Three-fingered proteins form a structurally related family of compounds that exhibit a great variety of biological properties. To address the question of the prediction of functional areas on their surfaces, we tentatively conferred the acetylcholinesterase inhibitory activity of fasciculins on a short-chain curaremimetic toxin. For this purpose, we assimilated the three-dimensional structure of fasciculin 2 with the one of toxin α. This comparison revealed that the tips of the first and second loops, together with the C terminus residue, deviated most. A first recombinant fasciculin/toxin α chimera was designed by transferring loop 1 in its entirety together with the tip of loop 2 of fasciculin 2 into the toxin α scaffold. A second chimera (rChII) was obtained by adding the point Asn-61 → Tyr substitution. Comparison of functional and structural properties of both chimeras show that rChII can accommodate the imposed modifications and displays nearly all the acetylcholinesterase-blocking activities of fasciculins. The three-dimensional structure of rChII demonstrates that rChII adopts a typical three-fingered fold with structural features of both parent toxins. Taken together, these results emphasize the great structural flexibility and functional adaptability of that fold and confirm that structural deviations between fasciculins and short-chain neurotoxins do indeed reflect functional diversity.

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The atomic coordinates and structure factors (code 1qm7) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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† The abbreviations used are: AChE, acetylcholinesterase; rFas, recombinant fasciculin; Fas, fasciculin; HPLC, high pressure liquid chromatography; EeAChE, E. electricus electrophoact acetylcholinesterase; BSA, bovine serum albumin; BTCh, butyrylthiocholine; BuChE, butyrylcholinesterase; rCh, recombinant Ch; nFas, native Fas.
We investigated the possibility of generating acetylcholinesterase inhibitory activity within the host curare-like toxin α through the progressive transfer of the most structural deviating regions from fasciculin 2. We show that the host protein cannot only accommodate the imposed structural modifications but also that the biological activity of the host short-chain toxin vanished, whereas nearly all the acetylcholinesterase-blocking activities of fasciculins emerged in a chimeric protein. The three-dimensional structure of the most active chimeric compound we engineered reveals a typical three-finger fold among which only transferred regions adopt a fasciculin-like conformation. Taken together, these results strongly suggest that structural deviations and functional topographies are intimately linked to each other. They also emphasize the great structural flexibility and functional adaptability of the three-finger fold. We propose this approach as a convenient alternative to systematic mutations in predicting a functional determinant at the surface of a toxin adopting a three-fingered scaffold. The evolutionary implications of these findings will be discussed.

**EXPERIMENTAL PROCEDURES**

**Genetic Constructions—**DNA fragments encoding recombinant fasciculin 1 (rFas1) and chimeric Tu/Fas proteins, named rChI and -II, were cloned into the bacterial expression vector pCP (23) and expressed as fusion proteins with two synthetic IgG binding domains derived from protein A (24). Extremities of the cDNA encoding fasciculin 1 were conveniently modified by polymerase chain reaction amplification to allow its oriented cloning. The synthetic genes encoding the two chimeras were derived from that initially built to express the native toxin α from *Naja nigricollis* (23) using the primary structures of native toxin α and fasciculin 2 (Fig. 1A). Briefly, the strategy for assembling the genes was as follows: first, 100 pmol of 12 complementary oligonucleotides with sizes ranging from 17 to 48 bases, covering both strands, and using high frequency codons in *Escherichia coli*, were annealed together; second, semisynthetic genes were generated after ligation together of the three N and C terminus cassettes, respectively, and third, semisynthetic genes were gel-purified and covalently associated. The cDNA and the two synthetic genes have at their 5′-extremities a *Kpn*I restriction site followed by a sequence encoding residues Asp-Asp-Lys specifically recognized by the bovine enterokinase. Two tandem stop codons TAA and a *Bam*HI restriction site were created de novo at the 3′-extremities (Fig. 1B). Finally, correctness of these constructions was checked by DNA sequencing of both strands.

