer. The two heterodimeric subunits A and B, each composed of linked chains 1 and 2, that form the tetramer are in yellow and blue, respectively. The three Ca ions are indicated as red balls. Ca²⁺-binding site 1 is localized within the dimerization interface while the two other equivalent Ca²⁺-binding sites 2 and 3 are symmetrically localized on each side of the dimer. The position of residue Asn103, a potential glycosylation site, is also indicated. The α -helix chains are annotated. A square indicates the region that is enlarged and displayed in (c). (b) Schematic view of the Fel d 1 tetramer following a rotation of about 90° around the horizontal axis. All the α -helix chains composing both subunits, including H5 and H8 that form the dimerization interface, are labeled. A square indicates the region that is enlarged and displayed in (d). (c) Calcium-binding sites 2 and 3. The Ca ions bind to the carbonyl groups of residues Asp46 and Met49 as well as to four and three water molecules in subunits A and B, respectively. The Ca²⁺ that binds to subunit A is presented in the Figure. (d) Calcium-binding site 1. The Ca²⁺ is localized centrally within the dimerization interface, and binding to OD1 atoms from residues Asn89 (in subunit A), Asn89 (B) and Asp130 (B) as well as to the carbonyl group of residue Ile125 (A) and three water molecules. (e) Secondary structure of a Fel d 1 (1+2) subunit. The secondary structure of a Fel d 1 (1+2) subunit is displayed above the sequence. The eight α -helical segments in each subunit of Fel d 1 (1+2) are indicated graphically. The cysteine residues that form disulfide bridges within each subunit are numbered below the sequence and indicated in italic and grey (for each of the three disulfide bridges). The residues that are involved in binding to Ca ions are indicated in pink. Finally the stretch of residues (121–131) which conformation differs between subunits A and B in Fel d 1 (1+2) is underlined. It should be noted that the conformation of the stretch of residues (121–131) in Fel d 1 (1+2) also differs with the corresponding stretch of residues (51–61) in Fel d 1 (2+1).

crystal structure of a tetrameric form of Fel d 1 (1+2) at a resolution of 1.6 Å (Figure 2(a) and (b)). The Fel d 1 tetramer displays a globular all helical fold, different from previously structurally characterized allergens.⁴⁹ Each subunit of the Fel d 1 (1+2) tetramer consists of eight helices named H1–H4 (corresponding to chain 1) and H5–H8 (corresponding to chain 2) (Figure 2(b) and (e)). The disulfide bridges present in the crystal structure of the Fel d 1 (1+2) tetramer correspond to those found in both the Fel d 1 (2+1) dimer¹⁴ and in native Fel d 1.^{6,13}

A total of four Ca ions are present in the crystal structure of the Fel d 1 (1+2) tetramer. One Ca²⁺ (site 1) is bound within the dimerization interface, resulting in profound local conformational changes (Figure 2(d)). Two Ca ions are bound in symmetrically positioned sites, one on each of the heterodimeric subunits A and B (sites 2 and 3, respectively; Figure 2(c)). Interestingly, these two latter sites correspond to the previously suggested putative Ca²⁺-binding site described for uteroglobin.³⁴ Finally, we determined a fourth Ca²⁺ site, but the position of this ion is most probably due to the packing of the molecules in the asymmetric unit (data not included).

Native Fel d 1 molecules are naturally glycosylated with a tri-antennary carbohydrate complex linked to Asn103 localized in the loop between helices H6 and H7.^{7,50} In the present crystal structure the side-chain of Asn103 is exposed to the solvent and is not localized in the proximity of either the dimerization interface of Fel d 1 (1+2), or the Ca²⁺-binding sites 2 and 3 (Figure 2(b)).

The two subunits A and B that form the final tetramer in Fel d 1 (1+2) are similar in structure, with a root-mean-square deviation (rmsd) of 0.5 Å for all C^α atoms except for a region corresponding to residues Glu121–Gly131 (Figure 3(a) and (b)). Here the C^α backbones of subunits A and B display large differences with a rmsd of 3.4 Å. In particular, the side-chains of residues Val128, Leu129 and Asp130 take very different conformations in the two subunits A and B (Figure 3(c)). Thus, this region folds in different ways in the two subunits that compose Fel d 1 (1+2) and breaks the otherwise nearly perfect symmetry of the tetramer.

Furthermore, the superimposition of each of the subunits (A and B) composing the Fel d 1 (1+2) tetramer with the molecule determined in the crystal structure of the Fel d 1 (2+1) dimer¹⁴ demonstrates their three-dimensional similarity, with an overall rmsd of 0.4 Å for the C^α backbones, except for the stretch of residues Glu121–Gly131 (corresponding to residues Glu51–Gly61 in Fel d 1 (2+1)) (Figure 3(d)). While residues Glu51–Gly61 form a loop followed by helix H4 at residue 54 in Fel d 1 (2+1), the corresponding loop extends to residues Leu129 and Gly131 in subunits A and B in Fel d 1 (1+2) (corresponding to residues 59 and 61 in Fel d 1 (2+1)), respectively (Figure 3(e)). One may speculate that binding of the Ca²⁺ caused the shortening of the helix H8 in Fel d 1 (1+2) corresponding to helix H3 in Fel d 1 (2+1).

The binding of a Ca²⁺ in the dimerization interface results in a profound local conformational change

The dimerization interface in the crystal structure of Fel d 1 (1+2), mainly composed of helices H5 and H8 from chain 2 in each subunit (Figure 2(a) and (b)), buries 1225 Å² of solvent-exposed molecular surface. This area represents 17% of the total accessible surface (7100 Å²), as calculated using the program Protein–Protein Interaction Server v1.5† and is in agreement with the mean ΔASA (1,685 Å²) for homodimers.^{51,52} The Fel d 1 (1+2) dimerization interface is composed of 65 residues, 31 and 34 from subunits A and B, respectively. Additionally, 46 water molecules and one Ca²⁺ are present between the two subunits. Six residues (Phe85, Gly131 and Leu132 from subunits A and B) form a hydrophobic inner core. Similarly, as a consequence of the symmetrical fold of the Fel d 1 (1+2) tetramer, equivalent residues in subunits A and B contribute with hydrogen donor and acceptor atoms, resulting in symmetrical hydrogen bond interactions between, e.g. the side-chains of residues Lys29–Asp82, Glu75–Lys141 and Thr135–Thr135 (Figure 4).

A well-defined Ca²⁺ is localized between the two subunits A and B, coordinated by seven oxygen ligands, comprising three OD1 atoms from residues Asn89 (subunit A), Asn89 (B) and Asp130 (B), a carbonyl group from residue Ile125 (A) and three water molecules (Figure 4). All side-chains in the contact region are well defined in the electron density map. The binding of the Ca²⁺ results in profound a conformational change that break the symmetry of the dimerisation interface, and directly governs the shape of the cavities in subunits A and B. In particular, the side-chain of Asp130 takes two very different conformations (Figure 3(c)). While Asp130 (B) binds to the Ca²⁺, the side-chain of Asp130 (A) projects towards the cavity in subunit A, making hydrogen bond interactions with the side-chain of Tyr119 and three water molecules. The main reason underlying the two diametrically opposed conformations observed for the side-chains of Asp130 (and as a consequence the side-chains of e.g. residue Leu129) is that otherwise the two Asp130 residues would sterically clash and repulse each other as their side-chains would take similar conformations. In conclusion, binding of the Ca²⁺ in this region of the Fel d 1 interface most probably stabilizes the tetrameric form, since the presence of such a positively charged ion allows for the packing of acidic residues (Asp130 in subunits A and B) in the Fel d 1 tetramer interface. However, it also results in profound local conformational changes that affect the size and form of the different cavities.

