

Molecular Dynamics of Major Allergens from *Alternaria*, Birch Pollen and Peach

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Abstract: In the search for factors that make a protein allergenic (an issue that remains so far elusive) some common features of allergens such as small size, high stability and lipid binding are recognized in spite of their structural diversity. Other relevant but still poorly understood feature is their capability to form homodimers. We investigated by means of Molecular Dynamics (MD) calculations the stability in solution of several dimers of three major allergens

from *Alternaria* mold, birch pollen, and peach fruit known to play essential roles in allergic disease. By running 20 ns MD simulations we found essential properties on solution that provide information of interest on their dimerization, stability of their epitopes and dynamical features of ligand binding cavities. Our results show that three essential allergen proteins display a distinct behavior on their trends to form homodimers in solution.

Keywords: Allergens · Epitopes · Molecular dynamics · Protein models · Protein structures

1 Introduction

Allergy is a health problem with about 25% of the population in developed countries affected. By the end of 2013, the Allergome database^[1,2] listed 2400 sources described as allergenic to humans and about 1000 allergens have been classified and assigned to a reduced number of protein families.^[3] Experimental structure is available for one hundred different allergen proteins which have been classified into 20 structural families.^[4] However, despite the increase of clinical, biological and chemical studies on allergy, the factors that make a protein allergenic remain elusive. Defining characteristic features of allergens is essential to discriminate the properties that distinguish them from structurally related innocuous proteins. Although known allergen proteins show structural diversity, some common features are recognized: most allergens are relatively small, rather stable and well-structured proteins^[4,5] and lipid binding is found for more than 50% of the major allergens.^[6]

Other relevant but still poorly understood feature of allergens is their capability to form homo-oligomers, particularly homodimers. A computational analysis of 55 allergen X-ray structures found that 80% of them can exist as symmetric dimers or oligomers in crystals.^[7] This report raised the suggestion that many of these molecular complexes aggregates could be transient dimers difficult to observe at normal concentrations in cells but becoming favored if concentration increases locally as it should occur on antigen-presenting cells or antigen-antibody complexes.^[7] We have recently extended the oligomerization analysis to 84 allergen X-ray structures using crystal information together with a new computational tool that uses evolutionary information to address dimer formation.^[8] Our study lowered the proportion of allergens with oligomeric states down to

66% although it still seems clear that oligomerization is a common feature of allergens.^[9]

While most studies to derive physicochemical or structural features of allergens have focused on their 3D structure, much less attention has been paid to their dynamic properties in solution. We address here the exploration of three major allergens from *Alternaria alternata* mold (Alt a 1), birch (*Betula verrucosa*) pollen (Bet v 1), and peach (*Prunus persica*) fruit (Pru p 3) by means of Molecular Dynamics (MD) simulations. The reasons why we chose these major allergens for our study are the following: (i) they are structurally different proteins that play roles in food allergy, a subject in which we have been working for years, (ii) they are major allergens that have been used as models in allergic disease, (iii) IgE-epitope regions have been reliably characterized in the three cases. *Alternaria* is one of the principal species associated with allergy, especially chronic asthma. Its major allergen, Alt a 1, is a protein with a unique dimeric β -barrel structure that appears to define a new protein family found in fungi with as yet unknown function.^[10] *Alternaria* infects a wide diversity of plants including fruits and recent results suggested the possibility that patients with chronic asthma could suffer from an al-

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lergic reaction when they eat contaminated fruit.^[11] The major birch pollen allergen Bet v 1 is the main elicitor of airborne allergies as 50–90% of patients affected by pollen allergies are sensitized to Bet v 1. Up to 90% of these patients also exhibit allergic cross-reactions to food allergens contained in fruits and vegetables. Bet v 1 is the most extensively studied allergen as it has been used as a model system in allergy (for a review see Reference^[12]). Experimental evidence on the existence of dimeric Bet v 1 has been very recently reported^[13] and its physiological ligand identified.^[12] The major peach allergen, Pru p 3, has been characterized as the major food allergen in the adult population in the Mediterranean area and it has been also used as a model system in food allergy.^[14,15] Pru p 3 is a prototypical member of nonspecific lipid transfer proteins (nsLTPs), a panallergen family widely distributed throughout plants with a particularly stable fold that explains their resistance to proteolysis.^[16] Although there is no evidence in vivo for Pru p 3 dimerization, the crystal structure of this protein is a dimer.^[17]

MD simulations on monomeric allergen proteins can be found in the literature (see for instance References^[16–23]) but these calculations have been customarily intended to assess the stability of ligand binding to allergens.^[17,18,20,22,23] However, to the best of our knowledge, no MD calculations for allergen dimers have been reported so far. In the current study we addressed the stability in solution of different oligomer structures of Alt a 1, Bet v 1, and Pru p 3 major allergens by means of 20 ns MD simulations. Besides addressing the stability of the different protein assemblies, we investigated also the structural flexibility of ligand binding sites in solution. A further relevant point in our MD calculations was the exploration of the dynamical stability of IgE-epitope regions identified before for these three allergens. We aimed thus to gain insight into the structure and dynamics properties of different oligomeric states in solution of representative proteins which play a major role in allergic diseases.

2 Computational Methods

2.1 Initial Geometries

The coordinates for initial geometries were the following. The crystal structure of Alt a 1, PDB entry 3 VOR,^[10] was used for the monomer. Dimer and tetramer geometries were generated with rotation matrices and translation vectors included in the PDB file. The dimer thus obtained shows two N-terminal cysteines at position 30 forming an intermolecular disulfide bridge. Whereas other four cysteines (residues 74, 89, 128, and 140) forming intramolecular disulfide bridges are conserved among all sequences of Alt a 1 homologs, Cys30 is only conserved among the closest homologs.^[10] For this reason, we decided to study a dimer with the constraint to keep the intermolecular disulfide bond along the MD simulation and a second dimer

leaving unbonded both Cys30 residues. Another crystal structure of an Alt a 1 variant, PDB entry 4AUD, already a dimer,^[24] was also used. This protein has a 97.6% sequence identity with 3 VOR but lacks six amino acids at the N-terminal segment that includes Cys30.

Three crystal structures of Bet v 1 differing in the amino acid at position 5 were used as initial geometries for monomers: PDB entry 1BV1 that is the protein with the genetically encoded tyrosine at position 5^[25] and PDB entries 4BK6 and 4BK7 that have cysteine and phenylalanine, respectively, at position 5.^[13] The dimer geometry constructed with the rotation matrix and translation vector given in the 1BV1 file displays the IgE epitope regions at the protein-protein interface (see below). Since allergens induce cross-linking of IgE antibodies bound to high affinity receptors on mast cells or basophil surfaces, one would expect that an allergen dimer that triggers cross-linking should have the antigenic regions located at opposite ends on the surface. For this reason, we used a second dimer predicted by the Evolutionary Protein-Protein Interface Classifier (EPPIC) approach^[8] that shows the proper orientation of epitopes (see below). A third Bet v 1 dimer corresponding to the dimeric structure of 4BK6 (cysteine at position 5) and showing proper orientation of epitopes was also utilized.

The crystal structure of Pru p 3, PDB entry 2ALG,^[17] was selected for the initial geometry of monomer. This PDB file has already two chains that were used for a first dimer of Pru p 3. Since this dimer has the IgE epitope regions near the protein-protein interface (see below), we used a second dimer predicted by EPPIC that has the proper orientation of epitopes at opposite ends of the protein surface. In addition, initial geometries for complexes of monomer and two dimers with palmitate were obtained upon superposition of palmitate molecule with the ligand in the 2ALG entry (laurate).

