Immunochemistry of Sea Anemone Toxins: Structure–Antigenicity Relationships and Toxin–Receptor Interactions Probed by Antibodies Specific for One Antigenic Region

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ABSTRACT: Two antibody subpopulations directed against *Anemonia sulcata* toxin I or II have been purified by immunoaffinity chromatography. These antibodies are specific for a single antigenic region and were used in a structure–antigenicity relationship study using homologous toxins and chemically modified derivatives of *A. sulcata* toxin II. Asp-7 and/or Asp-9 and Gln-47 of toxin II were found to be implicated in the antigenic region recognized by the two antibody subpopulations. On the contrary, Arg-14, Lys-35, -36, and -46, and α-NH_2 of the glycine residue of *A. sulcata* toxin II are not involved in the corresponding antigenic region. When assayed for interaction with the sodium channel, the antigenic region of toxin II, including Asp-9 and Gln-47, appeared fully accessible to its specific antibodies, suggesting that it is not involved in the binding of the toxin to its receptor.

Sea anemone and scorpion α toxins exert homologous pharmacological effects on the sodium channel of excitable membranes. They bind to a common site related to the action potential and are able to maintain the Na^+ channel open by decreasing its rate of inactivation (Catterall & Beress, 1978; Couraud et al., 1978; Vincent et al., 1980). Possible structural homologies that may account for a similar mode of action have not yet been defined. While little information is available for anemone toxins, X-ray crystallographic (Fontecilla et al., 1981) and chemical modification studies (Darbon et al., 1983; Darbon & Angelides, 1984; El Ayeb et al., 1986) have suggested that a conserved and hydrophobic but accessible surface might constitute the receptor binding site of scorpion toxins. In addition, recent data have indicated the respective locations of antigenic and receptor binding sites of scorpion toxins (El Ayeb et al., 1983b; El Ayeb et al., 1984; Bahraoui et al., 1986), but no comparable data are available for sea anemone toxins.

Anemone toxins are small polypeptides of 3000–5000 Da with a high content of hydrophobic amino acid residues, a strong basicity, and three disulfide bridges (Rathmayer, 1979). The sequences of seven of these polypeptide toxins are currently available (Wunderer et al., 1976; Beress et al., 1977; Martinez et al., 1977; Tanaka et al., 1977; Wunderer & Eulitz, 1978; Norton et al., 1978; Reimer et al., 1985). Some information on the conformational features of these polypeptides has been already reported (Prescott et al., 1976; Norton & Norton, 1979; Ishizaki et al., 1979; Norton et al., 1980; Nabiullin et al., 1982).

Two types of studies dealing with the immunogenicity and antigenicity of ATX I and ATX II are reported here. IgG and Fab fragment populations, specific to one antigenic region of ATX I and ATX II, were purified from rabbit immune antisera. These monoregion-specific populations were used in structure–antigenicity relationships with homologous toxins and ATX II chemically modified at known amino acid positions. In addition, the antigenic region of ATX II recognized by the monoregion-specific IgG subpopulations was investigated in terms of its capacity to participate in the receptor binding site of ATX II.

Materials and Methods

Materials. Protein A, protein A-Sepharose, CNBr-Sepharose Cl4B, Sephadex G-100, and SP-Sephadex were purchased from Pharmacia Fine Chemicals (Bois-d'Arcy, France). Bio-Gel P_2 was from Bio-Rad. Papain (EC 3.4.22.2) and lactoperoxidase (EC 1.11.1.7) were purchased from Sigma (St. Louis, MO). $Na^{125}I$ (13–17 mCi/μg) was obtained from Amersham. Serum against rabbit IgG's prepared in donkeys (RD_{17}) was purchased from Wellcome (Beckenham, U.K.). Nonimmune rabbit serum was prepared in our laboratory. Reagents for chemical modifications were from Roche (fluorescamine), Fluka (glycine ethyl ester, 1,2-cyclohexanedione, tyrosine), Merck [1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide], and Pierce (iodogen). Microtest flexible plates were from Falcon.

Methods. Toxins and Antitoxins. Anemone toxins were purified as previously described (Beress et al., 1975). Rabbits (blanc de Bouscat, Evic Ceba) were immunized with toxins I and II of *Anemonia sulcata* (ATX I, ATX II) following the standard procedure previously established for scorpion toxins (Delori et al., 1981). Serum titers were 100–250 μg of antibody/mL, as determined by immunoadsorption of the antibodies on ATX II or ATX I coupled to CNBr-activated Sepharose 4B.

Iodination of Proteins. Protein A was iodinated as described by Biberfeld et al. (1975). The iodinated protein was desalted from $Na^{125}I$ by filtration through a Sephadex G-25 column (1 × 20 cm). The specific radioactivity was 157 Ci/mmol. Of the iodinated protein 80–100% could bind specifically to rabbit nonimmune IgGs bound to Sepharose.

One nanomole of ATX II in 50 μL (final volume) of 10 mM Tris-HCl, pH 8.6, and 1 mCi of $Na^{125}I$ (13–17 mCi/μg) were added to 75 nmol of iodogen-coated tube and incubated for 15 min. The reaction was stopped by adding 100 μL of 0.1 M acetic acid. The iodinated protein was freed from excess of $Na^{125}I$ by filtration through a Bio-Gel P_2 column (1 × 20

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cm). The specific radioactivity of ATX II iodinated on histidine-32 and/or -37 was 200 Ci/mmol.

