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Unlocking the allergenic structure of the major house dust mite allergen Der f 2 by elimination of key intramolecular interactions

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Abstract We report on the structural background of the remarkable reduction of allergenicity in engineering of the major house dust mite allergen Der f 2. Disruption of intramolecular disulfide bonds in Der f 2 caused extensive conformational change that was monitored by circular dichroism and gel-filtration analysis. The degree of conformational change correlated well with the degree of reductions in the capacity to bind IgE and to induce histamine release from basophils in mite-allergic patients. Loosening the rigid tertiary structure by elimination of key intramolecular interactions is an effective strategy to reduce the number of high affinity IgE epitopes of allergen vaccine.

Key words: Allergen engineering; Major house dust mite allergen; Site-directed mutagenesis; Conformational change; IgE-binding

1. Introduction

Type I allergic diseases mediated by IgE against allergens such as asthma, rhinitis and atopic dermatitis affect more than 20% of the world's population. Allergen-specific immunotherapy involves repeated injection of allergen vaccine over prolonged periods to achieve a state of immunological tolerance [1] and has proven an effective treatment of allergic disease since the first trials in the early 1900s [2]. The major problem with allergen-specific immunotherapy, however, is the risk of an anaphylactic reaction. Recently, the use of hypoallergenic natural isoforms [3], engineered recombinant mutants [4–7], or non-anaphylactic recombinant fragments [8–10] of several allergens from house dust mite, tree pollen, and grass pollen has been proposed to reduce the allergenicity [11–13]. 'Allergen engineering' achieves a reduction of IgE-binding capacities of the allergen proteins by introducing site-directed mutations to the amino acid sequences of the recombinant allergens [4–7]. Ideally, the engineered allergens do not crosslink the complex of allergen-specific IgE and high affinity IgE receptor (FcεRI) on the surface of mast cells and basophils, nor induce the release of mediators from the cells [14,15].

The major causative factors associated with various allergic diseases are house dust mite (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) allergens [16–19]. The major allergens from house dust mite are classified as group 1 (Der f 1 and Der p 1, M_r 25 000) or group 2 (Der f 2 and Der p 2, M_r 14 000) [20–25]. Der f 2 is the major group 2 allergen from *D. farinae* [26,27], and consists of 129 amino acid residues with marked homology to Der p 2 [28], the group 2 allergen from *D. pteronyssinus*. We determined the location of three disulfide bonds of native Der f 2 (Cys8–Cys119, Cys21–Cys27 and Cys73–Cys78) [29] and the tertiary structure of recombinant Der f 2 by nuclear magnetic resonance [30].

Allergen engineering of Der f 2 has revealed that two disulfide bonds, Cys8–Cys119 and Cys73–Cys78, are critical to the IgE-binding capacity of Der f 2 [4,31,32]. Understanding of the structural background of the reduced allergenicity in engineered allergen mutants is important for development of effective strategies for allergen engineering. In this study, we focus on the background of the remarkable reduction of IgE-binding capacity in Der f 2 mutants. We demonstrate that disruption of disulfide bonds in Der f 2 caused remarkable conformational changes and that reductions in allergenic activities were dependent on the degree of change.

2. Materials and methods

2.1. Expression of the wild-type and cysteine mutants of Der f 2

Expression vectors for the wild-type Der f 2 and its three mutants, C21/27S, C73/78S, and C8/119S, were constructed as previously described [4]. Those for 8C-119C and ΔC were constructed by the use of restriction sites and site-directed mutagenesis in this study. The wild-type Der f 2 and its five mutants were expressed in *Escherichia coli* BL21 (DE3) transformed with the expression vectors.

2.2. Purification of the wild-type and cysteine mutants of Der f 2

The wild-type and mutants of Der f 2 produced as inclusion bodies in *E. coli* BL21 (DE3) were recovered, solubilized with buffer containing urea, refolded by dialysis against buffer, and purified by anion exchange column chromatography as described [4,31,33]. The disulfide bond formation of the purified recombinant wild-type and mutants of Der f 2 was confirmed correct by peptide mapping as described [29]. The purities were checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing or reducing conditions.