Protein–protein interfaces in all the dimers were analyzed with Protein Interfaces, Surfaces and Assemblies (PISA) software^[26,27] using the EBI-PISA server (www.ebi.ac.uk/msd-srv/prot_int/pistart.html) to compute interface areas as well as to determine residues and interactions (hydrogen bonds and salt bridges) in the interfaces.

2.2 Molecular Dynamics Calculations

MD calculations using the CHARMM 3.1 force field^[28] were performed with NAMD 2.9.^[29] Initial structures were immersed in periodic rectangular solvation boxes with water molecules added according to the TIP3P model^[30] together with Na⁺ and Cl[−] ions added to counter the total charge of the protein systems while providing 0.150 M salt concentration. The particle mesh Ewald summation method^[31] was used for long-range electrostatics and a 10 Å cutoff was set for short-range non-bonded interactions. The following set of calculations were performed for every system: (1) first optimization along 5000 conjugate gradient minimization steps; (2) equilibration of water for 100 ps at 2 fs time

steps, 298 K and 1 atm with all atoms fixed in proteins; (3) keeping same time step, T and P, simulation runs during 20 ns (10 million steps) in the NPT ensemble. Langevin dynamics for the T control and Nosé-Hoover Langevin piston method for the P control were employed. Output results were stored every 5000 steps rendering thus trajectories composed of 2000 frames which were processed and analyzed with VMD 1.9.1,^[32] Carma,^[33] and MDTRA^[34] programs. Molecular graphics were prepared and rendered with PyMOL 1.6.^[35]

3 Results and Discussion

Given that the aggregation states of the three allergens studied amount to a total of 17 protein systems, we resorted to a limited length of MD simulations in explicit solvent (20 ns) in order to get the total computational burden within affordable limits. Before proceeding further, we firstly assessed that this simulation time was adequate for our goals by testing in the main dimers of Alt a 1, Bet v 1, and Pru p 3 proteins (see below) that the trajectories provided stabilized values of the properties analyzed.

Data on protein-protein interfaces of the dimers investigated were obtained at three frames of MD simulations: initial (time = 0), middle (time = 10 ns), and final (time = 20 ns) structures. This information is summarized in Table 1 where we present interface areas, number and types of residues in the interface and number of intermolecular hydrogen bonds and salt bridges at the interface. Detailed data on the properties gathered in Table 1 are given in the Supporting Information where all the interacting residues, hydrogen bonds and salt bridges are specified in Tables S1 and S2 (Supporting Information). Root mean square fluctuations (RMSF) per residue computed in the MD simulations for all the aggregation states are shown in Figure 1. In what follows, MD results for every allergen together with their cor-

responding data in Table 1 and Figure 1 are presented and discussed separately.

3.1 Alt a 1 Allergen

Figure 2 displays initial geometries for monomer, dimer, and tetramer states of Alt a 1 protein from the crystal structure in PDB entry 3 V0R^[10] as well as the dimer in PDB entry 4AUD.^[24] This latter protein is a variant lacking the N-terminal tail that contains Cys30. In dimeric Alt a 1, this residue can be covalently linked to the equivalent residue from the second chain (Figure 2A). While it has been claimed that dimer stabilization should be due to this presumed disulfide linkage,^[10,36] mass spectra obtained under denaturing solution conditions have provided evidence that Alt a 1 dimerization is not mediated by the disulfide bond.^[7] It must be also noted that whereas four cysteines that make intramolecular disulfide bonds (residues 74, 89, 128, and 140) are conserved among all the Alt a 1 homologs, Cys30 is only conserved among the closest homologs. We studied (i) the dimer with the intermolecular disulfide bond kept along the MD simulation ("dimer SSbridge" in what follows), (ii) the equivalent dimer without that link (named just "dimer") and (iii) the dimer in PDB entry 4AUD that lacks six N-terminal amino acids including residue 30 (named "dimer (4AUD)"). Results are shown in Figure 2E–2H and Table 2.

The rather small changes in radii of gyration R_g (Figure 2E) indicate that protein size is steadily maintained over the simulation time, particularly for the three dimers that show small standard deviations about 0.1 Å, half those of monomer and tetramer (Table 2). While "dimer" and "dimer SSbridge" show virtually indistinguishable R_g values along the simulation time, "dimer (4AUD)" has a slightly greater size which in turn could indicate a slightly weaker interaction between monomers. This is confirmed by interface data in Table 1. Whereas "dimer" and "dimerSSbridge"

Table 1. Interface areas (Å²), number of residues, hydrogen bonds (HB) and salt bridges (SB) in protein-protein interfaces of dimers of Alt a 1, Bet v 1, and Pru p 3 allergen proteins at three frames of MD 20-ns simulations.

Dimer	Initial structure, time = 0				Middle structure, time = 10 ns				Final structure, time = 20 ns			
	Area	Residues[a]	HB	SB	Area	Residues[a]	HB	SB	Area	Residues[a]	HB	SB
Alt a 1 dimer	1361	39 (8c 12p 19np)	9	0	1590	45 (10c 14p 21np)	14	0	1333	41 (8c 13p 20np)	10	0
Alt a 1 dimer SSbridge	1361	39 (8c 12p 19np)	8	0	1427	41 (9c 12p 20np)	12	0	1393	41 (10c 11p 20np)	9	0
Alt a 1 dimer 4AUD	770.3	24 (5c 7p 12np)	1	0	852.0	28 (6c 8p 14np)	3	6	914.8	30 (7c 8p 15np)	4	8
Bet v 1 dimer1 Y5	822.0	27 (10c 7p 10np)	3	0	417.9	17 (7c 4p 6np)	4	0	312.2	16 (6c 3p 7np)	1	2
Bet v 1 dimer2 Y5	443.2	12 (6c 2p 4np)	3	1	388.1	12 (6c 2p 4np)	5	2	394.7	11 (4c 3p 4np)	5	2
Bet v 1 dimer C5	510.2	17 (8c 3p 6np)	6	2	650.9	18 (9c 2p 7np)	7	2	683.5	22 (12c 3p 7np)	6	3
Pru p 3 dimer1	453.8	16 (3c 7p 6np)	10	0	484.7	17 (3c 7p 7np)	13	0	469.1	17 (3c 7p 7np)	10	0
Pru p 3 dimer1 PLM	453.8	17 (3c 7p 7np)	10	0	432.5	19 (3c 7p 9np)	12	0	494.8	18 (3c 7p 8np)	12	0
Pru p 3 dimer2	330.6	11 (1c 4p 6np)	3	0	389.3	14 (1c 3p 10np)	2	0	371.8	13 (1c 4p 8np)	5	0
Pru p 3 dimer2 PLM	330.6	11 (1c 4p 6np)	3	0	114.2	8 (2c 2p 4np)	0	0	141.7	8 (3c 1p 4np)	0	4

