
Alteration of the tertiary structure of the major bee venom allergen Api m 1 by multiple mutations is concomitant with low IgE reactivity

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Abstract

We have engineered a recombinant form of the major bee venom allergen (Api m 1) with the final goal of reducing its IgE reactivity. This molecule (Api mut) contains 24 mutations and one deletion of 10 amino acids. The successive introduction of these sequence modifications led to a progressive loss of specific IgE and IgG reactivity and did not reveal any immunodominant epitopes. However, Api mut exhibited a clear loss of reactivity for Api m 1-specific IgE and IgG. Injection of Api mut into mice induced specific antibody production. This humoral response was as high as that induced by the Api m 1 but the cross-reactivity of the antibodies was weak. As inferred by far UV circular dichroism, this mutant was correctly folded. However, near UV circular dichroism and denaturation curves of Api mut showed that it exhibits a dynamic tertiary structure and that it is a highly flexible molecule. Finally, as all the sequence modifications have been introduced outside the human and murine T cell epitope regions, we investigated its T cell properties in mice. We showed that Api mut-specific T lymphocytes induced *in vivo* were stimulated *in vitro* by both proteins. These data provide new insights in the design of hypoallergenic molecules.

Keywords: IgE; protein engineering; allergy; immunotherapy

Specific immunotherapy is the only curative treatment of IgE mediated allergy. It is based on repeated injections of increasing doses of crude allergen extracts and exhibits variable efficacy. Crude allergen extracts have the major drawback of being highly allergenic. They present the risk of inducing severe anaphylactic side effects upon injection. Thus, there is a need for new molecules derived from al-

lergens to perform safer immunotherapy. Recent advances in the understanding of immunotherapy have underlined the major role played by the allergen-specific CD4⁺ T lymphocytes in the control of the allergic response and the induction of tolerance (Akdis and Blaser 1999). These cells recognize the allergens as 13 to 25 amino acid peptides, called T cell epitopes. These peptides are produced by the degradation of the allergen in the antigen presenting cells and are presented by HLA Class II molecules to CD4⁺ T cells. Their injection in mice in saline buffer and by different routes (Briner et al. 1993; Burkhart et al. 1999; Sundstedt et al. 2003) leads to T cell tolerance as a result of IL-10 secretion by antigen-specific CD4⁺ T lymphocytes (Burkhart et al. 1999; Sundstedt et al. 2003).

IL-10 is an immunosuppressive cytokine, which diminishes the T cell activation and IgE secretion by B lympho-

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Abbreviations: Api m 1, major bee venom allergen; Api wt, recombinant form of Api m 1; Api mut, polysubstituted mutant of Api wt; Api RCM, denatured and carboxymethylated form of Api m 1; HEL, hen egg lysozyme; GdnHCl, guanidium chloride; HLA II, Human Leukocyte Antigens of class II; PBMC, peripheral blood mononuclear cells.

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cytes (Jeannin et al. 1998; Akdis and Blaser 1999), as it increases IgG4 production (Jeannin et al. 1998). Conventional specific immunotherapy with crude allergen extracts seems to induce IgG4 by specific B lymphocytes and sometimes IFN γ by T lymphocytes. It is also followed by an IL-10 increase, which may account for the success of the treatment. Moreover, it was suggested by clinical studies performed in humans with short peptides (Muller et al. 1998; Oldfield et al. 2002) that the injected molecules do not necessarily require induction of protective antibodies. As a result, a new concept for successful immunotherapy has emerged: It should be possible to treat allergic patients with engineered molecules containing allergen-specific T cell epitopes but reacting weakly with specific IgE. These molecules could be either peptides or proteins. However, T cell epitopes vary from one individual to another and depend on the specificity of the HLA class II molecules that present them to the CD4⁺ T lymphocytes. Therefore, T cell epitopes containing peptides might be too short to cover all the T cell epitopes present in the entire population. In contrast, engineered proteins are expected to encompass most of the T cell epitopes of the native allergen. They are produced by recombinant technology, allowing a variety of sequence modifications and protein conjugations. Initially, most of these constructs have been engineered with the final attempt to affect the 3D structure of the molecule (Smith and Chapman 1996; Takai et al. 1997; Okada et al. 1998; Smith et al. 1998). Recent investigations have focused on mutated allergens with native-like structures. Based on the existence of natural variants, which weakly cross-react with Bet v1-specific antiserum, hypoallergenic mutants of this allergen have been proposed (Ferreira et al. 1998). Mutants of other allergens were constructed by site-directed mutagenesis (Swoboda et al. 2002). Other approaches consist in the construction of hybrids of homologous but noncross-reactive allergens (King et al. 2001) and in the oligomerization of the native monomeric molecules (Vrtala et al. 2001). Overall, a growing interest appears for the engineering of allergens and the design of hypoallergenic molecules.

