Structural Insights into Antibody Sequestering and Neutralizing of Na\(^+\) Channel \(\alpha\)-Type Modulator from Old World Scorpion Venom

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The Old World scorpion Androctonus australis hector (Aah) produces one of the most lethal venoms for humans. Peptidic \(\alpha\)-toxins Aah 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 \(\alpha\)-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 also preformed and crystallized 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-antibody orientation and main 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 \(\alpha\)-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 \(\alpha\)-helix and an antiparallel three-strand \(\beta\)-sheet, tightly reticulated by 3–4 disulfide bridges, and called the cystine-stabilized \(\alpha\)-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, antiserum 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...
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+) 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+ 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 congener 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, Aahl, 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 Na,1.2 and muscular Na,1.4 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 (Kd 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 Aahl. 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 Aahl 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 Aahl, and design a theoretical AahII-Fab9C2 complex. Striking conformational differences in the combining sites of the toxin-bound Fab4C1 versus unbound Fab9C2, and in the

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. Aahl residues whose side chains interact with side chains in the scFv 9004G CDRs (PDB code 2YC1) are indicated by circles below the alignment. The AahII C-terminal tripeptide that displays a 90° positional difference compared with unbound Aahl (1PTX (59)) is indicated by a bar above the alignment. The distinctive lengths of the β1–α1 segment and β2 and β3 strands in Cn2 and Aahl are evident. The belonging of α-toxins Aahl (from A. australis hector), Botll (from Buthus occitanus tunetanus; 95% sequence identity with Aahl), AmmVIII and AmmV (from A. mauretanicus mauretanicus; 89 and 75%, respectively), LqqV (from Leirus quinquenectriatus quinquenectriatus; 78%), of α-toxins AahII, AahIII, and AahIV (from A. australis hector; 44, 46, 44%, respectively), of α-like toxin LqqIII (from L. quinquenectriatus hebraeus; 39%), and of β-toxin Cn2 (from C. noxius Hoffmann; 45%) to distinct, sequence-dictated immunological and pharmacological groups is apparent.
Experimental AahII-Fab4C1 complex versus the theoretical Aahl-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 archetype Css2 (from the venom of Centruroides noxius Hoffmann, specific for the mammalian Na1.6 channel), bound to an engineered scFv antibody that also neutralizes the ~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 Aahl, 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 μL/mL). The purified IgGs were dialyzed against 0.02 M sodium phosphate, pH 7.0, and concentrated by ultrafiltration.

The Fabs were obtained by papain cleavage of the purified IgGs using a papain-to-IgG ratio of 1:10 (w/w) in the presence of 10 mM L-cysteine, 1 mM β-mercaptoethanol, 1 mM EDTA (~2 h, 37 °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 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 using 1 M Tris, pH 9.0 (55 μL/mL). The purified IgGs were dialyzed against 0.02 M sodium phosphate, pH 7.0, and concentrated by ultrafiltration.

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**Functional Analysis of Fabs**—The binding of Aahl, AahIII, and AahIV by IgG9C2 and Fab9C2 and of Aahll by IgG4C1 and Fab4C1 was analyzed by ELISA at 20 °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 μL/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). Incubation of the specific anti-toxin IgG (10⁻⁵ to 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 to 10⁻¹² M) was incubated along with the parental IgG at a concentration close to the previously determined half-effect (EC₅₀) value (10⁻⁷ M for IgG4C1, 10⁻⁹ for IgG9C2). Because of the limited coating capability of Aahl IV, presumably arising from its low pI value compared with the other Aah toxins (cf. the legend to Fig. 2), Fab9C2 binding to Aahl IV was assayed in competition with Aahl. Data were plotted and analyzed according to a sigmoidal equation.