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2.3. Gel-filtration analysis

The purified wild-type and mutants of Der f 2 (200 μl, approximate 100 μg/ml) were subjected to gel-filtration chromatography on a Superdex 75 HR 10/30 (1 cm × 30 cm) (Pharmacia, Uppsala, Sweden) at a flow rate of 1 ml/min with phosphate-buffered saline (pH 7.4). Absorbance at 280 nm was monitored. Molecular weight standards for gel-filtration (Bio-Rad, Richmond, CA, USA) were used for estimation of apparent molecular weights of the recombinant proteins.

2.4. Radioallergosorbent test-enzyme immunoassay (RAST-EIA)

IgE reactivities of the purified wild-type and mutants of Der f 2 were measured by RAST-EIA using a RAST-FEIA kit (Pharmacia) as previously described [10]. Briefly, serum of each allergic patient (1/2-1 dilution) was incubated with paper discs coupled with each of the purified antigens in various amounts for 3 h at 37°C. Then, the IgE that bound to the disc was detected with β-galactosidase-conjugated anti-human IgE antibodies by measuring the fluorescence. The sera from patients allergic to house dust mite were kindly provided by Dr. Hirokazu Okudaira, University of Tokyo.

2.5. Inhibition assay

The method was as described previously with some modifications [31]. Briefly, the serum of each allergic patient (1/2-1 dilution) was incubated with various concentrations of each of the purified wild-type Der f 2 and its mutants for 3 h at room temperature, and then the solution was added to a paper disc presoaked in wild-type Der f 2. The IgE that bound to discs was detected with β-galactosidase-conjugated anti-human IgE antibodies. The percentage of inhibition was expressed as the relative reduction of the fluorescence intensity in each sample to that when no inhibitors were added.