Antigenic Valence of ATX I and ATX II. The antigenic valence of ATX I and ATX II was checked by immunoprecipitation of ^{125}I-ATX II with ATX I and ATX II antitoxins and by the estimation of the number of anti-ATX I or anti-ATX II Fab fragments able to bind simultaneously on the ATX II surface.

Immunoprecipitation of a mixture of ^{125}I-ATX II (1.3×10^{-9} M) and ATX II (10^{-7} M) was carried out on a variable volume of ATX II antitoxin and ATX I antitoxin or a variable concentration of anti-ATX II IgGs and anti-ATX I IgGs that were purified on Sepharose-bound ATX II and ATX I, as previously described (El Ayeb et al., 1983b). Controls consisted of nonimmune IgGs and rabbit nonimmune serum. Incubation and separation conditions were those reported by El Ayeb et al. (1983a).

Anti-ATX I and anti-ATX II Fab fragments were prepared by papain proteolysis of the corresponding IgGs. They were then purified by filtration on a protein A-Sepharose column as reported (El Ayeb et al., 1983b). Fifty microliters of ^{125}I-ATX II (8×10^{-9} M) were incubated for 2 h at 37 °C with 400 μL of anti-ATX II or anti-ATX I Fab fragments at 1.4×10^{-6} and 3.8×10^{-6} M, respectively. The molecular weights of Fab toxin complexes were estimated by filtration through a calibrated Sephacryl S-300 column.

Competitive Radioimmunoassays. Competitive radioimmunoassay for ATX II using anti-ATX II IgGs, anti-ATX I IgGs, natural homologous and chemically modified toxins, were carried out as previously described (El Ayeb et al., 1984).

Solid-Phase Radioimmunoassays. They were performed on native or chemically modified toxin-coated (0–0.5 μg of toxin/well) U-bottom flexible microtiter plates by adding 50 μL of anti-ATX II IgGs or anti-ATX I IgGs at 2×10^{-8} M in 50 mM sodium phosphate buffer, pH 7.4, 0.1% BSA, followed by incubation for 3 h at 37 °C. Then, 50 μL of anti-IgG antiserum diluted 20 times was added, and a new incubation was carried out under the same conditions. Finally, 50 μL of 5×10^{-9} M ^{125}I-protein A (157 Ci/mmol) was added. After each addition the plates were washed five times with 50 mM phosphate buffer, pH 7.4, 0.1% BSA. The washed and dried wells were finally cut, and radioactivity was determined in a γ counter.

Chemical Modification of ATX II. Chemical modifications were performed as described by Barhanin et al. (1981): primary amines with fluorescamine; the guanidine group of the unique Arg-14 with 1,2-cyclohexanedione; carboxylic functions by reaction with glycine ethyl ester after activation with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide followed by purification of monomodified derivatives on SP-Sephadex C-25 column. The extent of reaction with fluorescamine and cyclohexanedione was determined by amino acid analysis of the derivatives.

Prediction of the Major Antigenic Sites Using the Method of Hopp and Woods. Hopp and Woods (1981) presented a method for predicting major antigenic sites of proteins by analyzing their amino acid sequence to determine the point of highest local hydrophilicity, which is invariably located in, or immediately adjacent to, an antigenic site. We have applied this methodology to the following available amino acid sequences: toxins I, II, and III of *A. sulcata* (ATX I, ATX II, ATX III); toxin A of *Anthopleura xantogrammica* (APA); toxin C of *Anthopleura elegantissima* (APC). All these toxins have the same effect on the sodium channel (Couraud et al., 1978; Catterall & Beress, 1978; Vincent et al., 1980). Physicochemical studies (Prescott et al., 1976; Norton and Norton, 1979; Ishizaki et al., 1979; Norton et al., 1980; Nabiullin et al., 1982) have demonstrated that the overall configuration of these toxins is similar, except for ATX III. This suggests that the homologous antigenic sites are located in the same or in very close regions.

Accessibility of the ATX II–Receptor Complex to Anti-ATX II IgGs Specific to One Antigenic Site. Synaptosomes (225 μL, 1 mg of protein/mL) prepared as previously described (Jover et al., 1980) were incubated for 30 min at 20 °C with 25 μL of ATX II (from 8.3×10^{-10} to 8.3×10^{-7} M final concentration) corresponding to total binding or with 25 μL of a mixture of equal volumes of ATX II from 1.66×10^{-9} to 1.66×10^{-6} M and AaH I (toxin I of *Androctonus australis* Hector) at 3.3×10^{-6} M for nonspecific binding. At the same time, 100 μL of anti-ATX II IgGs at 8.3×10^{-9} M (final concentration) was incubated for 2 h at 20 °C with 100 μL of ^{125}I-protein A at 6.5×10^{-9} M (final concentration). IgG–^{125}I-protein A complex (25 μL) was then added to the toxin–receptor complex, and incubation was prolonged for 1 h at 20 °C. The reaction was stopped by centrifugation at 11000*g* for 1 min. The pellets were washed with 3×1 mL of 25 mM HEPES, 140 mM choline chloride; 5.4 mM KCl; 1.8 mM $CaCl_2$; and 1 mM $MgSO_4$, pH 7.2, BSA 0.1%, and radioactivity was counted. The results were expressed as bound ^{125}I-protein A (cpm) vs. log ATX II concentration.

