Delineation of the Functional Site of α-Dendrotoxin

THE FUNCTIONAL TOPOGRAPHIES OF DENDROTOXINS ARE DIFFERENT BUT SHARE A CONSERVED CORE WITH THOSE OF OTHER Kv1 POTASSIUM CHANNEL-BLOCKING TOXINS*

We identified the residues that are important for the binding of α-dendrotoxin (αDTX) to Kv1 potassium channels on rat brain synaptosomal membranes, using a mutational approach based on site-directed mutagenesis and chemical synthesis. Twenty-six of its 59 residues were individually substituted by alanine. Substitutions of Lys5 and Leu9 decreased affinity more than 1000-fold, and substitutions of Arg3, Arg4, Leu6, and Ile8 by 5–30-fold. Substitution of Lys5 by norleucine or ornithine also greatly altered the binding properties of αDTX. All of these analogs displayed similar circular dichroism spectra as compared with the wild-type αDTX, indicating that none of these substitutions affect the overall conformation of the toxin. Substitutions of Ser38 and Arg46 were individually substituted by alanine. Substitutions caused no significant affinity change. This paper is available on line at http://www.jbc.org

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Venomous animals from four distinct phyla produce small toxic proteins that block a variety of Kv1 voltage-gated potassium channels. These are the scorpions (1), sea anemones (2–4), mambas, and anemones (5), and snakes (6–8), which are arthropods, cnidarians, molluscs, and chordates, respectively. At least four different folds, the sizes of which range from approximately 30 to 60 residues, are associated with these different potassium channel-blocking toxins. These are (i) the α/β-toxin fold, which is found in scorpion toxins, such as charybdotoxin (9); (ii) the fold that comprises two short helices and is only adopted by toxins from sea anemone toxins such as ShK (10) and BgK (11); (iii) the α/β-conotoxin fold, which has three β-sheet strands and is adopted by β-conotoxin from cone snails (12, 13); and (iv) the BPTI-type fold (14), composed of two short helices and a two-stranded β-sheet, which is adopted by the snake dendrotoxins (15–17) and probably by the sea anemone kalicudines (4).

Although structurally unrelated, the Kv1 channel-blocking toxins produced by scorpions, snakes, sea anemones, and snails all are likely to bind to the peptide loop between the membrane-spanning segments S5 and S6 of Kv1 channels (18–25, 11). Therefore, all of these toxins may possess a functional surface that is complementary to this loop, an observation that raises the question as to how similar these surfaces are from one toxin to another. The answer to such a question may not only shed light on the evolution of these toxins but should also help characterize the surface by which Kv1 channels interact with these toxins. Mutational analyses have finely delineated the functional sites of scorpion toxins (26) and sea anemone toxins (11, 25). Although the sea anemone and scorpion toxins are not structurally related, their functional sites share some similarities. They are all flat surfaces of comparable size (~700 Å2) with five functionally important residues, including a similar critical functional diad (11). This diad in both toxins comprises a lysine, which always plays a predominant part in binding, and an aromatic residue separated from the lysine by 6.6 Å. It was proposed that the lysine of scorpion toxins projects into the conduction pore of the channel (26–29), and the most critical lysine of sea anemone toxins was predicted to play a similar role (11, 25). Invertebrate toxins that have unrelated architectures but commonly block voltage-gated potassium channels therefore seem to display quite similar functional sites, suggesting that they underwent a convergent functional evolution. To investigate whether this proposal could be extended to toxins from vertebrates, we decided to delineate the functional site of α-dendrotoxin (αDTX), a well known voltage-gated potassium channel-blocking toxin, isolated from venom of the green mamba Dendroaspis angusticeps (30). This toxin is an extensively studied prototype of the family of

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The abbreviations used are: BPTI, bovine pancreatic trypsin inhibitor; DTX, dendrotoxin; Fmoc, fluorenylmethoxycarbonyl; Nle, norleucine; Orn, ornithine; Z or Glp, pyroglutamic acid.
**EXPERIMENTAL PROCEDURES**

**Materials—**Oligonucleotides were synthesized on an Applied Biosystems 381A synthesizer. Amino acid composition analyses were performed using an Applied Biosystems device (420A Derivatizer and 130A Separation system), and N-terminal sequencing was done using the Applied Biosystems sequencer (477A protein sequencer) on-line with the phenylthiohydantoin analyzer (120A analyzer). Dichoetric spectra were recorded on a Jobin-Yvon CD6 dichrograph. Expression vector pEZZ18 was generously provided by Prof. Mathias Uhlen (Royal Institute of Technology, Stockholm, Sweden). Mass determination was performed on an API III+ (Perkin-Elmer Sciex) mass spectrometer equipped with a nebulizer-assisted electrospray source.