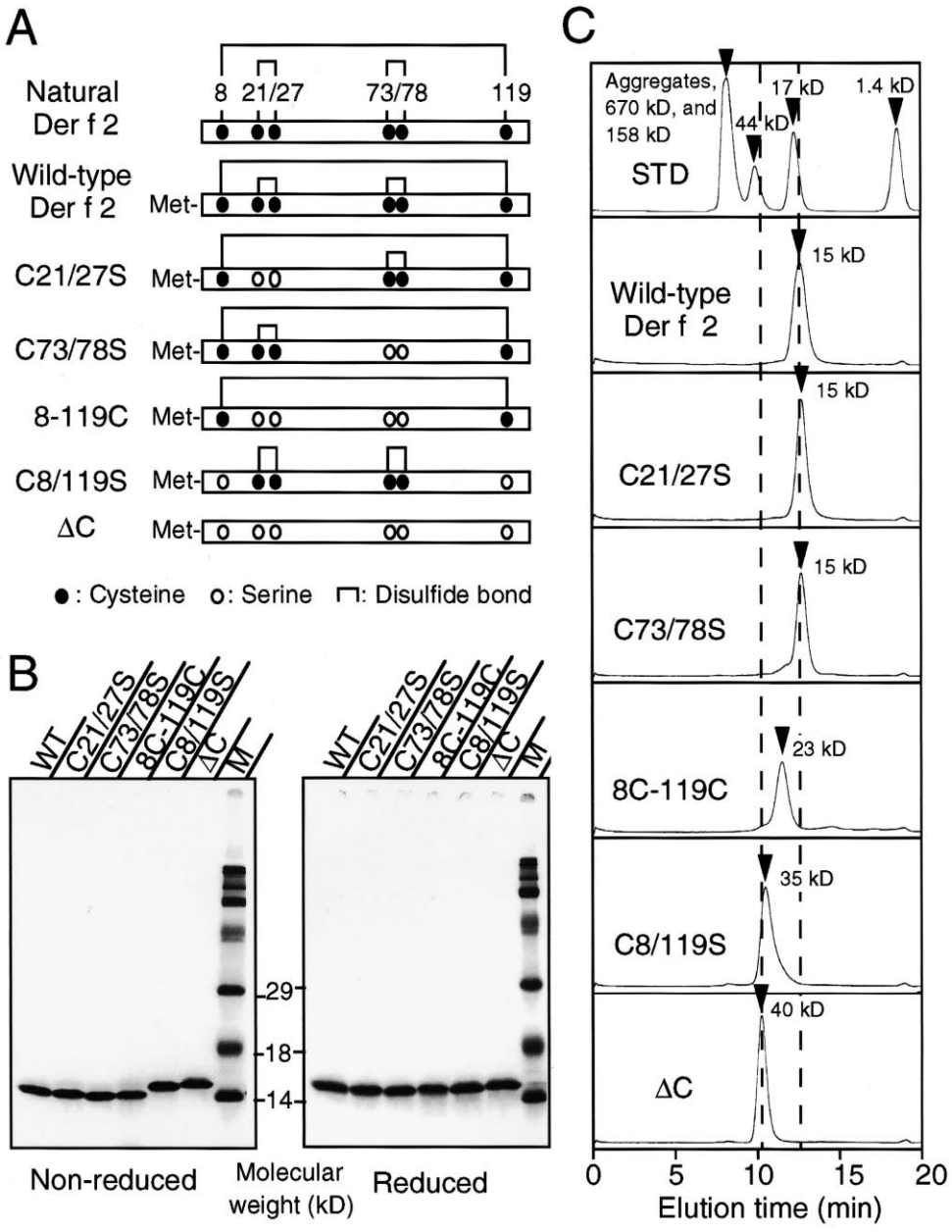

Fig. 1. Purified Der f 2 mutants. (A) Confirmation of intramolecular disulfide bonds. Schematic representation of native Der f 2 purified from mite extract and the purified wild-type and five mutants of Der f 2 expressed in *E. coli* with a methionine at the first N-terminal residue are shown with confirmed disulfide bonds. (B) SDS–PAGE analysis under non-reducing and reducing conditions. WT: wild-type Der f 2. M: molecular weight marker. (C) Elution profiles in gel-filtration analysis. Apparent molecular weights of the peaks of purified wild-type and mutants of Der f 2 are shown with arrows. The elution profile of molecular weight standards is also shown as a control (STD).

2.6. Histamine release

The method was as described previously except that a histamine enzyme-linked immunosorbent assay (ICN, Costa Mesa, CA, USA) was used [4]. The percentage of released histamine was calculated as the relative amount of histamine in the supernatant to the total of supernatant and cellular histamine.

2.7. Circular dichroism (CD)

CD spectra were measured on a JASCO J-700 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) using a 0.1 cm cell. Spectra were recorded at 25°C over 200–250 nm. Samples are 114 μg/ml in 20 mM Tris–HCl, pH 7.4, and 0.9% NaCl.

3. Results

3.1. Preparation of purified Der f 2 mutants with correct disulfide bonds

We generated five mutants of Der f 2, in which one (C21/27S, C73/78S, and C8/119S), two (8C-119C), or three (ΔC) pairs of cysteines forming the intramolecular disulfide bonds were changed to a pair of serines (Fig. 1A). Each of the purified wild-type Der f 2 and its mutants was detected as a single band on SDS–PAGE under both non-reducing and reducing conditions (Fig. 1B). Under non-reducing conditions, mobilities of C8/119S and ΔC, in both of which Cys8–Cys119 was disrupted, were smaller than those of the wild-type Der f 2, C21/27S, C73/78S, and 8C-119C, in all of which Cys8–Cys119 was conserved. Mobilities of the wild-type and all mutants of Der f 2 under reducing conditions were equivalent to those of C8/119S and ΔC under non-reducing conditions.