The effects of Fab4C1 onto Aahll binding to, and dissociation from, its binding site on rat brain synaptosomal Nav channel were analyzed by liquid-phase RIA (28) as briefly follows. Radioiodinated Aahll (2·10⁻¹⁰ M) was incubated in the presence of Fab4C1 in a range of concentrations (8·10⁻⁶ to 8·10⁻¹³ M) and rat brain synaptosomal membranes (20 μg) (30 min; 30°C). The unbound and membrane-bound toxin populations were separated by centrifugation (13,000 × g, 5 min, 4°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 radioiodinated Aahlll 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 Aahl-Fab9C2 and Aahll-Fab4C1 complexes were formed in solution at high Fab concentration (~100 μM, well above the toxin Kᵣ values, i.e. 8·10⁻¹⁰ for Aahll/IgG4C1 by RIA (28); 1.5·10⁻¹⁰ or 0.11·10⁻¹⁰ for Aahl/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°C). They were then buffer-exchanged for 0.01 M Hepes, pH 7.4, 0.05 M NaCl, 0.02% NaN₃ (4°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°C by vapor diffusion using 1–1.2 μl hanging drops and a protein-to-well solution ratio of 1:1 (v/v). For the Aahl-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 Aahl-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.
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-

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6 P. Marchot, manuscript in preparation.

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

TABLE 1
Data collection and refinement statistics

|                      | AahII-Fab4C1 | Fab9C2 |
|----------------------|--------------|--------|
| **Data collection**  |              |        |
| Beamline (ESRF)      | ID14-EH2     | ID23-1 |
| Resolution range (Å) | 13.2-3       | 15.1-6 |
| Space group          | P2₁         | P2₁   |
| Cell dimensions, a, b, c (Å): β (°) | 123.9, 84.7, 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 |
| Rsym (%)⁴           | 8.7 (43.1)   | 6.4 (42.3) |
| (||Fhkl|-|Fobs|)/|Fhkl|   | 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 (Å²) | 34.64 | 37.4 |

| **Refinement**       |            |        |
| Resolution (Å)       | 13.2-3     | 30.1-6 |
| Rcryst (%)           | 20.3 (26.9)| 17.4 (28.5)|
| Rfree (%)            | 25.8 (32.1)| 21.4 (29.3)|
| Number of reflections used in refinement | 62,651 | 59,927 |
| Number of water molecules | 210 | 614 |
| Root mean square deviation from ideal geometry | <0.001/0.001/0.001 | <0.001/0.001/0.001 |
| Chiral volume (Å³)   | 10.8 (3.45)| 11.26 (2.8) |
| Mean B factors (Å²)  | 38.5/39.7 | 19.4/21.1 |
| Ramachandran plot statistics⁷ | % of residues in favored/outlier regions | 97.6/0.07 | 97.4/0.07 |
| PDB accession code   | 4AEI       | 4AEH   |

⁴ Values in parentheses are for the highest resolution shell.
⁵ Rsym = ∑|Fobs|−|Fcal|/∑|Fobs|.
⁶ Rcryst = ∑|Fobs|−|Fcal|/∑|Fobs|.
⁷ Rfree is calculated for randomly selected reflections excluded from refinement.

α-Conformations 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; 2 aromatic residues; local pl 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 pl 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 Å (62 Cα atoms), and between bound AahII and the Aah model (cf. below) it is 0.78 Å (61 Cα atoms). Between Fab4C1 and Fab9C2 the r.m.s.d. is 0.65 Å (202 Cα atoms) for the constant domains and 1.36 Å (130 Cα atoms) for the variable domains. Comparison of the AahII-Fab4C1 complex with the structure of scorpion α-toxin Cn2 bound to scFv 9004G (PDB code 2YC1) yielded r.m.s.d. values of 1.84 Å (54 Cα atoms) between the bound toxins and 0.68 Å (107 Cα 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).
Theoretical Modeling—The AahI model was built with MODELLER (50) using the structure of the /H9251-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...
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 (AahI and AahII) or related antigen (AahIII and, as first shown here, AahIV).

The competitive effect of Fab4C1 toward binding of radio-labeled AahII to synaptosomal Na⁺ channel was found to occur with an IC₅₀ 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 comparable high affinities (0.8 nM for AahII binding to mAb 4C1 versus 0.2 nM for AahII binding to rat brain synaptosomes). This observation supports a previous assumption for fully or partially overlapping anti-IgG4C1 epitope and Na⁺ channel binding site at the surface of the AahII molecule (26, 28) and previous evidence for slow spontaneous dissociation of the toxin-channel complex (33, 53).