† <http://www.biochem.ucl.ac.uk/bsm/PP/server/>

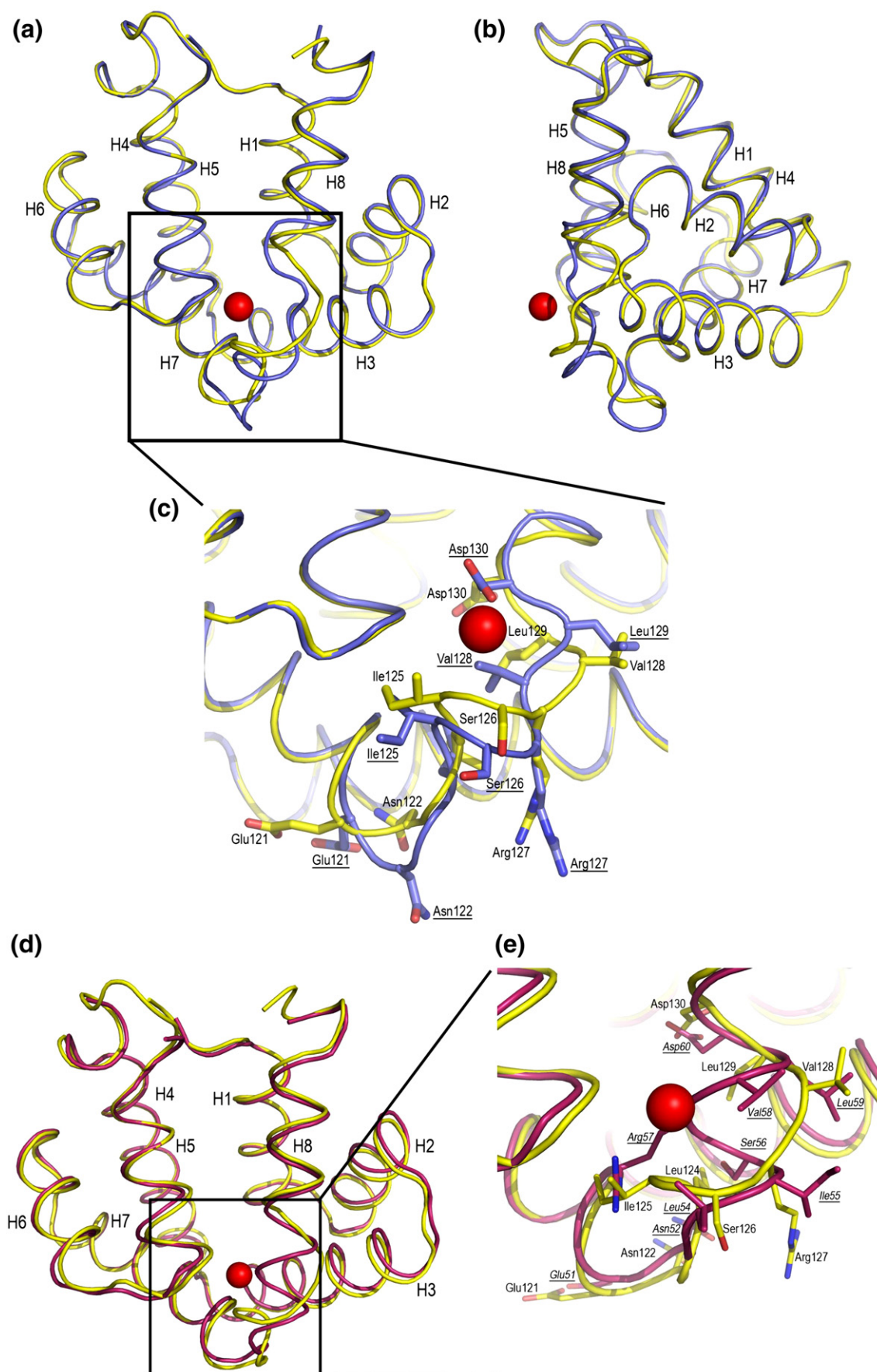


Figure 3 (legend on opposite page)

