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Structural Insights into Antibody Sequestering and Neutralizing of Na⁺ Channel α -Type Modulator from Old World Scorpion Venom^[S]

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Igor P. Fabrichny^{†1,2}, Grégoire Mondieilli^{‡2}, Sandrine Conrod[‡], Marie-France Martin-Eauclaire[‡], Yves Bourne^{§3}, and Pascale Marchot^{‡§4}

From the [†]Centre de Recherche en Neurobiologie-Neurophysiologie de Marseille (CRN2M, CNRS/Aix-Marseille Université UMR-6231), Institut Fédératif de Recherche Jean Roche, Faculté de Médecine Secteur Nord, CS80011, F-13344 Marseille cedex 15, France and [‡]Architecture et Fonction des Macromolécules Biologiques (AFMB, CNRS/Aix-Marseille Université UMR-7257), Campus Luminy-Case 932, F-13288 Marseille cedex 09, France

Background: The antigenic specificity of scorpion α -toxins, which target Na_v channels, hinders efficient antiserum production.

Results: Structures of two antibodies, which together neutralize the main toxins in a threatening venom, were solved in toxin-bound and unbound forms, respectively.

Conclusion: Selective toxin trapping involves distinctive molecular determinants and bound toxin orientations.

Significance: These complementary templates will help design new neutralizing molecules suitable for immunotherapy.

The Old World scorpion *Androctonus australis hector* (Aah) produces one of the most lethal venoms for humans. Peptidic α -toxins AahI to AahIV are responsible for its potency, with AahII accounting for half of it. All four toxins are high affinity blockers of the fast inactivation phase of mammalian voltage-activated Na⁺ channels. However, the high antigenic polymorphism of α -toxins prevents production of a polyvalent neutralizing antiserum, whereas the determinants dictating their trapping by neutralizing antibodies remain elusive. From an anti-AahII mAb, we generated an antigen binding fragment (Fab) with high affinity and selectivity for AahII and solved a 2.3 Å-resolution crystal structure of the complex. Sequestering of the C-terminal region of the bound toxin within a groove formed by the Fab combining loops is associated with a toxin orientation and main and side chain conformations that dictate the AahII antigenic specificity and efficient neutralization. From an anti-AahI mAb, we also preformed and crystallized a high affinity AahI-Fab complex. The 1.6 Å-resolution structure solved revealed a Fab molecule devoid of a bound AahI and with combining loops involved in packing interactions, denoting expulsion of the bound antigen upon crystal formation. Comparative analysis of the groove-like combining site of the toxin-

bound anti-AahII Fab and planar combining surface of the unbound anti-AahI Fab along with complementary data from a flexible docking approach suggests occurrence of distinctive trapping orientations for the two toxins relative to their respective Fab. This study provides complementary templates for designing new molecules aimed at capturing Aah α -toxins and suitable for immunotherapy.

Scorpion stings, the second most important cause of envenoming after snakebites, can cause severe systemic envenomation and generate a serious public life-threatening problem (1). Immunotherapy remains the only efficient treatment for envenomation, but its efficiency depends on both accurate identification of the scorpion species involved and timely anti-venom administration. The peptidic toxins found in venoms from different species or even in a single venom share high sequence homology throughout their 30–70 amino acid residues and an overall scaffold made of an α -helix and an antiparallel three-strand β -sheet, tightly reticulated by 3–4 disulfide bridges, and called the cystine-stabilized α -helix (or CSH) motif. Yet side chain variability at the antigenic surfaces of the toxins confers on them distinctive immunological properties and prevents cross-reactivity. When the case occurs, antigenic specificity hinders production of an antiserum able to neutralize at once all the toxins in a venom. Finally, antisera to be used as antidotes (antivenoms) by humans need to be highly potent and specific to trap the rapidly diffusing small toxins and still be devoid of potential secondary effects. Hence, and although some studies pointed to the higher functional stability and neutralizing capacity of antigen binding fragment (Fab)⁵ molecules

[S] This article contains supplemental Figs. 1 and 2.

The atomic coordinates and structure factors (codes 4AEI and 4AEH) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

¹ Alternate spelling: Fabrichniy. Present address: International Biotechnology Center GENERIUM, Vladimir region, Petushinskiy district, Volginskiy, Novosemenkovskaya Street 3, 601125 Russia. E-mail: fabri@ibcgenerium.ru.

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³ To whom correspondence may be addressed. Tel.: 33-0-491-825-566; E-mail: yves.bourne@afmb.univ-mrs.fr.

⁴ To whom correspondence may be addressed. Architecture et Fonction des Macromolécules Biologiques, CNRS/Aix-Marseille Université UMR-7257, Campus Luminy-Case 932, F-13288 Marseille cedex 09, France. Tel.: 33-0-491-825-579; E-mail: pascale.marchot@univ-amu.fr.

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⁵ The abbreviations used are: Fab, antigen binding fragment; Aah, *A. australis hector*; CDR, complementarity determining region; r.m.s.d., root mean square deviation; scFv, single-chain variable-fragment antibody; VH, CH, VL, CL, variable (V) and constant (C) regions of the heavy (H) and light (L) chains.

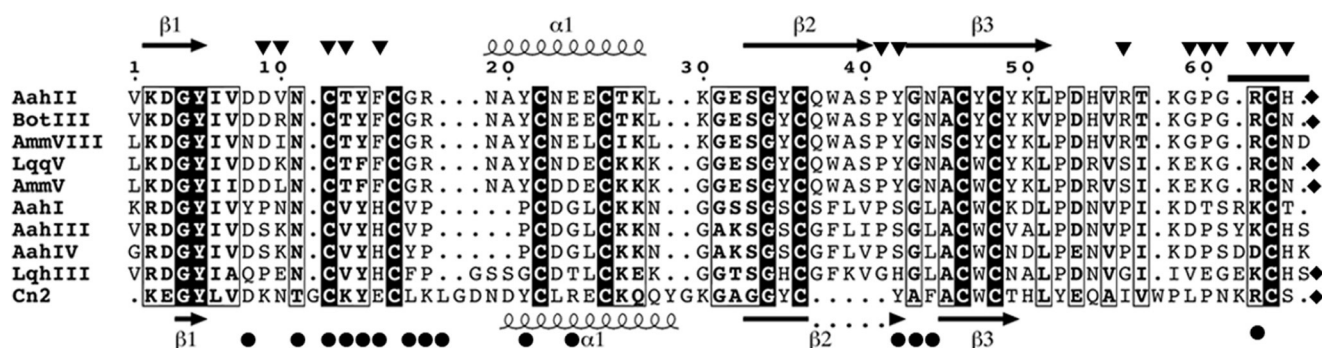


FIGURE 1. Sequences of the scorpion α -toxins used or cited in this study. The AahII and Cn2 residue numbering and secondary structure elements are indicated above and below the alignment, respectively. C-terminal amidation is indicated by a diamond. AahII residues whose side chains interact with side chains in the Fab4C1 CDRs are indicated by triangles above the alignment (cf. Table 2). Cn2 residues whose side chains interact with side chains in the scFv 9C2 CDRs (PDB code 2YC1 (31)) are indicated by circles below the alignment. The AahII C-terminal tripeptide that displays a 90° positional difference compared with unbound AahII (1PTX (59)) is indicated by a bar above the alignment. The distinctive lengths of the β 1- α 1 segment and β 2 and β 3 strands in Cn2 and AahII are evident. The belonging of α -toxins AahII (from *A. australis hector*), BotIII (from *Buthus occitanus tunetanus*; 95% sequence identity with AahII), AmmVIII and AmmV (from *A. mauretanicus mauretanicus*; 89 and 75%, respectively), LqqV (from *Leiurus quinquestriatus quinquestriatus*; 78%), of α -toxins AahI, AahIII, and AahIV (from *A. australis hector*; 44, 46, 44%, respectively), of α -like toxin LqhIII (from *L. quinquestriatus hebraeus*; 39%), and of β -toxin Cn2 (from *C. noxius Hoffmann*; 45%) to distinct, sequence-dictated immunological and pharmacological groups is apparent.

compared with their single-chain variable-fragment antibody (scFv) counterparts (2, 3), most strategies were aimed at generating, by protein and/or peptide engineering, new molecules such as recombinant Fab, scFv, tandem scFv, diabody or nanobody molecules, with enhanced recognition properties (Refs. 4–15; for review, see Refs. 16 and 17).