[a] Number of: c = charged, p = polar, np = nonpolar residues in parentheses

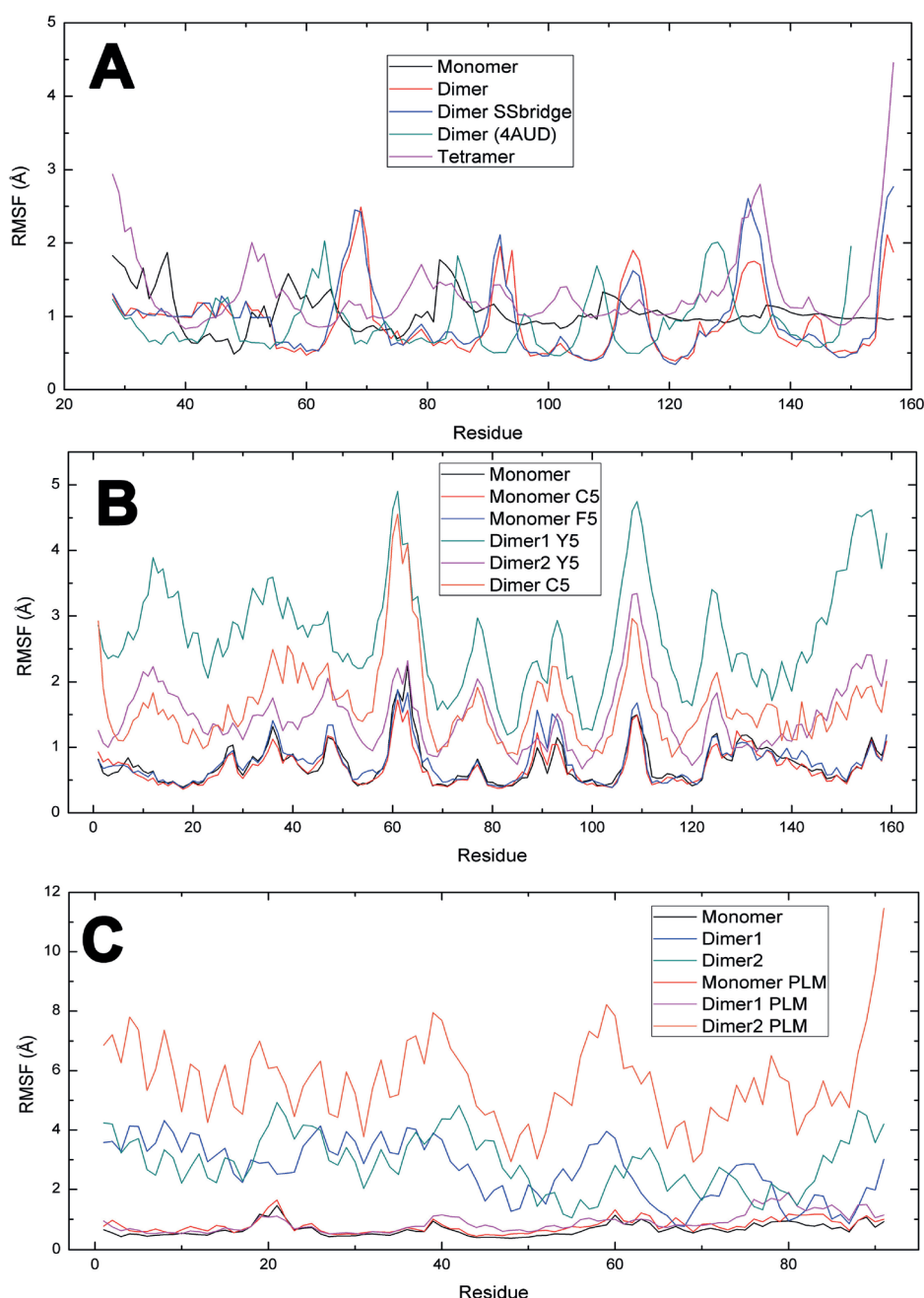


Figure 1. RMSF per residue of α carbons obtained in 20 ns MD simulations for oligomer states of (A) Alt a 1, (B) Bet v 1, and (C) Pru p 3 proteins.

have rather similar interface areas, numbers of interacting residues, and intermonomer hydrogen bonds, "dimer (4AUD)" shows much smaller values of these properties. However, the latter dimer presents a high number of salt bridges. The lack of six N-terminal residues in the 4AUD variant allows for a close proximity of two acidic (E35 and D37, 3 VOR numbering) and one basic (R103) residues in one chain and the counterpart residues in the other chain which permits formation of salt bridges (see Table S1 in the Supporting Information). As a consequence, interface area

and number of residues making contacts in the interface increase in "dimer (4AUD)" along the MD simulation although they remain below those of "dimer" or "dimerSS-bridge" (Table 1).

Unlike other proteins with a β -barrel fold or other major allergens such as Bet v 1 or Pru p 3, Alt a 1 lacks a clear binding cavity as its β -barrel core seems too narrow to harbor ligands. It has been speculated that the binding of a ligand might induce conformational changes resulting in the opening of the β -barrel or that in solution Alt a 1 had

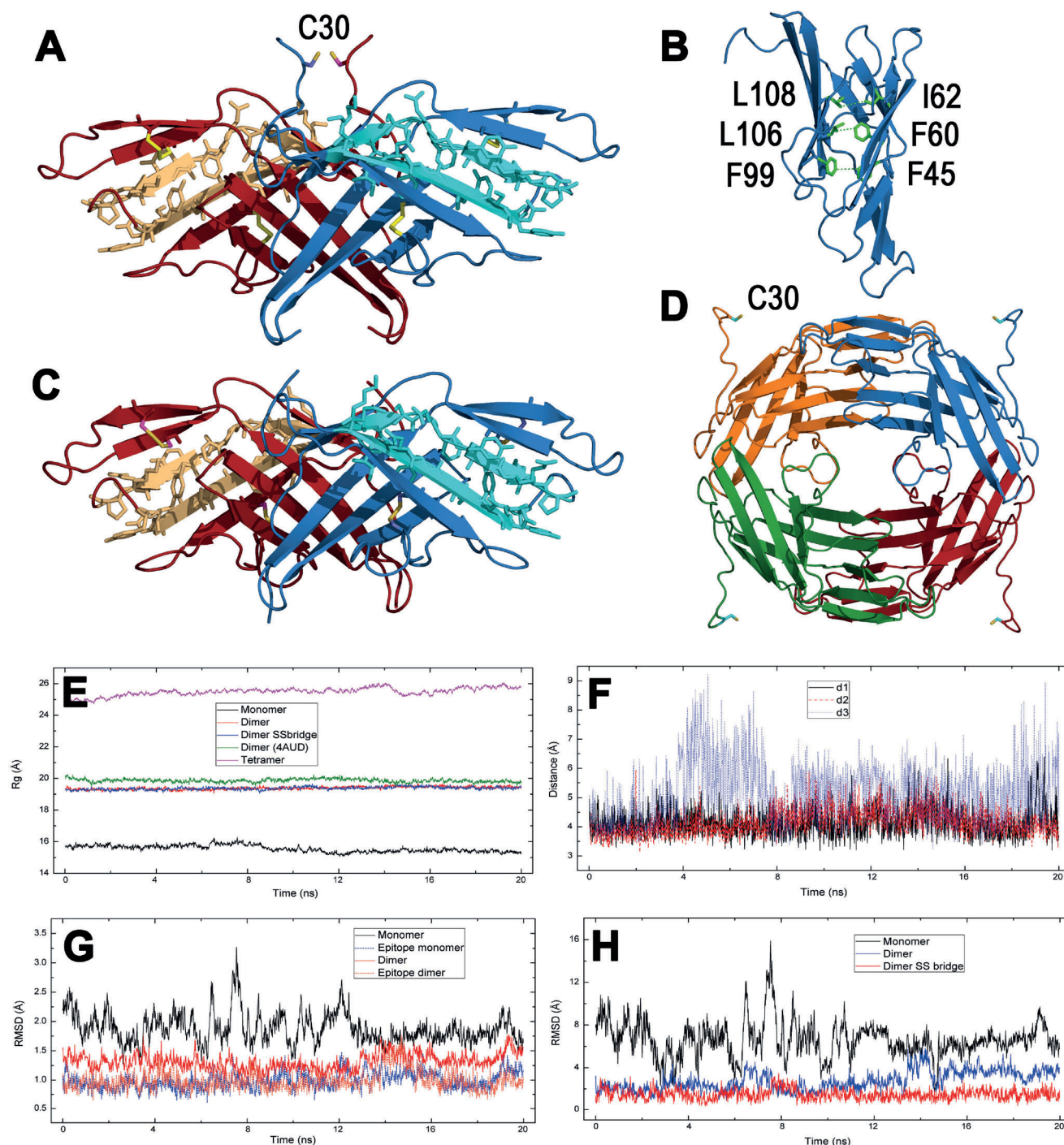


Figure 2. Oligomer states of Alt a 1 protein. (A) "Dimer": dimer of protein with PDB code 3 V0R. C30 and epitope residues shown as sticks. (B) Monomer with residues in the inner side of the β -barrel defining distances d1 (F45–F99), d2 (F60–L106), and d3 (I62–L108) shown as green sticks. (C) "Dimer (4AUD)": dimer of protein with PDB code 4AUD. Epitope residues shown as sticks. (D) Tetramer of protein 3 V0R. C30 residues shown as sticks. (E–H) Results of 20 ns MD simulations. (E) Radius of gyration. (F) Interatomic distances d1, d2, and d3 inside the β -barrel in the monomer. (G) *RMSD* of non-hydrogen atoms of proteins and epitope regions of Alt a 1 monomer and dimer. (H) *RMSD* of non-hydrogen atoms of C30 in monomer and dimers without (blue) and with (red) intermolecular disulfide bridge between C30 residues.