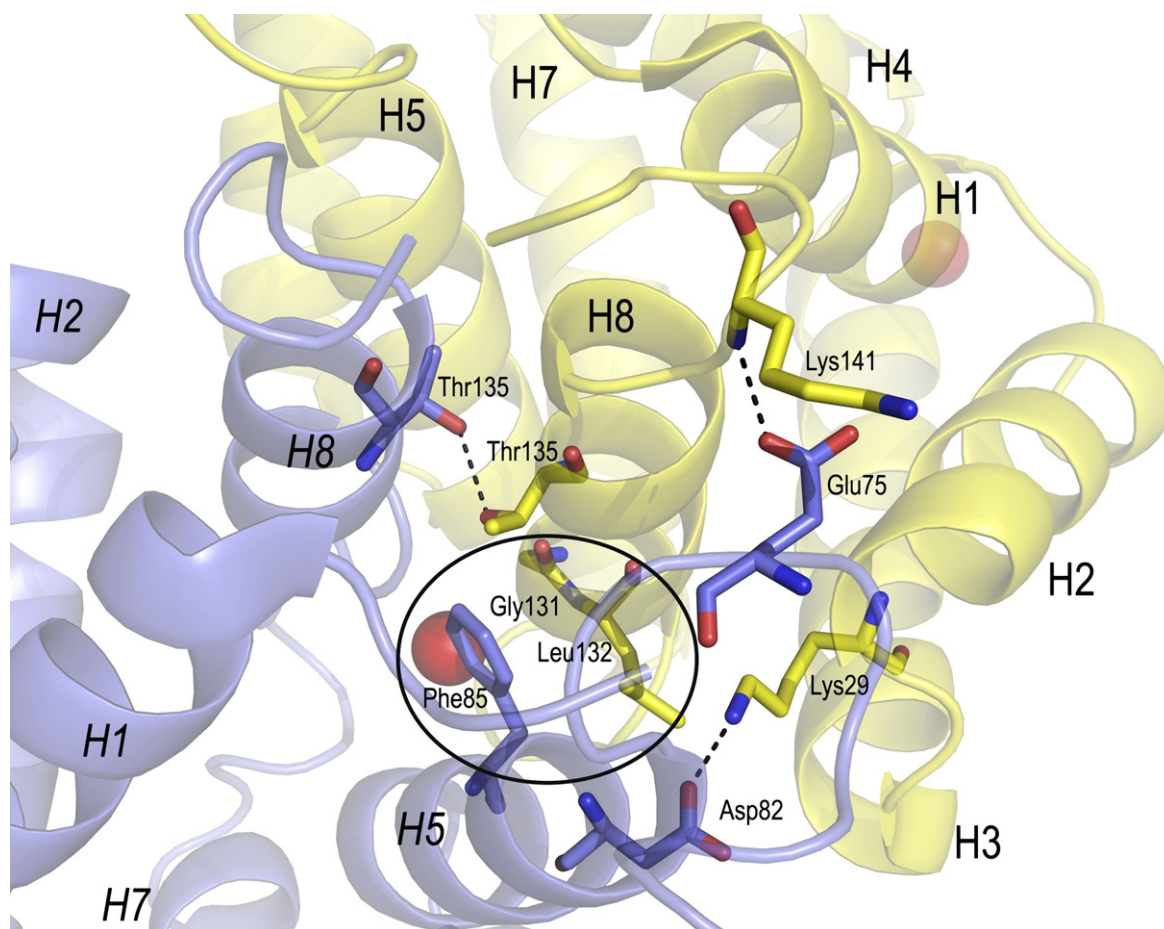


Figure 4. The tetramer interface is composed by a central hydrophobic core surrounded by charged residues that form hydrogen bond interactions. It is important to note that due to the 2-fold symmetry at the interface of the Fel d 1 (1+2), each subunit contributes to the interface with an identical residue. For simplicity, we have chosen to depict only half of the residues that form the interface in the present Figure. The central region of the interface is formed by a hydrophobic core, highlighted by a circular ring, and composed mainly of six residues Phe85, Gly131 and Leu132 provided by both subunits A (in yellow) and B (in blue). Hydrogen bond interactions are formed between the side-chains of charged residues that surround the central hydrophobic region. The Ca ions both at the interface and in the external site are depicted as red balls.

Two cavities of different size and form are present in the Fel d 1 tetramer

The Fel d 1 orthologues CC16, uteroglobin and ABP bind various ligands such as progesterone,

PCB-derivatives and androgen.^{17,23,53–55} The physiological role of these ligands has still to be determined. Interestingly, the previously reported crystal structure of Fel d 1 (2+1) revealed that the recombinant allergen bound an unknown ligand,

Figure 3. Subunits A and B are very similar throughout except for the dimerization-forming regions that are involved in binding to Ca^{2+} . The centrally localized Ca^{2+} is indicated as a red ball in all the five Figures. All the eighth helices in each subunit are indicated. (a) Superposition of Fel d 1 (1+2) subunit A on Fel d 1 (1+2) subunit B. The two subunits A and B (yellow and blue, respectively) that form the final tetramer are similar in structure, with a rmsd value of 0.5 Å for all C^α atoms, except for a region corresponding to residues Glu121–Gly131. Here the C^α backbones display large differences with a rmsd value of 3.4 Å. (b) View of the superposition of Fel d 1 (1+2) subunit A on Fel d 1 (1+2) subunit B following a rotation of about 90° around the horizontal axis. The position of the Ca^{2+} , localized within the dimerization interface, and mainly responsible for the observed differences, is indicated as a red ball. (c) The region corresponding to residues 121–131 is very different in subunits A and B in the Fel d 1 (1+2) tetramer. A majority of the side-chains of the residues 121–131 take different conformations in subunits A and B, due to the binding of a Ca^{2+} . In particular, the side-chains of the acidic residues Asp130 in subunits A and B take diametrically opposed conformations. While Asp130 in subunit B (underlined) binds to the Ca^{2+} , the side-chain of Asp130 in subunit A projects towards the cavity. (d) Superposition of Fel d 1 (1+2) subunit A on Fel d 1 (2+1). Superposition of subunit A from the crystal structure of Fel d 1 (1+2) (in yellow) on the previously determined crystal structure of Fel d 1 (2+1) (in red) reveals their structural similarity, except for a region corresponding to residues Glu121–Gly131 and residues Glu51–Gly61, in Fel d 1 (1+2) and Fel d 1 (2+1), respectively. (e) The region corresponding to residues 121–131 is very different in subunits A and B in the Fel d 1 (1+2) tetramer. Helix H8 in Fel d 1 (1+2) (in yellow) is shortened when compared to the corresponding helix H3 in Fel d 1 (2+1) (in red).

most probably derived from *Escherichia coli*.¹⁴ The Fel d 1 (1+2) crystal structure presented here does not contain any bound ligand. The size of the cavity found within the previously solved crystal structure of Fel d 1 (2+1) was 480 Å³. The sizes of the cavities in the Fel d 1 (1+2) subunits A and B are of 350 Å³ and 730 Å³, respectively, as calculated using the program SwissPDBViewer.⁴⁴ The difference in size between the two cavities present in Fel d 1 (1+2) is a direct result of the conformational change within the region corresponding to residues 121–131 (Figure 5). In subunit B, this stretch of residues is displaced out towards the dimerization interface, thereby creating a larger cavity (twice as big) when compared to subunit A. In particular, the side-chains of residues Leu129 and Asp130 point in opposite directions in subunits A and B, with a shift in C^α positions of

3.6 Å and 3.7 Å, respectively. Finally it should be noted that although of different size and shape, the two cavities within Fel d 1 (1+2) display a similar asymmetry (with one hydrophobic end and the other charged and polar) while, in contrast, the cavity in homodimeric uteroglobin is symmetrical, mainly composed of hydrophobic residues (data not included).⁵⁶ The cavities within Fel d 1 (1+2) subunits A and B contain three and seven water molecules, respectively. All of these water molecules are localized within the vicinity of the polar and charged residues at one end of the cavities.

The cavities observed in the crystal structure of the Fel d 1 tetramer permit localized large conformational changes that would not alter the overall fold of the tetrameric molecule. Molecular modeling suggested that the uteroglobin ligand progesterone may