Results

Isolation and Characterization of an Antibody Subpopulation Specific to One Antigenic Region of ATX I and ATX II. Sephacryl S-300 chromatography of ^{125}I-ATX II complexed with either anti-ATX II or anti-ATX I Fab fragments allowed the determination of molecular weights as 63 000 for the complex ^{125}I-ATX II–anti-ATX II Fab fragments and of 68 000 for the complex ^{125}I-ATX II–anti-ATX I Fab fragments (data not shown). These values are in good agreement with a complex of one Fab fragment and one toxin, whether anti-ATX I or anti-ATX II Fab fragments were used. Thus, ATX II and ATX I seem to share a minimum of one homologous antigenic region. However, ^{125}I-ATX II was immunoprecipitated by the ATX II antitoxin and not by the ATX I antitoxin (data not shown). Therefore, ATX II probably has several major antigenic regions, one of them being shared with ATX I. The screening of the anti-ATX I and anti-ATX II IgG populations specific to one antigenic region of ATX II may be due to the selective mode of linkage of these two toxins to the CNBr-activated Sepharose via their amino groups.

Cross-Antigenicity between Anemone Toxins. The antigenic homologies between ATX I, II, and III were assessed by competitive and solid-phase RIA using anti-ATX I IgG MR and Anti-ATX II IgG MR (i.e., specific to one antigenic region as demonstrated above). Figure 1 shows the competitive binding between ^{125}I-ATX II (10^{-9} M) and ATX I, II, and III for anti-ATX II IgG MR (7.2×10^{-8} M; Figure 1A) and anti-ATX I IgG MR (1.04×10^{-7} M, Figure 1B). The half-inhibition effects (K 0.5) obtained by using native ATX II against anti-ATX I IgG MR and anti-ATX II IgG MR were 8×10^{-9} and 4×10^{-8} M, respectively. ATX I slightly inhibited the binding of ^{125}I-ATX II to anti-ATX II IgG MR (15% at 1.5×10^{-5} M). However, ATX I completely inhibited the binding of ^{125}I-ATX II to anti-ATX I IgG MR with a $K_{0.5}$ of 1.6×10^{-8} M. These results suggest that ATX I and ATX II probably share only some homologous determinants within their common antigenic region. ATX III failed to inhibit the binding of ^{125}I-ATX II to anti-ATX I IgG MR or to anti-ATX

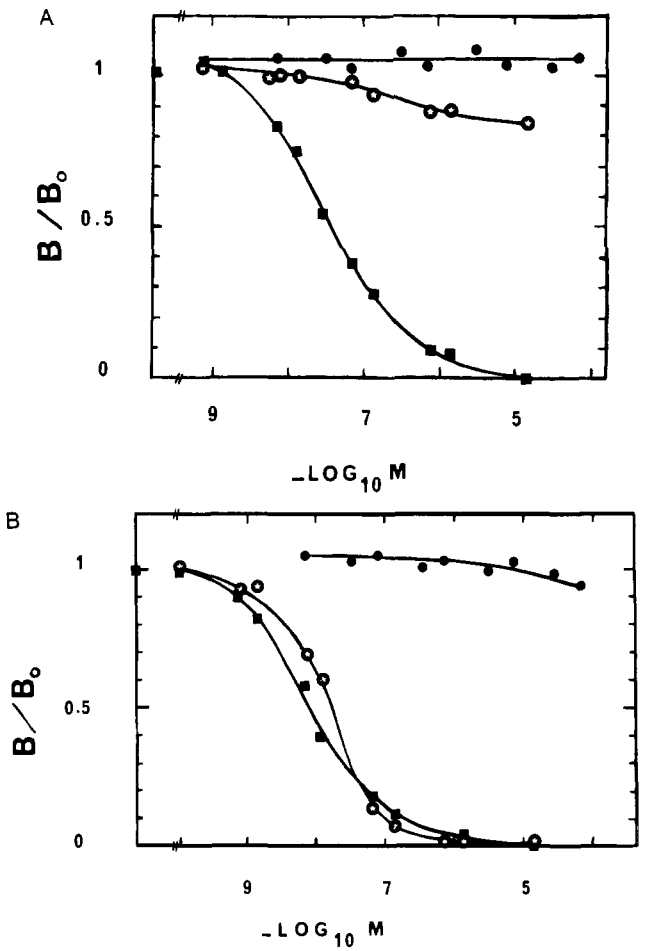

FIGURE 1: Effect of various anemone toxins on the binding of ^{125}I-ATX II to anti-ATX II IgG MR (A) and to anti-ATX I IgG MR (B) purified by immunoaffinity chromatography on ATX II and ATX I immobilized on Sepharose, respectively. The standard curves were based on ATX II (■) for anti-ATX II IgG MR (7.2×10^{-8} M) and for anti-ATX I IgG MR (1.04×10^{-7} M) and ^{125}I-ATX II (1×10^{-9} M). For ATX I (✪) and ATX III (●) incubation conditions were the same as for the standard curve, i.e., 60 min at 30 °C and overnight at 4 °C.