**Expression and Purification of Fused Proteins—** *E. coli* BL21 (DE3)LyS5S was used as a host strain for the expression of ZZ-Fas1 and ZZ Fas2. For this purpose cultures were grown to an optical density of 0.3 at 600 nm. Cells were harvested by centrifugation, resuspended in 500 ml of trypsin soy broth (Difco, Detroit, MI) supplemented with glucose (5 g/liter), ampicillin (200 mg/l), and chloramphenicol (30 mg/l). Induction of hybrid production was initiated by the addition of 0.1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside when the optical density of the culture incubated at 37 °C under aeration reached 0.5–0.6 at 600 nm. After 3 h of induction (A<sub>600</sub> = 1.8–2.0), cells were harvested by centrifugation, resuspended in 50 ml of TSE buffer (30 mM Tris-HCl, 20% (200 mg/l), and chloramphenicol (30 mg/l)). Induction of hybrid production was initiated by the addition of 0.1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside when the optical density of the culture incubated at 37 °C under aeration reached 0.5–0.6 at 600 nm. After 3 h of induction (A<sub>600</sub> = 1.8–2.0), cells were harvested by centrifugation, resuspended in 50 ml of TSE buffer (30 mM Tris-HCl, 20% sucrose, 5 mM EDTA, pH 8) supplemented with 0.1 mM of phenylmethylsulfonyl fluoride (final concentration), and disrupted by sonication at 0 °C, 5 × 3 min at 60% of full power. The supernatant was first purified on an IgG-Sepharose 4B-column (Amersham Pharmacia Biotech) according to Drevet et al. (20). Lyophilized hybrids eluted from the IgG column were further purified by chromatography on a reverse-phase HPLC column (Vydac, C<sub>18</sub> semipreparative) equilibrated in 0.1% trifluoroacetic acid. Elution was performed using a 0.1% trifluoroacetic acid/CH<sub>3</sub>CN/H<sub>2</sub>O gradient of 0–60% in 60 min. Protein concentration was determined spectrophotometrically, based on the following calculated extinction coefficients at 278 nm: ε<sub>a</sub> = 6200, 7500, and 8800 cm<sup>-1</sup>·mM<sup>-1</sup> for ZZ-Chl, -ChII, and -Fas1, respectively.

**Bovine Enteropontidase Cleavage and Renaturation of Recombinant Proteins—**1 mg of ZZ fusion protein purified as described above was combined with bovine enteropontidase (Invitrogen, EKMAX grade, specific activity = 8,500 units/ml in 50 mM Tris, pH 8, 10 mM CaCl<sub>2</sub>, 1% Tween-20, in a total volume of 0.83 ml, at an enzyme:substrate ratio of 1:19,000 (w/w) and incubated at 37 °C for 3 h. Resulting rFas1 or Tu/Fas chimeras were directly chromatographed on a reverse-phase column (Vydac, C<sub>18</sub>) using the following gradient: 0–5 min in 0.1% trifluoroacetic acid, 5–30 min to reach 24% of CH<sub>3</sub>CN, 10 min in trifluoroacetic acid, 0.1%/CH<sub>3</sub>CN 24%, and finally 40–70 min to obtain 60% of CH<sub>3</sub>CN. Products were analyzed using 20% polyacrylamide gel and submitted to amino acid composition and Edman degradation for peptide sequencing. In vitro renaturation of unfolded proteins was performed overnight at room temperature in a final volume of 1 ml using the following buffer: 0.1 x phosphate buffer, pH 8, containing 4 mM GSH and 2 mM GSSG. Refolded compounds were finally purified using conditions similar to those previously described. The dicroic spectra of refolded compounds were recorded at 20 °C using a CD VI Jobin-Yvon spectrophotometer, at a protein concentration of 5 × 10<sup>-5</sup> M.

**Radioimmunoassay and Inhibitory Activities—**The radioimmunoassays were carried out as described previously using 125I-labelled toxin α and two toxin-specific monoclonal antibodies, i.e. M<sub>α</sub> and M<sub>α</sub>-<sub>β</sub> (25).

**Electrophorus electricus** electroplax acetylcholinesterase (EeAChE) and human butyrylcholinesterase were used to assess the inhibitory activities of rFas1, rChI, and rChII. AChE activities were measured spectrophotometrically using acetylthiocholine iodide (1.0–0.05 mM) as substrate and 0.3 mM 5,5′-dithiobis(2-nitrobenzoic acid) as chromophore in 50 mM phosphate buffer, pH 7.5, 0.1 mg/ml BSA (26). Assay mixtures (including the different inhibitors) were preincubated for 60 min at 37 °C before starting the reaction by the addition of acetylthiocholine. K<sub>i</sub> values of rChI and native fasciculin 2 were estimated from Dixon plots according to Dixon (27). The lower K<sub>i</sub> value was estimated from slopes and intercepts of reciprocal 1/V Lineweaver-Burk plots versus various chimaera I concentrations. Human BChE activities were assayed in 50 mM phosphate buffer, pH 7.5, 0.1 mg/ml BSA at 37 °C with butyrylthiocholine (BTCh) as substrate, after a preincubation of 10 min. Hydrolysis of 1 mM BTCh was followed spectrophotometrically. Type and inhibition constants of BuChE for recombinant chimeras II were determined either using Dixon plots 1/V versus inhibitor concentration or according to Cornish-Bowden (30) by plotting S/V against S, at three BChE concentrations: 0.13, 0.25, and 0.50 mM.