The scorpion “long chain” toxins (60–70 residues, 4 disulfide bridges), which target the voltage-sensitive sodium (Na_v) channels of the nerve and muscle excitable cells and dramatically disrupt the neuromuscular, cardiovascular, and respiratory systems of the prey, are mainly responsible for the neurotoxic symptoms developed upon scorpion envenomation (18, 19). These toxins have been classified into α - and β -toxins, which respectively bind to site 3 and site 4 of the Na_v channel and distinctively alter the channel gating mechanism (for review, see Ref. 20). α -Toxins, mainly found in venoms from the Old World species, block the channel fast inactivation phase and inhibit the inactivation phase of the action potential, whereas the β -toxins, found in venoms from the New World, shift the membrane potential dependence of channel activation to more negative potentials and reduce the peak current amplitude, either of these two mechanism resulting in enhanced sodium entry into the cells. The α -toxins have been further classified into structural and immunological subgroups, numbered I–IV (21). Polyclonal antibodies raised against a toxin of one group fully neutralize congeners of the same group but not those whose sequences differ by more than 30–40% from that of the immunogen and who belong to another group (22).

The scorpion *Androctonus australis hector* (Aah) produces one of the most potent venoms. It is commonly found in Algeria and Tunisia, where it is responsible for almost all human casualties. Four α -toxins, AahI, AahII, AahIII, and AahIV, although they are minor components of the venom (a few percent, in weight), are responsible for up to 95% of the venom lethality, with AahII accounting for half of it (23). In fact, AahII displays the highest affinities described for site 3 of the neuronal $\text{Na}_v1.2$ and muscular $\text{Na}_v1.4$ channels in mammals (24), and it is considered a highly lethal α -toxin archetype.

AahII belongs to the structural and immunological group II, whereas the other three Aah toxins belong to group I (Fig. 1). This antigenic polymorphism hampers the rational production or design of a polyvalent and efficient antiserum against Aah venom. Immunochemical analyses of AahII have led to identify antigenic regions in the α -helix in the N and C terminus regions and in a surface loop specific to α -toxins (26–28). *In vitro*, mAbs 4C1 and 9C2, raised against AahII and AahI, respectively, and produced from mouse hybridoma, bind their respective toxin immunogen specifically and with high affinities (K_d values of 0.8 and 0.15 nM, respectively) (Refs. 7, 29, and 30; for review, see Ref. 17). mAb 9C2 also binds AahIII, although with a 10-fold lower affinity compared with AahI. *In vivo*, preincubation of mAb 4C1 with AahII neutralizes the intracerebroventricular toxin lethality in mice with a calculated protective capacity of 32,000 LD₅₀ per mg (28), whereas preincubation of mAb 9C2 with AahI results in a protective capacity of 1500 LD₅₀ per mg (29). This “neutralizing” capacity suggests that the epitopic and pharmacological sites overlap at least partly at the toxin surface and that mimics of the binding determinants of these antibodies could be engineered to generate antivenom molecules with improved recognition properties. Detailed information gathered by structural, mutagenesis, and epitope mapping analyses of the toxins along with channel binding assays is available (26, 27, 30). Yet the toxin and antibody structural determinants involved in toxin trapping and neutralization remain to be ascertained.

To precisely identify these determinants and compare the epitopic and pharmacological sites of the Aah toxins from mAbs 4C1 and 9C2, we generated Fab molecules with virtually unaltered binding affinities for their respective AahII and AahI immunogens and undertook a crystallographic analysis of the preformed, high affinity toxin-Fab complexes. This approach led us solve a 2.3 Å-resolution structure of the AahII-Fab4C1 complex and a 1.6 Å-resolution structure of Fab9C2 devoid of a bound AahI, and design a theoretical AahI-Fab9C2 complex. Striking conformational differences in the combining sites of the toxin-bound Fab4C1 versus unbound Fab9C2, and in the

experimental AahII-Fab4C1 complex *versus* the theoretical AahI-Fab9C2 complex, suggest the occurrence of a distinctive binding orientation of the two toxins relative to their respective trapping Fab.

Our study provides alternative templates for designing new neutralizing molecules aimed at capturing the Aah α -toxins in solution and offering enhanced suitability for therapeutic use. In conjunction with a structural analysis of the β -toxin Cn2 (main toxin in the venom of *Centruroides noxius* Hoffmann, specific for the mammalian Na_v1.6 channel), bound to an engineered scFv antibody that also neutralizes the \sim 90% homologous β -toxin archetype Css2 (from the venom of *Centruroides suffusus suffusus*) (31), our non-redundant and complementary data also highlight structural differences in the α - and β -toxins and their respective neutralizing antibodies that dictate their immunological specificities.

EXPERIMENTAL PROCEDURES

Protein Production and Purification—The toxins AahI, II, III, and IV were purified from the Aah venom by liquid chromatography, and their homogeneity was assessed using HPLC and amino acid analysis (23) and MALDI-TOF MS (Bruker Ultraflex II TOF/TOF; positive linear mode; m/z range 5000 to 8000). mAbs 4C1 (IgG1, κ (5)) and 9C2 (IgG2a, κ (6)), produced from murine hybridoma (22, 32), were purified from ascitic fluids in a single step of affinity FPLC on HiTrap protein-G (GE Healthcare) equilibrated with 0.02 M sodium phosphate, pH 7.0, and eluted with 0.1 M glycine, pH 2.7, with immediate neutralization of the eluant with 1 M Tris, pH 9.0 (55 μ l/ml). The purified IgGs were dialyzed against 0.02 M sodium phosphate, pH 7.0, and concentrated by ultrafiltration.

The Fabs were obtained by papaine cleavage of the purified IgGs using a papaine-to-IgG ratio of 1:10 (w/w) in the presence of 10 mM L-cysteine, 1 mM β -mercaptoethanol, 1 mM EDTA (\sim 2 h, 37 $^{\circ}$ C); the reaction was stopped with 6 mM iodoacetamide. The cleavage products and reactants were separated by gel-filtration FPLC on prepacked Superdex-200 (GE Healthcare) equilibrated and eluted with 0.02 M sodium phosphate, pH 7.2. The coeluting constant fragment and Fab were separated through several steps of affinity FPLC on HiTrap protein-A (GE Healthcare) equilibrated in the same buffer, with recovery of the non-retained Fab in the flow-through and expulsion of the retained constant fragment using 100 mM citric acid, pH 5.0. Homogeneity of the purified Fab was assessed by SDS-PAGE in reducing and non-reducing conditions and native-PAGE with migration toward the anode (12.5 and 7.5% homogenous PhastGels, respectively; GE Healthcare) and by MALDI-TOF MS (matrix: sinapinic acid 0.5 μ l at 10 mg/ml in TFA/acetonitrile/water 0.1:0.6:0.3 (v/v/v); dried-droplet method). The Fabs were concentrated by ultrafiltration and stored on ice.

Functional Analysis of Fabs—The binding of AahI, AahIII, and AahIV by IgG9C2 and Fab9C2 and of AahII by IgG4C1 and Fab4C1 was analyzed by ELISA at 20 $^{\circ}$ C (6) (Fig. 2). For IgG binding to the toxin, the toxin (10 nM in 0.1 M sodium bicarbonate, pH 9.8) was coated on a 96-well plate (100 μ l/well; overnight incubation). To preclude nonspecific IgG binding, the plate was saturated with a blocking solution (10 mM PBS, Tween 20, pH 7.4, 5% (w/v) powdered skim milk; 1 h). Incuba-

tion of the specific anti-toxin IgG (10^{-5} – 10^{-12} M; 1 h) was followed by incubation of an alkaline phosphatase-coupled rabbit IgG directed against mouse IgG (Sigma, A-1902) (90 min). Between each step the plate was extensively washed (10 mM PBS, Tween 20, pH 7.4, 0.1% (w/v) powdered skim milk). *p*-Nitrophenyl phosphate (Merck; 1 mg/ml in 10% diethanolamine, pH 9.8, 0.5 mM MgCl₂) was added, and absorbance measured at 405 nm every 30 min using a iEMS Reader MF (LabSystems). For Fab binding to the toxin, a competition protocol was set up where the specific Fab in a range of concentrations (10^{-5} – 10^{-12} M) was incubated along with the parental IgG at a concentration close to the previously determined half-effect (EC₅₀) value (1 10^{-7} M for IgG4C1, 1 10^{-8} for IgG9C2). Because of the limited coating capability of AahIV, presumably arising from its low pI value compared with the other Aah toxins (*cf.* the legend to Fig. 2), Fab9C2 binding to AahIV was assayed in competition with AahI. Data were plotted and analyzed according to a sigmoidal equation.