an internal cavity.^[10] We explored these possibilities by computing three distances within the β -barrel (two at the ends and one at the center, Figure 2B) along the MD simu-

lations. These interatomic distances show consistent values among aggregation states (Table 2). End distance d1 is between 4.1 and 4.9 Å with standard deviations ~ 0.5 Å, cen-

Table 2. Average values of *RMSD* of all non-hydrogen atoms, selected interatomic distances, and radius of gyration (R_g) for different assemblies of Alt a 1 allergen protein obtained in MD 20 ns simulations. Standard deviations in parentheses. All values in Å.

	<i>RMSD</i>			Interatomic distances inside the barrel [a]			
	Protein	Epitope [b]	C30 residue [c]	d1	d2	d3	R_g [d]
Monomer	1.880 (0.259)	0.987 (0.146)	6.620 (1.676)	4.136 (0.463)	4.156 (0.447)	5.125 (1.024)	15.56 (0.211)
Dimer chain A	1.317 (0.134)	0.984 (0.162)	2.748 (0.932)	4.351 (0.481)	3.978 (0.324)	4.563 (0.644)	19.39 (0.096)
Dimer chain B	1.265 (0.165)	0.880 (0.155)	2.676 (0.817)	4.065 (0.446)	3.853 (0.337)	4.769 (0.716)	
Dimer SSbr ch A	1.272 (0.145)	0.978 (0.200)	1.469 (0.508)	4.327 (0.473)	3.839 (0.303)	4.756 (1.028)	19.37 (0.095)
Dimer SSbr ch B	1.360 (0.153)	0.963 (0.153)	1.422 (0.483)	4.395 (0.444)	3.941 (0.315)	4.579 (0.769)	
Dimer (4AUD) ch A	1.470 (0.141)	1.069 (0.227)	–	4.815 (0.714)	4.101 (0.367)	3.894 (0.360)	19.86 (0.110)
Dimer (4AUD) ch B	1.314 (0.140)	1.040 (0.177)	–	4.905 (0.686)	4.193 (0.393)	4.126 (0.429)	
Tetramer chain A	1.964 (0.301)	–	6.112 (1.811)	4.187 (0.482)	3.957 (0.349)	5.067 (1.085)	25.49 (0.258)
Tetramer chain B	1.545 (0.278)	–	5.192 (1.867)	4.067 (0.445)	3.855 (0.294)	4.687 (0.799)	
Tetramer chain C	1.672 (0.381)	–	5.096 (2.134)	4.253 (0.432)	3.891 (0.297)	4.625 (0.694)	
Tetramer chain D	1.842 (0.351)	–	5.058 (2.294)	4.076 (0.469)	3.773 (0.256)	4.539 (0.539)	

[a] d1 = Cε1(F45)–Cζ(F99) distance, d2 = Cζ(F60)–Cδ1(L106) distance, d3 = Cδ(I62)–Cδ2(L108) distance: see Figure 2B. [b] Epitope *RMSD* not computed for the tetramer. [c] Alt a 1 protein with PDB code 4AUD lacks this residue. [d] Values refer to the whole molecular assembly.

tral distance d2 is between 3.8 and 4.2 Å with standard deviations ~0.3 Å, and end distance d3 is between 3.9 and 5.1 Å with greater standard deviations between 0.5 and 1.1 Å. As indicated by the latter deviations, d3 has much larger oscillations than d1 or d2 (Figure 2F), which suggests a greater opening of the β-barrel end corresponding to the I62–L108 pair at the cup-like region of the protein (Figure 2B). However, the overall magnitude of these distances (Table 2) along simulation time suggests that it is unlikely that in solution the cavity inside the β-barrel could harbor a ligand in any of the molecular aggregates analyzed.

Compared to other proteins, allergens are highly structured^[4–6] and it is also known that in contrast to IgG, IgE binds mainly to structured proteins^[4]. Therefore, one would expect IgE epitopes to locate in structured regions of allergens. Epitope location in β-strands^[10,36] (Figure 2) and our MD results show that this is indeed the case for Alt a 1. Average values of root means square deviations (*RMSDs*) and their standard deviations (Table 2) reveal a consistently smaller motion of epitope regions than the proteins as a whole along simulation time (Figure 2G). This is even more apparent in the monomer where the epitope region has an average *RMSD* about half that of the protein (Table 2, Figure 2G). The smaller *RMSD* values of epitope regions with respect to protein also holds for the dimer formed by the variant (“dimer (4AUD)” data in Table 2). The IgE epitope identified in Alt a 1 spans the sequence segments 41–50 and 54–63.^[36] The *RMSF* per residue plot (Figure 1A) shows that the regions covered by these residues display small fluctuations particularly in “dimer” and “dimerSSbridge” which would represent the preferred state for IgE cross-linking. The four regions showing large *RMSF* peaks in these dimers (sequence segments 64–70, 90–94, 112–116, and 131–136, Figure 1A) correspond to loops joining adjacent β strands that locate far from the protein-protein interface, specially the 131–136 region which is the farthest loop from the interface (horizontal extreme ends in Figure 2A). In contrast, all residues in tetrameric Alt

a 1 show overall larger fluctuations that indicate a greater mobility with respect to the remaining aggregation states. This is in agreement with the behavior revealed by the greater *RMSD* values (Table 2) and the larger oscillations of R_g in the tetramer (Figure 2E). Nevertheless, considering even the peaks associated to mobile loops in dimers or tetramers, the small *RMSF* values (Figure 1A) reveal a significant structural stability for the aggregation states of Alt a 1.

On the other side, the overall smaller *RMSD* values in dimers than in monomer or tetramer (Table 2) indicate that dimers are significantly more rigid in solution. Interestingly, the dimer with the disulfide bond between Cys30 residues (“dimer SSbr” data) is not more rigid than the dimer without this linkage (“dimer” data) a result that should confirm the earlier suggestion that Alt a 1 dimerization is not mediated by the disulfide bond.^[7] Even though Cys30 shows an obviously greater motion in the dimer without its disulfide bond (*RMSD* ~2.7 Å) than in the dimer with it (*RMSD* ~1.4 Å), this residue is much more flexible in monomer (*RMSD* = 6.6 Å) or in tetramer (*RMSD* > 5.1 Å) states, a result that indicates a far greater conformational freedom of the N-terminal tail in monomer or tetramer than in dimers (Figs. 2A–2D): the oscillations of *RMSD* for Cys30 are much greater in the monomer than in the dimers regardless disulfide bond formation (Figure 2H).