Bee venom immunotherapy is an efficient treatment for bee venom allergy, but provokes undesirable side effects for approximately 15% of patients (Muller et al. 1992). Among the different components of the bee venom, the PLA2 is recognized as the major allergen (Api m 1) since most prick-test positive patients possess Api m 1-specific IgE (King et al. 1976). Recognition by these antibodies is mainly dependent on the conformation of the allergen (Schneider et al. 1994). A number of T cell epitopes from Api m 1 have been already delineated by proliferative cellular assays performed with PBMC from allergic patients (Carballido et al. 1993; Kammerer et al. 1997; Texier et al. 2002). They appear to be localized mainly in the central and C-terminal parts of the molecule. In agreement with these data, we found that the sequences 76–106 and 111–134

comprise multiple HLA II binding regions, the sequence 81–97 being able to bind to 10 different HLA II molecules (Texier et al. 2000, 2001). Therefore, we used all these observations to create mutations and deletions in exposed areas of Api m 1 and outside the T cell epitope regions in order to reduce its IgE reactivity but not its capacity to stimulate T cells. Our data show that accumulation of mutations provokes a progressive loss of IgE reactivity although they do not disturb the secondary structure of the mutants. The mutant called Api mut combines all the mutations and one deletion. It exhibits an altered tertiary structure but also a strongly reduced antigenicity for polyclonal specific IgE from allergic patients.

Results

Construction and expression of Api m 1 mutants

We introduced 23 mutations in six different areas (A, B, C, D, G, H) of the surface of the molecule and one deletion between residues 106 and 115 (Fig. 1A,C). Mutated positions were selected outside the 81–106 region, which is rich in T cell epitopes (Texier et al. 2000). Residues conserved in the PLA2 family were not considered as they might be involved in the folding of the molecule. Most mutations correspond to size alteration or charge reversal of the amino-acid side chains (Fig. 1B). The deletion was introduced into a flexible loop and was designed to avoid local alteration of the structure of the molecule. After purification and *in vitro* refolding, homogeneity of all the mutants was analyzed by reversed-phase HPLC and their secondary structure was investigated by far UV CD spectroscopy (data not shown). Mutations impairing expression or folding were discarded. Then, selected mutated areas were progressively combined, leading to the Api mut protein harboring all the mutations and the deletion 106–115. As compared to the native molecule (Api m 1) purified from the bee venom and to the recombinant wild type protein (Api wt), the Api mut form displayed a slightly different CD spectrum (Fig. 1C). However, all spectra were characterized by a positive signal below 200 nm, a minimum at 208 nm, and a shoulder at 225 nm indicating they have adopted an ordered structure. This pattern can be attributed to a mixture of α -helices and β -sheets (Adler et al. 1973), in agreement with the crystal structure of Api m 1 (Scott et al. 1990). Thus, Api mut and the native molecule are folded and share similar elements of secondary structure.

Recognition of Api m 1 mutants by sera of patients allergic to bee venom

All mutants were investigated for their IgE and IgG reactivity toward a pool of four antisera collected from patients allergic to bee venom (Table 1). In competitive ELISA ex-

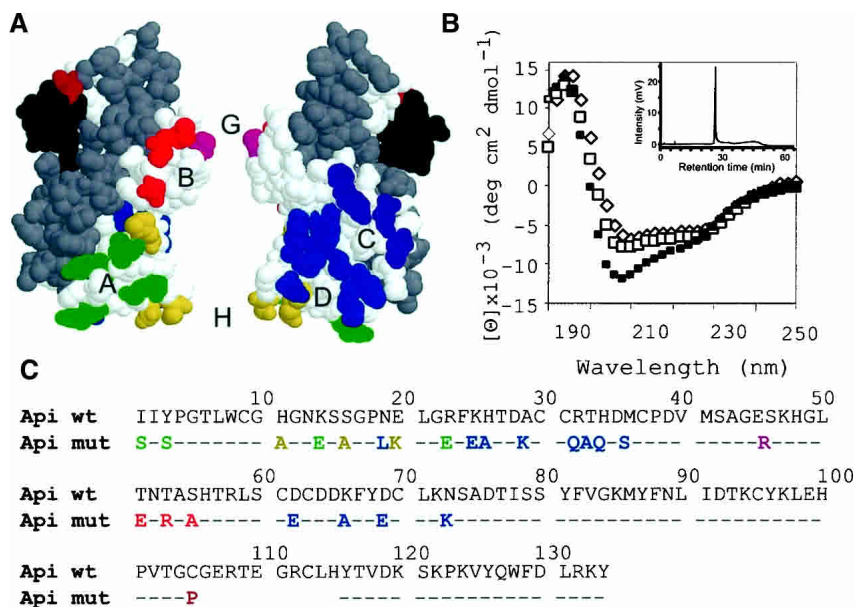


Figure 1. Mutations introduced in Api m 1 to generate Api mut. (A) Mutated residues are colored on the structure of Api m 1. Each letter and the corresponding color represent a single set of mutations separately introduced in Api m 1. In black is the deletion 106–115. In gray is the 81–106 region rich in T cell epitopes, which is not mutated. (B) Far UV CD spectra of Api mut (■), Api wt (□), and Api m 1 (◇) at 22°C. (Inset) HPLC profile of purified and refolded Api mut. (C) Alignment of the sequence of Api mut and Api m 1.