**Overall Description of AahII-Fab 4C1 Complex**—The overall crystal structure of the AahII-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 AahII molecule (the egg), shaped as a compact, somehow flat cone with dimensions ~26 × 24 × 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 αβ 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 ~70 × 40 × 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.

**AahII-Fab4C1 Interface**—The mode of binding of AahII 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 AahII molecular surface (~1000 of 4200 Å²) is bur-
ied to a 1.4 Å probe radius at the complex interface, whereas the Fab H and L chains contribute 465 and 325 Å², 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 \( \text{H} \text{H}_9 \text{252} - \text{H} \text{H}_9 \text{253} \) segment, and the \( \text{H} \text{H}_9 \text{252} - \text{H} \text{H}_9 \text{252} \) 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 \( \text{H} \text{H}_9 \text{252} - \text{H} \text{H}_9 \text{252} \) turn on the conserved hydrophobic surface of the toxin, whereas the long \( \text{H} \text{H}_9 \text{1} - \text{H} \text{H}_9 \text{1} \) segment and the \( \text{H} \text{H}_9 \text{2} - \text{H} \text{H}_9 \text{2} \) 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 \( \text{H} \text{H}_9 \text{1} - \text{H} \text{H}_9 \text{1} \) surface segment and the \( \text{H} \text{H}_9 \text{2} - \text{H} \text{H}_9 \text{2} \) 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 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 \( \sim 13 \) Å 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 \( \alpha \)-toxin from Buthus tamulus and the toxin chimera AahII\( ^{\text{LqhIT(face)}} \) (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

### TABLE 2

**Interactions at the AahII-Fab4C1 complex interface**

| AahII | Fab4C1 |
|-------|--------|
| **H-bonds / salt bridges** | **Distance (Å)** |
| Residue | Atom | Residue | Atom | CDR |
| 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 (Å) |
| 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 |

* Within 3.2 Å distance.
* For one of the two alternate conformations.
* Amide group.

**Fab-neutralized Scorpion α-Toxin**

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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 β2-β3 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-Naα 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 Naα 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 Naα channel (N-terminal region) and for Fab4C1 (C-terminal region) partially overlap.

Absence of Fab4C1 Reactivity for AahII Congeneres—On the basis of the structure of the AahII-Fab4C1 complex, a comparative sequence analysis of the other three Aah toxins, Aahl, 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 Aahl 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 Aahl 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 ~100-fold lower binding affinity compared with Aahl. 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 Aahl 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 Aahl Neutralization—As for the Aahl-Fab9C2 complex, a Aahl-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 crystallogenesis. 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 Aahl in solution may participate in complex instability, as suggested by the occurrence of three interconvertible peaks upon reverse phase HPLC purification of Aahl, not observed for Aahl.7 No experimental three-dimensional structure for a group I Aah toxin is available, although primary H+–NMR assignments and secondary structure of AahlIII have been reported (62). In fact, residue Pro-9, which is not found in the other Aahl α-toxins but is found in group III α-like toxins BmM1 M1 and M4, where it is followed by a non-proline peptide bond undergoing cis/trans isomerization (63, 64), may confer interconversion potency to Aahl. 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 AahII compared with its congeners.

The Fab9C2 molecule, of dimensions ~70 × 50 × 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 AahII 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 AahII 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 AahII as tightly as would be expected for this high affinity complex (data not shown). Software-assisted docking of the AahII 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 AahII 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, AahII is rotated (by ~90°) 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 AahII loop regions compared with the starting structures, suggesting genuine complementarity of the partners before complex formation. Almost 27% of the AahII 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 IgG4C2 toward AahII 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 AahII 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 Nav 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 complementary 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 β1-α1 segment, part of the α1 helix, and the tip of the β2-β3 turn within the scFv paratype (Fig. 1). The C-terminal region of Cn2, with a nonvisible 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 β1-α1 and shorter β2 and β3 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 (α1-β2 turn and beginning of the β2 strand), 36–46 (end of the β2 strand, β2-β3 turn, and beginning of the β3 strand), 50–59 (third type I β-turn), 19–28 (α1 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\(_\alpha\) 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, these residues only AahII Phe-15 is buried at the Fab4C1 complex interface, whereas in Aahhl, 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 suitability for therapeutic use (10), prediction of potential epitope regions through bioinformatics approaches (75), or the tralizing antibodies that dictate their immunological specifici-