3.2 Bet v 1 Allergen

Although birch pollen Bet v 1 is probably the best characterized allergen as it has been used as a model system in research on allergy, the allergenic significance of its dimerization has been controversial (see reviews in References^[12,13]). Very recently, a combination of crystallographic, mass spectrometric, and immunological methods has provided evidence on dimeric Bet v 1 having a non-canonical incorporation of cysteine at position 5 instead of genetically encoded tyrosine.^[13] In addition, a crystal structure for a variant with phenylalanine at position 5 was also report-

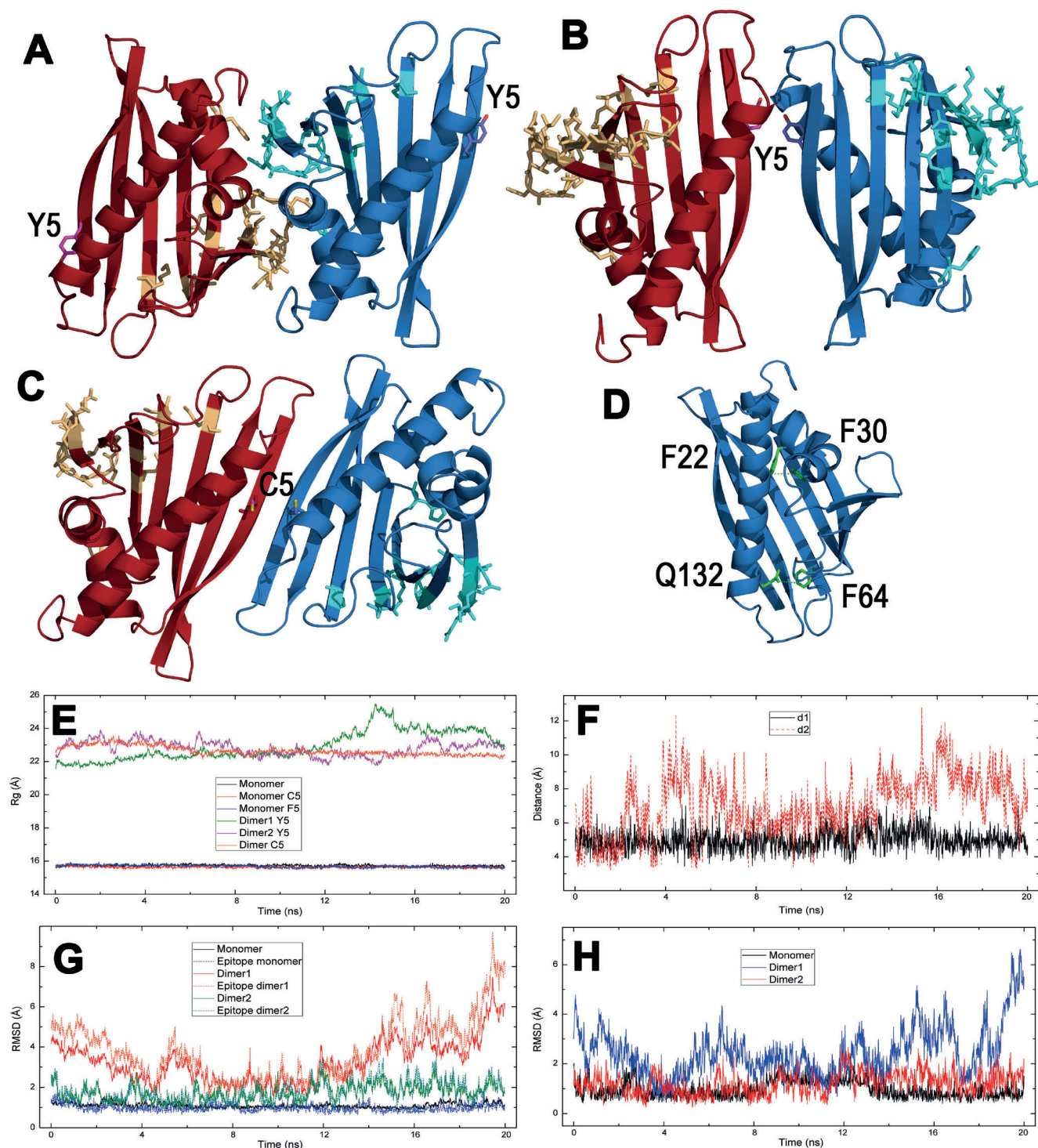


Figure 3. Dimers and monomer of Bet v 1 protein. (A) "Dimer1 Y5": crystallographic dimer of protein with PDB code 1BV1. Y5 and epitope residues shown as sticks. (B) "Dimer2 Y5": same as (A) for the EPPIC-predicted dimer. (C) "Dimer C5": crystallographic dimer of protein with PDB code 4BK6. C5 and epitope residues shown as sticks. (D) "Monomer": canonical monomer with residues inside the cavity defining distances d1 (F22–F30) and d2 (F64–Q132) shown as green sticks. (E–H). Results of 20 ns MD simulations. (E) Radius of gyration. (F) Interatomic distances d1 and d2 inside the cavity in the monomer. (G) *RMSD* of non-hydrogen atoms of proteins and epitope regions of Bet v 1 monomer and dimers. (H) *RMSD* of non-hydrogen atoms of Y5.

ed. Based upon a number of experimental results, the authors proposed that residue 5 should play a relevant role in

the allergenic properties of this protein.^[13] Figure 3 displays initial geometries for two dimers of canonical Bet v 1 pro-

tein (PDB code 1BV1), the crystallographic one ("dimer1 Y5" in what follows, Figure 3A) and that predicted by EPPIC^[8] on evolutionary data grounds ("dimer2 Y5", Figure 3B), the non-canonical dimer ("dimer C5", PDB code 4BK6^[13], Figure 3C) and the canonical monomer ("monomer", Figure 3D). Residues corresponding to the reported IgE epitope of Bet v 1^[25] are located near the protein-protein interface in "dimer1 Y5" whereas they are properly located for cross-linking of IgE antibodies in both "dimer2 Y5" and "dimer C5" (Figures 3A–3C). MD results for these dimers together with "monomer" and non-canonical monomers with cysteine ("monomer C5") and phenylalanine ("monomer F5") at position 5 are presented in Table 3 and Figures 3E–3H.

Changes in radii of gyration R_g for the three monomers are indistinguishable (average $R_g = 15.7$ Å with standard deviations below 0.1 Å) but clear differences are found for the three dimers, not in their average R_g values between 22.7 and 22.9 Å but in their oscillations (Figure 3E) reflected into their large standard deviations (Table 3). Only non-canonical "dimer C5" shows a steady behavior while both "dimer1 Y5" and "dimer2 Y5" display large oscillations of R_g that suggest floppy structures, particularly "dimer1 Y5" which has a standard deviation ~ 1 Å. These results point to a stronger inter-monomer attraction in the structure of "dimer C5" than in both "dimer1 Y5" and "dimer2 Y5" structures. Interface data in Table 1 confirm this point. Although the initial structure of the crystallographic "dimer1 Y5" has a greater interface area than "dimer2 Y5" and "dimer C5", this latter dimer increases its interface area and number of interacting residues, hydrogens bonds and salt bridges as the simulation progresses (Table 1). Motion in solution brings together side chains of C5 residues in the two protein chains and "dimer C5" becomes linked by an intermolecular disulfide bridge that forms at about 5 ns (Figure 3E). In contrast, many intermolecular contacts are broken along the simulation in the crystallographic "dimer1 Y5" and the interface area at the final structure is only 38% of the area at the initial structure (Table 1). The EPPIC-predicted "dimer2 Y5" shows a proper location for epitope residues

but it has a geometry with a smaller protein-protein interface (Figure 3B) which in turn involves less intermolecular contacts than in crystallographic dimers. However, the formation of five hydrogen bonds and two salt bridges (Table 1 and Table S1 in the Supporting Information) along the simulation gives an increased stability to "dimer2 Y5" with respect to "dimer1 Y5".