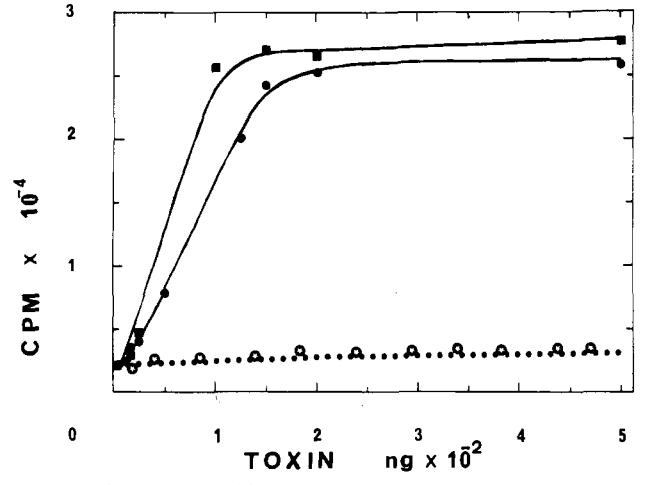

FIGURE 2: Saturation solid-phase radioimmunoassay binding curves when using anti-ATX II IgG MR (2×10^{-8} M), ATX II (■), ATX I (●), ATX III (✪), and rabbit nonimmune IgGs for nonspecific binding (•). Binding was revealed by using 50 μL of 5×10^{-9} M ^{125}I-protein A (157 Ci/mmol).

II IgG MR. In the solid-phase radioimmunoassay, ATX III similarly failed to bind to anti-ATX II IgG MR (Figure 2) and to anti-ATX I (not shown). These results show that the

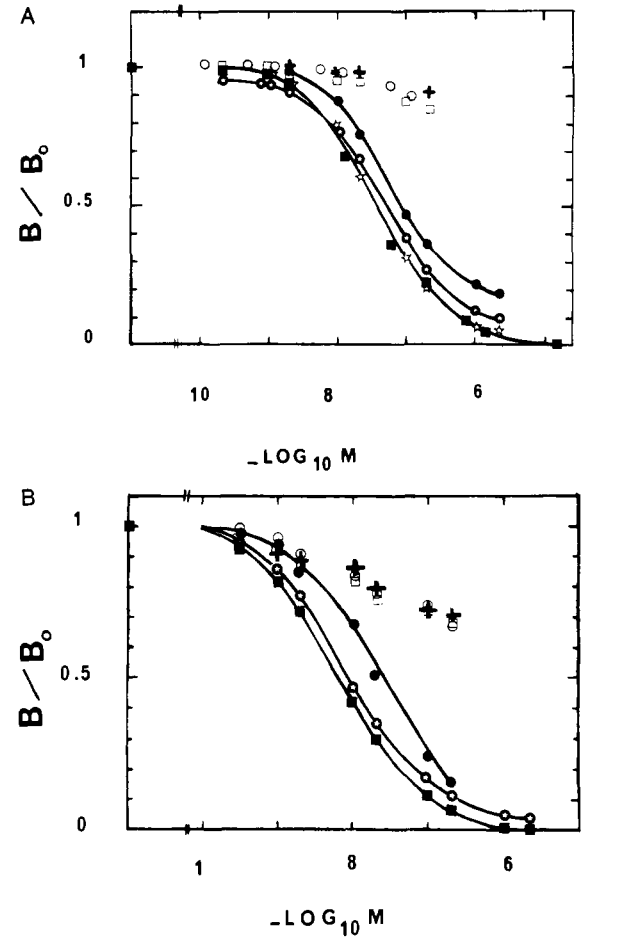

FIGURE 3: Effect of different chemical modifications of ATX II on the binding of ^{125}I-ATX II to anti-ATX II IgG MR (A) and to anti-ATX I IgG MR (B). The standard curves were based on ATX II (■, A and B), ^{125}I-ATX II at 1×10^{-9} M, and anti-ATX II IgG MR (7.2×10^{-8} M) or anti-ATX I IgG MR (1.04×10^{-7} M). Inhibition was by cyclohexanedione-modified Arg-14 ATX II (✪), ATX II trated in the same conditions but without cyclohexanedione (☆), fluorescamine-modified ATX II (●), carboxylate-monomodified ATX II (□, ○) and carboxylate-dimodified ATX II (+). Incubation conditions were the same as Figure 1.

homologous region recognized by anti-ATX I IgG MR and anti-ATX II IgG MR populations has no equivalent on the ATX III surface. Moreover, the homologous antigenic region of ATX I and ATX II does not seem to overlap the toxic site since ATX III, not recognized by anti-ATX I IgGMR or by anti-ATX II IgGMR, is able to bind to the same sodium channel site recognized by the two other *A. sulcata* toxins (Vincent et al., 1980).

Figure 2 shows solid-phase radioimmunoassays for ATX I and ATX II. These two toxins bound the same amount of anti-ATX II IgG MR (2×10^{-8} M) or anti-ATX I IgG MR (data not shown) at the plateau. This finding, in combination with that obtained by competitive assays, indicates that the different antibody subpopulations contained in anti-ATX I IgG MR or anti-ATX II IgG MR may recognize multiple overlapping determinants.

Critical Residues of the Antigenic Region Common to ATX I and ATX II. We attempted to identify amino acids involved or not in the common antigenic region of ATX I and ATX II on the surface of ATX II by using arguments drawn from the two following approaches. The first is concerned with the measurement of the effects of appropriate chemical modifications of ATX II on its antigenicity. Figure 3 gives the inhibition effects of ATX II and its derivatives on the binding