**Production of dDTX Analogs in Escherichia coli—**Recombinant dDTX analogs were produced using the periplasmic expression system previously described (32). As for previous recombinant dDTXs (32), each analog was recovered as two isoforms: one with an N-terminal glutamine residue and the other, as found in wild-type dDTX, with an N-terminal pyroglycamic residue. The latter was used for structural and functional characterization.

**Chemical Synthesis of dDTX and dDTX Analogs—**The synthesis of native dDTX and of analogs P2A, K5A, K5Ne, K5Orn, L6A, I8A, L9A, E33A, R34A, and I58A was performed using the Fmoc/Gly-4-hydroxymethylphenoxy resin, 20-fold excess of each amino acid, dicyclohexylcarbodiimide/1-hydroxy-7-azabenzotriazole activation. Deprotection (1.5 h) and cleavage (300 mg of peptide + resin) was achieved using the mixture trifluoroacetic acid/triisopropylsilane/water (90/0.5/0.5, v/v/v). The acidic mixture was then precipitated in 100 ml of cold diethylether, redissolved in 50 ml of 10% acetic acid in water, and lyophilized. Oxidation of the reduced peptide was achieved at 0.1 mg/ml in degassed potassium phosphate buffer (100 mM, pH 7.5) using the redox couple reduced glutathione (5 mM)/oxidized glutathione (0.5 mM). The disappearance of the reduced peptide was monitored by reverse phase high-pressure liquid chromatography on a C18 analytical column (4.6 x 250 mm, Vydac) using a linear gradient of 40 min starting from 0.1% trifluoroacetic acid in water to 90% acetonitrile/0.1% trifluoroacetic acid in water. The crude oxidized peptide was purified using a reverse phase high-pressure liquid chromatography on a C18 semipreparative column (10 x 250 mm, Vydac) in the same conditions as above followed by a final purification on a MonoS column (Amersham Pharmacia Biotech) in the conditions used for recombinant analogs (32).

**Characterization of dDTX Analog—**For all dDTX analogs, the protein concentration was estimated using molar extinction coefficients determined by amino acid composition analyses. The presence of the desired substitution in the dDTX analogs produced in *E. coli* was checked by Edman degradation of the N-terminal glutamine isofrom, or, for analogs S44A, K48A, E51A, R54A, and R55A, by sequencing peptides resulting from endoproteinase Lys-C (Boehringer Mannheim) tryptic digestion and alkylated cysteine (32). For the chemically synthesized dDTX analogs, the substitution was assessed by mass spectrometry.

**Disulfide Bridge Assignment of Chemically Synthesized Analogues—**70 μg of each analog and wild-type dDTX purified from venom were cleaved by trypsin. The resulting peptides were separated by reverse phase high-pressure liquid chromatography at a flow rate of 0.8 ml/min (300 mm column, Vydac) using a 65-min gradient of 10–60% eluent B (0.085% trifluoroacetic acid, 50% acetonitrile in water) in eluent A (0.1% trifluoroacetic acid in water). Peptides were identified by means of amino acid composition analyses. For the analog R34A, the peptide containing half-cysteines 16, 32, 40, and 53 was further cleaved by chymotrypsin.

**Preparation of [125I]dDTX—**Synthetic dDTX (10 μg) was incubated at room temperature in a micro test tube (Eppendorf) coated with 1 μg of iodogen (Pierce) with 2 mCi of [125I] (Amersham Pharmacia Biotech) in 200 μl of 0.1 M sodium phosphate. After 15 min, 20 μl of 0.1 M sodium thiosulfate were added, and the reaction mixture was injected onto a C18 column (Vydac). After washing the column with 25% eluent B (0.085% trifluoroacetic acid, 50% acetonitrile) in eluent A (0.1% trifluoroacetic acid in water), separation was achieved using a 40-min gradient of 25–60% eluent B in eluent A (1 ml/min). The fraction containing pure monoiiodinated dDTX (2000 Ci/mmol) was kept at 4 °C for the addition of bovine serum albumin (1 mg/ml).

**RESULTS**

**Choice of Substitutions—**In the early stages of this study, there was no clear indication to suggest which analogs should be prepared. It was only established (32) that substitutions D12N and K28A/K29A/K30G had little effect on the biological activity of the toxin, suggesting that these positions were unlikely to be important in the toxin binding to Kv1 channels, whereas the substitution Z1Q caused affinity to increase 5-fold.