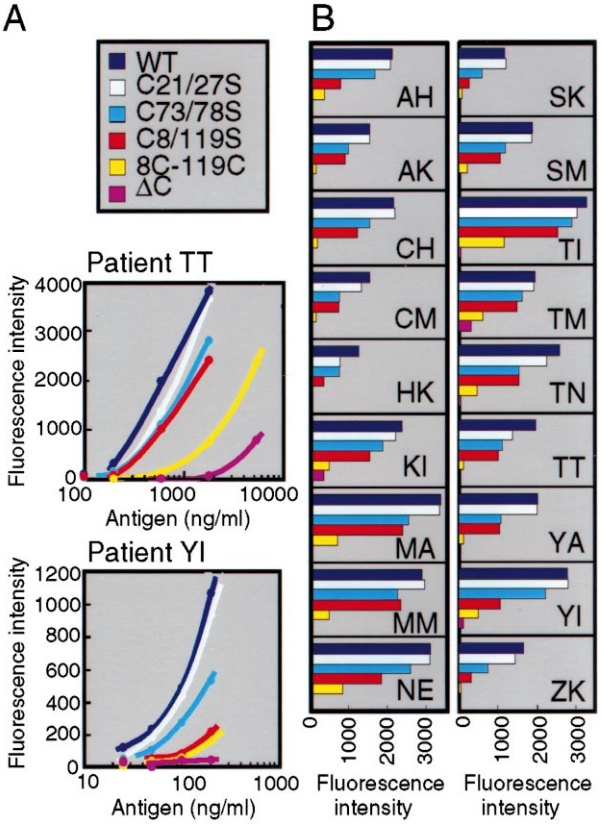

Fig. 2. Reactivities of purified Der f 2 mutants to IgE in mite-allergic patient sera. (A) IgE-binding profiles of two patients. (B) IgE-binding profiles of 18 patients. The concentration of antigens in the immobilization step was 600 ng/ml. Initials of the patients' names are indicated. WT: wild-type Der f 2.

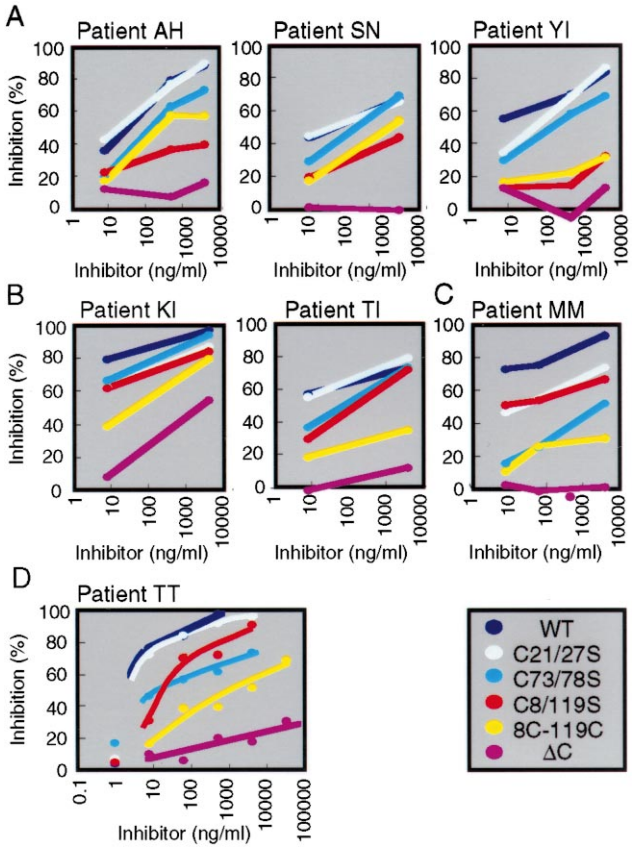

Fig. 3. Inhibition of IgE-binding to the immobilized wild-type Der f 2 by addition of Der f 2 mutants. (A) Group A: C8/119S was remarkably more reduced than C73/78S. (B) Group B: C8/119S was slightly more reduced than C73/78S. (C) Group C: C73/78S was more reduced than C8/119S. (D) Group D: C8/119S was weaker at low concentrations but stronger at high concentrations than C73/78S. The concentration of the wild-type Der f 2 in the immobilization step was 100 ng/ml. Initials of the patients' names are indicated. WT: wild-type Der f 2.