periments, IC_{50} values obtained for recombinant Api wt were equivalent to those obtained for Api m 1, although the N-terminal His-Tag was not cleaved from Api wt. Preliminary experiments showed that Api wt devoid of His-tag has the same pattern of recognition toward both IgE and IgG as Api m 1 and Api wt bearing the His-tag (data not shown). Thus, the His-tag was not removed from the mutants. Re-

Table 1. Capacity of the Api m 1 variants to inhibit m 1 recognition by IgE and IgG

Protein	IC_{50} (nM)	
	IgE	IgG
Api m 1	150 (\pm 40)	25 (\pm 8)
Api wt	250 (\pm 35)	20 (\pm 6)
Api AB	150 (\pm 15)	45 (\pm 7)
Api ABC	1500 (\pm 900)	450 (\pm 140)
Api ABD	2000 (\pm 0)	150 (\pm 280)
Api ABG	200 (\pm 35)	60 (\pm 15)
Api ABH	200 (\pm 300)	60 (\pm 40)
Api ABCDGH	2500 (\pm 1700)	1000 (\pm 700)
Api AB Δ 106–115	1500 (\pm 40)	450 (\pm 76)
Api mut	>10000	>1000
HEL	>10000	>1000

ELISA assays were performed on Api m 1 coated plates using a constant dilution of a pool of antisera from patients allergic to bee venom and a serial dilution of each mutant. Bound IgE and IgG were revealed with appropriate antihuman isotype antibodies. Data are expressed as IC_{50} and correspond to average and standard deviation values of three independent experiments.

activity for IgE of the three mutants Api AB, Api ABG, and Api ABH was very close to that of Api wt (Table 1). This reactivity was only slightly reduced for the mutants Api ABC, Api ABD, and Api AB Δ 106–115. Therefore, local alteration of the surface of the molecule does not affect recognition by antibodies. Strikingly, even Api ABCDGH, which contains 23 mutations, was almost as antigenic as the native molecule, with a loss of recognition of about 15-fold only. In sharp contrast, deletion 106–115 applied to this mutant, giving Api mut, completely abrogated antibody recognition. No reactivity was found at the maximal concentration of 10,000 nM, the difference being estimated as greater than a factor of 500. A very similar pattern of loss of recognition was seen for the specific IgG.

Then, using the same pool of sera, we investigated the IgE and IgG antigenicity of Api mut by a direct ELISA in which Api m 1, Api wt, Api mut, or HEL were coated on different ELISA plates (Fig. 2A,B). Almost no IgE-specific signal was detected on HEL ELISA plates, whereas a strong signal was observed on Api m 1 and Api wt ELISA plates, up to a serum dilution of 1/1600. In contrast, IgE reactivity for Api mut-coated plates was low at the minimal serum dilution of 1/100 and negligible below 1/400. IgG signals for Api m 1 and Api wt reached very high values although it was very weak to negligible for Api mut and not significant for HEL. We also compared by direct ELISA the reactivity of sera from nine different patients toward Api wt and Api mut (Fig. 2C,D). Eight out of nine sera showed a clear reactivity for Api wt whereas only three exhibited

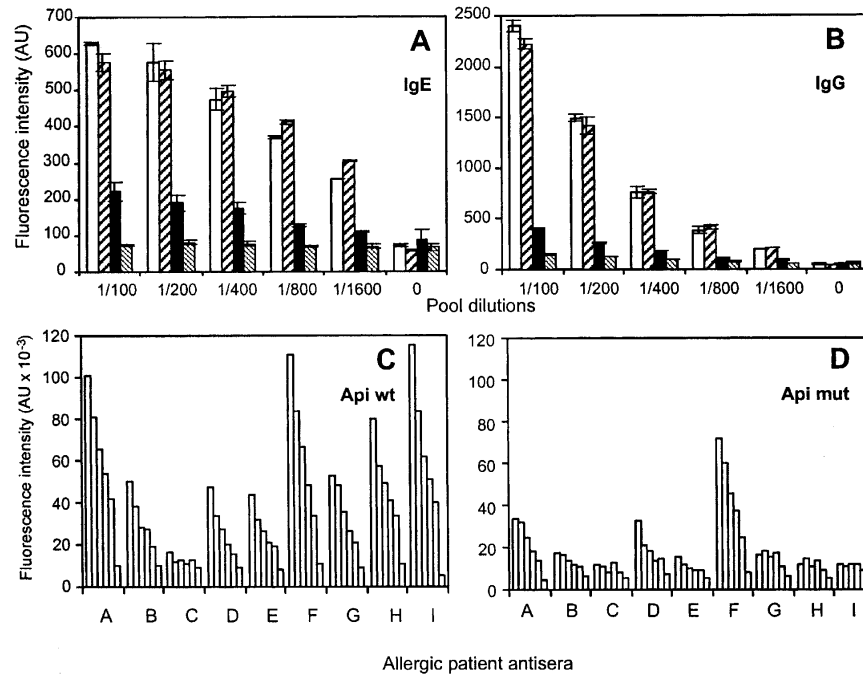


Figure 2. Recognition of Api mut by human IgE and IgG. (A,B) A pool was made with four antisera from patients allergic to bee venom (patients A, F, H, and I). Dilutions of the pool were assayed on ELISA plates coated with, from left to right, Api m 1 (white), Api wt (hatchings), Api mut (black), and HEL (small hatchings). Bound IgE and IgG were revealed with appropriate anti-human isotype antibodies in A and B, respectively. (C,D) Individual sera (designated by the letters A, B, C, D, E, F, G, H, I) were assayed at serial dilutions (1/100, 1/200, 1/400, 1/800, 1/1600, and none) on ELISA plates coated with (C) Api wt or (D) Api mut. Bound IgE were revealed as in A. Data from this figure are representative of two independent experiments.

some signal toward Api mut. However, these signals were weaker as compared to those obtained with Api wt ELISA plates. Overall, Api mut is much less antigenic than the Api wt allergen.