In the present study, therefore, selection of the positions of dDTX to be substituted was based on a comparison of the primary structures of dendrotoxins and functionally different proteins possessing the same fold (Fig. 1). First, we expected that functionally important features of dendrotoxins would emerge from comparison of their primary structures with those of structurally related proteins. Five residues (Lys5, Lys19, 25394
Lys\textsubscript{28}, Gln\textsubscript{31}, and Ser\textsubscript{38}) are more specific to dendrotoxins. Four (Lys\textsubscript{5}, Lys\textsubscript{19}, Gln\textsubscript{31}, and Ser\textsubscript{38}) were substituted in this study, and one (Lys 28) in our previous work (32). Asp\textsubscript{36}, which is present in three of the four DTXs, was also substituted. Second, according to their primary structures, two groups of dendrotoxins can be distinguished: DTX-K and \(\alpha\)DTX, and \(\alpha\)DTX and DTX-I, which possess two additional residues in their N-terminal. Because this structural partition corresponds to distinct functional properties of DTXs (see under "Discussion"), we anticipated that residues uniquely conserved in both \(\alpha\)DTX and DTX-I but not in DTX-K and \(\delta\)DTX may be associated with specific behavior of the first two toxins. Thirteen residues (Pro\textsubscript{2}, Arg\textsubscript{4}, Leu\textsubscript{6}, Ile\textsubscript{8}, His\textsubscript{10}, Arg\textsubscript{11}, Tyr\textsubscript{17}, Gln\textsubscript{27}, Lys\textsubscript{29}, Glu\textsubscript{33}, Trp\textsubscript{37}, Ser\textsubscript{44}, and Ile\textsubscript{58}) were therefore substituted either in this study or in the previous one (Lys\textsubscript{29}) (32). Third, because dendrotoxins are more basic than any other Kunitz-type proteins, we substituted the positively charged residues not yet included by the above selection procedure (Arg\textsubscript{3}, Arg\textsubscript{15}, Arg\textsubscript{34}, Arg\textsubscript{46}, Lys\textsubscript{48}, Arg\textsubscript{54}, and Arg\textsubscript{55}). In addition, Asp\textsubscript{18}, which is specific to \(\alpha\)DTX, was also substituted. We also mutated Glu\textsubscript{51} and, finally, Leu\textsubscript{9} and Asn\textsubscript{43} because they were found to be close to functionally important residues identified during this study.

**Production of \(\alpha\)DTX Analogs**—Using the procedure previously described (32), the production of \(\alpha\)DTX analogs in \textit{E. coli} yielded only 10–90 \(\mu\)g of each isoform per liter of culture. Additionally, attempts to produce the mutant \(\alpha\)DTX-W37A failed, probably because Trp\textsubscript{37} belongs to a small hydrophobic core (15). To increase analog yields, we decided to produce them by a chemical approach (43). Peptide synthesis led to milligram amounts of synthetic \(\alpha\)DTX, with a 6% yield from the starting resin. We identified the disulfide bonds of the resulting synthetic \(\alpha\)DTX by submitting it to trypsin digestion. The peptide map obtained with the resulting fragments was identical to that of wild-type \(\alpha\)DTX (not shown), indicating that the disulfide bridges are identical in the wild-type and synthetic toxins. In mice, intracerebroventricular injections of synthetic \(\alpha\)DTX indicated an LD\textsubscript{50} of 5 ng/g, a value that is similar to that of the toxin isolated from venom (32). Furthermore, the synthetic and native toxins displayed a similar ability to inhibit the binding of radiolabeled \(\alpha\)DTX to rat brain synaptosomal membranes (not shown). Using the chemical approach, we therefore synthesized chemically the analogs possessing the substitutions P2A, K5A, L6A, I8A, L9A, E33A, R34A, and I58A; all other alanine analogs were produced by the recombinant approach. We introduced nonnatural residues at position 5 (K5Nle and K5Orn) to probe in more detail the role of lysine 5, which was...
found to be critical for toxin binding (see below). The analog N43A was also chemically synthesized, but we could not oxidize it, suggesting that the introduced substitution prevented the correct folding of the protein.