**RESULTS**

**Structural Characteristics of Short-chain Curaremimetic Toxins and Fasciculins—**To examine the structural homogeneity of these two toxin families, we compared the x-ray three-dimensional structures of representative members using the iterative program ALIGN (31). Curaremimetic toxins clearly form a homogenous structural family, with a calculated average r.m.s. co deviation value of 1.2 Å. However, the tips of loop 1 (residues 7–12), of loop 2 (residues 29–35), and the two C-terminal residues deviate by between 3 and 5 Å (not shown). It is noteworthy, that six of the ten functional residues forming the toxic site of this family of toxins are located in the two most variable regions (19, 20). It has been suggested that the flexibility of the tip of loops 1 and 2 is necessary to fit the nicotinic acetylcholine receptor (32).

Fasciculin 1 and 2 from the venom of the green mamba *Dendroaspis angusticeps* are two potent inhibitors of acetylcholinesterases (8) whose high resolution three-dimensional structures have been solved using x-ray crystallography (9, 10). Their amino acid sequences differ only by a single substitution at position 47, where a tyrosine or an asparagine is found in fasciculin 1 and 2, respectively (33). Superimposition of their backbones reveals r.m.s. co deviation values mostly comprised between 0.3 and 2 Å. Only residues 6–13 of loop 1 have distinct conformations with r.m.s. deviations ranging between 2 and 10 Å (not shown). It has been proposed that this unusual loop flexibility might be important in the binding to AChEs. Interestingly, elucidation of the x-ray structures of two fasciculin 2 AChE complexes shows that the conformation of the free fasciculin 2 resembles that of the bound state and differs from that of free fasciculin 1 (22). Taken together these data support the idea that loop 1 in fasciculins displays considerable mobility, which is important for tight binding to AChEs. The identification of interacting residues at the tip of loop 1 further emphasizes its functional importance. Even if characterized by a similar fold, curaremimetic toxins and fasciculins therefore possess a number of specific structural features.
Structural Deviations between Short-chain Curaremimetic Toxins and Fasciculins—To identify precisely the regions where these structural deviations occur, we carried out a detailed structural comparison of toxin $\alpha$ from *Naja nigricollis*, a typical member of short-chain toxins (34, 35), and fasciculin 2. Though both toxins possess 61 residues, toxin $\alpha$ and fasciculin 2 each differ by a specific insertion, which causes a numbering shift from position 18 that is only restored at position 56 (see Fig. 1). Using the iterative program ALIGN, we performed a superimposition of the backbone of the x-ray structure of fasciculin 2 and toxin $\alpha$ (Fig. 2, lower views), revealing an average r.m.s. deviation of 2.82 Å for 226 of the 244 atom pairs of the backbone used. The best superimpositions include most of the major $\beta$-sheet strands: 3–5, 14–17, 23–26, 34–41, and 49–55, together with the turn linking loops 2 and 3, and the upper part of loop 3, which includes residues 56–60. However, distances greater than 2.0 Å were observed for regions 5–12, 18–21, 27–34, 41–47, 50, 55–56, and 61 (Fas numbering). Therefore, the regions with the largest structural deviations between toxin $\alpha$ and fasciculin 2 correspond to almost all of the first loop, the following turn, the lower part of the second loop, and the C terminus residue. Interestingly, except for segment 18–21, the functional residues of these two molecules are included in these areas. That segment 18–21 did not superimpose well is not surprising because it corresponds to the turn connecting loops 1 and 2, where the additional proline $\alpha_{18}$ in toxin $\alpha$ is located. The deviating region 5–12 forms a $\beta$-hairpin at the tip of loop 1 of both toxins. However, their primary structures are totally different resulting in highly distinct organizations in the two toxins, the hairpin looks longer in toxin $\alpha$ and broader in fasciculin 2 (Fig. 2). Amino acid residues 27–34 constitute the second $\beta$-hairpin localized at the extremity of the central loop displays opposite orientations in the two toxins, inducing an inversion of their overall concavity (Fig. 2). Thus, the fine comparison of the structures adopted by fasciculin 2
versus toxin α reveals three significant structural deviating regions corresponding to the tips of loops 1 and 2 together with residue 61.