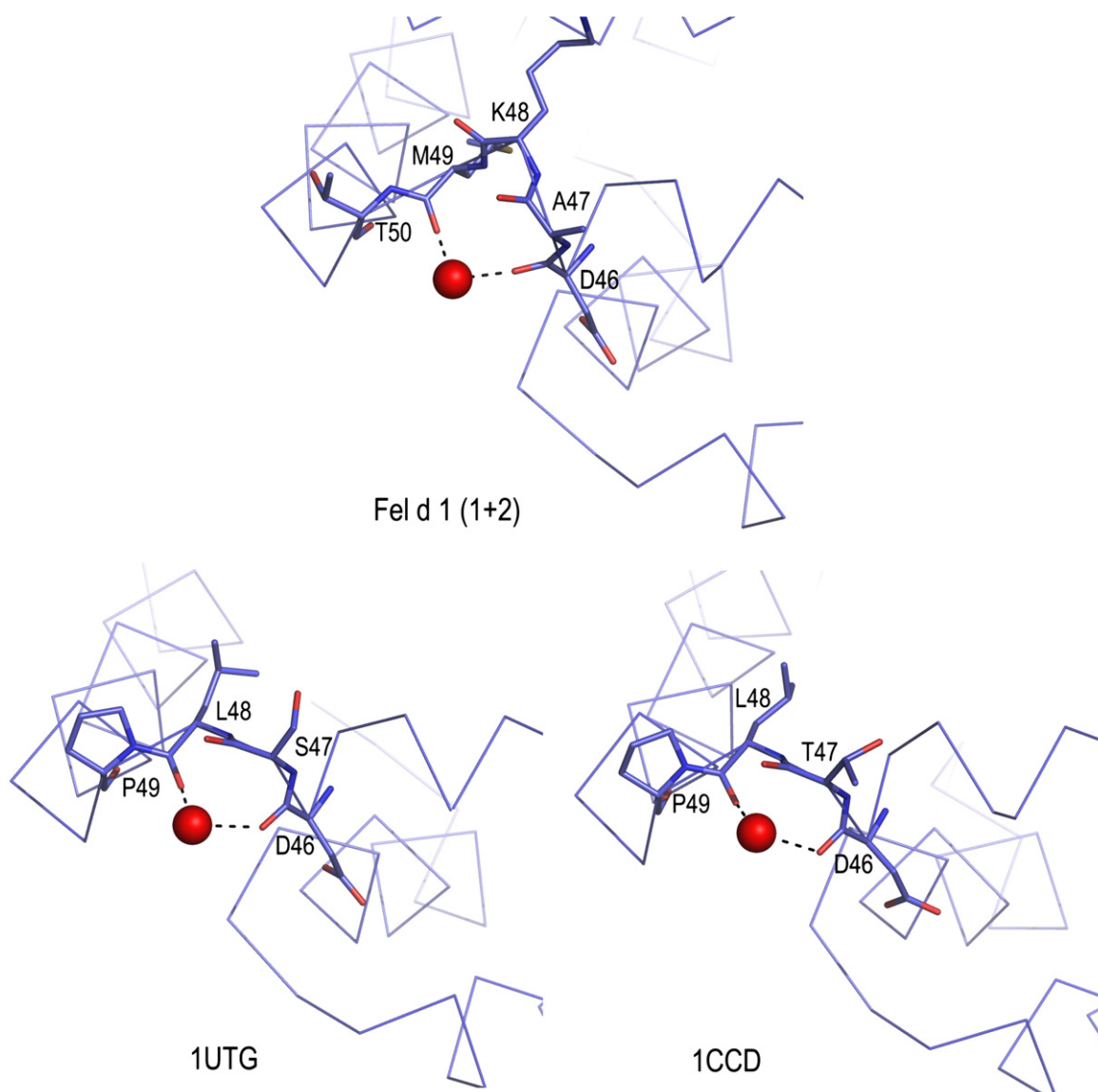


Figure 5. The Ca²⁺-binding site localized on each side of the Fel d 1 tetramer is similar to the putative Ca²⁺-binding site in uteroglobins. The Ca²⁺ (Red ball) binds to the carbonyl groups of residues Asp46 and Met49 in Fel d 1 (1+2). Four water molecules that also bind to the Ca²⁺ (as shown in Figure 2(c)) are omitted in the present Figure. The other pictures provide a view of the putative Ca²⁺-binding site previously identified in uteroglobin (PDB codes 1UTG and 1CCD). A Ca²⁺ was modeled in each putative site.

fit within both cavities of the crystal structure of the Fel d 1 (1+2) tetramer, and that such binding would not be affected by the conformation of residue Asp130 (data not included). This is in agreement with previous predictions by Pattabiraman *et al.* who modeled progesterone in the cavity of uteroglobin.⁵⁷ Interestingly, Fel d 1 tyrosine residue Y21, that forms hydrogen bond interactions with a hydroxyl group in the modeled progesterone, corresponds to the tyrosine residue suggested to be critical for binding of these ligands in uteroglobin.⁵⁷ Thus, one may speculate that besides potentially binding different ligands, the role of the cavities present in the structure of the major cat allergen is critical for the proper tetramerization of Fel d 1 (as a dimer of two heterodimeric subunits A and B), allowing for large local conformational changes of some specific residues while keeping the overall fold of the allergen Fel d 1.

Two symmetrically positioned calcium sites on the surface of the Fel d 1 tetramer correspond to the previously described putative Ca^{2+} binding site in uteroglobin

In addition to the Ca^{2+} binding site localized as described above within the dimerization interface, two additional well-defined Ca^{2+} -binding sites were also identified in the crystal structure of Fel d 1 (1+2), symmetrically localized on each side of the tetramer. In each site the Ca^{2+} binds to the carbonyl groups of residues Asp46 and Met49, as well as to four water molecules (Figures 2(c) and 6). All side-chains in the contact region are well defined in the electron density map. These two Ca^{2+} -binding sites correspond to the putative Ca^{2+} -binding site previously identified by Barnes *et al.* using a hydrophobic contrast function.³⁴ Comparison of the Ca^{2+} -binding site in Fel d 1 with the corresponding sites in the crystal structures of uteroglobin (PDB codes 1UTG and 2UTG)^{56,58} as well as rat Clara Cell 17 (PDB code 1CCD)⁵⁹ suggests that these proteins bind Ca^{2+} in a very similar way (Figure 6). Interestingly Chowdhury *et al.* demonstrated that residues Lys43 and Asp46 in the α -helix 3 of uteroglobin are essential for its PLA_2 -inhibitory activity.^{29,31} The independent or simultaneous mutation of Lys43 and Asp46 to a glutamate and a lysine, respectively, resulted in the alteration of the capacity of uteroglobin to inhibit PLA_2 .³¹ Molecular modeling of the substitution of Asp46 to a lysine in the crystal structure of Fel d 1 suggested that this residue confers the right conformation to the calcium-binding site (data not included). Residue Asp46 contacts the side-chains of two adjacent lysine residues (Lys42 and Lys54), stabilizing this region. Substitution of Asp46 to a lysine residue would disrupt the native conformation due to electrostatic and steric repulsion. Similarly the mutation of Asp46 to a lysine in uteroglobin would most probably also result in steric clashes in addition to electrostatic repulsions with the side-chains of residues Lys42, Lys43 and Arg53 (data not included). Thus, despite the fact that Fel d 1 and

uteroglobin share an overall sequence identity of only 20%, the crystal structures of Fel d 1 presented here and previously published¹⁴ reveal a striking similarity to the three-dimensional structure of uteroglobin. Furthermore, the present crystal structure presented here demonstrates the presence of a Ca^{2+} -binding site in each subunit of the Fel d 1 tetramer, equivalent to the putative Ca^{2+} -binding site in uteroglobin, suggesting a potential mimicry of uteroglobin by the major cat allergen Fel d 1.

Concluding remarks

The crystal structure of the Fel d 1 tetramer presented here provides novel information regarding the overall structure of this important allergen. The structure suggests that Ca^{2+} plays a key role in the formation of the tetrameric form of Fel d 1 (site 1). Considering the striking overall similarity of Fel d 1 to uteroglobin (despite very limited sequence identity), combined with the identification in the present structure of two additional Ca^{2+} -binding sites (sites 2 and 3) that correspond to the previously described putative Ca^{2+} -binding site in uteroglobin, we may speculate that: (1) the major cat allergen Fel d 1 may be able to sequester Ca ions from PLA_2 in a similar manner to uteroglobin, potentially resulting in the induction of platelet-activating factors and the activation of an allergic response; (2) that the cavities observed in the crystal structures of Fel d 1 may play a role in allowing for the conformational rearrangement of side-chains present within the dimerization interface as an alternative to binding of a ligand; and (3) that the precise knowledge of the Fel d 1 structure will open for new possibilities to engineer improved cat allergens for immunotherapy.