The effects of Fab4C1 onto AahII binding to, and dissociation from, its binding site on rat brain synaptosomal Nav channel were analyzed by liquid-phase RIA (28) as briefly follows. Radio-iodinated AahII (2 10^{-10} M) was incubated in the presence of Fab4C1 in a range of concentrations (8 10^{-6} –8 10^{-13} M) and rat brain synaptosomal membranes (20 μ g) (30 min; 30 $^{\circ}$ C). The unbound and membrane-bound toxin populations were separated by centrifugation (13,000 $\times g$, 5 min, 4 $^{\circ}$ C) and quantified on a γ counter. Data points corresponding to fractional toxin binding (B/Bo) were plotted according to a sigmoidal equation. Dissociation of the bound radio-iodinated AahII induced by an excess of Fab4C1 (8 10^{-7} M, leading to near-complete competition in the above assay) was recorded at regular time intervals over 30 min and compared with dissociation induced by an excess of unlabeled toxin (5 10^{-7} M) (33, 34).

Complex Formation, Crystallization, Data Collection, and Processing—The AahI-Fab9C2 and AahII-Fab4C1 complexes were formed in solution at high Fab concentration (\sim 100 μ M, well above the toxin K_d values, *i.e.* 8 10^{-10} for AahII/IgG4C1 by RIA (28); 1.5 10^{-10} or 0.11 10^{-10} for AahI/IgG9C2 by RIA (29) or ELISA (7), respectively) and using a slight molar excess of the toxin over the Fab to preclude stoichiometric deficiency (incubation was for 3 h at room temperature then overnight at 4 $^{\circ}$ C). They were then buffer-exchanged for 0.01 M Hepes, pH 7.4, 0.05 M NaCl, 0.02% NaN₃ (4 $^{\circ}$ C) in conditions precluding complex dissociation and concentrated to 10–20 mg/ml by ultrafiltration. Full occupancy of the Fab by bound toxin was verified by native-PAGE (7.5% homogeneous PhastGels; GE Healthcare) with migration toward the anode (35) (Fig. 2). The complexes were filtered on sterile cellulose acetate and stored on ice.

Crystallization of the toxin-Fab complexes was achieved at 20 $^{\circ}$ C by vapor diffusion using 1–1.2 μ l hanging drops and a protein-to-well solution ratio of 1:1 (v/v). For the AahII-Fab4C1 complex, large plate-like crystals appeared within a week with 15% PEG 2000 monomethyl ether, 0.1 M Hepes, pH 8.0, 0.1 M NaCl as the well solution; similar crystals were obtained in the presence of 0.1 M MgCl₂ or CaCl₂. For the AahI-Fab9C2 complex, rod-like crystals were obtained within a few days with 12.5% PEG 4000, 0.1 M imidazole-malate, pH 7.0, NaCl 50 mM. Single crystals were briefly transferred to the well

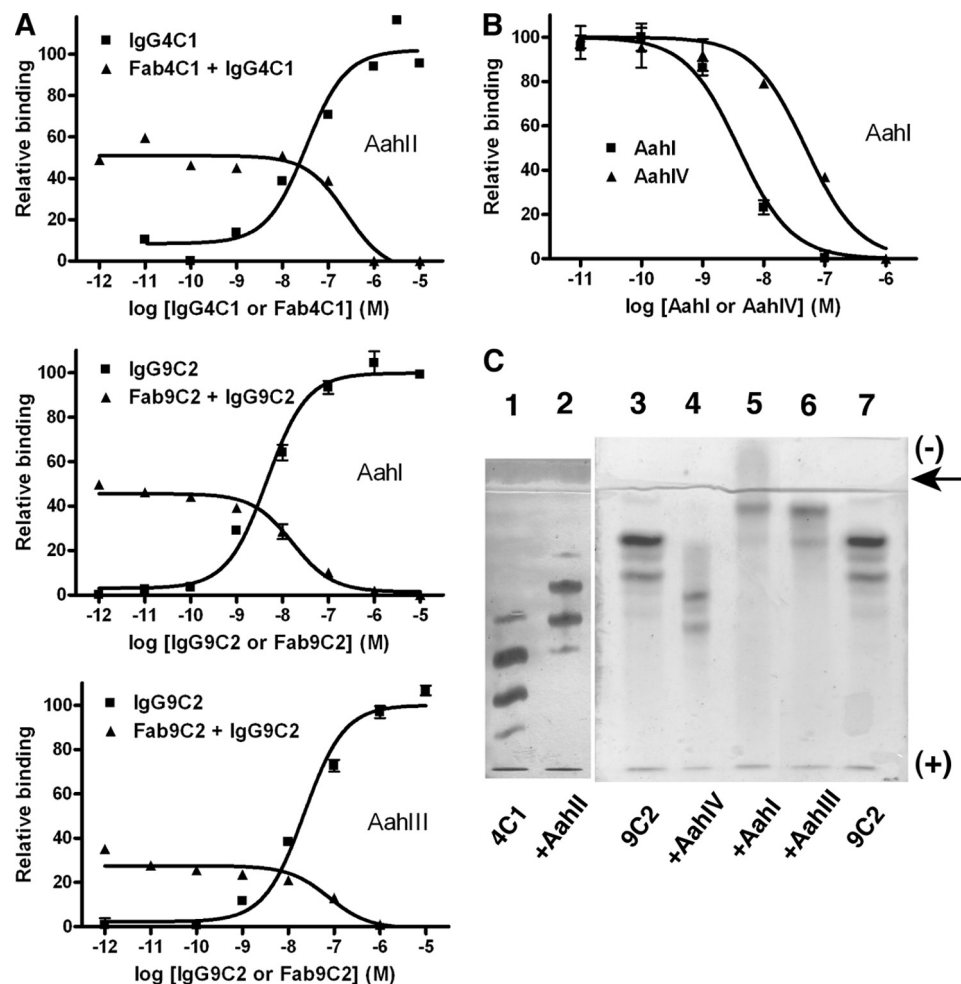


FIGURE 2. Functional quality of the purified Fabs. *A*, shown is ELISA analysis of binding of coated AahI, AahII, and AahIII by IgG4C1 and IgG9C2 (direct binding; squares) and by Fab4C1 and Fab9C2 (competitive binding; circles). The competition assays used IgG9C2 at 10^{-8} and IgG4C1 at 10^{-7} M. In each case, the Fab concentration at the intersection of the direct and competition curves, close to the IgG EC₅₀ value, assess for Fab retention of the IgG binding capacity. *B*, shown is ELISA analysis of Fab9C2 binding to AahI and AahIV in solution, competitively with coated AahI. Fab9C2 was 10^{-8} M. *C*, shown is native-PAGE analysis of the Fabs and toxin-Fab complexes, with migration toward the anode (bottom). Lanes 1 and 2, Fab4C1 unbound and bound to AahIII, respectively; lanes 3 and 7, Fab9C2 alone; lanes 4–6, Fab9C2 bound to AahIV, AahI, and AahIII, respectively. The charge heterogeneity of the purified Fabs, otherwise homogenous in their mass (cf. “Results”), and the shift toward the cathode of the AahI-, AahII-, and AahIII-bound versus unbound Fabs are evident. The unbound toxins AahI, AahII, and AahIII in excess migrate toward the cathode and off the gel, as partially does the AahI-Fab9C2 complex, of a higher pI compared with the AahII-Fab4C1 and AahIII-Fab9C2 complexes. Only the AahIV-Fab9C2 complex further migrates toward the anode, as does unbound AahIV, of a lower pI compared with the other toxins. (Theoretical pI values are: Fab4C1, 7.16; Fab9C2, 8.15; AahI, 8.47; amidated AahII, 8.15; AahIII, 8.17; AahIV, 6.03.)

solution supplemented with 20–30% (v/v) glycerol and flash-cooled in liquid nitrogen. Diffraction data were collected at 100 K at the European Synchrotron Radiation Facility (Grenoble, France) and processed and scaled with XDS (36). Despite the numerous attempts, no suitable crystals were obtained from unliganded Fab4C1 or the preformed AahIII- and AahIV-Fab9C2 complexes.

Structure Solution and Refinement—The protein sequences of the variable domains of the two Fabs had been determined by PCR-mediated cDNA cloning (EMBL data bank accession numbers Y17588 and Y17589 for the 4C1 VH and VL regions, respectively (5); 9C2 sequences were not deposited (6)). The sequences of the constant domains were retrieved from the IMGT repertoire for proteins and alleles.