The correct disulfide bond formations of the purified wild-type and mutants of Der f 2 were confirmed by peptide mapping [29] (Fig. 1A).

Each of the purified mutants was detected as a single peak in gel-filtration analysis (Fig. 1C). Interestingly, 8C-119C, C8/119S and ΔC had prolonged elution times compared with the wild-type Der f 2, C21/27S, and C73/78S. Apparent molecular weights of the wild-type and mutants of Der f 2 in gel-filtration were calculated. The apparent molecular weights of the wild-type Der f 2, C21/27S and C73/78S were approximately 15 kDa. The apparent molecular weights of 8C-119C, C8/119S and ΔC were approximately 23 kDa, 35 kDa, and 40 kDa, respectively.

3.2. Reactivities of purified Der f 2 mutants to IgE in allergic patient sera

The IgE reactivities of the homogeneously purified recombinant proteins with correct disulfide bonds were determined by RAST-EIA [10,31]. In two patients, TT and YI, the most remarkable reduction was in ΔC followed by 8C-119C and no or a significant but not remarkable reduction in C21/27S (Fig. 2A). In the patient TT, C8/119S and C73/78S showed a moderate reduction. In the patient YI, C8/119S showed a remarkable reduction and C73/78S showed a moderate reduc-

tion. Similar results were obtained in analysis of the IgE-binding profiles of 18 patients (Fig. 2B). A tendency existed in the IgE-binding profiles; in order of IgE reactivity, the mutants ranked C21/27S > C73/78S ≥ C8/119S > 8C-119C > ΔC. Although there was a tendency that the reactivity of C8/119S was lower than that of C73/78S, the range of the relative IgE reactivity of C8/119S to C73/78S differed to some extent; the reactivity of C8/119S was equivalent with or slightly higher than that of C73/78S in patients such as CM, MM, and TN.

3.3. Activities of purified Der f 2 mutants to inhibit IgE-binding to immobilized wild-type Der f 2

The capacities of the mutants to inhibit IgE from binding to the wild-type Der f 2 were also examined using the sera of seven patients by inhibition assay (Fig. 3). In all patients, the inhibitory effect by ΔC was the most reduced and that by C21/27S was equivalent to that by the wild-type Der f 2 or relatively strong among the mutants. However, the inhibitory profiles of the patients for the other three mutants, C73/78S, C8/119S, and 8C-119C, were not the same. The patients were classified into four groups based on a comparison of C8/119S and C73/78S in inhibitory activity; group A: C8/119S was remarkably weaker than C73/78S (three patients) (Fig. 3A), group B: C8/119S was slightly weaker than C73/78S (two patients) (Fig. 3B), group C: C73/78S was weaker than C8/119S (one patient) (Fig. 3C), and group D: C8/119S was weaker at low concentrations but stronger at high concentrations than C73/78S (one patient) (Fig. 3D). Group D was considered a mix of groups A, B, and C. In patients belonging to group A, C8/119S was weaker than or equivalent to 8C-119C in the inhibitory activity (Fig. 3A). On the other hand, in patients belonging to groups B, C, or D, 8C-119C was the second weakest after ΔC (Fig. 3B–D).

3.4. Activities of purified Der f 2 mutants to induce histamine release from basophils

Peripheral blood basophils from an allergic patient, TT, were stimulated with the wild-type and mutants of Der f 2, and histamine release was measured (Fig. 4). Activities of all the mutants were lower than that of the wild-type Der f 2. The activity of ΔC was the most reduced. The threshold concentration of ΔC (approximately 1 μg/ml) to stimulate peripheral basophils to release histamine was about 1000 times more than that of the wild-type Der f 2 (approximately 0.001 μg/ml), that of 8C-119C and C8/119S (0.1–1 μg/ml) about 100–1000 times more, that of C73/78S (0.01–0.1 μg/ml) about 10–100 times more, and that of C21/27S (0.001–0.01 μg/ml) about 1–10 times more.