**Binding of [125I]αDTX to Rat Brain Synaptosomal Membranes**—Rat brain synaptosomal membranes are frequently used to study the binding properties of various ligands toward Kv1 channels, using radiolabeled αDTX as a tracer (11, 25, 32, 44). High proportions of synaptosomal membranes are usually used in such experiments. Because our mutational analysis required numerous competitive binding experiments, we established new binding conditions using the lowest possible proportions of membranes. A typical saturation binding experiment is shown in Fig. 2A. Scatchard analysis (Fig. 2B) indicated that [125I]αDTX binds to a single class of sites with an apparent dissociation constant \(K_d\) of 5.4 pm. The \(B_{\text{max}}\) value was 1.1 pmol/mg of protein, in agreement with previously published values (6). The mean \(K_d\) and \(B_{\text{max}}\) values (± S.D.) calculated from all individual experiments \((n = 5)\) were 4.0 ± 1.4 pm and 2.8 ± 1.7 pmol/mg of protein, respectively. Because our results differed from those previously reported (45), we assessed the validity of the apparent \(K_d\) value, by determining the kinetics of binding of [125I]αDTX binding to rat brain synaptosomal membranes. Dissociation was initiated by adding a 1000-fold molar excess of αDTX (Fig. 3). The mean value (± S.D.) from all individual experiments \((n = 3)\) for the dissociation rate constant \(k_{\text{off}}\) was 3.8 ± 1.2 \(\times 10^{-4}\) s\(^{-1}\). The association rate constant of [125I]αDTX binding was determined using an apparent concentration of synaptic membranes and three different [125I]αDTX concentrations (Fig. 4A). The \(k_{\text{on}}\) was deduced from the plot of \(k_{\text{obs}}\) versus [125I]αDTX concentrations (Fig. 4B). The mean value of \(k_{\text{on}}\) (± S.D.) from two individual experiments was 2.0 ± 1.2 \(\times 10^8\) M\(^{-1}\) s\(^{-1}\). The dissociation constant \(K_d\) calculated from the ratio of these values is 1.9 pm, a value that agrees with that determined by saturation binding to equilibrium. The values of the apparent \(K_d\) and \(k_{\text{on}}\) deduced from our experiments differed from those reported in Ref. 45, probably as a result of experimental differences in protein concentrations and/or binding sites (46–48).

**Binding Properties of αDTX Analogues**—Fig. 5A shows typical plots of the competitive binding of [125I]αDTX and wild-type αDTX and three analogs with lower relative affinities to rat brain synaptosomal membranes. Using similar experiments, we investigated the inhibitory potency of 28 analogs of αDTX (26 alanine analogs plus K5Nle and K5Orn). The resulting \(K_i\) values are listed in Table I, and the relative affinities are shown in Fig. 5B. Clearly, some mutations caused marked decreases in affinity, whereas several others did not. Similar changes in affinity were found when binding experiments were performed with higher concentrations of binding sites (not shown). The results of these mutations will now be presented by dividing the toxin structure into four regions: (i) 1–18, which contains a 3\(_{10}\) helical structure between residues 5 and 9; (ii) 19–38, with a double-stranded antiparallel \(\beta\)-sheet and a \(\beta\)-turn between residues 27 and 31; (iii) 39–49, with no canonical secondary structure; and (iv) 50–58, which adopts a helical structure (15).

Twelve of the 18 positions of the N-terminal region have been explored (Fig. 5B), revealing that substitutions of six nearly consecutive residues (Arg\(^3\), Arg\(^4\), Lys\(^5\), Leu\(^6\), Ile\(^8\), and Leu\(^9\)) caused affinity decreases. K5A and L9A seem to be critical, and they lowered affinity by more than three orders of magnitude. This is in agreement with recent data that indicated that the selective modification of Lys\(^5\) also greatly affected the toxin affinity of the highly analogous dendrotoxin I (49). In addition, we observed that substitution of Lys\(^8\) by ornithine ((CH\(_2\))\(_3\)-NH\(_2\); 5 Å) and norleucine (which lacks the e-amino) lowered affinity by approximately two and three orders of magnitude, respectively (Fig. 5C). The substitution R3A only induced a less than 10-fold decrease, whereas the three substitutions R4A, L6A, and I8A resulted in 10–30-fold decreases in affinity. The other substitutions (P2A, H10A, R11A, R15A, Y17A, and D18A) and the previously described mutation D12N (32) did not significantly reduce the affinity. In the region 19–38, the substitutions K19A, Q27A, R31A, E34A, and D36A had no significant effect on the affinity of αDTX, in agreement with previous results that showed that substitutions in this region had little effect on the biological properties of αDTX (32). In contrast, the substitution S38A was associated with a 7-fold decrease in affinity. Of the three substitutions that were introduced in the 39–50 region, S44A and K48A caused no change in binding properties, whereas R46A induced a nearly 200-fold affinity decrease.
decrease. Finally, although the C-terminal helix 50–58 is spatially close to the critical N-terminal region, none of the substitutions E51A, R54A, R55A, and I58A caused any significant change in binding affinity.