In this context, efficient serotherapy against Aah envenomation would require concomitant capture and neutralization of all four toxins before they first reach the Na\(_\alpha\) channel target, or as soon as they spontaneously dissociate to prevent immediate reassociation. IgG4C1 binds AahII, whereas IgG9C2, generated against AahII, 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 LD\(_{50}\) of the Aah venom (11).\(^8\) Hence, in conjunction with recent strategies involving selection of scFvs using phage-display technology (74), production of cameldi antibodies because of their particular suitability for therapeutic use (10), prediction of potential epitope 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-

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\(^8\) N. Aubrey, personal communication.

**REFERENCES**

1. Chippaux, J. P., and Goyffon, M. (2008) Epidemiology of scorpionism. A global appraisal. *Acta Trop.* 107, 71–79

2. Quintero-Hernández, V., Júdez-González, V. R., Ortiz-León, M., Sánchez, R., Possani, L. D., and Becerril, B. (2007) The change of the scFv into the Fab format improves the stability and in vivo toxin neutralization capacity of recombinant antibodies. *Mol. Immunol.* 44, 1307–1315

3. Sami-Merah, S., Hammondi-Triki, D., Martin-Eauclaire, M. F., and Laraba-Djebbar, F. (2008) Combination of two antibody fragments F(ab’)(2)/Fab. An alternative for scorpion envenoming treatment. *Int. Immunopharmacol.* 8, 1386–1394

4. Yahni, N., Devaux, C., Mansuelle, P., Defendini, M. L., and Granier, C. (1992) Monoclonal antibodies to toxin II from the scorpion *Androctonus australis hector*. Further characterization of epitope specificities and neutralizing capacities. *Toxicon* 30, 723–731

5. Mousli, M., Devaux, C., Rochat, H., Goyffon, M., and Billiald, P. (1999) A recombinant single-chain antibody fragment that neutralizes toxin II from the venom of the scorpion *Androctonus australis hector*. *FEBS Lett.* 442, 183–188

6. Devaux, C., Moreau, E., Goyffon, M., Rochat, H., and Billiald, P. (2001) Construction and functional evaluation of a single-chain antibody fragment that neutralizes toxin Aah from the venom of the scorpion *Androctonus australis hector*. *Eur. J. Biochem.* 268, 694–702

7. Devaux, C., Clot-Faybesse, O., Pugnière, M., Mani, J. C., Rochat, H., and Granier, C. (2002) A strategy for inducing an immune response against *Androctonus australis* scorpion venom toxin I in mice. Production of high affinity monoclonal antibodies and their use in a sensitive two-site immuno-nometric assay. *J. Immunol. Methods* 271, 37–46

8. Aubrey, N., Devaux, C., Sizaret, P. Y., Rochat, H., Goyffon, M., and Billiald, P. (2003) Design and evaluation of a diabody to improve protection against a potent scorpion neurotoxin. *Cell Mol. Life Sci.* 60, 617–628

9. Aubrey, N., Muzard, J., Christophe Peter, J., Rochat, H., Goyffon, M., Devaux, C., and Billiald, P. (2004) Engineering of a recombinant Fab from a neutralizing IgG directed against scorpion neurotoxin Aah and functional evaluation versus other antibody fragments. *Toxicon* 43, 233–241

10. Meddeh-Mouelhi, F., Boushaouala-Zahar, B., Benlasfar, Z., Hammadi, M., Mejri, T., Moslah, M., Karoui, H., Khorchani, T., and El Ayeb, M. (2003)
Immunized camel sera and derived immunoglobulin subclasses neutralizing *Androctonus australis hector* scorpion toxins. *Toxicon* **42**, 785–791

Juste, M., Martin-Eauclaire, M. F., Devaux, C., Billiard, P., and Aubrey, N. (2007) Using a recombinant bispecific antibody to block Na⁺ channel toxins protects against experimental scorpion envenoming. *Cell Mol. Life Sci.* **64**, 206–218