Bet v 1 has a huge hydrophobic cavity that runs parallel to the large α -helix (Figure 3D). This protein is known to bind a great diversity of ligands in vitro such as flavonoids and fatty acids^[37,38], and quercetin-3-O-sophoroside has been recently identified as its natural ligand.^[12] With the aim to assess the flexibility of this pocket in solution, we computed two interatomic distances at both ends of the cavity (Figure 3D) along the MD simulation (Figure 3F). The four residues chosen to monitor both distances are involved in binding different ligands within the hydrophobic pocket.^[38] The innermost distance d1 corresponding to the F22-F30 pair has values between 4.8 and 5.4 Å with standard deviations between 0.4 and 0.8 Å. The outermost distance d2 corresponding to the F64-Q132 pair shows much greater values between 7.0 and 8.1 Å and larger standard deviations between 1.3 and 2.2 Å (Table 3). While d1 remains steady over simulation time, d2 shows large oscillations (Figure 3F) suggesting a greater flexibility in the outer end of the cavity. In agreement with available evidence, both distances show values compatible with binding ligands of moderate-large size.^[37,38] However, our MD results suggest that an end of the ligands should remain anchored at the narrower inner end of the cavity (d1 distance) while being permitted by the protein structure to move at the opposite end (d2 distance). It must be noted that F30 has been identified as a key residue in the coordination of distinct ligands.^[38] Our results also show that cavity properties in the dimers are comparable to those of monomeric forms (Table 3).

In agreement with that anticipated by R_g values and confirmed by *RMSF* plots (Figure 1B), the low average *RMSDs* ~ 1 Å and their small standard deviations ~ 0.1 Å reveal a very low mobility in the three monomers. Residues in

Table 3. Average values of *RMSD* of all non-hydrogen atoms, selected interatomic distances, and radius of gyration (R_g) for different assemblies of Bet v 1 allergen protein obtained in MD 20 ns simulations. Standard deviations in parentheses. All values in Å.

	RMSD			Interatomic distances inside cavity [a]		
	Protein	Epitope	Residue 5	d1	d2	Rg[b]
Monomer	1.103 (0.120)	0.991 (0.185)	0.964 (0.349)	4.967 (0.594)	7.005 (1.735)	15.73 (0.060)
Monomer C5	1.036 (0.120)	1.190 (0.179)	0.908 (0.346)	4.767 (0.422)	7.055 (1.846)	15.66 (0.055)
Monomer F5	1.103 (0.106)	1.138 (0.207)	1.336 (0.840)	5.033 (0.526)	7.817 (1.760)	15.68 (0.078)
Dimer1 Y5 chain A	3.237 (1.032)	3.809 (1.490)	2.439 (1.007)	5.268 (0.775)	8.114 (1.841)	22.94 (0.887)
Dimer1 Y5 chain B	2.790 (0.932)	2.973 (0.960)	2.240 (0.838)	4.881 (0.622)	7.416 (1.819)	
Dimer2 Y5 chain A	1.736 (0.395)	1.792 (0.477)	1.199 (0.432)	5.386 (0.703)	7.202 (2.120)	22.80 (0.437)
Dimer2 Y5 chain B	1.646 (0.405)	1.760 (0.436)	1.176 (0.453)	4.907 (0.473)	8.030 (1.683)	
Dimer C5 chain A	1.702 (0.446)	1.733 (0.504)	0.963 (0.462)	4.780 (0.531)	7.645 (1.264)	22.66 (0.279)
Dimer C5 chain B	1.872 (0.476)	1.993 (0.525)	1.048 (0.409)	5.067 (0.551)	8.054 (2.217)	

[a] d1 = C ζ (F22)–C ζ (F30) distance, d2 = C ζ (F64)–O ϵ 1(Q132) distance: see Figure 3D. [b] Values refer to the whole molecular assembly.

them exhibit *RMSF* values below 1 Å with the exception of sequence segments 59–64, 90–93, and 107–109 that correspond to loops joining adjacent β strands. In the dimers, these loops happen to locate at positions far from the dimerization interface (loops at vertical extreme ends in Figures 3A–3C) and hence they show also *RMSF* peaks in the dimers though with far greater values than in monomers (Figure 1B). The main IgE epitope identified in Bet v 1 is composed of residues 42–52 plus 70, 72, 76, 86, and 97.^[25] In contrast to Alt a 1, *RMSD* values for epitope residues are now similar to those of whole proteins although with slightly greater standard deviations ~ 0.2 Å (Table 3). A common feature is that epitope residues are located in regions with low structural fluctuations (Figure 1B). Both *RMSD* changes (Figure 3G) and *RMSF* values (Figure 1B) reveal a greater structural mobility for the crystallographic “dimer1 Y5” and much lower for the EPPIC-predicted “dimer2 Y5” in agreement with the discussion on protein-protein interfaces above. Interestingly, although some epitope residues locate on loop regions (Figure 3B and 3C), the dimers with proper orientation of epitopes (“dimer2 Y5” and “dimer C5”) have average *RMSDs* similar for proteins and epitopes (Table 3): “dimer2” and “epitope dimer2” plots in Figure 3G are indistinguishable. On the contrary, the dimer with epitopes located near the protein-protein interface (“dimer1 Y5”) has greater *RMSDs* and larger oscillations of epitope residues (Figure 3G). Given the location of these residues in this dimer and its evolution along the simulation discussed above with regard to protein-protein interfaces, this is another result associated to the greater instability of “dimer1 Y5”.

Finally, residue at position 5 in monomers has different features when it is tyrosine or cysteine (*RMSD* < 1.0 Å, 0.35 Å standard deviation, Table 3) and when it is phenylalanine (*RMSD* = 1.34 Å, 0.84 Å standard deviation). In dimers with properly oriented epitopes (“dimer2 Y5” and “dimer C5”), the residue at position 5 locates at the protein-protein interface (Figure 3B and 3C) and it therefore moves with less flexibility (smaller *RMSD* values, Table 3) than in “dimer1 Y5” where residue 5 locates at outer sides of the structure (Figure 3A). As a consequence, residue 5 has far greater oscillations (Figure 3H) and greater *RMSF* values (Figure 1B) in “dimer1” than in “dimer2”. On the basis of these MD results, it could be conjectured that the presumed allergenic role of residue 5^[13] might involve stabilization of dimeric structures that allow for IgE cross-linking. This should be supported by the smaller mobility suggested by *RMSD* data of this residue as compared to that of proteins as a whole (Table 3).

3.3 Pru p 3 Allergen

Major peach allergen, Pru p 3, has been used as a model in food allergy^[15,39] and is a prototypical member of nsLTPs, proteins widely distributed in plants with a particularly stable fold and a large hydrophobic tunnel with open ends

at opposite sides of the surface able to harbor a great variety of lipidic ligands.^[18,40] The allergenic properties of Pru p 3 have been thoroughly studied^[14–17] and its IgE epitopes have been characterized by using different techniques.^[14] Figure 4 displays initial geometries of dimeric crystal structure of Pru p 3^[17] (“dimer1” in what follows) that has epitope residues at a location not suitable for IgE cross-linking (Figure 4A), a dimer predicted by EPPIC^[8] (“dimer2” with the proper orientation of epitope residues (Figure 4C), the monomer (Figure 4B), and the protein-palmitate complex (Figure 4D). MD results for monomer, “dimer1”, and “dimer2” together with their corresponding complexes with palmitate (PLM) chosen as a representative ligand of nsLTPs are presented in Table 4 and Figures 4E–4J.