Specificity of antibodies induced by injection of Api mut in mice

We compared the specificity of antibodies induced by Api wt and Api mut injected into BALB/c mice. Figure 3A shows that antibodies raised against Api m 1 or Api wt preferentially reacted with ELISA plates coated with these antigens. Nine out of ten sera (five Api m 1 and five Api wt) showed no reactivity with ELISA plates coated with Api mut, whereas none reacted with HEL. Both Api mut and HEL induced a good specific antibody response, equivalent to that observed with Api m 1 and Api wt. However, Api mut-specific antibodies weakly recognized the wild type forms. Accordingly, competitive experiments performed with the Api mut-specific antisera (Fig. 3C) showed a dose dependent inhibition with Api mut whereas Api m 1, Api wt, and the negative control HEL were unable to inhibit the binding of the specific antibodies to adsorbed Api mut. In contrast, Api m 1 and Api wt efficiently competed with Api wt-specific antibodies (Fig. 3B), whereas Api mut and HEL

did not. Therefore, Api mut elicited antibodies that reacted much more weakly with the native molecule, as compared to Api m 1- and Api wt-specific antibodies. It is poorly antigenic for Api m 1- and Api wt-specific antisera as found previously for sera from allergic patients.

T cell reactivity of Api mut

BALB/c mice (Texier et al. 1999) as well as humans (Carballido et al. 1993) recognize a common T cell epitope in the P81–98 region of Api m 1, which is known to bind efficiently to I-E^d (Texier et al. 1999) and to multiple HLA-DR molecules (Texier et al. 2000, 2001). Considering this common epitope, we investigated the capacity of Api mut to be recognized by T lymphocytes specific for Api m 1 and to induce a specific T cell response. As shown in Figure 4, A and B, the T cell hybridomas 5G5.7 and 3A2.10 specific for the Api m 1 peptide 81–98 presented by I-E^d molecules (Texier et al. 1999) were stimulated by Api m 1, Api wt, Api RCM (a reduced form of Api m 1), Api mut, and the peptide 81–98, but not by HEL. Api mut appeared more efficiently presented to the T cells by 10-fold. In contrast, Api mut did not stimulate the 6A4.4 T cell hybridoma (Texier et al. 1999), which was specific for the Api m 1 peptide 21–38 (data not shown). This part of the molecule was indeed

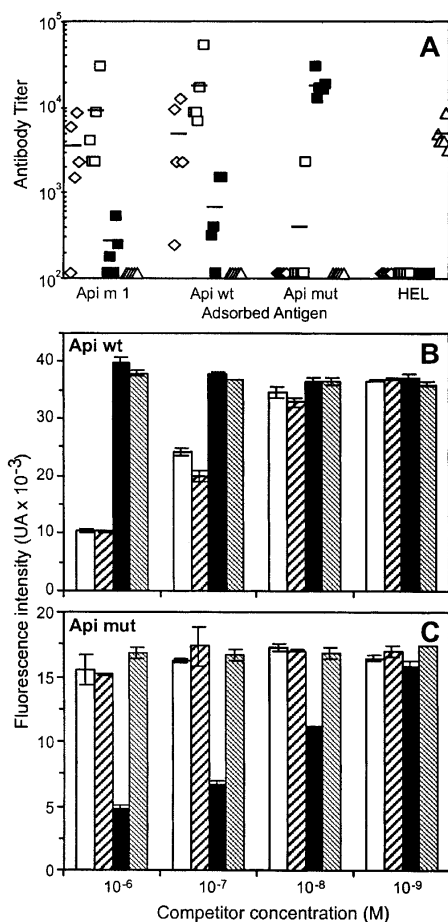


Figure 3. Antibody response against Api mut in BALB/c mice. (A) Specificity of sera from mice immunized with Api m 1 (\diamond), Api wt (\square), Api mut (\blacksquare), or HEL (\triangle) was investigated on the different antigens adsorbed on ELISA plates. Antibody titers were defined as the highest serum dilution corresponding to one-third of the maximal signal. Bars indicate the mean for each group of mice. (B,C) Antisera from mice immunized with (B) Api wt or (C) Api mut were incubated on (B) Api wt- or (C) Api mut-coated plates, respectively. Capacity of each protein, Api m 1 (white), Api wt (hatchings), Api mut (black), and HEL (small hatchings) to inhibit the signal was evaluated at different dilutions.