The structure of the AahII-Fab4C1 complex was solved by molecular replacement with MOLREP (37) using the AahII structure (PDB accession code 1PTX) and the pair of constant (CL, CH) and variable (VL, VH) regions (without the variable

loops) of another IgG1-issued Fab⁶ as search models. Automatic building of the initial model with ARP/wARP (38) yielded a virtually complete model consisting of three toxin-Fab complexes with well defined density maps except for the disordered loop region 133–142 in two CH domains, consistent with inherent flexibility of these domains. The ARP/wARP model was improved by manual adjustment with the graphics program COOT (39) and was refined with REFMAC (40) including TLS refinement, with each toxin molecule and each variable and constant domain defining a TLS group and NCS restraints. Data collection and refinement statistics are reported in Table 1.

The structure of Fab9C2 devoid of a bound AahI was solved by molecular replacement and refined using the same strategy as above described and taking into account the sequence differ-

⁶ P. Marchot, manuscript in preparation.

TABLE 1

Data collection and refinement statistics

	AahII-Fab4C1	Fab9C2
Data collection		
Beamline (ESRF)	ID14-EH2	ID23-1
Resolution range (\AA) ^a	13–2.3 (2.35–2.3)	15–1.6 (1.65–1.6)
Space group	P2 ₁	P2 ₁
Cell dimensions, <i>a</i> , <i>b</i> , <i>c</i> (\AA); β ($^\circ$)	123.94, 38.74, 154.07; 91.36	55.93, 71.0, 65.96; 111.82
No. of observations	267,030	230,560
No. of unique reflections	65,949	63,057
<i>R</i> _{sym} (%) ^b	8.7 (43.1)	6.4 (42.3)
$\langle (I/\sigma(I)) \rangle$	10.8 (3.45)	11.26 (2.8)
Redundancy	4.0 (4.1)	3.7 (3.7)
Completeness (%)	99.2 (99.9)	99.5 (99.7)
B factor from Wilson plot (\AA^2)	34.64	37.4
Refinement		
Resolution (\AA)	13–2.3	30–1.6
<i>R</i> _{cryst} ^c	20.3 (26.9)	17.4 (28.5)
<i>R</i> _{free} (%) ^d	25.8 (32.1)	21.4 (29.3)
Number of reflections used in refinement	62,651	59,927
Number of reflections for <i>R</i> _{free}	3,298	3,155
Number of water molecules	210	614
r.m.s.d. ^e		
Bond length/ angles (\AA) / ($^\circ$)	0.009/1.37	0.010/1.37
Chiral volume (\AA^3)	0.095	0.095
Mean B factors (\AA^2)		
Main/side chain	38.5/39.7	19.4/21.1
Solvent/other ligands	31.4/46.0	32.9/-
Ramachandran plot statistics ^f		
% of residues in favored/outlier regions	97.6/0.07	97.4/0.07
PDB accession code	4AEI	4AEH

^a Values in parentheses are for the highest resolution shell.

^b $R_{\text{sym}} = \sum_{\text{hkl}} (\sum_i |I_{\text{hkl}} - \langle I_{\text{hkl}} \rangle|) / \sum_{\text{hkl}} \langle I_{\text{hkl}} \rangle$.

^c $R_{\text{cryst}} = \sum_{\text{hkl}} \|F_o - |F_c|/\sum_{\text{hkl}} |F_o|\|$.

^d *R*_{free} is calculated for randomly selected reflections excluded from refinement.

^e Root mean square deviation from ideal geometry.

^f Ramachandran plot statistics have been calculated with the MolProbity server.

ences between the HC chains of IgG1 and IgG2a. Automatic building of the initial model with ARP/wARP yielded a virtually complete model consisting of a Fab molecule with well defined density maps except for the disordered Cys-134–Gly-139 loop region in the heavy (H) chain.

Structure Analysis and Comparison with Other Structures—The Fab complementarity determining region (CDR) boundaries were defined according to the IMGT standards (supplemental Fig. 1). However, to avoid virtual gaps in the structure coordinates and inconsistencies related to β -strands, the consecutive numbering of residues and Greek letter labeling of β -strands and α -helices are used herein (supplemental Fig. 1). The final structure of the AahII-Fab4C1 complex comprises 64 residues for the bound toxin, 220 and 218 residues for the H and light (L) chains, respectively, and 210 water molecules. Fab4C1 CDRs L1 (Gln-27–Tyr-37), L2 (Lys-55–Ser-57), L3 (Phe-94–Thr-102), H1 (Gly-26–Tyr-33; 4 aromatic residues; local pI 5.52), and H2 (Ile-51–Thr-58; local pI 3.80) belong to canonical structural classes 4, 1, 1, 1, and 3A, respectively, whereas CDR H3 (Ser-97–Tyr-109; 4 aromatic residues; local pI 3.93) shows the common stem conformation as determined from the length and conformation of the hypervariable loops (41). A Thr instead of a Val was found at position 117 in the hinge region between the VH and CH domains.

The final structure of Fab9C2 comprises 219 and 211 residues for the H and L chains, respectively, and 611 water molecules. High temperature factors and weak electron densities are associated with residues Cys-134–Gly-139 in the H chain. Fab9C2 CDRs L1 (Glu-27–Asn-32), L2 (Ala-50–Thr-52), L3 (Gln-89–Thr-97; 3 aromatic residues), H1 (Gly-26–Trp-33; 2

aromatic residues; local pI 5.52), and H2 (Ile-51–Thr-58) belong to canonical structural classes 2, 1, 1, 1, and 2, respectively, whereas CDR H3 (Ala-97–Tyr-108; 4 aromatic residues; local pI 3.93) is 1 residue shorter than its Fab4C1 counterpart. H chain residue Asp-50 bears no glycan moiety despite its belonging to a consensus sequence for *N*-glycosylation.

Stereochemistry of the two structures was analyzed with COOT and MOLPROBITY (42); no residues were found in the disallowed regions of the Ramachandran plot. The Fab elbow angles were calculated using a web-based applet (43).

Search for the closest structural homologues of AahII, Fab4C1, and Fab9C2 used secondary-structure matching (44) (see supplemental Experimental Procedures/Results/Discussion). Least-square structural superpositions were calculated with LSQMAN (45). The r.m.s.d. between bound and unbound AahII is 0.63 \AA (62 C α atoms), and between bound AahII and the AahI model (*cf.* below) it is 0.78 \AA (61 C α atoms). Between Fab4C1 and Fab9C2 the r.m.s.d. is 0.65 \AA (202 C α atoms) for the constant domains and 1.36 \AA (130 C α atoms) for the variable domains. Comparison of the AahII-Fab4C1 complex with the structure of scorpion β -toxin Cn2 bound to scFv 9004G (PDB code 2YC1) yielded r.m.s.d. values of 1.84 \AA (54 C α atoms) between the bound toxins and 0.68 \AA (107 C α atoms) between the variable domains of the bound Fab and scFv.

Electrostatic surface potentials were calculated using APBS (46) with the PyMOL APBS tools. Fig. 1 and supplemental Fig. 1 were generated with ESPript (47) and MUSCLE (48), and Figs. 3 and 4 and supplemental Fig. 2 were generated with PyMOL (49).