### Structural Properties of α-DTX Analogs—

Before concluding that an affinity decrease reflects the functional contribution of a substituted residue, it is essential to establish that the substitution has not altered the toxin structure. To probe the conformational state of the toxin analogs displaying weaker binding affinity, we compared their content in secondary structure with that of the wild-type protein, by monitoring their circular dichroic spectra (Fig. 6). None of the substitutions introduced at positions 5 and 9 were associated with any detectable change of the dichroic content of the toxin, indicating that no major conformational change occurred upon either of these substitutions. A similar conclusion was reached with the analogs R3A, R4A, L6A, and I8A (data not shown). Therefore, the decreases in affinity observed upon substitutions at positions 3, 4, 5, 6, 8, and 9 (Fig. 5B and Table I) probably do not reflect a structural role of these side chains.

The dichroic spectrum of the analog S38A (Fig. 6) was markedly different from that of the wild-type toxin; the band at 210 nm observed for the native toxin was shifted to 205 nm, and a shoulder became observable around 225 nm. Such a change is difficult to interpret, but it may suggest that the helical structure is reinforced and/or that the β-sheet content of the toxin is decreased. Such a finding is not too surprising because Ser 38 establishes critical interactions with other parts of the toxin, its side chain oxygen atom being involved in hydrogen bonds with main-chain nitrogen atoms of Cys16, Cys40 and in a bifurcated hydrogen bond with carbonyl oxygen atoms of Pro13 and Cys40 (15). Thus, its substitution by alanine may prevent formation of these bonds and hence alter the toxin structure. Similarly, the circular dichroic spectrum of αDTXR46A was different from that of wild-type αDTX (Fig. 6), suggesting that the substitution also induced structural perturbations. Exam-
ination of the tertiary structure of αDTX (15) suggests that the side chain of Arg46 interacts with the aromatic ring of Trp37, as observed in BPTI between the corresponding residues Asn44 and Tyr35 (50). In BPTI, substitution of Tyr35 by a glycine suppresses this interaction, with striking structural consequences (51). Furthermore, substitution of Asn44 by an alanine

![Graph](image_url)

**Fig. 5. Inhibition of [125I]αDTX binding to rat brain synaptosomal membranes by wild-type αDTX and αDTX analogs.** A, 1 ml of membranes (0.5 μg protein/ml) were incubated with [125I]αDTX (12 pM) in the presence of increasing concentrations of either wild-type αDTX or αDTX analogs. Under these conditions and in the absence of any competitor, total binding represents less than 10% of total [125I]αDTX and nonspecific binding represents 10% of total binding. Ki values of the competitors (C) were determined by fitting the data with Kaleidagraph using the equation: bound [125I]αDTX (%B/Bo) = 100 × (1 + (L*/Kd)/(1 + (L*/Kd) + (C/Ki))), where L* = total [125I]αDTX and C = total competitor concentration. All of the data could be fitted with a correlation coefficient of 0.97. B and C, relative affinity of αDTX analogs with respect to wild-type αDTX.
decreased the stability of an analog of BPTI in which only the disulfide 5–55 was present (52). The consequences may be similar in aDTX upon substitution of Arg46 by an alanine. We therefore suggest that the affinity decreases noted with substitutions at positions 38 and 46 may result from perturbation of aDTX conformation.

**DISCUSSION**

The Functional Site of aDTX—Although aDTX has been widely studied for more than 15 years (reviewed in Ref. 8), little is known regarding the molecular features that are associated with its capacity to recognize Kv1 channels. We used extensive mutational analysis to delineate its functional site. Twenty-six positions were individually substituted by an alanine. This suppresses the side chain beyond the β-carbon and does not introduce a new chemical function. Such an approach proved to be successful in identifying functionally important residues in various proteins (53), but it provides little information on how the residue contributes to the binding of the protein to its target. If one includes the substitutions described in our previous report (32) (K28A/K29A/K30G, D12N, and Z1Q), we have now probed 31 positions of aDTX.