mutated at the positions 23, 25, 26, 29, 32, 33, 34, and 36, which evidently disturb the T cell stimulation. To compare the T cell response induced by Api wt and Api mut, we immunized BALB/c mice (H-2^d) with each protein. As described previously for Api m 1 (Texier et al. 1999), Api wt recruited *in vivo* T lymphocytes that were stimulated *in vitro* by Api wt but not by the negative control HEL (Fig. 4C). These cells were also activated by the peptide 81–98, by Api RCM and by Api mut. The latter was as efficient as Api wt to stimulate the T cells. The peptide 81–98 appeared less active and might be presented with a lesser efficiency as shown in T cell hybridomas experiments. Upon immunization with Api mut, a specific T cell response was raised against Api mut (Fig. 4D). These T cells were also stimu-

lated by Api wt, Api RCM, and the peptide 81–98, but with a lower efficiency. This indicates that Api mut recruited 81–98 specific T cells *in vivo*, which were specific for the native allergen. It seemed to induce also the activation of specific T cells that reacted exclusively with this modified form. Overall, the mutations and the deletion of the allergen did not prevent the presentation of Api mut to Api wt-specific T cells. Api mut shares with Api wt a common repertoire of specific T cells in BALB/c mice. In particular, these cells reacted with the peptide 81–98, which is a T cell epitope in humans (Carballido et al. 1993; Kammerer et al. 1997; Texier et al. 2002).

Spectroscopic analysis of Api mut

To understand the basis of the lack of antibody cross-reactivity between Api wt and Api mut, we have investigated the stability of the tertiary structure of both molecules as a function of temperature and GdnHCl concentration. Near-UV CD spectroscopy detects aromatic residues constrained in rigid chiral environments, revealing their embedding in tertiary structure (Adler et al. 1973). Any decrease of a near-UV CD signal reflects reduction in the order of tertiary structure. The spectra of Api wt at 10°C, 22°C, and 37°C are near identical and display a strong positive signal (Fig. 5A). This suggests that Api wt possess a rather rigid tertiary structure whatever the temperature studied. At 10°C and 22°C, the spectra of Api mut show signals of similar shape but of lower intensities. This indicates that below

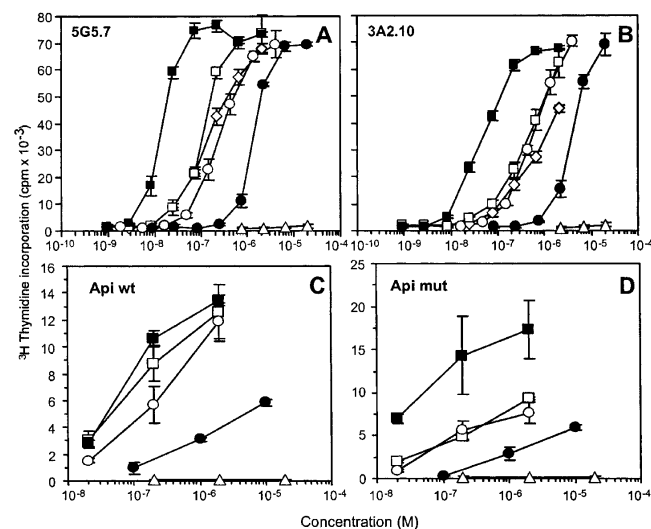


Figure 4. T cell reactivity of Api mut. (A,B) Api m 1 (\diamond), Api wt (\square), Api mut (\blacksquare), Api RCM (\circ), peptide 81–98 (\bullet), or HEL (\triangle) were incubated with specific T cell hybridomas (A) 5G5.7 and (B) 3A2.10 and APC (BALB/c splenocytes). Activation of T cell hybridomas was assessed by IL-2 secretion. (C,D) Spleen cells from BALB/c mice immunized with (C) Api wt and (D) Api mut were cultured in the presence of the different antigens described above. T cell activation was assessed by IL-2 secretion.

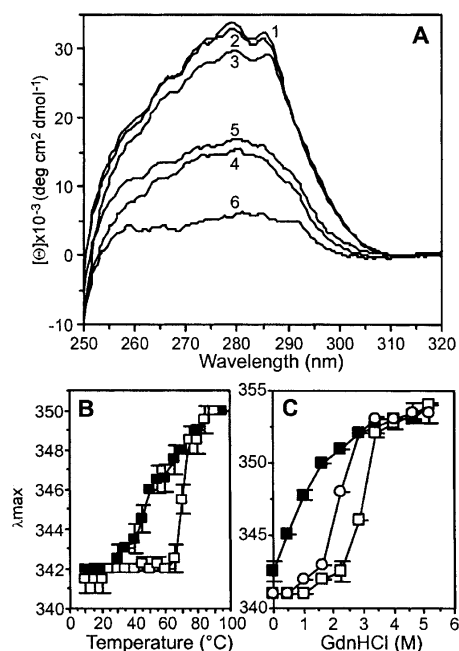


Figure 5. Stability of Api wt and Api mut. (A) Near UV CD spectra of Api wt at 10°C, 22°C, and 37°C (1, 2, 3) and Api mut at 10°C, 22°C, and 37°C (4, 5, 6). (B) Trp fluorescence of Api wt (□) and Api mut (■) as a function of temperature. (C) Trp fluorescence of Api wt (open symbols) and Api mut (closed symbols) as a function of GdnHCl concentration at 22°C (□, ■) and 37°C (○).