Materials and Methods

Crystallization and data collection

Protein expression, purification and crystallization of the recombinant Fel d 1 (1+2) construct have been described.¹⁵ Briefly, Fel d 1 (1+2) was expressed in *E. coli* as a construct where chain 2 is directly linked to the C-terminal of chain 1 using PCR. A C-terminal six histidine tag was added to the Fel d 1 (1+2) construct for purification purposes. The protein was purified by metal affinity, size exclusion and ion-exchange chromatography and concentrated to 5 mg/ml. Natural Fel d 1 was purified as described.³⁶ Rod-shaped crystals appeared after four weeks in 22.5% (w/v) PEG3350 and 0.5 M CaCl_2 at 20 °C using the hanging drop vapor diffusion technique. The crystals belonged to space group *P*1 and diffracted to 1.6 Å resolution.³⁷ A total of 253 images were collected using beam-line I711 at MAX-lab (Lund, Sweden) with 1.5° oscillations per image. The space group and unit cell parameters were determined using the auto-indexing option of MOSFLM,³⁸ and scaling and reduction of the data were performed using SCALA from the CCP4 suite of programs³⁹ (Table 1). The unit cell contained two molecules of Fel d 1 with a molecular mass of 19,177 Da, giving a solvent content of 37%.

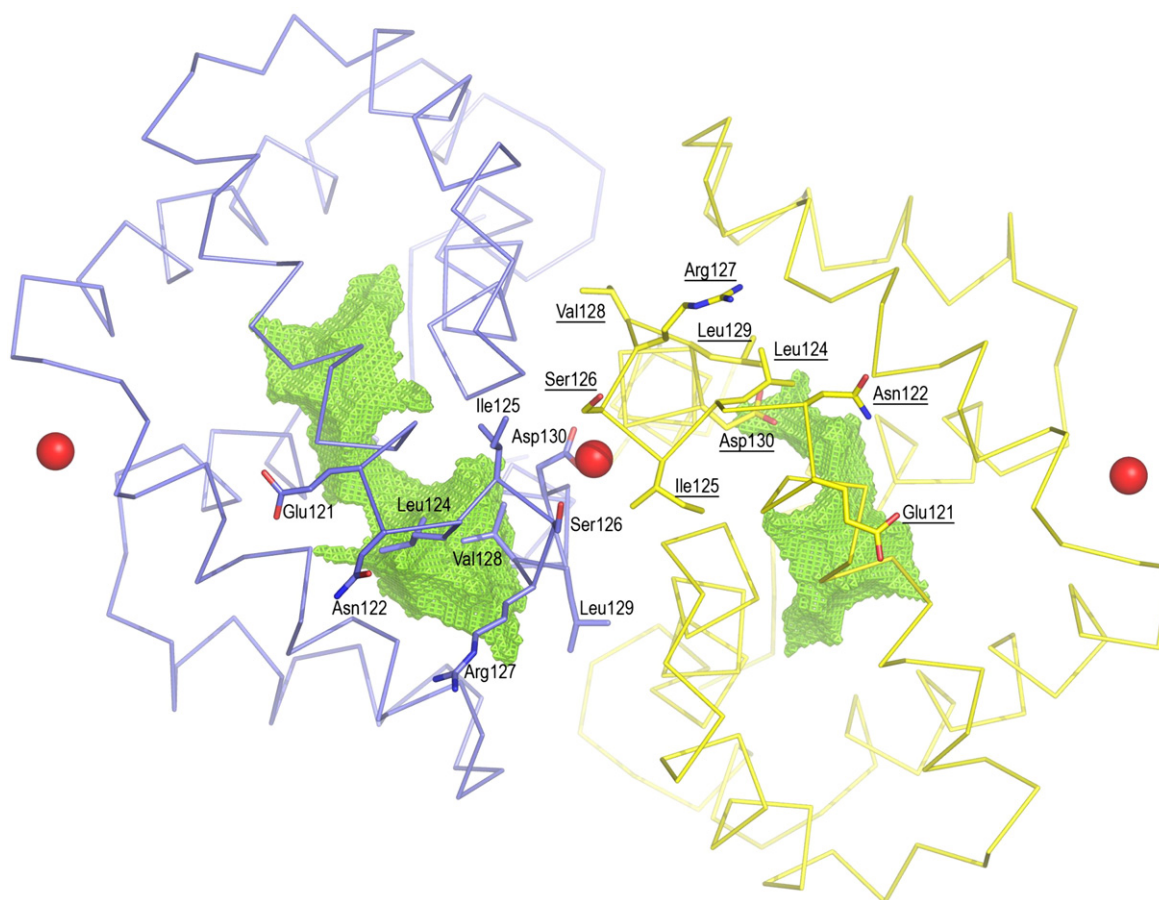


Figure 6. The shape of the two cavities is different, directly governed by the conformation of centrally localized side-chains. The shape of the two cavities (in lime-green) is directly governed by the conformation of residues Leu129 and Asp130. The Ca^{2+} plays a key role in the conformation of the side-chains of these residues. The side-chain of Asp130 (underlined) does not interact with the Ca^{2+} and projects in the cavity in subunit A (in yellow), while the side-chain of Asp130 (subunit B) binds to the Ca^{2+} . The two external Ca ions are also indicated.

Structure determination and refinement

The structure was determined by molecular replacement using the program MolRep.⁴⁰ The atomic coordinates of the Fel d 1 (2+1) (PDB code 1PUO)¹⁴ were used as the search model. Two solutions were unambiguously identified. Further crystallographic refinement was conducted using the program REFMAC from the CCP4 package.³⁹ Five percent of the reflections were set aside for validation. The structure was first subjected to rigid body refinement. After each round of refinement, errors in the model were identified by careful examination of the $(2F_o - F_c)$ and $(F_o - F_c)$ maps with the program O.⁴¹ Isotropic, individual *B*-factor refinement was used throughout the procedure. Refinement cycles of TLS parameters were also performed using REFMAC5.⁴² The final model consists of 291 residues. A total of 270 water molecules were added to well-defined peaks (2.0σ and greater in $F_o - F_c$ maps) found between 2 Å and 4 Å from O or N atoms in the protein, using the program ARP in REFMAC. Within the set of water molecules, those having very low *B* factors and residual density were examined as possible Ca ions, and if there were appropriate interactions with amino acid residues, they were refined as Ca ions. The stereochemistry of the model was validated using the program PROCHECK.⁴³ Structural comparisons were carried out with Swiss PDBViewer using default parameters.⁴⁴ The

final refinement parameters are presented in Table 1. The Figures were created using the program PyMOL§.

Protein Data Bank accession code

The atomic coordinates and structure factors (PDB code 2EJN) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ, USA.