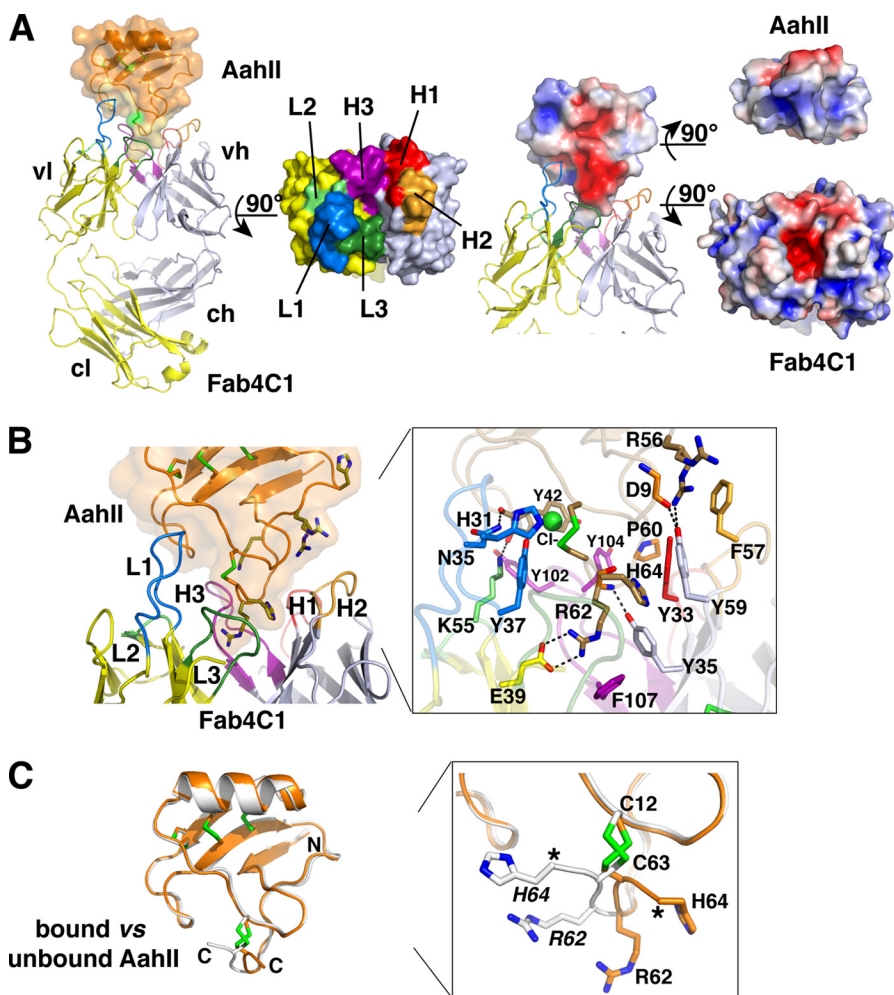


FIGURE 3. **Structure of the AaHII-Fab4C1 complex.** *A*, shown are the overall features of the complex, oriented as to look at the toxin face opposite to the conserved hydrophobic surface. The AaHII molecule is displayed with a transparent molecular surface in orange with residues buried at the binding interface in yellow. The Fab L chain is displayed in yellow with CDRs L1, L2, and L3 in blue, light green, and dark green and the Fab H chain in white with CDRs H1, H2, H3 in red, orange, and purple, respectively. The disulfide bridges are in green. The shape and charge complementarities of the binding interfaces, with the protruding, electropositive AaHII C-terminal region and the concave, electronegative Fab4C1 binding pocket, is evident when both molecules are rotated 90° from the overall view. The electrostatic potentials mapped on the molecular surfaces are shown at -3 kT/e (blue) to $+3$ kT/e (red). (*Right view*, only the VL and VH domains of Fab4C1 are shown.) *B*, shown are close-up views of the complex interface, highlighting the exquisite complementarities in shape and chemistry of the two partners. The toxin orientation and fully buried C-terminal region are consistent with the extremely high neutralizing capacity of IgG4C1 toward AaHII binding onto the Na_v channel *in vivo* (cf. Introduction). The dashed lines denote hydrogen bonds. The label sizes are related to the side chain proximity to contribute a perspective effect. The main and side chains are colored as in panel *A*, with red oxygens and blue nitrogens. *C*, shown is a comparison of AaHII in the Fab4C1-bound (orange) and unbound (white) conformations. The 180° switch of the C-terminal amidated tripeptide and associated torsion of the Cys-12–Cys-63 disulfide bridge are evident. The stars denote the amide groups.

Theoretical Modeling—The AahI model was built with MODELLER (50) using the structure of the α -like toxin LqhIII as a template (PDB code 1BMR; 61% identity; *cf.* Fig. 1) and based on the TM-score and the HHPred server (51). The models of the AahI-Fab9C2 complex were generated with HADDOCK 2.1 using default parameters (52) and, as possible interfacial active residues, 28 residues of the Fab9C2 CDRs (14 from the H-chain and 14 from the L-chain) and 30 residues of AahI, randomly distributed at the toxin surface but including the C-terminal Gly-57–Thr-63 peptide. Neighboring solvent-accessible residues (four for AahI, eight for Fab9C2) that could be indirectly involved in the binding were defined as passive residues. Eight distinct runs of protein-protein docking were computed from randomly oriented AahI molecules placed at the proximity of the Fab9C2 CDRs. For each run, the top 200 complexes generated after rigid-body energy minimization

were subjected to flexible simulated annealing in torsion angle space and flexible water refinement in Cartesian space, and the three energetically best models scored by HADDOCK were comparatively analyzed. As a control, the same strategy and criteria were applied for *ab initio* docking of AahII onto Fab4C1 using the two partners isolated from the structure of the complex and randomly oriented relative to each other.

RESULTS AND DISCUSSION

Chemical and Functional Quality of Fab4C1 and Fab9C2—The protein sequences of the two Fabs were published earlier (5, 6) (supplemental Fig. 1). SDS-PAGE in reducing and non-reducing conditions and mass spectrometry analyses showed each of the two purified Fabs to be of a proper mass, slightly below 50 kDa, and high homogeneity (data not shown). Native-PAGE analysis revealed greater average mobility for Fab4C1

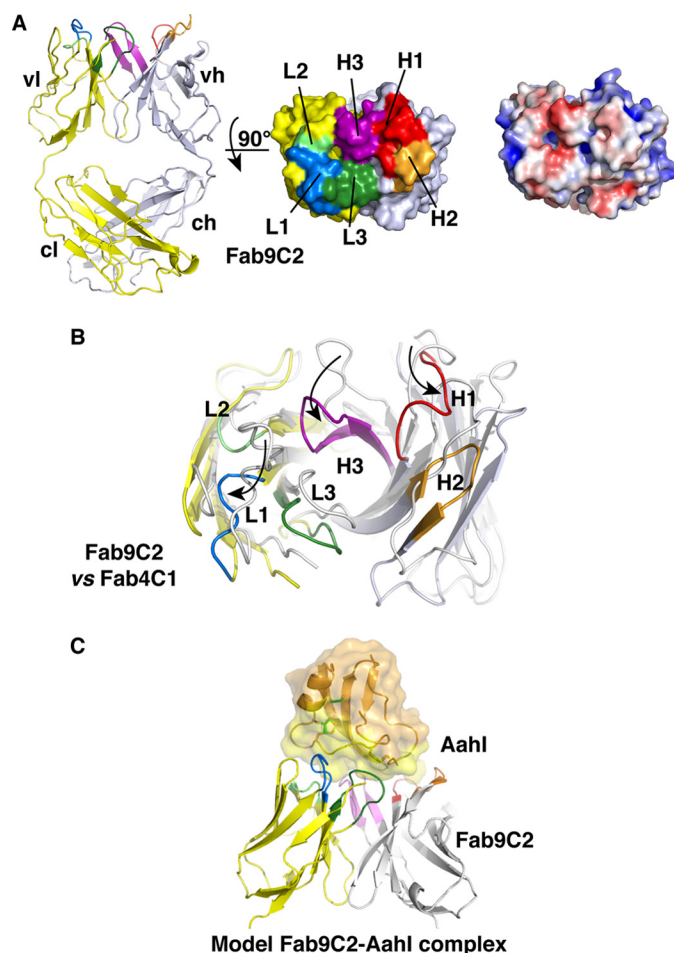


FIGURE 4. Structure of Fab9C2 and model of its Aahl complex. A, shown are the overall features of unbound Fab9C2 with the same orientation and color codes as for Fab4C1 in Fig. 3. In Fab9C2, compared with Fab4C1, the much shorter CDR L1 and distinctive conformation of CDR H3 (cf. supplemental Fig. 1) along with the flat interacting surface and absence of an electronegative patch generate distinctive topographies for the combining site. B, shown is superimposition of Fab9C2 and Aahl-bound Fab4C1 according to the two Fab L chains (r.m.s.d. 2.7 Å for 180 C α atoms) with Fab4C1 colored as in panel A and Fab9C2 in white (same orientation as in the central and right panels in A). The arrows point to the major conformational differences found in CDRs L1, H1, and H3 (only the VL and VH domains of the two Fabs are shown). C, shown is the most representative docking model of the Aahl-Fab9C2 complex as obtained by HADDOCK. The Aahl molecule is displayed with a transparent molecular surface in orange with residues buried at the binding interface in yellow. The toxin orientation and partly buried C-terminal region observed in this model are consistent with the neutralizing capacity of IgG9C2 toward Aahl binding onto the Na $_v$ channel *in vivo*, 20-fold lower than that of IgG4C1 toward Aahl binding (cf. Introduction). (Only the Fab9C2 VL and VH domains are shown).

compared with Fab9C2, consistent with their respective sequence-based theoretical pI values (Fig. 2). The presence of four (for Fab4C1) and two to three (Fab9C2) isoforms differing in their net charges reflects the high content in Lys and Arg residues at the H chain C terminus and the limited specificity of papain cleavage, likely to generate sequence variability in this region. Yet all isoforms displayed equal capacity for toxin binding, as assessed by mobility shift assays and competitive ELISA titrations (Fig. 2). Hence, each Fab retained the distinctive binding properties of the parental IgG toward its respective immunogen (Aahl and AahlII) or related antigen (AahlIII and, as first shown here, AahlIV).