22°C Api mut has a tertiary fold related to that of Api wt, but its amino-acid side chains are less packed within the structure. At 37°C, the near UV CD signal vanishes, revealing that the tertiary structure of Api mut becomes highly flexible. It is noteworthy that although no tight tertiary structure is detected, the secondary structure of the protein remains native-like (Fig. 1; data not shown). To further analyze the difference in stability of both molecules, we have investigated their unfolding by temperature and GdnHCl by monitoring the intrinsic fluorescence of their tryptophans (Trp). The maximum emission wavelength (λ_{max}) of a Trp reflects the polarity of its environment. In the nonpolar core of a globular protein, λ_{max} is around 330 nm, whereas in a hydrophilic environment λ_{max} reaches 355 nm. Thus, any change in λ_{max} reveals a conformational change in a protein. Figure 5B shows the λ_{max} of Api wt and Api mut as a function of temperature. The λ_{max} of both proteins shifts from 342 nm to 350 nm upon denaturation. This suggests that the Trp are in a similar environment in both proteins below 25°C. However, the thermal unfolding curves are dramatically different. That of Api wt exhibits a sharp transition starting above 65°C, with a mid transition at about 70°C. In contrast, the denaturation of Api mut is progressive, starting above 25°C with a mid transition at about 50°C. A sharper slope and a higher mid-transition point indicate a stronger stability of the structure. Hence, the

results clearly indicate that Api mut is much less stable than Api wt. Accordingly, the unfolding of both proteins by GdnHCl at 10°C, 22°C, and 37°C lead to a similar conclusion (Fig. 5C; data not shown). Whatever the temperature, the denaturation of Api mut occurs at lower concentrations of GdnHCl than those required to unfold Api wt. These results further demonstrate that the mutations and the deletion introduced in Api mut decrease its stability.

Overall, Api mut is folded in physiological conditions as shown by far UV CD (Fig. 1) as well as Trp fluorescence (Fig. 5B), but it has a more dynamic and flexible tertiary structure than Api wt as shown by near UV CD and denaturation experiments (Fig. 5A,B).

Discussion

The globular structure of the major bee venom allergen Api m 1 (Scott et al. 1990) is organized in three helices from which protrude three loops of different sizes. Its recombinant form produced in *Escherichia coli* was found as allergenic as the wild-type molecule (Forster et al. 1995) in contrast to the denatured protein or internal fragments (Schneider et al. 1994; Texier et al. 2002). Therefore, IgE epitopes appear to be contained mainly in the polypeptide side chains (and not in the glycosylated moieties) and to be conformational (Schneider et al. 1994). In this study, the successive introduction of 24 mutations and one deletion of 10 amino acids in the C-terminal part of the molecule results in a progressive loss of recognition by specific antibodies. Surprisingly, however, no set of mutations induces any important loss of binding, although each set involves several residues and covers a significant surface of the molecule (Fig. 1; Table 1). Mutations C and D provoke the main effects whereas mutations AB, G, and H provoke no direct effect and seem to be less recognized. This contrasts with the results obtained using human monoclonal antibodies for which a dominant epitope is controlled by the residue Lys 25 (Dudler et al. 1994). In our assays this modification did not provoke a substantial effect. The reason for this discrepancy is probably that we used whole sera of allergic patients and not monoclonal antibodies. Binding of monoclonal antibodies could be dramatically affected by the mutation of a key residue in the recognition site. In contrast, the binding of a mixture of polyclonal antibodies with different epitope specificities is not affected by a single mutation, as most of the interacting surfaces remain unchanged. Therefore, our data strongly suggest that no immunodominant epitope exists for Api m 1. We also found that IgE and IgG recognition patterns were very similar, as the relative binding loss did not significantly differ between IgE and IgG experiments. Differences of IC₅₀ values in these experiments may result from either the difference of sensitivity of the assays or from the difference of affinity between IgG and IgE. These

observations strongly suggest that the B cell repertoire against Api m 1 is independent from the antibody isotype.

As the B cell epitopes of Api m 1 were essentially of the conformational type (Schneider et al. 1994; Texier et al. 2002), we investigated the secondary and tertiary structure of the molecules by CD and fluorescence spectroscopy. We observed that Api wt and Api mut shared a similar content of secondary structure (Fig. 1), whatever the temperature investigated. In contrast, at around 37°C, Api mut progressively loses a tight packing of the tertiary structure. Thus, it acquires precisely the structural features of the so-called molten globule state. As conformational epitopes generally involve amino acids located on separate strands of the molecule, the loss of recognition of Api mut by Api m 1-specific antibodies may not only result from the direct contribution of the changes of amino acids we have introduced, but also from the indirect effect of the mutations on the stability of the molecule. However, it is not possible to discriminate which of these two effects contributes the most to the loss of recognition of Api mut. Interestingly, such alterations of the tertiary structure by point mutations have been observed in molecules different from Api m 1, namely the bovine pancreas PLA2 (Yuan et al. 1999), the AB1-1 allergen of the nematode ascaris (McDermott et al. 2001), and IL-6 (Matthews et al. 2000). Although we destabilized the tertiary structure by extensively mutating the surface of the protein, we can speculate that introduction of a limited number of mutations at key positions could also affect the tertiary structure in a similar way. Such positions are expected to be mainly buried in the core of the molecule.