Lymphoproliferation assay in PBMC cultures

Peripheral blood mononuclear cells (PBMCs) were isolated from five cat-allergic patients, all with allergen-specific IgE to cat (Pharmacia CAP System, Phadia AB, Uppsala, Sweden) by Ficoll-Paque (GE Healthcare Biosciences, Uppsala, Sweden) density gradient centrifugation. PBMCs (2×10^5) were cultured in triplicate in 96 well microtiter plates (Nunc, Roskilde, Denmark) in 200 µl of complete RPMI medium (i.e. RPMI 1640 supplemented with 10% (v/v) bovine growth serum (Hyclone, Utah, USA), 2 mM glutamine (Sigma), 50 µM β-mercaptoethanol (Sigma), 25 µg/ml of gentamicin, 100 IU/ml of penicillin

§ <http://pymol.sourceforge.net/>

and 100 µg/ml of streptomycin (Gibco, Invitrogen Corporation, UK) at 37 °C and 5% CO₂ in a humidified atmosphere. Cells were stimulated with different concentrations of Fel d 1 (1+2) (1, 10 and 25 µg/ml) or left unstimulated with medium alone in triplicates. After six days, proliferative responses were measured by [³H] thymidine incorporation and expressed as stimulation indices, as described.⁴⁵

Flow-cytometry assisted cell proliferation assay (FASCIA) in whole blood

To compare biological activities of Fel d 1 (1+2) and (2+1) we applied a method that was described for cell-mediated immune responses after stimulation with viral antigens (flow-cytometric assay of specific cell-mediated immune response in activated whole blood; FASCIA).⁴⁶ Briefly, whole blood collected in heparinized tubes from two cat allergic patients (ImmunoCAP-positive to cat) and one healthy control (ImmunoCAP-negative for serum-IgE to cat) was diluted eight times in complete RPMI medium. The diluted blood was stimulated in FACS tubes for seven days with 20 µg/ml of Fel d 1 (1+2), Fel d 1 (2+1) or incubated in medium only. One µg/ml of tuberculin purified protein derivate (PPD) was used as a positive control (Statens Serum Institut, Copenhagen, Denmark). After incubation the cells were washed and stained with anti-CD3-FITC and anti-CD4-PerCP (BD Pharmingen, San José, CA, USA) at room temperature for 20 min. Erythrocytes were lysed twice with 1× Pharm lyse (BD Pharmingen), washed in phosphate-buffered saline (PBS) and fixed with 450 µl of Cellfix (BD Biosciences, San José, CA, USA). Cells were analyzed by flow cytometry on a FACSCalibur instrument (BD Biosciences) using the Cellquest software. As a measure of stimulation, the number of proliferating CD3⁺/CD4⁺ cells is expressed as the number of proliferating cells/ml of blood.

Endotoxin test

Endotoxin activity in the Fel d 1 (1+2) and (2+1) preparations was determined as endotoxin units (EU)/ml based on the limulus amebocyte lysate endochrome assay (Charles River Endosafe), according to the manufacturer's instructions.

Basophil activation assay

The activation of basophils was measured by analysis of CD63 on CD203 positive cells as described.⁴⁷ Briefly, heparinized blood samples were taken from three cat-allergic patients (ImmunoCAP-positive for serum-IgE to cat) and the aliquots (100 µl) incubated with dilutions of natural Fel d 1, Fel d 1 (1+2) and Fel d 1 (2+1) antigens, anti-IgE antibody (1 µg/ml), or PBS for 15 min (37 °C). After incubation, cells were washed in PBS/EDTA and then incubated with 10 µl of phycoerythrin-labeled CD203c monoclonal antibody 97A6 (Immunotech, Marseille, France) for 15 min at room temperature. Thereafter samples were subjected to erythrocyte lysis with 2 ml of lysing solution. Cells were then washed, resuspended in PBS, and analyzed by two-color flow cytometry using a FACSCalibur instrument (BD Biosciences). The basophil marker CD203c was used to set a basophil gate including only CD203c⁺ cells. The cells were further analyzed for granule release using an anti-CD63 antibody. The magnitude of basophil activation was calculated as the percentage of CD63⁺ cells among the gated basophils (CD63⁺/CD203c⁺).

Direct ELISA

Serum specimens from four individuals were selected on the basis of positive IgE responses to cat dander (ImmunoCAP-positive to cat). For control purposes serum from one non-cat-allergic control was used. The serum samples were analyzed in duplicate by ELISA for IgE antibody binding to recombinant Fel d 1 (1+2) or Fel d 1(2+1). The assay was performed as a sequential, solid phase adsorption of allergens, serum sample, primary antibody, antibody conjugate and finally substrate, and included rinsing four times with 250 µl of PBS containing 0.05% (v/v) Tween 20 (PBS-T) between incubations. Unless stated otherwise all steps were performed at room temperature. Microtiter plates (96 wells) were coated with 100 µl of Fel d 1(1+2) and Fel d 1 (2+1) to final concentrations of 5 µg/ml in 0.1 M carbonate buffer (pH 9.6). After overnight adsorption at 4 °C, the plates were emptied, and the remaining protein binding sites were blocked with 200 µl of PBS-T containing 1% (w/v) bovine serum albumin for 2.5 h at room temperature. Each serum sample (100 µl) was diluted 1:1 in PBS (duplicates) and incubated for 2 h, after which 100 µl of rabbit anti-human IgE (Miab, Uppsala, Sweden; diluted 1:2000 (v/v)) was added, and incubation was continued for 2 h. Finally, 100 µl of goat anti-rabbit antibody (Dako, Denmark; diluted 1:2000 (v/v)) conjugated to alkaline phosphatase was added, and incubation continued for 1 h. Alkaline phosphatase substrate tablets (Sigma 104® Diagnostics) were used for color development, and the color reaction monitored at 405 nm was registered using an automated ELISA reader (Multiskan RC, Labsystems, Helsinki, Finland).

Competition ELISA

A competition assay of serum IgE was performed using pooled sera from three individuals sensitized to cat (ImmunoCAP-positive to cat) and the serum from the non-allergic control. Microtiter plates (96 wells) were coated with 100 µl of 5 µg/ml Fel d 1 (1+2). Three-fold serial dilutions in PBS-T of Fel d 1(1+2), Fel d 1 (2+1) and natural Fel d 1, were incubated at a 1:1 volume ratio with the serum pool diluted 1:2 (v/v) in PBS for 2 h at room temperature and thereafter added to the wells. The dilutions ranged from 3000 ng/ml to 0.46 ng/ml and the incubation was performed over night at 4 °C. The absorbance value for the serum pool without inhibitor protein and that for the negative control serum were regarded as no inhibition and complete inhibition, respectively. The subsequent steps were as described for the direct ELISA.