All of the analogs were tested for their capacity to inhibit the binding of iodinated aDTX to rat brain synaptosomal membranes. aDTX is known to recognize various subtypes of Kv1 channels with different affinities. Using cloned Kv1 channels, electrophysiological data revealed that aDTX blocks Kv1.1, Kv1.2, and Kv1.6 with comparable affinities, but has much lower affinities for Kv1.3, Kv1.4, and Kv1.5 (reviewed in Refs. 7–8 and 31). Furthermore, the aDTX-specific Kv1 channels from rat brain can be heteromeric and may form a heterogeneous population of molecules (54, 55). Almost all aDTX acceptors contain Kv1.2 α-subunits, and 50% of them also possess Kv1.1 α-subunits. In contrast, the aDTX-sensitive potassium channels containing Kv1.6 or Kv1.4 α-subunits appear to be much less abundant (54). Despite this heterogeneity, our present and previous binding experiments suggest the presence of a predominant single class of binding sites.

Six residues (Arg3, Arg4, Lys5, Leu6, Ile8, and Leu9) were identified as being functionally important because their substitution caused a substantial decrease in the binding affinity of aDTX to rat brain synaptosomal membranes without affecting the toxin conformation. These residues are represented in Fig. 7, where they have been colored red, orange, and yellow for substitutions causing affinity decreases of above 100-fold, be-

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**TABLE I**

Affinity of aDTX analogs determined by their ability to compete with [125I]aDTX for binding to rat brain synaptosomal membranes

| aDTX | \( K_i \) pM | \( K_i \) analog/WT |
|------|-------------|------------------|
| WT | 4 ± 1 | 1 |
| P2A | 10.6 ± 1.1 | 2.9 ± 1.0 |
| R3A | 28.5 ± 4.6 | 7.9 ± 3.1 |
| R4A | 35 ± 12 | 11 ± 5.75 |
| K5A | 5800 ± 700 | 1594 ± 574 |
| K5Orn | 471 ± 42 | 128 ± 43 |
| K5Ne | 5700 ± 400 | 1547 ± 487 |
| L6A | 43.4 ± 12 | 12.4 ± 6.1 |
| I6A | 115 ± 26 | 33.2 ± 14.8 |
| I6A | 4160 ± 900 | 170 ± 510 |
| H10A | 1.6 ± 0.2 | 0.45 ± 0.15 |
| R11A | 3.0 ± 0.1 | 0.8 ± 0.2 |
| R15A | 4.6 ± 0.1 | 1.22 ± 0.33 |
| Y17A | 3.7 ± 0.1 | 1.0 ± 0.27 |
| D18A | 3.4 ± 0.1 | 0.91 ± 0.25 |
| K19A | 2.3 ± 0.1 | 0.63 ± 0.17 |
| Q27A | 2.9 ± 0.1 | 0.78 ± 0.21 |
| Q31A | 3.65 ± 0.05 | 0.98 ± 0.26 |
| E35A | 5.3 ± 0.05 | 1.42 ± 0.37 |
| R34A | 7.1 ± 0.1 | 1.9 ± 0.5 |
| D36A | 4.35 ± 0.1 | 1.30 ± 0.35 |
| S38A | 23.5 ± 6.5 | 6.64 ± 3.24 |
| S44A | 6.7 ± 0.1 | 1.8 ± 0.45 |
| R46A | 56 ± 2 | 160 ± 78 |
| K48A | 2.8 ± 0.05 | 0.75 ± 0.2 |
| R51A | 3.65 ± 0.05 | 0.98 ± 0.26 |
| R54A | 5.4 ± 0.05 | 1.44 ± 0.38 |
| R55A | 4.0 ± 0.21 | 1.08 ± 0.3 |
| I58A | 3.45 ± 0.25 | 1.27 ± 0.63 |

\( a \) \( K_i \) ± S.E. values are calculated from competition experiments as described in the legend of Fig. 5.

\( b \) WT, wild type.

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**Fig. 6.** Far ultraviolet circular dichroic spectra of wild-type aDTX and some aDTX analogs. The spectra were recorded at 20 °C in 0.1-cm cuvettes, with a protein concentration of 5–10 μM.
tween 10- and 100-fold, and between 5- and 10-fold, respectively. Glp1, the glutamine substitution of which was previously shown to increase the affinity by 5-fold (32), is shown in yellow. All of these residues are concentrated in one region. However, before concluding that the functional site is thus delineated, the borders of the site also had to be identified. We found that 18 other positions, i.e. Pro2, His10, Arg11, Arg15, Tyr17, Asp18, Lys27, Lys31, Glu33, Arg34, Asp36, Ser44, Lys48, Glu51, Arg54, Arg55, and Ile58, can be substituted without causing affinity changes, suggesting that they are not major binding participants. These functionally excluded residues are colored green in Fig. 7. Arg46 and Ser38, the alanine substitution of which induced affinity decreases and structural perturbations, are also colored green. All of these residues are not only widely spread on the toxin surface but a number of them, such as Pro2, Gln27, Gln31, Ser44, and Arg55, also closely surround the six functionally important residues. We therefore suggest that most of the side chains by which aDTX binds to potassium channels from rat brain synaptosomes have been identified.