The competitive effect of Fab4C1 toward binding of radio-labeled AahlII to synaptosomal Na $_v$ channel was found to occur with an IC $_{50}$ value of 8 nM (data not shown), a value fully consistent with that reported for IgG4C1 (26, 28). However, in contrast to non-purified IgG4C1, reported to slightly destabilize the toxin-receptor complex (28), purified Fab4C1 was found not to affect dissociation of the channel-bound toxin (data not shown). Hence, *in vitro*, the capability of Fab4C1 to prevent toxin binding to its receptor site is not accompanied by the capability to draw the bound toxin out of this site, despite the comparably high affinities (0.8 nM for AahlII binding to mAb 4C1 versus 0.2 nM for AahlII binding to rat brain synaptosomes). This observation supports a previous assumption for fully or partially overlapping anti-IgG4C1 epitope and Na $_v$ channel binding site at the surface of the AahlII molecule (26, 28) and previous evidence for slow spontaneous dissociation of the toxin-channel complex (33, 53).

Overall Description of AahlII-Fab 4C1 Complex—The overall crystal structure of the AahlII-Fab4C1 complex shows a toxin molecule captured by the Fab variable (VH and VL) domains through multiple interactions mediated by the six CDRs, consistent with a canonical antigen-Fab complex (Fig. 3). The complex mostly resembles an egg inserted “small end first” in the eggcup. The AahlII molecule (the egg), shaped as a compact, somehow flat cone with dimensions $\sim 26 \times 24 \times 12$ Å, consists of a 3-stranded antiparallel β -sheet (residues Lys-2–Gly-4, Glu-32–Gln-37, and Ala-45–Leu-51; Fig. 1) that defines the conserved hydrophobic surface of the toxin (54). This β -sheet is flanked by a single α -helix (residues Asn-19–Lys-28) that caps the opposite face of the toxin and defines the “large end” of the egg. The overall fold is stabilized by four disulfide bridges (Cys-12–Cys-63, Cys-16–Cys-36, Cys-22–Cys-46, and Cys-26–Cys-48) forming a cysteine-stabilized $\alpha\beta$ motif common to all scorpion α - and β -type toxins, some small proteins with toxic properties such as blockers of potassium and chloride channels, and insect and plant defensins (55). The Fab4C1 molecule (the eggcup), with dimensions $\sim 70 \times 40 \times 35$ Å, shows the canonical β -sandwich Ig fold and is characterized by an elbow angle of 140.5° between the variable and constant domains of the H and L chains. The six CDRs forge a binding pocket 13 Å deep and 12 Å wide at the molecular surface of the variable region and partially wrap around the bound toxin. In fact, the extended CDR L1 (Gln-27–Tyr-37; supplemental Fig 1) and the short anionic CDR H2 (Ile-51–Thr-58) located on opposite edges of the binding site, along with the long, anionic, and hydrophobic CDR H3 (Ser-97–Tyr-109) located midway between the other two, are suitably positioned to serve as boundary clamps for trapping the bound cationic toxin (Table 2). CDR L3 (Phe-94–Thr-102), the hydrophobic CDR H1 (Gly-26–Tyr-33), and the very short CDR L2 (Lys-55–Ser-57) contribute complementary anchoring points to complex stabilization.

AahlII-Fab4C1 Interface—The mode of binding of AahlII onto the Fab4C1 variable region (VL+VH domain) is associated with remarkable complementarity in both the shape, chemistry, and electrostatic potentials of the negatively charged Fab paratope surface and the positively charged toxin epitope surface, consistent with a high affinity complex (Fig. 3). Almost 25% of the AahlII molecular surface (~ 1000 of 4200 Å 2) is bur-

TABLE 2
Interactions at the AahII-Fab4C1 complex interface

AahII		Fab4C1			
H-bonds / salt bridges ^a					
Residue	Atom	Residue	Atom	CDR	Distance (<i>Å</i>)
Asp9	O	Tyr59	OH	H2	2.8
Pro41	O	Lys55	Nζ	L2	3.0
Tyr42	O	Asn35	Nδ2	L1	2.9
Arg56 ^b	NH2	Tyr59	OH	H2	3.1
Pro60	O	Tyr33	N	H1	2.8
Arg62	N	Tyr35	OH	H1	2.9
	NH1	Glu39	Oε2	L1	3.0
	NH2	Glu39	Oε1	L1	2.9
His64	Nε2	Ser50	Oγ	H2	2.8
	NH2*	Gly96	O	L3	2.9
	NH2*	Ser97	O	L3	3.0
Non-bonded					
Residue		Residue		CDR	Distance (<i>Å</i>)
Val10		Tyr59		H2	~3.8
Asn11		Tyr33		H1	~3.8
Cys12		His31		L1	~3.8
Thr13		Asn33		L1	~3.6
Phe15		Asn33		L1	~3.8
Pro41		Tyr102		H3	~3.8
Arg56		Phe57		H2	~3.9
Gly59		Tyr33		H1	~3.9
Pro60		Tyr104		H3	~3.7
Gly61		Tyr33		H1	~3.7
Arg62		Phe107		H3	~3.8
Cys63		Tyr37		L1	~3.8
His64		Tyr59		H2	~4.0

^a Within 3.2 \AA distance.^b For one of the two alternate conformations.

* Amide group.

ied to a 1.4 \AA probe radius at the complex interface, whereas the Fab H and L chains contribute 465 and 325 \AA^2 , respectively. Both the number of residues and total surface area buried at the interface are well in the range of general patterns for antigen-antibody complexes (56) and, more generally, high affinity peptide-protein complexes (57). Bound AahII is oriented with its C-terminal region, which includes the C-terminal pentapeptide, the β 1- α 1 segment, and the β 2- β 3 turn, deeply buried in the binding pocket. The significant contribution of this region of the AahII molecule to its antigenic activity had been anticipated using peptidic mapping strategies (27). The tip of CDR H3 in Fab4C1 is ideally positioned to face the β 2- β 3 turn on the conserved hydrophobic surface of the toxin, whereas the long CDR L1 on the adjacent Fab domain wraps around midway the opposite face of AahII, made by the long β 1- α 1 segment and the β 2- β 3 turn. Of the 16 AahII and 18 Fab4C1 residues

buried at the complex interface, 15 AahII and 17 Fab residues form 11 hydrogen bonds/salt bridges and numerous non-polar interactions and dictate the binding pattern with respect to the AahII-Fab4C1 complex stability. Four water molecules involved in water-mediated contacts and a chloride ion cement the AahII-Fab4C1 interactions in optimizing further their complementarity.

The AahII C-terminal region, which encompasses all residues from Arg-56 to the amidated His-64 and contributes 68% to the binding surface area defines an “anchor” region that dominates the complex interface (Fig. 3). The solvent-accessible Cys-12–Cys-63 disulfide bridge, which is packed against the side chains of His-31 and Tyr-37 in CDR L1, also contributes to the interface. Other contact points in AahII involve non-continuous residues from the long β 1- α 1 surface segment and the β 2- β 3 turn. Details of the AahII contacts with the Fab CDRs are summarized in Table 2.

At the center of the combining site, the Arg-62 guanidinium, which protrudes from the toxin core, is sequestered deep within the pocket where it forms a salt bridge with Glu-39 at the base of CDR L1 and cation- π interactions with Phe-107 in CDR H3 (Fig. 3). The imidazole ring of the neighboring His-64 is ideally positioned to stack against the Tyr-59 indole, located at the base of CDR H2 and contributing a cluster of aromatic residues with Tyr-33, Tyr-35, and Trp-47 and to hydrogen bond to Ser-50 at the base of CDR H2. At the periphery of the combining site, the Arg-56 guanidinium adopts two conformations; in conformer A it forms polar interactions with Tyr-59 at the base of CDR H2 and with Tyr-33 in CDR L1 and at the rim of the pocket and stacks against Phe-57 (CDR H2), whereas in conformer B it is directed toward the solvent. Finally, Pro-60, which is directly opposite to Arg-62 at the bottom of the pocket, promotes a weak proline-aromatic interaction with Tyr-104 in CDR H3 (58). Although the main chain carbonyl and nitrogen atoms of the AahII C-terminal region make polar interactions with Fab4C1 CDRs H1 and H3, the major contribution of the five side chains for binding to Fab4C1 argues for a core epitope responsible for antibody specificity.