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References

- Freidhoff, L. R., Meyers, D. A. & Marsh, D. G. (1984). A genetic-epidemiologic study of human immune responsiveness to allergens in an industrial population. II. The associations among skin sensitivity, total serum IgE, age, sex, and the reporting of allergies in a stratified random sample. *J. Allergy Clin. Immunol.* **73**, 490–499.
- Johansson, S. G., Nopp, A., Florvaag, E., Lundahl, J., Soderstrom, T., Guttormsen, A. B. *et al.* (2005). High prevalence of IgE antibodies among blood donors in Sweden and Norway. *Allergy*, **60**, 1312–1315.
- Roost, H. P., Kunzli, N., Schindler, C., Jarvis, D., Chinn, S., Perruchoud, A. P. *et al.* (1999). Role of current and childhood exposure to cat and atopic sensitization. European Community Respiratory Health Survey. *J. Allergy Clin. Immunol.* **104**, 941–947.
- Ohman, J. L., Lowell, F. C. & Bloch, K. J. (1974). Allergens of mammalian origin. III. Properties of a major feline allergen. *J. Immunol.* **113**, 1668–1677.
- van Ree, R., van Leeuwen, W. A., Bulder, I., Bond, J. & Aalberse, R. C. (1999). Purified natural and recombinant Fel d 1 and cat albumin in *in vitro* diagnostics for cat allergy. *J. Allergy Clin. Immunol.* **104**, 1223–1230.
- Kristensen, A. K., Schou, C. & Roepstorff, P. (1997). Determination of isoforms, N-linked glycan structure and disulfide bond linkages of the major cat allergen Fel d1 by a mass spectrometric approach. *Biol. Chem.* **378**, 899–908.
- Duffort, O. A., Carreira, J., Nitti, G., Polo, F. & Lombardero, M. (1991). Studies on the biochemical structure of the major cat allergen *Felis domesticus* I. *Mol. Immunol.* **28**, 301–309.
- Bond, J. F., Brauer, A. W., Segal, D. B., Nault, A. K., Rogers, B. L. & Kuo, M. C. (1993). Native and recombinant Fel dI as probes into the relationship of allergen structure to human IgE immunoreactivity. *Mol. Immunol.* **30**, 1529–1541.
- Keating, K. M., Segal, D. B., Craig, S. J., Nault, A. K., Semensi, V., Wasserman, A. S. *et al.* (1995). Enhanced immunoreactivity and preferential heterodimer formation of reassociated Fel d I recombinant chains. *Mol. Immunol.* **32**, 287–293.
- Slunt, J. B., Rogers, B. L. & Chapman, M. D. (1995). IgE antibodies to recombinant forms of Fel d I: dichotomy between fluid-phase and solid-phase binding studies. *J. Allergy Clin. Immunol.* **95**, 1221–1228.
- Vailes, L. D., Sun, A. W., Ichikawa, K., Wu, Z., Sulahian, T. H., Chapman, M. D. & Guyre, P. M. (2002). High-level expression of immunoreactive recombinant cat allergen (fel d 1): targeting to antigen-presenting cells. *J. Allergy Clin. Immunol.* **110**, 757–762.
- Seppälä, U., Hägglund, P., Wurtzen, P. A., Ipsen, H., Thorsted, P., Lenhard, T. *et al.* (2005). Molecular characterization of major cat allergen Fel d 1: expression of heterodimer by use of a baculovirus expression system. *J. Biol. Chem.* **280**, 3208–3216.
- Grönlund, H., Bergman, T., Sandström, K., Alvelius, G., Reininger, R., Verdino, P. *et al.* (2003). Formation of disulfide-bonds and homodimers of the major cat allergen Fel d 1 equivalent to the natural allergen by expression in *Escherichia coli*. *J. Biol. Chem.* **278**, 40144–40151.
- Kaiser, L., Grönlund, H., Sandalova, T., Ljunggren, H.-G., van Hage-Hamsten, M., Achour, A. & Schneider, G. (2003). The crystal structure of the major cat allergen Fel d 1, a member of the secretoglobulin family. *J. Biol. Chem.* **278**, 37730–37735.
- Kaiser, L., Grönlund, H., Sandalova, T., Ljunggren, H.-G., Schneider, G., van Hage-Hamsten, M. & Achour, A. (2003). Production, crystallization and preliminary crystallographic study of the major cat allergen Fel d 1. *Acta Crystallog. sect. D*, **59**, 1103–1105.
- Klug, J., Beier, H. M., Bernard, A., Chilton, B. S., Fleming, T. P., Lehrer, R. I. *et al.* (2000). Uteroglobin/ Clara cell 10-kDa family of proteins: nomenclature committee report. *Ann. N Y Acad. Sci.* **923**, 348–354.
- Emes, R. D., Riley, M. C., Laukaitis, C. M., Goodstadt, L., Karn, R. C. & Ponting, C. P. (2004). Comparative evolutionary genomics of androgen-binding protein genes. *Genome Res.* **14**, 1516–1529.
- Mukherjee, A. B., Kundu, G. C., Mantile-Selvaggi, G., Yuan, C. J., Mandal, A. K., Chattopadhyay, S. *et al.* (1999). Uteroglobin: a novel cytokine? *Cell Mol. Life Sci.* **55**, 771–787.
- Peri, A., Cordella-Miele, E., Miele, L. & Mukherjee, A. B. (1993). Tissue-specific expression of the gene coding for human Clara cell 10-kD protein, a phospholipase A2-inhibitory protein. *J. Clin. Invest.* **92**, 2099–2109.
- Pilon, A. L. (2000). Rationale for the development of recombinant human CC10 as a therapeutic for inflammatory and fibrotic disease. *Ann. N Y Acad. Sci.* **923**, 280–299.
- Benson, M., Jansson, L., Adner, M., Luts, A., Uddman, R. & Cardell, L. O. (2005). Gene profiling reveals decreased expression of uteroglobin and other anti-inflammatory genes in nasal fluid cells from patients with intermittent allergic rhinitis. *Clin. Exp. Allergy*, **35**, 473–478.
- Doull, I. J., Lawrence, S., Watson, M., Begishvili, T., Beasley, R. W., Lampe, F. *et al.* (1996). Allelic association of gene markers on chromosomes 5q and 11q with atopy and bronchial hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* **153**, 1280–1284.
- Hård, T., Barnes, H. J., Larsson, C., Gustafsson, J. Å. & Lund, J. (1995). Solution structure of a mammalian PCB-binding protein in complex with a PCB. *Nature Struct. Biol.* **2**, 983–989.
- Umland, T. C., Swaminathan, S., Singh, G., Warty, V., Furey, W., Pletcher, J. & Sax, M. (1994). Structure of a human Clara cell phospholipid-binding protein-ligand complex at 1.9 Å resolution. *Nature Struct. Biol.* **1**, 538–545.
- Charpin, C., Mata, P., Charpin, D., Lavaut, M. N., Allasia, C. & Vervloet, D. (1991). Fel d I allergen distribution in cat fur and skin. *J. Allergy Clin. Immunol.* **88**, 77–82.
- Miele, L., Cordella-Miele, E., Mantile, G., Peri, A. & Mukherjee, A. B. (1994). Uteroglobin and uteroglobin-like proteins: the uteroglobin family of proteins. *J. Endocrinol. Invest.* **17**, 679–692.
- Zielonka, T. M., Charpin, D., Berbis, P., Luciani, P., Casanova, D. & Vervloet, D. (1994). Effects of castration and testosterone on Fel dI production by sebaceous glands of male cats: I-Immunological assessment. *Clin. Exp. Allergy*, **24**, 1169–1173.