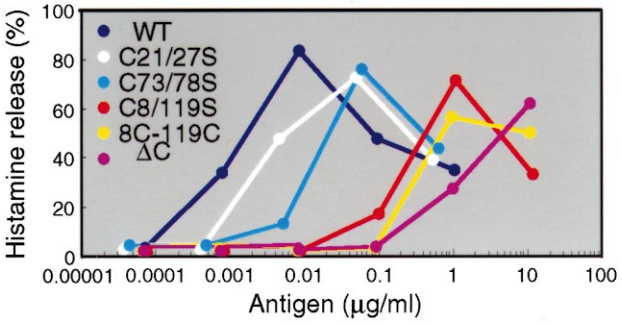

Fig. 4. Histamine release from basophils stimulated with the purified Der f 2 mutants. Peripheral blood leukocytes were obtained from a mite-allergic patient TT. WT: wild-type Der f 2.

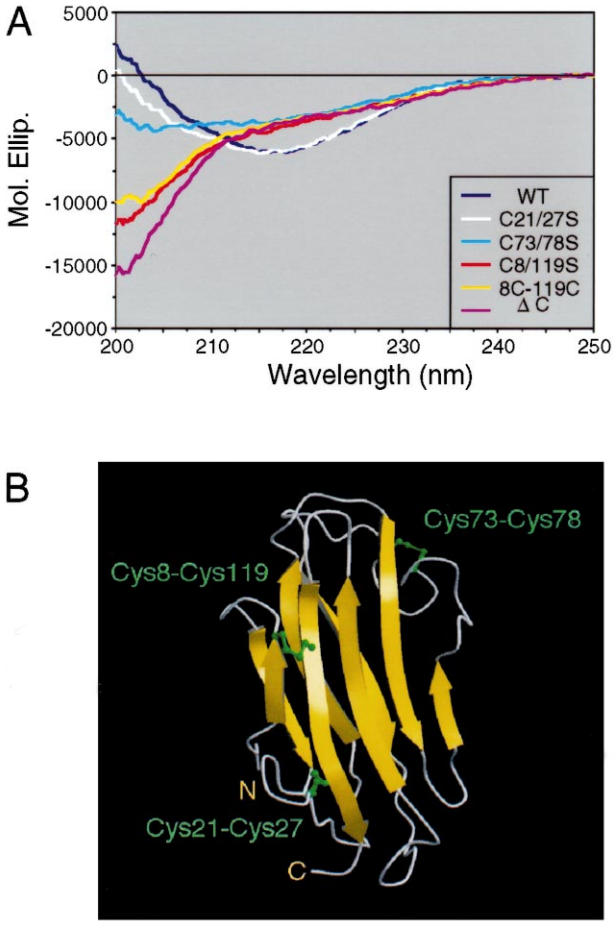

Fig. 5. (A) CD spectra of the purified Der f 2 mutants. The experimental parameters are: bandwidth 1.0 nm, sensitivity 20 m°, step resolution 0.2 nm/datum, scan speed 50 nm/min, and number of scans 8. (B) Ribbon diagram of the mean structure of Der f 2 [30]. The disulfide bonds and β-strands are shown. This figure was produced by the program MOLSCRIPT [42] and Raster 3D [43].

3.5. Secondary structures of purified Der f 2 mutants

CD spectra of all the Der f 2 mutants were different from that of the wild-type Der f 2 (Fig. 5A). The fractions of helical structures were estimated by using the ellipticity at 222 nm [34]. The calculated values of the helical content are as follows: wild-type Der f 2: 8.7%, C21/27S: 8.8%, C73/78S: 2.3%, C8/119S: 3.7%, 8C-119C: 2.7%, and ΔC: 2.9%. Thus, the estimated helical content of C21/27S was comparable with that of the wild-type, and in the other four mutants disruption of the helices was estimated to be similar to each other. Therefore, the differences in the region of 200–210 nm among these four mutants could be attributed to transitions from β-structures to random coils, i.e. they ranked ΔC > C8/119S, 8C-119C > C73/78S in order of disruption of the two β-sheets, the main components of the wild-type Der f 2 [30].