Compared with unbound AahII (59), the overall conformation of bound AahII is virtually unchanged except for the C-terminal Arg-62 to His-64 region, where an $\sim 75^\circ$ rotation of the Φ torsional angle for Cys-63 reduces conformational flexibility of the Cys-12–Cys-63 disulfide bridge and drastically modifies the backbone direction, resulting in an $\sim 13 \text{\AA}$ distance between the positions of the His-64 imidazole ring centroids (Fig. 3). In fact, a search for structural AahII homologues reveals that the orientation of the C-terminal region found in bound AahII is conserved in other scorpion toxins, as exemplified by the acidic α -toxin from *Buthus tamulus* and the toxin chimera AahII^{LqhaIT(face)} (52 and 86% sequence identity, respectively) (cf. r.m.s.d. values in the supplemental Experimental Procedures/Results/Discussion). However, the distinctive conformational constraints elicited by, or associated with, crystal packing for unbound AahII and Fab trapping for bound AahII suggest inherent flexibility of the C-terminal region of the toxin that could modulate both receptor recognition and antibody complex formation. This feature should be considered when using the structure of AahII as a template for theoretical modeling of

a toxin congener whose three-dimensional structure is not available.

At the periphery of the complex interface, Asp-9 and the Pro-41–Tyr-42 residue pair, located on opposite sides of the cavity, contribute polar interactions with Fab Tyr-59 at the base of CDR H2, Lys-55 in CDR L2, and Asn-35 in CDR L1 through their main chain carbonyl atoms only (Fig. 3). Moreover, Pro-41 at the tip of the β 2– β 3 turn inserts between Tyr-33 and Tyr-104 and promotes proline-aromatic interactions, suggesting that it may confer toxin specificity for Fab4C1 binding.

The structure of the AahII–Fab4C1 complex is in overall good agreement with earlier mapping data suggesting the presence of discontinuous toxin epitopes for IgG binding and the essential role of the C-terminal region of the toxin (28). The four AahII regions identified as being responsible for antigenic reactivity (segments Val-1–Asp-8, Gly-4–Cys-12, Thr-27–Tyr-35, Ala-39–Ala-45, Pro-52–Lys-58, and Val-55–Gly-61 (27)) and for receptor binding (residues around the disulfide bridge Cys-12–Cys-63 and segment Lys-50–Gly-59, non-accessible to the antibodies in the AahII–Na_v complex (25)) are consistent with the binding pattern that emphasizes the central role of the C-terminal end in contrast to the N-terminal and helical regions.

Moreover, although single modification of Arg-56 decreased both AahII binding to Fab4C1 (*cf.* above) and its receptor, chemical modification of antigenic residue Arg-62 had a low effect on the pharmacological activity of AahII (60). However, in both unbound and Fab-bound AahII, the Lys-58 side chain points toward the toxin core and largely contributes to the toxin structural integrity. Hence, despite previous assumptions for an active role (28, 61), this residue cannot contribute direct interaction either with the antibody or with the Na_v channel. Although the structure confirms the predominant role of His-64 for Fab4C1 binding, the remote location of His-54 on the opposite face of the binding interface is inconsistent with previous binding studies (28). Altogether these data demonstrate that the respective AahII binding sites for the Na_v channel (N-terminal region) and for Fab4C1 (C-terminal region) partially overlap.

Absence of Fab4C1 Reactivity for AahII Congeners—On the basis of the structure of the AahII–Fab4C1 complex, a comparative sequence analysis of the other three Aah toxins, AahI, AahIII, AahIV, that belong to the structural and immunological group I, and of four AahII-related toxins, AmmV, AmmVIII, BotIII, and LqqV, of group II, clearly establishes that the determinants dictating the fine specificity of AahII toward Fab4C1 are clustered within the C-terminal region of the toxin molecule (Fig. 1). Indeed, in each of the three group I toxins, the Gly-61 to Ser substitution and its flanking one-residue insertion likely affect the conformation of the C-terminal region. The non-conservative Arg-56 to Pro substitution should significantly affect the hydrogen-bonding pattern, whereas the conservative substitutions of Arg-62 by Lys in AahI and AahIII and by Asp in AahIV would weaken the salt bridge interaction with Fab4C1 Glu-39. Substitution of the C-terminal His-64 by Thr in AahI should have a dramatic effect on the hydrogen-bonding pattern and stacking interactions, whereas the C terminus extension by a Ser in AahIII and a Lys in AahIV likely disrupts

both the bonding pattern mediated by the His-64 imidazole ring and the polar interactions mediated by the amide group. Other sequence differences are either conservative substitutions or associated with a non-interactive region of the toxin molecule, and they do not seem to be crucial for interaction with Fab4C1.

The capability of IgG4C1 for binding potent group II α -toxins BotIII, LqqV, and AmmV, as well as AmmVIII that is devoid of toxicity to mammals, has also been documented (24, 28). BotIII, the closest AahII homologue with Arg, Val, and Asn substitutions for AahII residues Val-10, Leu-51, and His-64, respectively, and an amidated C terminus (Fig. 1), is the only other toxin that can be neutralized by IgG4C1, although with \sim 100-fold lower binding affinity compared with AahII. In contrast to the conservative Val substitution to Leu-51, which is buried within the toxin core, the Arg substitution to Val-10, which is solvent-exposed and vicinal to the Fab4C1 binding interface, may generate steric clashes with Val-99 in CDR L3. Hence, both this mutation and the drastic H64N mutation, central to the interface, likely contribute for the lower affinity of BotIII. The other three toxins, LqqV, AmmV, and AmmVIII, which are more distant from AahII (89–75% sequence identity; Fig. 1), possess several non-conservative substitutions in their C-terminal regions that are incompatible with IgG4C1 binding. In particular, in place of His-64 they all have an Asn, which in LqqV and AmmV is amidated while in AmmVIII it is followed by a supplementary Asp residue. Also, LqqV and AmmV have the same Arg-56 to Ser substitution as found in AahI and AahIII, while their G59E and P60K substitutions could cause steric clashes and electrostatic repulsion with interacting side chains from adjacent CDRs of the Fab4C1 H-chain.

Structure of Fab9C2 and Implications for AahI Neutralization—As for the AahII–4C1 complex, an AahI–Fab9C2 complex was preformed in solution in conditions insuring total occupancy of the Fab by bound toxin, as assessed by a mobility shift assay (Fig. 2), before being subjected to crystallogensis. Yet the structure shows a Fab9C2 molecule devoid of a bound toxin and whose CDR loops are tightly involved in crystal packing interactions (Fig. 4). Considering the rather neutral crystallization conditions, unlikely to promote complex dissociation in the crystallization drop (*cf.* “Experimental Procedures”), this suggests that the toxin was expelled from its binding site at the surface of the Fab combining region during crystal formation. Conformational equilibrium of AahI in solution may participate in complex instability, as suggested by the occurrence of three interconvertible peaks upon reverse phase HPLC purification of AahI, not observed for AahII.⁷ No experimental three-dimensional structure for a group I Aah toxin is available, although primary H⁺-NMR assignments and secondary structure of AahIII have been reported (62). In fact, residue Pro-9, which is not found in the other Aah α -toxins but is found in group III α -like toxins BmK M1 and M4, where it is followed by a non-proline peptide bond undergoing cis/trans isomerization (63, 64), may confer interconversion potency to AahI. In addition, the particular sequence of the Gly-28–Ser-35 octapeptide,

⁷ M. F. Martin-Eauclaire, unpublished data.

which resembles a “linker” sequence but is predicted to form β -strand 2, may also provide greater conformational flexibility to AahI compared with its congeners.

The Fab9C2 molecule, of dimensions $\sim 70 \times 50 \times 40$ Å, is characterized by an elbow angle of 171° , a value supporting flexibility in the overall Fab topology. Most of the Fab9C2 CDRs, defined as a short polar CDR L1 (Glu-27–Asn-32), a very short and apolar CDR L2 (Ala-50–Thr-52), hydrophobic CDRs L3 (Gln-89–Thr-97) and H1 (Gly-26–Trp-33), an apolar CDR H2 (Ile-51–Thr-58), and a hydrophobic and anionic CDR H3 (Ala-97–Tyr-108), significantly differ from those in AahII-bound Fab4C1 in their lengths, sequences, and conformations (Fig. 4; supplemental Fig 1). As a result, they form a planar and largely hydrophobic combining site surface with a neutral electrostatic potential instead of the negatively charged groove found at the molecular surface of Fab4C1 (Fig. 4). Compared with the twisted conformation of CDR H3 in Fab4C1, CDR H3 in Fab9C2 extends toward the L chain as to occupy the central binding pocket formed by the VH and VL domains. Moreover, compared with the long CDR L1 in Fab4C1 that wraps around the bound AahII, the much shorter and non-protruding CDR L1 in Fab9C2 appears to be unable to tightly interact with a bound toxin, a feature that may drastically modify the binding position and orientation of AahI compared with those of AahII onto Fab4C1. Similarly, the CDR H2 in Fab9C2 is dominated by small and apolar side chains in place of the aromatic and polar side chains found in the corresponding region of Fab4C1.