Hmla, I., Abdallah B. A., Saerens, D., Benlasfar, Z., Conrath, K., El Ayeb, M., Muylermans, S., and Bouhouala-Zahar, B. (2008) VHH, bivalent domains, and chimeric Heavy chain-only antibodies with high neutralizing efficacy for scorpion toxin AahI. *Mol. Immunol.* **45**, 3847–3856

Hmla, I., Saerens, D., Ben Abderrazek, R., Vincke, C., Abidi, N., Benlasfar, Z., Govaert, I., El Ayeb, M., Bouhouala-Zahar, B., and Muylermans, S. (2010) A bispecific nanobody to provide full protection against lethal scorpion envenoming. *FASEB J.* **24**, 3479–3489

Baghaoui, E., Pichon, J., Muller, J. M., Darbon, H., Elayeb, M., Granier, C., Marvaldi, J., and Rochat, H. (1988) Monoclonal antibodies to scorpion toxins. Characterization and molecular mechanisms of neutralization. *J. Immunol.* **141**, 214–220

29. Clot-Faybesse, O., Juin, M., Rochat, H., and Devaux, C. (1999) Monoclonal antibodies against the *Androctonus australis hector* scorpion neurotoxin I. Characterization and use for venom neutralization. *FEBS Lett.* **458**, 313–318

30. Alvarez, A., Moreau, V., Feliciori, L., Nguyen, C., Duarte, C., Chavez-Olortegui, C., Molina, F., Martin-Eauclaire, M. F., and Granier, C. (2010) Design of antibody-reactive peptides from discontinuous parts of scorpion toxins. *Vaccine* **28**, 970–980

31. Canul-Tec, J. C., Riaño-Umbarila, L., Rudino-Piñera, E., Becerril, B., Possani, L. D., and Torres-Larios, A. (2011) Structural basis of neutralization of the major toxic component from the scorpion *Centruroides noxius* Hofmann by a human-derived single-chain antibody fragment. *J. Biol. Chem.* **286**, 20892–20900

32. Devaux, C., Clot-Faybesse, O., Juin, M., Mabrouk, K., Sabatier, J. M., and Rochat, H. (1997) Monoclonal antibodies neutralizing the toxin II from *Androctonus australis hector* scorpion venom. Usefulness of a synthetic, non-toxic analog. *FEBS Lett.* **412**, 456–460

33. Jover, E., Martin-Moutot, N., Couraud, F., and Rochat, H. (1978) Scorpion toxin specific binding to rat synaptosomes. *Biochim. Biophys. Res. Commun.* **85**, 377–382

34. Martin-Eauclaire, M. F., Alami, M., Giamarchi, A., Missimilli, V., Rosso, J. P., and Bougis, P. E. (2006) A natural anatoxin, Amm VIII, induces neutralizing antibodies against the potent scorpion α-toxins. *Vaccine* **24**, 1990–1996

35. Marchot, P., Ravelli, R. B., Raves, M. L., Bourne, Y., Vellom, D. C., Kanter, J., Camp, S., Sussman, J. L., and Taylor, P. (1996) Soluble monomeric acetylcholinesterase from mouse. Expression, purification, and crystallization in complex with fasciculin. *Protein Sci.* **5**, 672–679

36. Kabasch, W. (1993) Automatic processing of rotation diffraction data from crystals of initially unknown symmetry and cell constants. *J. Appl. Cryst.* **26**, 795–800

37. Collaborative Computational Project, Number 4 (1994) The CCP4 suite, programs for protein crystallography. *Acta Crystallogr. D Biol. Crystallogr.* **50**, 760–763

38. Perrakis, A., Morris, R., and Lamzin, V. S. (1999) Automated protein crystals of initially unknown symmetry and cell constants. *Acta Crystallogr. D Biol. Crystallogr.* **55**, 456–460

39. Emsley, P., and Cowtan, K. (2004) Coot. Model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2126–2132

40. Marshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr. D Biol. Crystallogr.* **53**, 240–255

41. Al-Lazikani, B., Lesk, A. M., and Chothia, C. (1997) Standard conformations for the canonical structures of immunoglobulins. *J. Mol. Biol.* **273**, 927–948

42. Davis, I. W., Leaver-Fay, A., Chen, V. B., Block, J. N., Kapral, G. J., Wang, X., Murray, L. W., Aren dall, W. B., 3rd, Snoeyink, J., Richardson, J. S., and Richardson, D. C. (2007) MolProbity. All-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Res.* **35**, W375–W383