28. Levin, S. W., Butler, J. D., Schumacher, U. K., Wightman, P. D. & Mukherjee, A. B. (1986). Uteroglobin inhibits phospholipase A2 activity. *Life Sci.* **38**, 1813–1819.
29. Chowdhury, B., Mantile-Selvaggi, G., Kundu, G. C., Miele, L., Cordella-Miele, E., Zhang, Z. & Mukherjee, A. B. (2000). Amino acid residues in alpha-helix-3 of human uteroglobin are critical for its phospholipase A2 inhibitory activity. *Ann. N Y Acad. Sci.* **923**, 307–311.
30. Miele, L., Cordella-Miele, E., Facchiano, A. & Mukherjee, A. B. (1988). Novel anti-inflammatory peptides from the region of highest similarity between uteroglobin and lipocortin I. *Nature*, **335**, 726–730.
31. Chowdhury, B., Mantile-Selvaggi, G., Miele, L., Cordella-Miele, E., Zhang, Z. & Mukherjee, A. B. (2002). Lys43 and Asp46 in alpha-helix 3 of uteroglobin are essential for its phospholipase A2 inhibitory activity. *Biochem. Biophys. Res. Commun.* **295**, 877–883.
32. Dennis, E. A. (1994). Diversity of group types, regulation, and function of phospholipase A2. *J. Biol. Chem.* **269**, 13057–13060.
33. Andersson, O., Nordlund-Moller, L., Barnes, H. J. & Lund, J. (1994). Heterologous expression of human uteroglobin/polychlorinated biphenyl-binding protein. Determination of ligand binding parameters and mechanism of phospholipase A2 inhibition *in vitro*. *J. Biol. Chem.* **269**, 19081–19087.
34. Barnes, H. J., Nordlund-Moller, L., Nord, M., Gustafsson, J., Lund, J. & Gillner, M. (1996). Structural basis for calcium binding by uteroglobins. *J. Mol. Biol.* **256**, 392–404.
35. Nord, M., Gustafsson, J. A. & Lund, J. (1995). Calcium-dependent binding of uteroglobin (PCB-BP/CCSP) to negatively charged phospholipid liposomes. *FEBS Letters*, **374**, 403–406.
36. Dandeu, J. P., Rabillon, M., Beltrand, M. J., Lux, M. & Duval, R. (1990). Immobilized metal ion affinity chromatography for the purification of Fel d I, a cat major allergen, from a house-dust extract. *J. Chromatog.* **512**, 177–188.
37. Kaiser, L., Gronlund, H., van Hage-Hamsten, M. & Achour, A. (2005). Crystallization and preliminary crystallographic data of a Fel d 1 (1+2) construct corresponding to the major allergen from cat. *Acta Crystallog. sect. F*, **61**, 232–234.
38. Leslie, A. G. (1999). Integration of macromolecular diffraction data. *Acta Crystallog. sect. D*, **55**, 1696–1702.
39. CCP4 (1994). The CCP4 suite: programs for protein crystallography. *Acta Crystallog. sect. D*, **50**, 760–763.
40. Vagin, A. A. & Isupov, M. N. (2001). Spherically averaged phased translation function and its application to the search for molecules and fragments in electron-density maps. *Acta Crystallog. sect. D*, **57**, 1451–1456.
41. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard (1991). Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallog. sect. A*, **47**, 110–119.
42. Winn, M. D., Isupov, M. N. & Murshudov, G. N. (2001). Use of TLS parameters to model anisotropic displacements in macromolecular refinement. *Acta Crystallog. sect. D*, **57**, 122–133.
43. Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). PROCHECK: a program to check the stereochemical quality of pprotein structures. *J. Appl. Crystallog.* **26**, 283–291.
44. Guex, N. & Peitsch, M. C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis*, **18**, 2714–2723.
45. Eriksson, T. L., Gafvelin, G., Elfman, L. H., Johansson, C., Van Hage-Hamsten, M. & Olsson, S. (2001). T cell responses to recombinant isoforms, synthetic peptides and a mutant variant of Lep d 2, a major allergen from the dust mite *Lepidoglyphus destructor*. *Clin. Exp. Allergy*, **31**, 1881–1890.
46. Svahn, A., Linde, A., Thorstensson, R., Karlen, K., Andersson, L. & Gaines, H. (2003). Development and evaluation of a flow-cytometric assay of specific cell-mediated immune response in activated whole blood for the detection of cell-mediated immunity against varicella-zoster virus. *J. Immunol. Methods*, **277**, 17–25.
47. Saarne, T., Kaiser, L., Gronlund, H., Rasool, O., Gafvelin, G. & van Hage-Hamsten, M. (2005). Rational design of hypoallergens applied to the major cat allergen Fel d 1. *Clin. Exp. Allergy*, **35**, 657–663.
48. Trivedi, B., Valerio, C. & Slater, J. E. (2003). Endotoxin content of standardized allergen vaccines. *J. Allergy Clin. Immunol.* **111**, 777–783.
49. Aalberse, R. C. (2000). Structural biology of allergens. *J. Allergy Clin. Immunol.* **106**, 228–238.
50. Morgenstern, J. P., Griffith, I. J., Brauer, A. W., Rogers, B. L., Bond, J. F., Chapman, M. D. & Kuo, M. C. (1991). Amino acid sequence of Fel dI, the major allergen of the domestic cat: protein sequence analysis and cDNA cloning. *Proc. Natl Acad. Sci. USA*, **88**, 9690–9694.
51. Jones, S. & Thornton, J. M. (1995). Protein-protein interactions: a review of protein dimer structures. *Prog. Biophys. Mol. Biol.* **63**, 31–65.
52. Lo Conte, L., Chothia, C. & Janin, J. (1999). The atomic structure of protein-protein recognition sites. *J. Mol. Biol.* **285**, 2177–2198.
53. Beato, M. (1976). Binding of steroids to uteroglobin. *J. Steroid Biochem.* **7**, 327–334.
54. Gillner, M., Lund, J., Cambillau, C., Alexandersson, M., Hurtig, U., Bergman, A. *et al.* (1988). The binding of methylsulfonyl-polychloro-biphenyls to uteroglobin. *J. Steroid Biochem.* **31**, 27–33.
55. Lopez de Haro, M. S., Perez Martinez, M., Garcia, C. & Nieto, A. (1994). Binding of retinoids to uteroglobin. *FEBS Letters*, **349**, 249–251.
56. Bally, R. & Delettre, J. (1989). Structure and refinement of the oxidized P21 form of uteroglobin at 1.64 Å resolution. *J. Mol. Biol.* **206**, 153–170.
57. Pattabiraman, N., Matthews, J. H., Ward, K. B., Mantile-Selvaggi, G., Miele, L. & Mukherjee, A. B. (2000). Crystal structure analysis of recombinant human uteroglobin and molecular modeling of ligand binding. *Ann. N Y Acad. Sci.* **923**, 113–127.
58. Morize, I., Surcouf, E., Vaney, M. C., Epelboin, Y., Buehner, M., Fridlansky, F. *et al.* (1987). Refinement of the C222(1) crystal form of oxidized uteroglobin at 1.34 Å resolution. *J. Mol. Biol.* **194**, 725–739.
59. Umland, T. C., Swaminathan, S., Furey, W., Singh, G., Pletcher, J. & Sax, M. (1992). Refined structure of rat Clara cell 17 kDa protein at 3.0 Å resolution. *J. Mol. Biol.* **224**, 441–448.

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