When designing the Api mut protein, we also aimed to maintain the specific T cell activity of the native molecule. A number of studies on allergic patients have shown that the two main stimulating regions are concentrated in the C-terminal half of this allergen (Carballido et al. 1993; Kammerer et al. 1997; Texier et al. 2002) and correspond to regions rich in HLA-DR binding sequences: 76–106 and 111–134 (Texier et al. 2000, 2001). Based on all the binding data, mutations were introduced outside the 76–106 and 116–134 regions. As the 81–98 human T cell epitope (Carballido et al. 1993; Kammerer et al. 1997) is also an epitope in BALB/c mice (Texier et al. 1999), we investigated its presentation to T cells in this mouse model and showed that Api mut and Api wt share a common repertoire of specific T cells. It is likely that this would be also the case for CD4⁺ lymphocytes from allergic patients.

Finally, the loss of recognition of Api mut by IgE and its inability to induce Api wt-specific antibodies may be sufficient to reduce the risk of anaphylactic shock, at least for the majority of the patients. Considering the number of side effects observed during immunotherapy of patients allergic to hymenoptera venom (around 15%) (Muller et al. 1992), this is the major property expected for a future treatment. As suggested by mice immunization experiments, Api mut

should not be able to elicit Api m 1 neutralizing antibodies in contrast to conventional immunotherapy. However, as the major region that contains the human T cell epitopes has been kept free of mutations, we expect that Api mut would be recognized by human T cells specific for Api wt. This property should contribute to the efficacy of the treatment as suggested by several authors (Smith and Chapman 1996; Takai et al. 1997; Ferreira et al. 1998; Muller et al. 1998; Okada et al. 1998; Smith et al. 1998; Vrtala et al. 2001; Oldfield et al. 2002; Swoboda et al. 2002). Recent works performed in humans with short peptides containing T cell epitopes have been found to provide some clinical benefits (Muller et al. 1998; Oldfield et al. 2002) although they probably did not elicit neutralizing antibodies. They seem to favor the appearance of IL-10-secreting T lymphocytes and hence a decrease of T cell activation and IgE secretion (Akdis and Blaser 1999). It is expected to be sufficient to efficiently reduce the allergic symptoms but this needs to be demonstrated in large clinical trials.

Materials and methods

Construction of the mutants

All PLA2 mutants were derived from the plasmid pQWT. This plasmid was constructed by insertion of a PLA2 synthetic gene in the expression vector pQE-9 (Qiagen) derived from plasmid pDS56/RBSII (Dudler et al. 1992). Mutants were obtained by site-directed mutagenesis using the kit (QuikChange, Stratagene) and cassettes insertion (Eurobio). The *E. coli* strain XL1-Blue (Novagen, VWR) was used for cloning.

Expression, purification and in vitro refolding of the recombinant proteins

Expression was done in the *E. coli* strain M 15 pREP4 (Qiagen) at 37°C. Bacteria were grown in Terrific Broth (Difco) supplemented with 0.4% glycerol (v/v), 200 mg/L ampicillin and 30 mg/L kanamycin. Protein expression was induced with 0.05 mM IPTG (Eurobio). Inclusion bodies were recovered from the bacteria and finally solubilized in 10 mL of denaturing buffer (7 M Gdn-HCl, 0.1 M NaH₂PO₄, 10 mM Tris-HCl at pH 8). Inclusion bodies were sulfonated by adding 0.3 M anhydrous Na₂SO₃ and 0.5 mL of Thannhauser reagent (Thannhauser et al. 1984) and dialysed twice against 500 mL of UNTI 10 buffer (8 M urea, 0.1 M NaH₂PO₄, 10 mM Tris-HCl at pH 8, 10 mM imidazole). The proteins were then purified by immobilized metal ion (Ni²⁺) affinity chromatography (IMAC) using a HiTrap Chelating Sepharose Fast Flow gel (Pharmacia) according to the manufacturer's instructions. The eluted proteins were precipitated overnight by dialysis against 5 mM acetic acid and the precipitate was extensively washed with water. The sulfonated protein was resuspended in the denaturing buffer GT (6 M Gdn-HCl, 0.1 M Tris-HCl at pH 8) at 0.5 mg/mL (A₂₈₀) and refolded by dialysis against 25 volumes of renaturation buffer (0.9 M GdnHCl, 0.1 M Tris-HCl at pH 8.3, 10% glycerol (v/v), 8 mM cysteine, 1 mM cystine) for 48 h at 4°C in the dark. The refolded, soluble proteins were separated from protein aggregates by centrifugation at 4500 rpm for 1 h at 4°C. The sample was dialysed for 24 h against 25 volumes of PG buffer (20 mM

Na_2HPO_4 at pH 8.5, 10% glycerol) at 4°C. The protein was then concentrated by ultrafiltration on an Amicon YM 10 kDa membrane (Amicon, Millipore) to a concentration of approximately 1.5 mg/mL. The concentrated protein was dialysed against 100 volumes of 20 mM Na_2HPO_4 at pH 8.5 to remove glycerol and was stored at -20°C. Approximately 100 mg/L of recombinant protein was recovered after purification and the refolding yield varies from 5% to 30%.