The six functional residues identified in this study are nearly consecutively located along the peptide stretch 2–8 (Fig. 7A). They form a surface that covers approximately 700 Å², a value that is compatible with those usually observed at the interface
of protein-protein complexes (56). The center of this surface is occupied by Lys5, one of the two most important residues, which protrudes from the functional surface (Fig. 7B). To investigate the role of the different parts of its side chain, we substituted Lys5 into ornithine and norleucine. The results (Fig. 5C) indicated a critical role of both its ε-amine group and, to a lesser extent, the distance between its α-carbon and this amine. The second most important residue is Leu9, the δ-carbon of which is 6.9 Å from the α-carbon of Lys5. The three moderately important residues Arg4, Leu6, and Ile8 surround Lys5 in a rather compact manner, whereas the less important residue (Arg3) and Glp are more remote from the two important residues Lys5 and Leu9, and are presumably at the border of the functional site.

Dendrotoxins Do Not All Possess the Same Functional Site—As mentioned above, dendrotoxins can be divided into two structural subclasses. One of them includes αDTX and DTX-I, and the other comprises βDTX and DTX-K (see Fig. 1). Dendrotoxins from the two groups are characterized by different biological properties. Thus, αDTX is relatively selective in blocking slow-inactivating K⁺ currents in rat dorsal root ganglion cells, whereas βDTX, which belongs to the DTX-K group, is more selective against non-inactivating currents (57). DTX-I, of the αDTX group, blocks channels formed by expression in Xenopus oocytes of a-subunits Kv1.1, Kv1.2, and Kv1.6, whereas DTX-K is highly selective for Kv1.1 (58). However, despite 95% sequence identity between DTX-K and βDTX, DTX-K is much more potent than βDTX in blocking Kv1.1 channels (59). Therefore, although all members of the dendrotoxin family bind to voltage-gated potassium channels, they display slightly distinct biological activities, suggesting differences in their functional sites.

Recently, nine variants of DTX-K were produced and tested for their capacity to inhibit the binding of [125I]DTX-K to rat brain synaptic membranes (33). Four residues were identified as playing a major role in the toxin binding function: Lys8 and Lys6 in the N-terminal part of the toxin, and Trp25 and Lys26, which are located in the β-turn that joins the two antiparallel β-strands. Comparison of the functional sites of αDTX and DTX-K (Fig. 8) revealed that they share only a common lysine (Lys8 in DTX-K and Lys6 in αDTX). Interestingly, the affinity decrease observed upon the substitution K3A in DTX-K (1250-fold) is similar to that induced by the substitution K5A in αDTX, suggesting that these residues play a similar important role in the two toxins. Because the second most important residue of αDTX (Leu9) is found in all dendrotoxins, we suspect that this residue, which has not yet been probed in DTX-K, may also be functionally important in this toxin. All of the other functional residues of DTX-K differ in αDTX and most are specific to one dendrotoxin subclass. Thus, among the four additional functional residues in αDTX (Arg3, Arg4, Leu6, and Ile8), Arg3 is only found in αDTX, whereas the three others (Arg4, Leu6, and Ile8) are also found in DTX-I but not in the other dendrotoxins. Similarly, among the additional functional residues identified in DTX-K (Lys8, Trp25, and Lys26), Trp25 and Lys8 are also found in βDTX, but not in αDTX or DTX-I. Therefore, dendrotoxins from the same subclass may share similar functional sites, which, however, may differ from one subclass to the other. Only a common core, composed of Lys8 (or Lys9 in DTX-K) and, probably, Leu9 (or Leu7 in DTX-K), seems to be shared by all dendrotoxins. This could explain the heterogeneity in biological properties observed between the two subclasses. A similar situation was recently observed with curarimimetic toxins. On the basis of their primary structures, these toxins are currently classified as long-chain and short-chain toxins, the longer toxins possessing, in particular, an extra small cyclic loop (60). Both long and short chain toxins bind to muscular nicotinic acetylcholine receptors with high affinities (61), whereas the presence of the extra loop uniquely provides long-chain toxins with a high affinity for α7 neuronal receptors.