of ^{125}I-ATX II to anti-ATX II IgG MR (Figure 3A) and to anti-ATX I IgG MR (Figure 3B). The modification of ϵ-amines of lysines-35, -36, and -46 and the α-amine of glycine-1 (Figure 5) by fluorescamine decreased the ability of modified ATX II to compete with the native toxin by a factor of 2 ($K_{0.5}$ ratio of 8.3×10^{-8} to 3.8×10^{-8} M) and 4 times ($K_{0.5}$ ratio of 2.5×10^{-8} to 6.3×10^{-9} M) when anti-ATX II IgG MR and anti-ATX I IgG MR were used, respectively. These results indicate that lysines-35, -36, and -46 and the α-NH_2 terminal glycine are not involved in the antigenic region of ATX II recognized by anti-ATX I IgG MR and anti-ATX II IgG MR. This interpretation is supported by the data obtained by solid-phase radioimmunoassays (Figure 4) using anti-ATX I IgG MR at 10^{-7} M (Figure 4A) and anti-ATX II IgG MR at 2×10^{-8} M (Figure 4B) since ATX II and fluorescamine modified ATX II bound roughly the same amount of IgGs. Furthermore, lysines-35, -36, and -46 and the α-NH_2 terminal glycine residue are probably not involved in the antigenicity of ATX II, since native and modified toxins bound the same amount of unfractionated anti-ATX II serum (Figure 4C).

ATX II contains a single arginine residue at position 14 (Figure 5). As shown by amino acid analysis, the reaction of ATX II with 1,2-cyclohexanedione led to a derivative in which arginine-14 is selectively and completely modified. Other amino acid residues, including lysines, remained intact. This derivative inhibited the binding of ^{125}I-ATX II to anti-ATX II IgG MR as efficiently as ATX II ($K_{0.5}$ ratio of 5.25×10^{-8} to 3.8×10^{-8} M; Figure 3B) and to anti-ATX I IgG MR ($K_{0.5}$ ratio of 9.5×10^{-9} to 6.3×10^{-9} M; Figure 3A). All these results demonstrate that arginine-14 is not involved in the antigenic regions of ATX II recognized by anti-ATX I IgG MR and anti-ATX II IgG MR.

ATX II contains three carboxylate groups: the β-carboxylic functions of aspartates-7 and -9 and the C-terminal α-carboxylic function of glutamine-47 (Figure 5). Purification of ATX II modified by glycine ethyl ester on a SP-Sephadex C-25 column yielded mainly three different modified derivatives that were shown by Barhanin et al. (1981) to incorporate one and two glycine ethyl ester moieties per molecule of toxin, respectively. The capacity of mono- and dimodified derivatives to inhibit the binding of ^{125}I-ATX II to anti-ATX I IgG MR and anti-ATX II IgG MR is shown in Figure 3, parts A and B, respectively. Mono- and dimodified derivatives inhibited 15 and 30% of the binding of ^{125}I-ATX II to anti-ATX II IgG MR and to anti-ATX I IgG MR, respectively. The binding capacity of these modified derivatives was also tested by solid-phase radioimmunoassay using anti-ATX I IgG MR (Figure 4A) and anti-ATX II IgG MR (Figure 4B). Dimodified toxin did not bind a significant amount of anti-ATX I IgG MR or anti-ATX II IgG MR. The modification of one carboxylate group resulted in a 50% decrease of the binding capacity to anti-ATX II IgG MR (Figure 4B), whereas that to anti-ATX I IgG MR was almost completely lost (Figure 4A). These data show the involvement of a minimum of two carboxylate groups in the ATX II antigenic region recognized by anti-ATX I IgG MR and anti-ATX II IgG MR. However, the different reactivity pattern of anti-ATX I IgG MR and anti-ATX II IgG MR for ATX II, revealed by modification of the carboxylate groups, may be related to a restricted number of anti-ATX I IgG MR subpopulations that recognize ATX II, compared to that present in anti-ATX II IgG MR. Otherwise, affinity differences between anti-ATX II IgG MR and anti-ATX I IgG MR for ATX II can also account for the decreased binding capacities.