Changes in radii of gyration R_g (Figure 4E and Table 4) and *RMSF* values (Figure 1C) are indistinguishable for the monomer irrespective of the presence of PLM. For “dimer1”, R_g results with and without palmitate are also similar but the standard deviations are slightly smaller in the presence of the ligand and in fact, *RMSF* values show that the complex has smaller fluctuations in this dimer. On the contrary, while uncomplexed “dimer2” present R_g changes and *RMSF* per residue values not very different from “dimer1”, the presence of palmitate yields a larger size and far greater oscillations: compare “dimer2” and “dimer2 PLM” plots in Figure 1C and in Figure 4E or the corresponding average values in Table 4. These results point to a destabilizing effect of the ligand on the geometry of “dimer2”. Protein-protein interface data (Table 1) show that whereas interface areas, number of residues making contacts and hydrogen bonds in the interface of “dimer1” are very similar with and without the ligand, the presence of palmitate in “dimer2” gives place to far smaller interface areas and less interacting residues as the simulation progresses. Moreover, while a few more contacts and slightly greater interface areas occur in uncomplexed “dimer2” along the simulation, just the opposite happens in the presence of the ligand. The geometry of “dimer2” predicted by EPPIC on evolutionary grounds has a weak intermolecular attraction noticed in the small interface area and the low number of interacting residues in the initial structure (Table 1) that turns out even weaker with palmitate. Since no particular feature is seen in the structure of this system that could explain this effect, it seems just a consequence of the increased motion produced by the presence of a flexible ligand (see next paragraph) in an otherwise weak protein-protein aggregate. The large hydrophobic cavity (tunnel) is one of the main features of nsLTPs.^[18,40] This tunnel runs parallel to the larger α -helix and is flanked by the unstructured C-terminal tail (Figure 4). In order to assess its flexibility, we computed along the MD simulation two distances between residues that are involved in the interaction with palmitate and have besides their side chains at opposite locations at both ends of the cavity (Figure 4B and 4D). Depending on the structure considered, the outermost distance d1 between

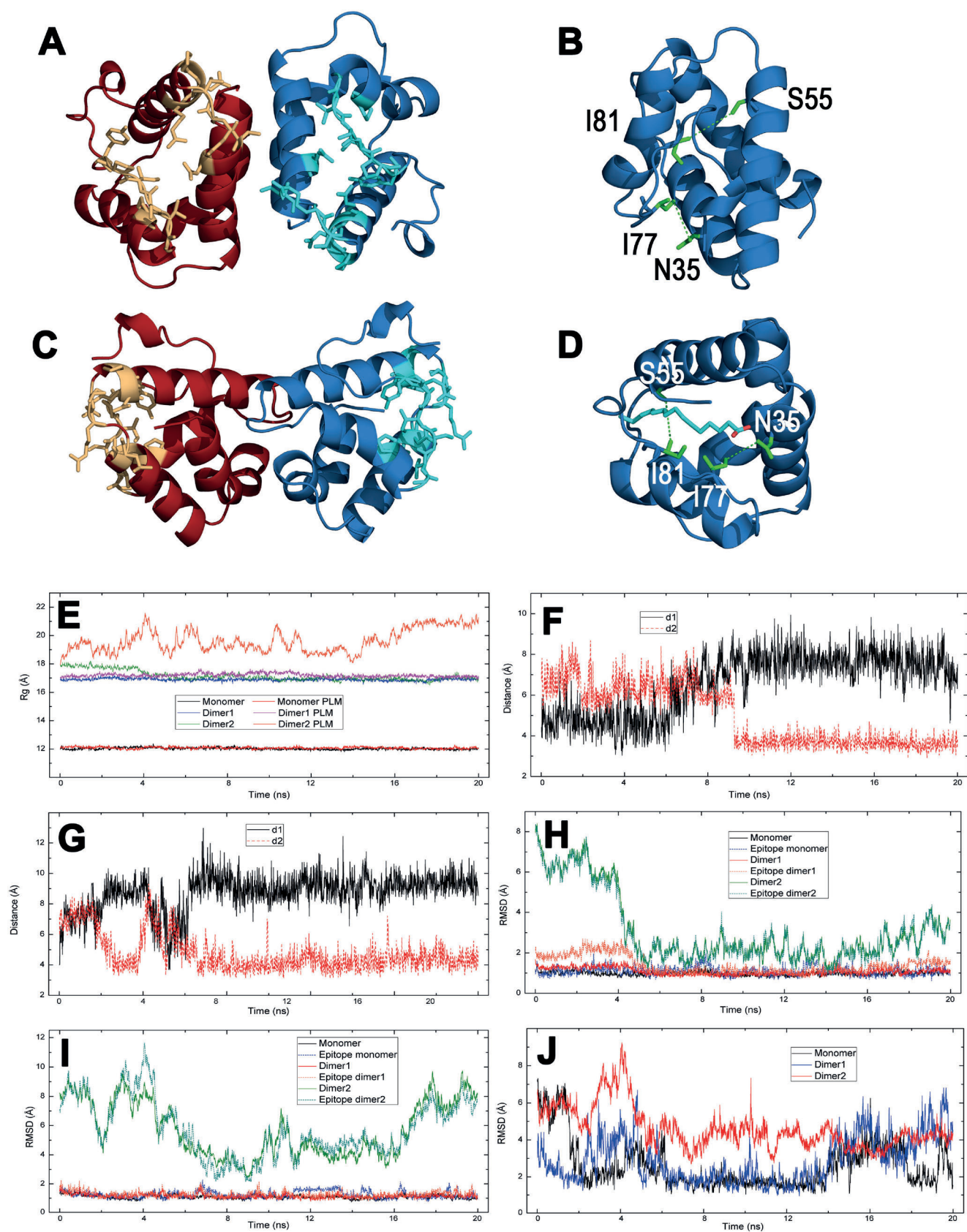


Table 4. Average values of *RMSD* of all non-hydrogen atoms, selected interatomic distances, and radius of gyration (R_g) for different assemblies of Pru p 3 allergen protein obtained in MD 20 ns simulations. Standard deviations in parentheses. All values in Å.

	<i>RMSD</i>			Interatomic distances inside the tunnel[a]		
	Protein	Epitope	PLM	d1	d2	R_g [b]
Monomer	0.974 (0.105)	1.096 (0.216)	–	6.576 (1.498)	4.933 (1.455)	12.03 (0.077)
Dimer1 chain A	1.075 (0.175)	1.392 (0.432)	–	5.213 (0.952)	3.968 (0.611)	16.89 (0.089)
Dimer1 chain B	0.994 (0.145)	1.081 (0.342)	–	5.535 (0.763)	4.472 (1.033)	
Dimer2 chain A	3.128 (1.759)	3.071 (1.719)	–	5.158 (1.025)	3.945 (0.831)	17.15 (0.346)
Dimer2 chain B	2.785 (1.548)	2.688 (1.516)	–	6.057 (1.084)	4.389 (1.217)	
Monomer PLM	1.077 (0.137)	1.245 (0.260)	2.603 (1.278)	8.727 (1.217)	4.764 (1.201)	12.08 (0.074)
Dimer1 PLM ch A	1.147 (0.152)	1.255 (0.213)	2.878 (1.240)	7.311 (1.665)	5.320 (1.717)	17.19 (0.130)
Dimer1 PLM ch B	1.124 (0.236)	1.378 (0.239)	2.661 (1.047)	7.901 (1.174)	6.362 (1.437)	
Dimer2 PLM ch A	5.732 (1.895)	5.763 (1.975)	4.608 (1.124)	6.876 (2.374)	6.519 (2.078)	19.63 (0.819)
Dimer2 PLM ch B	5.635 (1.986)	5.506 (1.955)	4.385 (1.264)	6.443 (2.826)	5.964 (1.101)	

[a] d1 = N δ 2(N35)–C δ 1(I77) distance, d2 = O γ (S55)–C γ 2(I81) distance: see Figure 4B. [b] Values refer to the whole molecular assembly.