Design of Chimeric Proteins—A first chimera (ChI) was designed by transferring in toxin α the two major deviating regions 5–12 and 27–34 from fasciculin 2 (Fig. 1). As deduced from the above comparison plus the sequence alignment, these two sequences belong to the first and second loops. Moreover, it is known that the adjacent residues of an amino acid sequence region are often critical for this stretch to adopt the expected secondary structure (36). In addition, the structure superimposition revealed conserved residues surrounding the deviating regions (Fig. 1), possibly playing the role of hinges to naturally accommodate the transferred segments into the host toxin.

Therefore, to preserve both the β-sheet and the loop flexibility, we transferred the loop 1 in its entirety, together with residues 27–37 (toxin α numbering) in the first construction (Fig. 1). To further attenuate the structural deviations between the two toxin conformations, we designed a second chimeric construction (Fig. 1). In the latter, we additionally substituted the C-terminal asparagine residue of toxin α for a tyrosine. This was motivated by the observation that within the complex formed with AChE, the C terminus of Fas2 is stabilized by Tyr-4, inducing a substantial increase of the interface (21). By doing this, we anticipated that all residues involved in the stabilizing intramolecular hydrogen bonding between both loops 1 and 2 and the C terminus of native fasciculin 2 may be correctly transferred. We therefore designed two recombinant chimeric proteins, rChI and rChII, by substituting into toxin α 41 and 42, 6% of fasciculin 2 residues, respectively. Among the 45 remaining amino acid residues of rChII, 13, the four pairs of cysteine residues, Tyr-23, Arg-37, Gly-38, Pro-42, and Asn-59, have been described as forming the core responsible for the common three-fingered architecture (13), 15 are specific to...
Structural Deviations between Toxins

apparent molecular mass of 14 and 7–8 kDa, corresponding to those expected for the ZZ domains and cleaved chimaera, respectively. The recombinant proteins were purified by reverse-phase HPLC, leading to a homogeneous moiety as shown in Fig. 3 (lane 5). Sequencing of the first 15 amino acid residues and amino acid analysis of the recombinant proteins, confirmed the specificity of the cleavage and the absence of any side product (not shown). On average, we obtained 10–14 mg of each cleaved and purified recombinant protein/liter of bacterial culture.

Owing to the strategy of expression, disulfides bridges of unfolded native or recombinant fasciculin 1 and chimeric recombinant polypeptides were formed in vitro using similar redox conditions. The most appropriate conditions to refold reduced native fasciculin 1 (rFas1) required a ratio of 4 GSH:2 GSSG. Under these conditions, the refolded fasciculin 1 recovered full inhibitory activity toward acetylcholinesterase (not shown), whereas its reduced and carboxymethylated form was inactive. The same conditions were found to be most appropriate for the rFas1 and both chimeric proteins. No further improvement was seen in the presence of enzymatic catalysts such as protein disulfide isomerase, alone or in combination with thioredoxin (data not shown). No free thiol group was detected in either recombinant protein, using Ellman reagent, indicating that the 8 cysteines were involved in disulfide bridges. We noted no difference between the refolding capacities of the recombinant fasciculin 1 and the chimaera as compared with the native fasciculin 1. It is noteworthy that the refolding conditions we used are opposite to those used to refold toxin α (23), with GSSG/GSH ratios of 2/4 versus 4/2, respectively. This was surprising, because rChI and -II contain the globular core where the disulfides of toxin α, a region which has been several times associated with conformational features of the short-chain three-fingered fold and in particular with its folding rate efficiency, are located (46). This suggests that the role played by the upper part in the folding of short-chain toxins might be influenced by sequence variations in the two first loops, and/or that at least one of the four intramolecular disulfide bonds within these three recombinant compounds might be characterized by a higher redox potential.