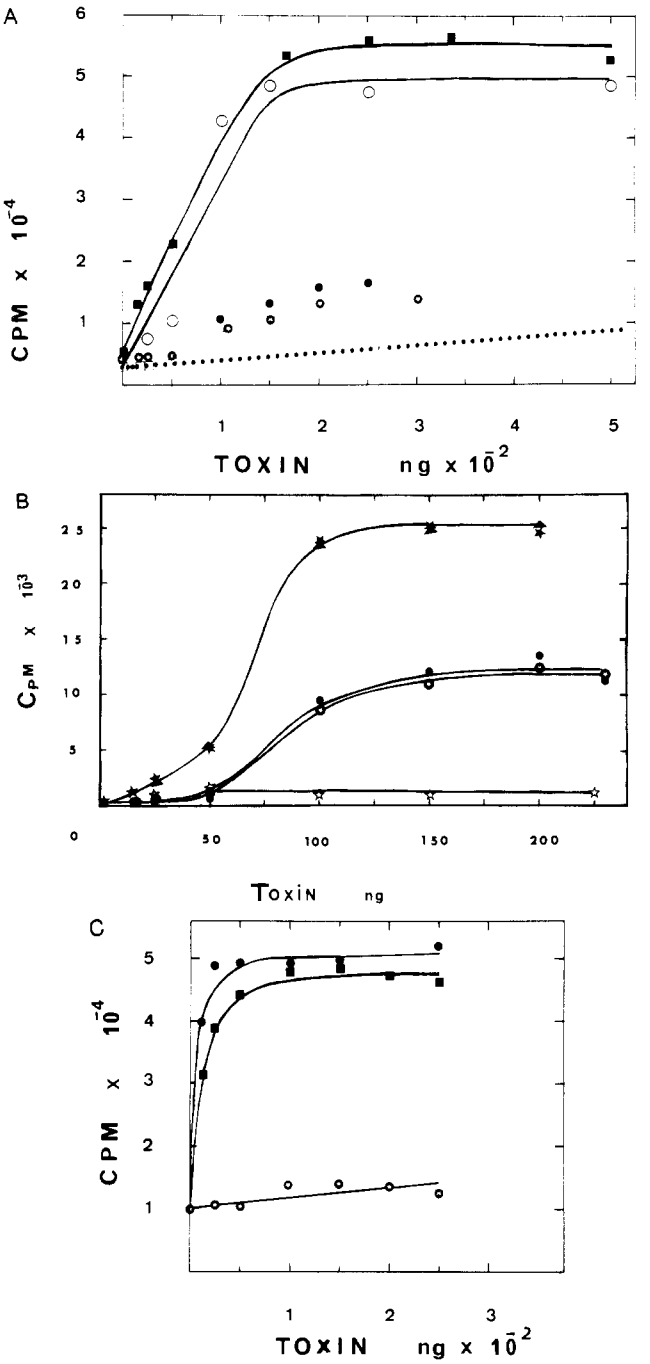

FIGURE 4: Comparative solid-phase radioimmunoassay binding curves obtained by using anti-ATX I IgG MR (10^{-7} M, A), anti-ATX II IgG MR (2×10^{-8} M, B) and ATX II antitoxin (diluted 250 times, C), and native or chemically modified ATX II. A: (■) ATX II, (○) fluorescamine-modified ATX II, (●) carboxylate-monomodified ATX II, (□) carboxylate-monomodified ATX II, (◘) carboxylate-dimodified ATX II, (•) nonspecific binding obtained by using rabbit nonimmune IgGs. B: (▲) ATX II, (★) fluorescamine-modified ATX II, (●) carboxylate-monomodified ATX II, (◘) carboxylate-monomodified ATX II, (☆) carboxylate-dimodified ATX II. C: (●) ATX II, (■) fluorescamine-modified ATX II, (◘) nonspecific binding obtained by using rabbit nonimmune serum.

In conclusion, anti-ATX I IgG MR and anti-ATX II IgG MR specific to one antigenic region recognize an ATX II surface that does not involve lysine residues, the α-NH_2 glycine terminal residue, and arginine-14 residue but includes Asp-7 and/or Asp-9 and/or Gln-47.

The second approach was to submit the amino acid sequences of ATX II, ATX I, ATX III, APA, and APC (Figure 5) to the Hopp and Woods predictive method. The hydro-

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        1      5              10              15              20              25      29
             Ala
ATXI    Gly-Ala-    -Cys-Leu-Cys-Lys-Ser-Asp-Gly-Pro-Asn-Thr-Arg-Gly-Asn-Ser-Met-Ser-Gly-Thr-Ile-Trp-Val-    -    -Phe-Gly-Cys
             Pro

        Ile
ATXII   Gly-    -Pro-Cys-Leu-Cys-Asp-Ser-Asp-Gly-Pro-Ser-Val-Arg-Gly-Asn-Thr-Leu-Ser-Gly-Ile-Ile-Trp-Leu-    -    -Ala-Gly-Cys
        Val

AP-C    Gly-Val-Pro-Cys-Leu-Cys-Asp-Ser-Asp-Gly-Pro-Ser-Val-Arg-Gly-Asn-Thr-Leu-Ser-Gly-Ile-Ile-Trp-Leu-    -    -Ala-Gly-Cys

AP-A    Gly-Val-Ser-Cys-Leu-Cys-Asp-Ser-Asp-Gly-Pro-Ser-Val-Arg-Gly-Asn-Thr-Leu-Ser-Gly-Thr-Leu-Trp-Leu-Tyr-Pro-Ser-Gly-Cys

ATXIII  Arg-Ser-Cys-Cys-Pro-Cys-Tyr-Trp-Gly-Gly-Cys-Pro-Trp-Gly-Gln-Asn-Cys-Tyr-Pro-Glu-Gly-Cys-Ser-Gly-    -    -Pro-Lys-Val


        30          35          40          45          49
ATXI    Pro-Ser-Gly-Trp-Asn-Asn-Cys-Glu-Gly-Arg-Ala-    -Ile-Ile-Gly-Tyr-Cys-Cys-Lys-Gln

ATXII   Pro-Ser-Gly-Trp-His-Asn-Cys-Lys-Lys-His-Gly-Pro-Thr-Ile-Gly-Trp-Cys-Cys-Lys-Gln

AP-C    Pro-Ser-Gly-Trp-His-Asn-Cys-Lys-Ala-His-Gly-Pro-Thr-Ile-Gly-Trp-Cys-Cys-Lys-Gln

AP-A    Pro-Ser-Gly-Trp-His-Asn-Cys-Lys-Ala-His-Gly-Pro-Thr-Ile-Gly-Trp-Cys-Cys-Lys-Gln
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FIGURE 5: Amino acid sequence of different toxins: ATX I, II, and III from *A. sulcata*; APA toxin from *A. xanthogrammica*; APC toxin from *A. elegantissima*. References: ATX I (Wunderer & Eulitz, 1978); ATX II (Wunderer et al., 1976); ATX III (Beress et al., 1977; Martinez et al., 1977); APA (Tanaka et al., 1977); APC (Norton et al., 1978).