N35 and I77 shows values between 5.2 and 6.6 Å in the absence of ligand and between 7.3 and 8.7 Å in the presence of PLM. The innermost distance d2 corresponding to the S55–I81 pair has values between 3.9 and 4.5 Å in the absence of ligand and between 4.8 and 6.5 Å in the presence of PLM (Table 4). Both ends display thus an opening in the presence of ligand though it is noticeably greater at the outer end corresponding to d1. A feature of both distances is their great standard deviations that in turn suggest a great mobility. In fact, plots in Figs. 4F and 4G reveal large oscillations, particularly for the outermost distance d1. In the absence of ligand (Figure 4F), the innermost distance d2 starts with large oscillations but it stabilizes with a sudden decrease at a conformational change of I81 occurring between 9.26 and 9.27 ns (Figure 5) and remains steady afterwards oscillating with small amplitude around a distance below 4 Å. In the presence of ligand (Figure 4G), both distances separate at about 6 ns and remain oscillating steadily around values d1 ~9 Å and d2 ~4.5 Å. Compared with the uncomplexed monomer, these larger values indicate a considerable opening of the outermost end of the tunnel.

RMSD values (Figure 4H and 4I and Table 4) reveal again a completely different behavior of “dimer2” as compared to monomer and “dimer1”. In the absence of PLM (Figure 4H), “dimer2” stabilizes at about 4 ns and remains with large oscillations around *RMSD* ~2 Å. In the presence of ligand (Figure 4I), *RMSD* of “dimer2” displays large oscillations without signs of stabilization. The IgE-epitope is composed of residues 35, 37, 39, 40, 42–44, 70, and 74–79.^[14] *RMSF* plots (Figure 1C) show no particular features for these residues.

Figure 4. Dimers and monomer of Pru p 3 protein. (A) “Dimer1”: crystallographic dimer of protein with PDB code 2ALG. Epitope residues shown as sticks. (B) Monomer protein with residues inside the tunnel defining distances d1(N35–I77) and d2 (S55–I81) shown as green sticks. (C) “Dimer2”: same as (A) for the EPPIC-predicted dimer. (D) Same as (C) with a palmitate molecule shown as cyan sticks inside the tunnel. (E–J). Results of 20 ns MD simulations. (E) Radius of gyration. (F) Interatomic distances d1 and d2 inside the tunnel in the uncomplexed monomer. (G) Same as (F) in the protein-palmitate complex. (H) *RMSD* of non-hydrogen atoms of proteins and epitope regions of Pru p 3 monomer and dimers for uncomplexed systems. (I) Same as (H) for protein-palmitate complexes. (J) *RMSD* of non-hydrogen atoms of palmitate.

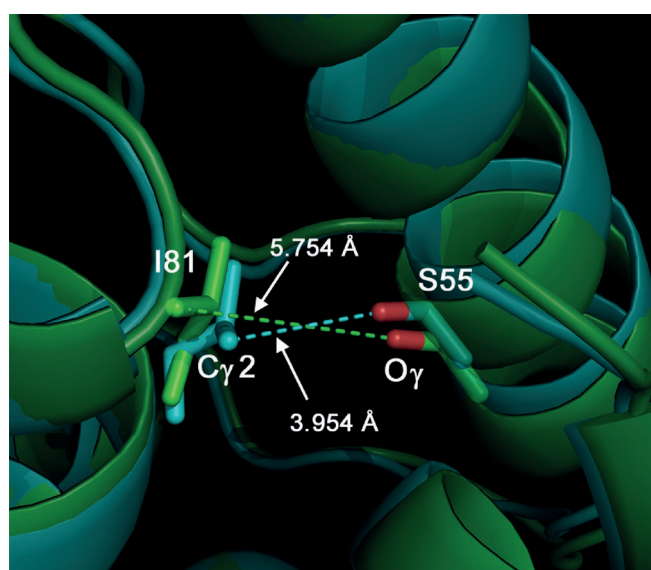


Figure 5. Change of interatomic distance d2 inside the tunnel in uncomplexed Pru p 3 monomer. Decrease in d2=O γ (S55)–C γ 2(I81) distance at the geometry change of side chains occurring between 9.26 ns (green) and 9.27 ns (cyan).

In both “dimer1” and “dimer2”, *RMSD* changes of epitope residues are rather similar to those of the dimers as a whole. In agreement with the discussion above, high average *RMSDs* ~3 Å for uncomplexed “dimer2” and ~5.6 Å for its complex with PLM as well as great standard deviations near 2 Å in both cases reveal that “dimer2” is a clearly unstable structure. In contrast, the monomeric form and

"dimer1" exhibit low *RMSDs* with small standard deviations (Table 4) and a steady behavior over the simulation time which is nearly the same for epitope residues. These features agree with the well-known structural stability characteristic of nsLTPs.^[16] As for the mobility of palmitate (Figure 4J and Table 4), *RMSD* values show no clear stabilization although in both monomer and "dimer1" average values ~ 2.7 Å indicate less flexibility than in "dimer2" where again greater average values ~ 4.5 Å (Table 4) point to an unstable structure.

4 Conclusions

MD simulations aimed at examining the stability in solution of oligomeric states of three major allergen proteins draw the following conclusions.

Alt a 1 forms a rather stable dimer in solution without formation of inter-monomer disulfide linkage between Cys30 residues. The identified epitope of Alt a 1 locates in a structured region with greater rigidity than the dimer as a whole. The narrow cavity inside the β -barrel remains steadily small over the simulation time which makes unlikely that it might harbor any ligand.

Bet v 1 forms dimers in solution with proper orientation of epitopes for IgE cross-linking and similar stability to that of monomer. Even though some epitope residues locate in loop regions, their mobility is comparable to that of proteins as a whole. The large hydrophobic pocket has an inner end with less flexibility than its outer end which shows a large opening, features present in monomeric and dimeric forms. As for residue 5 for which a role in the allergenic properties of Bet v 1 has been proposed, our results point to a stabilizing effect on inter-monomer attraction. MD data suggest a greater stability of the dimer with cysteine instead of genetically encoded tyrosine at position 5.

Pru p 3 in solution forms no stable dimers with proper orientation of epitopes for IgE cross-linking. The hydrophobic tunnel of Pru p 3 shows an inner end less flexible than its outer end which displays a large opening in solution, a feature that holds in the presence of palmitate although in this case, a larger opening is found in the outer end. MD results indicate that the dimer with proper orientation of epitopes is not stable in solution, a feature which also holds in the complex with palmitate. Since the stable crystallographic dimer has epitope residues at a location not suitable for IgE cross-linking our MD study hints at a monomeric form as the more likely with regard to the allergenic properties of Pru p 3.

Summarizing, MD results presented in this work show that Alt a 1, Bet v 1, and Pru p 3, three proteins that play an essential role in allergy disease, have in common (i) very stable monomeric structures, (ii) epitope residues with flexibility lower than or similar to that of proteins as a whole in spite of spanning part of loop regions, and (iii) formation of homodimers. The three allergens differ in (i) the stability of

the dimers presenting epitopes with a proper orientation for IgE cross-linking: great in Alt a 1, moderate in Bet v 1, and extremely low in Pru p 3 and (ii) the flexibility of internal cavities: rigid and small in Alt a 1, rigid at an end and flexible at the opposite end in Bet v 1, and overall flexible in Pru p 3. Three essential allergens are thus shown to display a distinct behavior on their trends to form homodimers in solution.

Competing Interests

The authors declare that no competing interests exist.

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