4. Discussion

The set of five Der f 2 mutants each differing in choice of three intramolecular disulfide bonds (Figs. 1A and 5B) used in

this study was a suitable model with which to examine the structural background of the remarkable reduction of allergenicity in mutant allergens engineered for improved allergen-specific immunotherapy. The mutants were purified to homogeneity, and confirmed to have the correct disulfide bonds (Fig. 2).

We have reported that two disulfide bonds, Cys8–Cys119 and Cys73–Cys78, are critical to the IgE-binding capacity of Der f 2 by analyzing the allergenicities of C21/27S, C73/78S, and C8/119S [4]. In CD spectra, C8/119S and C73/78S differed remarkably from that of the wild-type Der f 2 but C21/27S less so (Fig. 5A). Additional disruption of disulfide bonds in ΔC, in which all three disulfide bonds were disrupted, and 8C-119C, in which only one disulfide bond, Cys8–Cys119, was conserved, led to disruption of the secondary structure and further reductions in IgE reactivity (Figs. 2, 3 and 5). Furthermore, increases of the apparent molecular weight in ΔC, C8/119S, and 8C-119C were observed in gel-filtration analysis (Fig. 1C). ΔC, C8/119S, and 8C-119C have apparent molecular weight of approximately 2.7-, 2.3-, and 1.5-fold that of wild-type Der f 2, respectively. The major house dust mite group 2 allergens, Der f 2 and Der p 2, are single-domain proteins which are mainly composed of two β-sheets (Fig. 5B) [30,35]. The loosening of the rigid structure in the three mutants corresponds well to the disruption of the β-sheets detected by CD (Fig. 5). Additionally, Der p 2 was reported to have a short helix at residues 72–75 [35], and Der f 2 also has a helix-like structure at the corresponding position (residues 72–76) although it is not explicitly described [30]. We conclude that, in ΔC, C8/119S, and 8C-119C, global structural change caused the remarkable reduction of IgE reactivity, while in C73/78S, the disruption of the helix 72–76 affected the IgE reactivity.

IgE reactivity as detected by binding assay is considered dependent on the number of IgE epitopes immobilized on the disc which have enough affinity not to be washed out during the assay (Fig. 2). A tendency existed in the IgE-binding profiles, i.e. in order of IgE reactivity the mutants ranked C21/27S > C73/78S ≥ C8/119S > 8C-119C > ΔC (Fig. 2B). But the range of the relative IgE reactivities of C8/119S to C73/78S differed to some extent. These results suggest that the IgE recognition profiles of patients allergic to Der f 2 have similarity but are not completely uniform. The lower IgE-binding of C8/119S than C73/78S in the majority of sera is considered to result from remarkable disruption of the β-sheet structure and the global folding (Figs. 1C and 5A). IgE-binding of C73/78S equivalent to or lower than C8/119S in patients such as CM, MM, and TN is considered to result from more complete disruption of IgE epitopes around Cys73–Cys78 in C73/78S (Figs. 2 and 3C). However, in ΔC and 8C-119C, on the disruption of one more disulfide bond to C8/119S and C73/78S, the diversity was lost and a much more complete elimination of the IgE reactivity was achieved in all patients (Fig. 2).