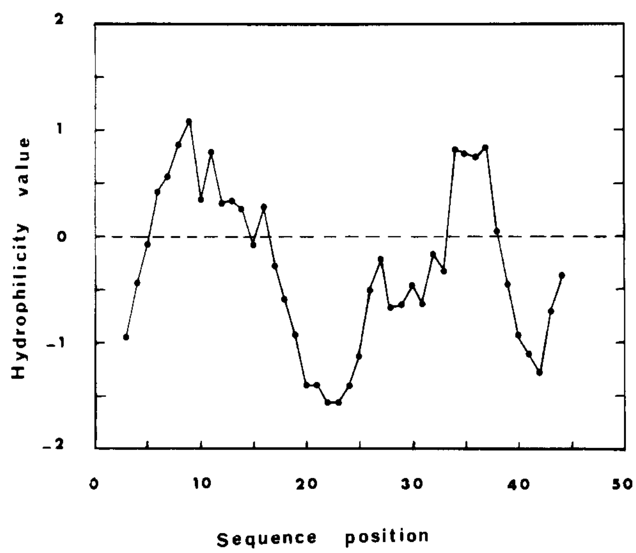

FIGURE 6: Hydrophilicity of toxin II of *A. sulcata*. The averaged antigenicity values are plotted vs. position along the amino acid sequence. The *x* axis contains 47 increments, each representing an amino acid in the sequence of the toxin. The *y* axis represents the range of hydrophilicity (from +2 to −2). The data points are plotted at the center of the averaging group from which they were derived.

philicity diagram of ATX II (Figure 6) showed a first peak at position 9, which was the highest for all toxins examined, and a second one at position 37, but less hydrophilic. This calculation supports the involvement of carboxylate groups (Asp-7, Asp-9, Gln-47) in the antigenicity of ATX II since cysteine-4 is linked to cysteine-44. However, we have shown that lysine-35 and -36 residues are not antigenic, although probably contained in the second hydrophilic region.

Accessibility of the Antigenic Region Containing Asp-7 and/or -9 and/or Gln-47 in ATX II–Rat Brain Synaptosome Complex. To determine whether or not anti-ATX II IgG MR

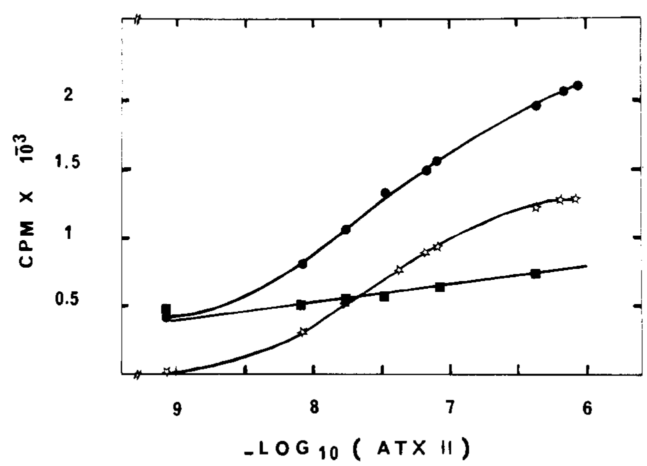

FIGURE 7: Binding of anti-ATX II IgG MR to toxin-bound rat brain synaptosomes. Portions of 225 μL of synaptosome at 1 mg/mL are incubated for 30 min at 20 °C with 25 μL of ATX II (from 8.3×10^{-10} to 8.3×10^{-7} M final concentration) (●) or with 25 μL of a mixture of volumes of ATX II (from 1.66×10^{-9} to 1.66×10^{-6} M) and AaH I (3.3×10^{-6} M) (■) for nonspecific binding. In a parallel experiment 100 μL of anti-ATX II IgG MR (8.3×10^{-9} M final concentration) was incubated for 2 h at 20 °C with 100 μL of ^{125}I-protein A (6.5×10^{-9} M final concentration). A 25-μL portion of ^{125}I-protein A anti-ATX II IgG MR complex was added to the toxin–receptor complex, and the incubation was prolonged for 1 h at 20 °C. The reaction was then stopped by centrifugation at 11000*g* for 1 min, and the pellet was washed with 3 × 1 mL of HEPES–choline buffer and counted for radioactivity. ☆: specific binding of ^{125}I-protein A anti-ATX II IgG MR on ATX II rat brain synaptosomes complex.

could recognize synaptosomes-bound ATX II, we measured the ability of ^{125}I-protein A–IgG complexes to bind to synaptosomes preincubated with ATX II (total binding) or a mixture of ATX II and AaH I. Toxins AaH I and ATX II are antigenically unrelated (nonspecific binding). Figure 7 shows the specific binding of ^{125}I-protein A–anti-ATX II IgG MR to rat brain synaptosomes bound ATX II. The nonspecific

binding of ATX II to synaptosomal membranes was controlled by using an excess of AaH I (3.3×10^{-6} M, final concentration). The elevated nonspecific binding of ATX II to synaptosomes (30%) was similar to that described by Vincent et al. (1980). Despite this fact, a good specific binding signal was obtained, suggesting that the ATX II antigenic region encompassing Asp-7 and/or -9 and/or Gln-47 remained accessible to its specific IgG's when the toxin was bound to its receptor. Taking into account the specific radioactivity of ^{125}I-protein A (157 Ci/mmol), the probable equimolar stoichiometry of the ^{125}I-protein A–IgG MR/ATX II (Langone, 1982), and the amount of proteins in synaptosomal preparations (225 μg/assay), the amount of sodium channel per milligram of protein at the plateau can be estimated to be ~95 fmol/mg. This value is in good agreement with the estimation obtained by using an IgG subpopulation specific for the α helix region (19–28) of toxin II of *A. australis* Hector, for which the amount of sodium channel was found to be ~75 fmol/mg of protein (El Ayeb et al., in press).