αDTX Shares a Functional Anatomy with Other Potassium Channel Inhibitors—The binding sites of three types of structurally unrelated Kv1 channel blocking toxins are known. These are those of αDTX (this work) and DTX-K (33), scorpion toxins (26–28), and sea anemone toxins (11, 25). Although these toxins have distinct architectures, they are all likely to bind to the same region of Kv1 channels (11, 18–24) and display functional surfaces that share several molecular features. Their functional surfaces have a comparable size of approximately 700 Å². They all have a protruding lysine, which appears to be a key center associated with the binding to Kv1 channels (11, 25–28, 33). In scorpion toxins, the positive charge of the key lysine (Lys27) is thought to mimic the positive charge of potassium ions (26–29). Furthermore, in all models aiming at docking the position of a scorpion toxin molecule bound to a potassium channel, the side chain of Lys27 projects into the conduction pore of the channel, whereas the other important residues interact with residues of the outer wide vestibule (26–29, 62–64). That Lys8 of αDTX may play a role similar to that of Lys27 of scorpion toxins was suggested by mutational data of Lys8 into norleucine and ornithine. Clearly, its ε-amine group, presumably positively charged, as well as the distance between the α-carbon and this amine group, are important factors. Comparable results were obtained with the most critical lysine of BgK for binding to rat brain Kv1 channels. Thus, we propose that Lys8 of αDTX, the side chain of which protrudes from the functional site (Fig. 7B), is a key residue that may fit into the pore of the vestibule of voltage-gated potassium channel.
channels, as does Lys27 of scorpion toxins and, presumably, the key lysine of sea anemone toxins (11, 25).

The key lysine of both sea anemone and scorpion toxins is associated with a 6.6 ± 1 Å distant key aromatic residue (α-carbon of lysine-center of aromatic ring), either a tyrosine or a phenylalanine. Such a diad of residues was proposed to form a conserved functional core in potassium channel-blocking toxins from invertebrates (11). The functional site of αDTX does not possess such an aromatic residue, but it does have the highly critical and hydrophobic Leu9, the distance from Lys8 of which (α-carbon lysine-δ-carbon leucine: 6.9 Å) is comparable to that observed in the diads of invertebrate toxins. Therefore, all toxins that block Kv1 channels seem to possess a comparable functional diad, which, however, should be more broadly defined as being composed of a hydrophobic residue separated from the key lysine by 6.7 ± 0.9 Å (α-carbon of lysine-center of aromatic ring or δ-carbon of the aliphatic side chain) (Fig. 9). Enzymes with comparable functions possess critical catalytic diads or triads, although they adopt different overall architectures. We find it striking that structurally distinct toxins may possess binding diads with comparable recognition functions.

It is remarkable that the functional site of αDTX, which is produced by a venomous vertebrate (a mamba), shares some features with the functional sites of structurally unrelated potassium channel-blocking toxins produced by venomous invertebrates. This observation suggests that all of these toxins, irrespective of the phylogentic origin of the venomous animals that produce them, underwent a comparable convergent evolution, the Kv1 channels of the prey possibly acting as evolutionary sieves.

αDTX and BPTI Have Topographically Unrelated Functional Sites—As already mentioned, the fold adopted by αDTX is also observed in proteins with unrelated functions. This is the case, in particular, of BPTI (14), which blocks the catalytic action of proteases. That a conserved protein fold is capable of exerting unrelated functions is not uncommon. Thus, it is well known that antibodies (65) or enzymes (66) with conserved overall scaffolds can exert distinct functions. In these cases, however, the respective functional sites, i.e., paratopes or catalytic cavities, are confined in similar areas. Here, we are facing a completely different situation because the functional sites of BPTI and αDTX are located in topographically unrelated regions of the protein. The “antiprotease site” region of BPTI (67) comprises residues Pro13, Lys15, Arg17, Ile19, and Arg39, which are located on “top” of the BPTI structure (Fig. 10), whereas the αDTX functional site is at the opposite end of the fold. Clearly, therefore, the surface of the BPTI/DTX fold can accommodate distinct functional sites in unrelated regions, a scenario that agrees with previous proposals made for other toxin folds (68–70). It will now be interesting to examine whether additional regions of the DTX/BPTI fold are associated with other functions, such as the calcium channel-blocking activity of calcidiun (34). In any case, the available data suggest that the DTX/BPTI fold undergoes a natural “engineering” resulting in divergent functional topographies. As a result, the DTX/BPTI fold appears to be a promising additional template for engineering of novel functions, as initiated with the scorpion toxin fold (71–72).

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Delineation of the Functional Site of α-Dendrotoxin

25403