Manual docking of an AahI model onto the Fab9C2 combining site, performed in retaining the relative positions and orientations of the related partners in the AahII–Fab4C1 complex, confirmed that the Fab9C2 variable domains lack the shape and electrostatic complementarities required to accommodate AahI as tightly as would be expected for this high affinity complex (data not shown). Software-assisted docking of the AahI model in eight random orientations led to eight ensembles of three to four energetically best models of a complex, of which one was found in each of the ensembles. In this complex, residue Lys-61 of AahI is anchored at the center of the binding interface (Fig. 4), as is residue Arg-62 of AahII relative to the Fab4C1 combining surface. However, compared with the orientation of AahII bound to Fab4C1, AahI is rotated (by $\sim 90^\circ$) and translated (by 15 Å) as to bury its Cys-12–Val-17, Pro-39–Leu-42, and Ser-59–Thr-63 regions at the complex interface. Only limited conformational changes are observed in the Fab9C2 CDRs and AahI loop regions compared with the starting structures, suggesting genuine complementarity of the partners before complex formation. Almost 27% of the AahI molecular surface (~ 1070 Å² of 4000 Å²) is buried at the complex interface, whereas the Fab H and L chains contribute 500 and 560 Å², respectively, a distribution comparable to that found in the Fab4C1–AahII complex. Hence, only the flat shape and neutral charge of the combining site may be responsible for the lower protective capacity of IgG9C2 toward AahI compared with that of IgG4C1 toward AahII despite the comparable affinities (*cf.* Introduction). Similar docking of AahII onto the Fab4C1 combining site using the same criteria as for docking of AahI onto Fab9C2 led to a single ensemble of complexes with bound AahII inserted within the Fab combining site in similar

binding positions and orientations as observed in the crystal structure of the AahII–Fab4C1 complex.

Structural Comparisons with Cn2–scFv9004G Complex—In North America, scorpions of medical importance belong to the genus *Centruroides*. Many very potent β -toxins have been identified from the venoms of various subspecies (65, 66). Differently from α -toxins, which preferentially bind site 3 of Na_v channels and modify their inactivation, the β -toxins specifically bind site 4 and modify the channel activation process (20). The distinctive pharmacological properties are accompanied by distinctive immunological properties, as illustrated by the total absence of cross-reactivity between the α - and β -toxin classes (65). Moreover, unlike α -toxins, the β -toxins share greater structural homologies, as exemplified by significant cross-reactivity of a serum raised against a *Centruroides* immunogen with the main toxin from another *Centruroides* species (67, 68). Hence, the availability of a crystal structure for a β -toxin bound to an antibody fragment is more likely to provide a canonical template for studying immunoreactivity of diverse members in this class of toxins than it is for α -toxins.

The recently reported crystal structure of the β -toxin Cn2 bound to the engineered antibody scFv 9004G highlights the shape and electrostatic complementarity of the binding interfaces, to which the scFv contributes five of its six CDRs (31) (supplemental Figs. 1 and 2). The scFv variable domains define a planar combining site characterized by a non-protruding CDR L1 and a short and untwisted CDR H3, more similar to their counterparts in Fab9C2 than those in AahII-bound Fab4C1. Bound Cn2 inserts one side of its cone-shaped core, with the side chain of Glu-15 as the central anchor, into the combining site, thereby clustering its long β 1– α 1 segment, part of the α 1 helix, and the tip of the β 2– β 3 turn within the scFv paratope (Fig. 1). The C-terminal region of Cn2, with a non-visible Ser-66, is positioned at the periphery of the complex interface and does not contribute direct interaction with the scFv (supplemental Fig 2). This binding orientation results in the bound Cn2 toxin being rotated by 90° and flipped upside-down from the orientation of AahII bound to Fab4C1. Compared with AahII, the longer β 1– α 1 and shorter β 2 and β 3 segments found in Cn2 also contribute to the distinctive positioning of the toxin relative to the Fab. Superimposition of the two complexes (*cf.* r.m.s.d. values under “Experimental Procedures”) further reveals large conformational differences in the C-terminal regions of the two bound toxins after position 59, again arguing for conformational flexibility of this main epitope.

Implications of the Structures for Immunotherapy of Scorpion Envenomation—A polyclonal serum raised against AahII was found to recognize simultaneously four major antigenic regions at the surface of the toxin molecule (22, 69). These regions roughly encompass residues at positions 29–36 (α 1– β 2 turn and beginning of the β 2 strand), 36–46 (end of the β 2 strand, β 2– β 3 turn, and beginning of the β 3 strand), 50–59 (third type I β -turn), 19–28 (α 1 helix), and the Cys-12–Cys-63 bond region (70, 71). In addition to mAb 4C1, a second mAb, named 3C5, was generated that was found to partly overlap with mAb 4C1 but to display a much lower affinity, by almost 3 orders of magnitude, and no neutralizing capacity (28). This suggests

that AahII trapping by mAb 3C5 involves either a bound toxin orientation or a conformation of the antibody binding pocket distinct from those observed with the AahII-Fab4C1 complex (or a combination of both).

The bioactive surface of α -toxins, studied over three decades through various complementary approaches, encompasses two regions corresponding to one side of the molecule core, and the N- and C-terminal region, respectively (72). Recent initial modeling of the interaction of α -toxin LqhII, a close homologue of AahII, with the Na_v channel has led to suggest that residues Phe-15, Arg-18, Trp-38, and Asn-44 at the side of the toxin core would recognize the voltage-sensing (gating) module in domain IV of the channel, whereas residues Lys-2, Thr-57, and Lys-58 in the toxin N- and C-terminal region would recognize the pore module in domain I of the channel (73). In fact, of these residues only AahII Phe-15 is buried at the Fab4C1 complex interface, whereas in AahI, His-15, Pro-18, and Phe-36 contribute interactions with Fab9C2 (Figs. 3 and 4). Considering the extended toxin surface area buried at the Fab4C1 complex interface (Fig. 3), such a very limited overlap of the bioactive and epitopic surfaces of AahII would not explain the remarkable protective capacity of mAb 4C1 relative to toxin binding to the channel and its inability to accelerate dissociation of the preformed toxin-channel complex.

In this context, efficient serotherapy against Aah envenomation would require concomitant capture and neutralization of all four toxins before they first reach the Na_v channel target, or as soon as they spontaneously dissociate to prevent immediate reassociation. IgG4C1 binds AahII, whereas IgG9C2, generated against AahI, also binds AahIII (29) and AahIV (this work) with high affinities. The intraperitoneal injection of a bispecific tandem-scFv combining the variable domains of IgG4C1 and IgG9C2 or the concomitant intraperitoneal injections of single-chain homomeric diabodies also derived from these IgGs were found to protect experimentally envenomed mice against the overall toxicity of subcutaneous injection of up to 3 LD₅₀ of the Aah venom (11).⁸ Hence, in conjunction with recent strategies involving selection of scFvs using phage-display technology (74), production of camelid antibodies because of their particular suitability for therapeutic use (10), prediction of potential epitopic regions through bioinformatics approaches (75), or the raising of antibodies against discontinuous epitopes (30, 76), our structural data provide new templates for further enhancement of the binding affinity and neutralizing capacity of anti-Aah antivenoms to reach more efficient immunotherapy in humans.

In summary, the crystal structure of the high affinity AahII-Fab4C1 complex unambiguously reveals the position and orientation of the bound α -toxin immunogen relative to its antibody and those of the interacting side chains at the complex interface. The structure also points to the prominent role of the long CDR L1, the short anionic CDR H2, and the long anionic and hydrophobic CDR H3 in forming a groove-like, charge-complementary combining site to sequester the compact, cationic toxin through its C-terminal region as required for effi-

cient *in vivo* neutralization toward Na_v channel binding. In contrast, the accompanying structure of Fab9C2 without a bound AahI immunogen displays a distinctive combining site conformation that appears barely compatible with its high affinity for the toxin. In fact, docking analysis suggests that AahI trapping by Fab9C2 involves a bound toxin orientation distinctive from that observed for bound AahII, thereby supporting total absence of cross-reactivity for the two complexes and milder neutralization of AahI, compared with AahII, toward Na_v channel binding. Finally, structural comparison of the AahII-Fab and Cn2-scFv complexes highlights structural determinants in the α -toxin and β -toxin and their respective neutralizing antibodies that dictate their immunological specificities. Hence, our study provides complementary templates for designing new molecules aimed at neutralizing all four α -toxins in the Aah venom and suitable for therapeutic use.

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