IgE reactivity detected by inhibition assay is considered dependent on the relative number and relative affinity of serum heterogeneous IgE molecules which bind the inhibitor to those of the IgE molecules which bind wild-type Der f 2 (Fig. 3). At higher inhibitor concentrations, where the number of mutant Der f 2 molecules can override attenuation of binding affinity, the reactivity is considered to reflect mainly the relative number of IgE molecules that each inhibitor can absorb. ΔC had no or very little inhibitory activity in all patients. A similar phenomenon was observed in 8C-119C. We considered that the disruption of three or two disulfide bonds in ΔC or 8C-119C synergistically perturbed or disrupted the conformations of many IgE epitopes. In patients belonging to group A, inhibitory activity of 8C-119C was higher than or equivalent to C8/119S (Fig. 3A). In these patients, the number of IgE epitopes maintained by Cys8–Cys119 in 8C-119C is considered to be larger than or equivalent to that of IgE epitopes maintained by Cys73–Cys78 in C8/119S.

In one patient, TT, C8/119S had less inhibitory activity than C73/78S at low concentrations but more at high concentrations (Fig. 3D). In this patient, C8/119S had significantly less activity to release histamine from basophils than C73/78S (Fig. 4). These results suggest that the number of IgE epitopes with high affinity is critical to whether the cells degranulate in the immediate phase. Torigoe et al. reported that aggregation of IgE–FcεRI complexes on mast cells with low affinity ligands for IgE was ineffective in completing the signal transduction cascades to induce degranulation of the cells [36,37]. The activity of ΔC to stimulate basophils to release histamine was about 1000 times less than that of the wild-type Der f 2, and the activity of 8C-119C and of C8/119S about 100–1000 times less (Fig. 4). The number of IgE epitopes with affinity high enough to induce cell degranulation was considered to be markedly reduced in the mutants, ΔC, 8C-119C, and, C8/119S, in which global compactness was disrupted (Figs. 1C and 5A).

Smith et al. reported that a mutant of Der p 2 from *D. pteronyssinus*, C73R, expressed as a fused form with a histidine-tag in *E. coli*, had around 10 times less activity than wild-type Der p 2 to induce an immediate reaction in a skin test in three patients and around 100 times less activity in two patients [7]. This is consistent with our result that the histamine-releasing activity of a mutant of Der f 2, C73/78S, was about 10–100 times less than that of wild-type Der f 2 in this study (Fig. 4). Disruption of the helix 72–75 might have occurred in their Der p 2 mutant, C73R, as was confirmed in our Der f 2 mutant, C73/78S (Fig. 5A).

The structural changes to engineered allergen mutants were defined for the first time in this study. Ferreira et al. reported on a hypoallergenic mutant of the major birch pollen allergen Bet v 1, in which six amino acid substitutions were introduced [5]. Their strategy is based on the comparative analysis of sequences of natural hypoallergenic variants [3], and therefore, the engineered hypoallergenic mutant maintained a secondary structure similar to that of the allergenic wild-type Bet v 1. However, natural hypoallergenic variants such as those of Bet v 1 are not observed in general [13]. On the other hand, the disruption of tertiary and/or secondary structures of allergens defined in this study is considered a strategy widely adaptive to various allergens. Furthermore, the disruption of tertiary and/or secondary structures would particularly reduce the number of epitopes with high affinity to IgE and be a more effective strategy than direct targeting of each IgE epitope on the surface of the allergen. The tertiary and secondary structures of proteins are maintained by various intramolecular interactions. Some of these interactions might be critical to maintaining the correct folding or for the folding process itself. Disulfide bond formation targeted in this study is a typical intramolecular interaction in protein. In allergen

engineering based on this strategy, information on the tertiary structure of allergens might be useful for screening key intramolecular interactions other than disulfide bonds [30,38].

We showed that the degree of the tertiary and/or secondary conformational change correlated well with the degree of reduction in the capacity to bind IgE and to induce histamine release from basophils. A mutant, C8/119S, in which compactness of the global structure is disrupted has been analyzed for T-cell reactivity [4], IgE-binding, and skin prick tests [39,40] in a larger population of patients and the administration of C8/119S has been shown to suppress an immediate allergic reaction in mice [41]. Loosening of the rigid tertiary structure by elimination of key intramolecular interactions in the allergen molecule would be an effective strategy in allergen engineering adaptive to various allergens for safe and effective allergen-specific immunotherapy.

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