43. Stanfield, R. L., Zemla, A., Wilson, I. A., and Rupp, B. (2006) Antibody elbow angles are influenced by their light chain class. *J. Mol. Biol.* **357**, 1566–1574

44. Kleywegt, G. J. (1996) Use of non-crystallographic symmetry in protein structure refinement. *Acta Crystallogr. D Biol. Crystallogr.* **52**, 842–857

45. Baker, N. A., Sept, D., Joseph, S., Holst, M. J., and McCammon, J. A. (2001) Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10037–10041

46. Gouet, P., Courcelle, E., Stuart, D. I., and Mézou, F. (1999) ESPript. Analysis of multiple sequence alignments in PostScript. *Bioinformatics* **15**, 305–308

47. Edgar, R. C. (2004) MUSCLE. A multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **5**, 113

48. Delano, W. L. (2002) in *The PyMOL Molecular Graphics System* (DeLano Scientific LLC, eds), Palo Alto, CA
Fab-neutralized Scorpion α-Toxin

50. Saló, A., and Blundell, T. L. (1993) Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. 234, 779–815
51. Söding, J. (2005) Protein homology detection by HMM-HMM comparison. Bioinformatics 21, 951–960
52. Dominguez, C., Boelens, R., and Bonvin, A. M. (2003) HADDOCK. A protein-protein docking approach based on biochemical or biophysical information. J. Am. Chem. Soc. 125, 1731–1737
53. Granier, C., Moreau, V., Chavez-Olortegui, C., and El-Ayeb, M. (2009) in Animal Toxins: State of the Art. Perspectives in Health and Biotechnology (De Lima, M. E., Pimenta, A., Martin-Eauclaire, M. F., and Benedeta Zingali, R., eds) pp. 123–136. Belo-Horizonte, Editora UFMG, Brazil
54. Sun, Y. M., Bosmans, F., Zhu, R. H., Goudet, C., Xiong, Y. M., Tytgat, J., and Wang, D. C. (2003) Importance of the conserved aromatic residues in the scorpion α-like toxin BmK M1. The hydrophobic surface region revisited. J. Biol. Chem. 278, 24125–24131
55. Cornet, B., Bonmatin, J. M., Hetru, C., Hoffmann, J. A., Ptak, M., and Vovelle, F. (1995) Crystal structure of toxin II from the scorpion Androctonus australis hector. J. Mol. Biol. 243, 83–137
56. Lo Conte, L., Chothia, C., and Janin, J. (1999) The atomic structure of protein-protein recognition sites. J. Mol. Biol. 285, 2177–2198
57. Janin, J., and Chothia, C. (1990) The structure of protein-protein recognition sites. J. Biol. Chem. 265, 16027–16030
58. Chakrabarti, P., and Bhattacharyya, R. (2007) Geometry of nonbonded interactions involving planar groups in proteins. Prog. Biophys. Mol. Biol. 95, 83–137
59. Housset, D., Habersetzer-Rochat, C., Astier, J. P., and Fontecilla-Camps, J. C. (1994) Crystal structure of toxin II from the scorpion Androctonus australis hector refined at 1.3 A resolution. J. Mol. Biol. 238, 88–103
60. Kharrat, R., Darbon, H., Granier, C., and Rochat, H. (1990) Structure-activity relationships of scorpion α-neurotoxins. Contribution of arginine residues. Toxicon 28, 509–523
61. Legros, C., Céard, B., Vacher, H., Marchot, A., Bougis, P. E., and Martin-Eauclaire, M. F. (2005) Expression of the standard scorpion α-toxin AaH II and AaH II mutants leading to the identification of some key bioactive elements. Biochim. Biophys. Acta 1723, 91–99
62. Mikou, A., LaPlante, S. R., Guittet, E., Lallemand, J. Y., Martin-Eauclaire, M. F., and Rochat, H. (1992) Toxin III of the scorpion Androctonus australis hector. Proton nuclear magnetic resonance assignments and secondary structure. J. Biomol. NMR 2, 57–70
63. He, X. L., Li, H. M., Zeng, Z. H., Liu, X. Q., Wang, M., and Wang, D. C. (1999) Crystal structures of two α-like scorpion toxins. Non-proline cis peptide bonds and implications for new binding site selectivity on the sodium channel. J. Mol. Biol. 292, 125–135