Elisa assays

Purified PLA2 from bee venom (Api m 1) (Sigma) was purified on a C_4 Vydac column using a water / acetonitrile / trifluoroacetic acid gradient to eliminate melittin. Sera from allergic patients were a kind gift of Prof. F. Leynadier (Hôpital Tenon, Paris). Female BALB/c mice were immunized subcutaneously with 1.6 nmole of Api wt or Api mut emulsified in CFA and with the same amount of peptide in IFA three weeks later. Mice were bled two weeks after the last injection and individual titers were measured by ELISA. Microtiter ELISA plates were coated overnight with a 63 nM solution of Api m 1, Api wt, Api mut, or HEL in adsorption buffer (0.1 M borate at pH 8.2) and then saturated with saturation buffer (0.1 M Tris-HCl at pH 7.5, 0.3% BSA, 0.003% thimerosal) for 5 h at room temperature. Before use, the plates were washed with washing buffer (10 mM Tris-HCl at pH 7.5, 0.05% Tween). All incubations were made at room temperature in dilution buffer (10 mM Tris-HCl at pH 7.4, 1 M NaCl, 0.05% Tween, 0.2% BSA, 0.003% thimerosal). Dilutions of a pool of sera from four allergic patients, or individual sera, were added with or without a competitor protein. After incubation for 2 h, bound specific IgE or IgG were detected by biotinylated anti-human IgE (1/250 final dilution; Southern Biotechnology Associates, Interschim), anti-human IgG (1/8000 final dilution; Southern Biotechnology Associates) or anti-mouse IgG (Sigma). Detection was done with a streptavidin phosphatase conjugate (1/2000 final dilution; Amersham) and 4-methylumbelliferyl phosphate substrate (Sigma) diluted in detection buffer (50 mM carbonate at pH 9.8, 1 mM MgCl_2). After 30 min of incubation, plates were analyzed on a spectrofluorimeter Fluorolite 1000 (Dyner) or Victor2 (Perkin Elmer) with an excitation at 365 nm and an emission at 450 nm.

Stimulation of murine T cells with the Api m 1 mutants

Api m 1-specific T cell hybridomas (5G5.7 and 3A2.9) are specific for the peptide 81–98/I-E^d combination (Texier et al. 1999). They were maintained in DMEM-supplemented medium (DMEM [Sigma], 10% FCS, 4 mM glutamine, 2 mM sodium pyruvate, 100 µg/mL gentamycin, 1% nonessential amino acids, 10 mM HEPES, 5×10^{-5} M 2-ME). T cell stimulating assays were performed in flat-bottomed 96-well tissue culture plates (Nunc) in a final volume of 0.2 mL. Hybridoma cells (5×10^4 cells) were mixed with splenocytes from BALB/c mice (5×10^5 cells) and appropriate concentrations of the proteins. Cultures were incubated 24 h at 37°C and supernatants were subsequently harvested and frozen. The supernatants were thawed and subjected to a CTLL assay. Briefly, IL2/IL4 dependent CTLL cells were cultured in RPMI-supplemented medium (RPMI 1640 [Sigma], 5% FCS, 2 mM glutamine, 10 mM HEPES, 100 µg/mL gentamycin, 5×10^{-5} M 2-ME). After extensive washing, 10^4 cells were distributed into each well containing 50 µL of supernatant prewarmed at 37°C and plates were placed in the CO₂ incubator for 24 h. Tritiated thymidine (1 µCi/well, 5 Ci/mole; Amersham) was added and the cells were harvested 6 h later by aspiration on

glass fiber filters. Incorporated thymidine was detected by scintillation counting using a β-counter (Perkin Elmer). T cell stimulation of bulk populations was performed as follows: Female BALB/c mice 8 to 12 wk old were immunized subcutaneously with 1.6 nmole of Api mut or Api wt emulsified in CFA and with the same quantity emulsified in IFA 15 d later. Twelve days later, spleen cells were suspended in RPMI-supplemented medium containing 1% of autologous normal mouse serum instead of FCS. They were plated at 10^6 cells/well in triplicate in 96-well microtiter plates (Nunc) with and without antigens. T cell stimulation was monitored by CTLL assay as described above.

CD spectropolarimetry and fluorescence spectroscopy

CD experiments were performed on a CD6 spectrodichrograph (Jobin-Yvon Instruments) as previously described (Chenal et al. 2002). Far-UV and near-UV CD spectra were measured in 0.5 mm and 10 mm path length quartz cells. Api wt and Api mut concentrations were 30 µM and 24.3 µM, respectively. The samples were buffered using 20 mM sodium phosphate (pH 8). Each far-UV and near-UV spectrum represents the average of 10 and 30 scans, respectively. Fluorescence measurements were performed with a FP-750 spectrofluorometer (Jasco) in a thermostated cell holder as previously described (Chenal et al. 2002). The protein concentration was 1.2 µM. Each maximal emission wavelength (λ_{max}) is the average calculated from three emission spectrum measurements.

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