Discussion

The comparison of amino acid sequences of sea anemone and scorpion α toxins did not reveal any significant homology. Nevertheless, these two toxins bind to the same receptor site at the sodium channel (Catterall & Beress, 1978; Couraud et al., 1978; Vincent et al., 1980). Consequently, both toxin families are thought to share a structurally homologous receptor binding site. Information on the structural localization of this homologous receptor binding site on the anemone toxin surface is scarce. Only arginine-14 is known to be involved in the binding site of ATX II to rat brain synaptosomes. On the contrary, many approaches have been followed in an attempt to localize this receptor binding site on the surface of scorpion toxin. Crystallographic data (Fontecilla et al., 1981) and chemical modifications (Sampieri & Habersetzer-Rochat, 1978; Darbon et al., 1983; Darbon & Angelides, 1984; El Ayeb et al., 1986) have localized the receptor binding site on a flattened, conserved, hydrophobic but exposed surface of scorpion toxins. Moreover, this conserved region is shown to be nonimmunogenic when immunizing rabbits with the native toxin (El Ayeb et al., 1986). Immunochemistry studies have recently shown that the α-helix region of toxin II of *A. australis* Hector (AaH II), which is located at the opposite side of the conserved region, remains accessible to antibodies when AaH II is receptor bound (El Ayeb et al., in press). Thus, we have extended these immunochemical approaches to anemone toxins to gain new valuable structure–activity information.

To reach this goal, it was first necessary to purify antibody populations that recognize single antigenic regions and then identify to which portion of the molecule surface they are directed. Consequently, anti-ATX I IgG MR and anti-ATX II IgG MR used in the present study were obtained by immunoaffinity purification of anti-ATX I and anti-ATX II serum on ATX I and ATX II linked to CNBr-Sepharose, respectively. They have been shown to be specific to one antigenic region, since their respective Fab fragments are able to bind to ATX II in a 1:1 ratio. This indicates that (1) subpopulations contained in these single antigenic region specific IgGs may recognize overlapping antigenic determinants and (2) ATX I and ATX II exhibit a minimum of one common major antigenic region. In addition, anti-ATX I serum is unable to precipitate ^{125}I-ATX II without the addition of double antibody, as does anti-ATX II serum. This can be interpreted as a minimum of two major antigenic regions on the surface of ATX II, only one being common with ATX I.

The identification of the corresponding region on the ATX II surface was then attempted by chemical modifications; arginine-14, lysines-35, -36, and -46 and the α-NH_2 of terminal glycine are not contained in the corresponding antigenic region. However, the β-carboxylate of aspartic-7 and/or -9 and α-carboxylate of glutamine-47 are antigenic. ^{13}C NMR studies (Norton et al., 1980) have indicated that, in APA, aspartic-7 or -9 forms a salt bridge with lysine-35 or -36. These findings may hold for ATX II since it was demonstrated by NMR that APA and ATX II have a very similar conformation (Norton & Norton, 1979; Norton et al., 1980). These findings demonstrate that Asp-7 or -9 and Gln-47 form a discontinuous antigenic region. The precise delimitation of this region will become possible when crystallographic data (Smith et al., 1984) will furnish more indications on the three-dimensional structure of this type of molecule.

Using anti-ATX II IgG MR specific to the antigenic region encompassing probably Asp-9 and Gln-47, we tested the accessibility of this region when ATX II is bound to its receptor. Similar approaches have been successfully applied by Moyle et al. (1982) and Milius et al. (1983) using either monoclonal or polyclonal antibodies, respectively, and Arnheiter et al. (1983) to examine the orientation of human chorionic gonadotropin and a human leukocyte interferon molecule on their respective receptor. Although steric constraints between antibody–toxin and toxin–receptor interactions probably impair the use of selected antibodies to examine the location of the toxic site, they are particularly useful for determining antigenic regions that remain accessible when the toxin is receptor bound. Accessibility of small proteins to their antibodies when they are bound to their respective receptor was also observed for α-bungarotoxin (Merlie et al., 1981) and α toxin of *Naja nigricollis* (Boulain & Menez, 1982), two snake toxins with molecular weights close to scorpion toxins. In the case of α-bungarotoxin, its specific antibodies are able to specifically immunoprecipitate the toxin–receptor complex, whereas a monoclonal antibody specific for toxin α of *N. nigricollis* was able to bind to the toxin–receptor complex and promote the acceleration of its dissociation, probably by changing the conformation of the toxin (Rousselet et al., 1984).

Our estimation of the amount of sodium channel per milligram of protein at the plateau was about 95 fmol/mg. This value is on the same order of magnitude as that obtained by a different approach, using direct titration of the receptor with toxin II of *A. australis* Hector (Jover et al., 1980) and different from that published by Vincent et al. (1980) who reported a binding capacity 10 times higher using ^{125}I-ATX II. However, the accessibility of the region containing Asp-7 and Gln-47 to their antibodies when ATX II is bound to its receptor is compatible with results reported by Barhanin et al. (1981), who showed that monocarboxylate-modified ATX II could still bind to rat brain synaptosomes.

It would be worthwhile purifying anti-ATX II IgGs specific for other regions of the toxin molecule by immunoaffinity chromatography, implicating carboxylate groups as coupling reactant groups. These populations would be helpful to identify new relationships between the toxic and antigenic regions of anemone toxins.

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