64. Guan, R. J., Xiang, Y., He, X. L., Wang, C. G., Wang, M., Zhang, Y., Sundberg, E. J., and Wang, D. C. (2004) Structural mechanism governing cis and trans isomeric states and an intramolecular switch for cis/trans isomerization of a non-proline peptide bond observed in crystal structures of scorpion toxins. J. Mol. Biol. 341, 1189–1204
65. Martin, M. F., Garcia y Perez, L. G., El Ayeb, M., Kopeyan, C., Bechis, G., Jover, E., and Rochat, H. (1987) Purification and chemical and biological characterizations of seven toxins from the Mexican scorpion, Centruroides suffusus suffusus. J. Biol. Chem. 262, 4452–4459
66. de la Vega, R. C., and Possani, L. D. (2007) Novel paradigms on scorpion toxins that affects the activating mechanism of sodium channels. Toxicon 49, 171–180
67. Garcia y Perez, G., Martin, M. F., and Rochat, H. (1988) Preparation of a polyvalent antivenom against various Mexican scorpion Centruroides species. Toxicon 26, 1102–1106
68. Rian˜o-Umbarila, L., Contreras-Ferrat, G., Olamendi-Portugal, T., Morelos-Júa´rez, C., Corzo, G., Possani, L. D., and Becerril, B. (2011) Exploring cross-reactivity to neutralize two different scorpion venoms with one single chain antibody fragment. J. Biol. Chem. 286, 6143–6151
69. El Ayeb, M., Bahraoui, E. M., Granier, C., Delori, P., Van Rietschoten, J., and Rochat, H. (1984) Immunoochemistry of scorpion α-toxins. Purification and characterization of two functionally independent IgG populations raised against toxin II of Androctonus australis hector. Mol. Immunol. 21, 223–232
70. Bahraoui, E. M., El Ayeb, M., Van Rietschoten, J., Rochat, H., and Granier, C. (1986b) Immunoochemistry of scorpion α-toxins. Study with synthetic peptides of the antigenicity of four regions of toxin II of Androctonus australis hector. Mol. Immunol. 23, 357–366
71. Bahraoui, E. M., Granier, C., Van Rietschoten, J., Rochat, H., and El Ayeb, M. (1986a) Immunoochemistry of scorpion α-toxins. Elucidation of the molecular basis of selective recognition of specific human antibodies elicited by a synthetic peptide of scorpion toxin. J. Immunol. 136, 3371–3377
72. Ye, X., Bosmans, F., Li, C., Zhang, Y., Wang, D. C., and Tytgat, J. (2005) Structural basis for the voltage-gated Na+ channel selectivity of the scorpion α-like toxin BmK M1. J. Mol. Biol. 353, 788–803
73. Guir, M., Kahn, R., Karbat, I., Regev, N., Wang, J., Catterall, W. A., Gordon, D., and Guervitz, M. (2011) Elucidation of the molecular basis of selective recognition uncovers the interaction site for the core domain of scorpion α-toxins on sodium channels. J. Biol. Chem. 286, 35209–35217
74. Rian˜o-Umbarila, L., Júrrez-González, V. R., Olamendi-Portugal, T., Ortiz-León, M., Possani, L. D., and Becerril, B. (2005) A strategy for the generation of specific human antibodies by directed evolution and phage display. An example of a single-chain antibody fragment that neutralizes a major component of scorpion venom. FEBS J. 272, 2591–2601
75. Moreau, V., Granier, C., Villard, S., Laune, D., and Molina, F. (2006) Discontinuous epitope prediction based on mimotope analysis. Bioinformatics 22, 1088–1095
76. Duarte, C. G., Alvarenga, L. M., Dias-Lopes, C., Machado-de-Avila, R. A., Nguyen, C., Molina, F., Granier, C., and Chávez-Olótegui, C. (2010) In vivo protection against Tityus serrulatus scorpion venom by antibodies raised against a discontinuous synthetic epitope. Vaccine 28, 1168–1176