Secondary Structure, Antigenic, and Functional Properties of Recombinant Fas 1 and Chimeras I and II—Refolded recombinant fasciculin 1 and chimeras I and II display similar far-UV CD spectra indicating that they have similar secondary structures, which are absent in reduced/carboxymethylated rChII (not shown). Furthermore, the simultaneous presence of a positive band around 197 nm and a negative one at 215 nm, both absent in denatured chimaera II, strongly suggests that the dominant β-sheet structure characterizing the three-fingered fold was present in the two recombinant chimeric proteins. Native toxin α, which naturally interacts with high affinity with the nicotinic acetylcholine receptor (47), is also specifically recognized by two anti-short-chain neurotoxins: monoclonal antibodies called Mo1 (48) and Mo2–3 (49). However, the molecular topographies recognized on the surface of short-chain neurotoxins by Mo1 (50), Mo2–3 (51), and the receptor (19, 20, 51) have been deeply altered to design rChI and -II. Thus, as expected, competition binding experiments with tritiated toxin α demonstrated that in contrast to the wild-type toxin α, neither of the two chimeras was able to bind anymore to either monoclonal antibodies or to the acetylcholine receptor, even at concentration as high as 10 μM (not shown).

Fig. 4 shows the inhibition patterns of AchE from EeAChE by increasing concentrations of nFas1 and -2, rFas1, and rChI and -II. More precisely, the data demonstrate that: i) rFas1 has a similar inhibitory activity as compared with nFas1 or nFas2; ii) rChI inhibits hydrolysis of acetylthiocholine with an IC50 value approximately 150-fold higher than that of native fasciculin; iii) rChII is only 15-fold less potent than native fasciculins; and iv) reduced/carboxymethylated rChII has virtually no activity at concentrations nearly four orders of magnitude greater than the lowest active concentration of native fasciculin. To gain a more precise view of the relative potencies of these compounds that act as tight inhibitors (28), the data have been represented in typical Dixon (Fig. 5) and Lineweaver-Burk (Fig. 6) plots. The K, values thus calculated are respectively equal to 5500, 680, 38, and 42 pm for rChI, rChII, rFas1, and native fasciculin 2. Lineweaver-Burk representations (Fig. 6) also demonstrate that chimeras I and II are noncompetitive inhibitors of EeAChE, suggesting that both compounds bind to the peripheral site of the enzyme as do native fasciculins (29).

Finally, we studied the effects of our two recombinant chimeras on hydrolysis of BTC by human butyrylcholinesterase. As shown in Fig. 7, upper panel, human butyrylcholinesterase was inhibited dose dependently by rChI and rChII in the micromolar range, with an IC50 value slightly increased as compared with nFas2. The type of inhibition of human BuChE by the most potent chimera rChII, and its constant, were determined according to the methods of Dixon (27) and Cornish-Bowden (30). The intersection points of the plots of S/V (Fig. 7, lower left panel) or 1/V (Fig. 7, lower right panel), as a function of varying concentrations of rChII, reflect the dissociation constants of the EI complex (K1) and ternary complex ESI (K1′), respectively. We therefore conclude that rChII is a mixed or a noncompetitive inhibitor of human butyrylcholinesterase with K1 and K1′ values of 1.27 and 1.13 μM, respectively. These results are in agreement with those reported by others (52).

Structural Analysis of rChII—In agreement with the results provided by circular dichroism, rChII adopts a typical three-fingered fold (Fig. 8). Indeed, the three-dimensional structure of rChII clearly demonstrates that most of its β-sheet regions and disulfides superimpose well on those of fasciculin 2 and/or toxin α (Fig. 8). Interestingly, the three-dimensional structure

FIG. 4. Inhibition of E. electricus AChE. The inhibition induced by different inhibitors was measured after 60 min at 37 °C as described under “Experimental Procedures.” Data points correspond to duplicates that differed by less than 5%. •, fasciculin 1; △, recombinant fasciculin 1; ■, fasciculin 2; •, chimaera II; ○, chimaera I; ▲, unfolded chimaera II.
of rChII appears as a combination of specific structural features of fasciculins and short-chain toxins (53). For instance, loop 1 perfectly superimposed on loop 1 of fasciculin 2, as well as loop 2. In contrast, regions 16–21 and 55–57 are typically toxin a, with the presence of an additional proline in position 18 and a deletion between residues 56 and 57 in rChII. These two regions keep within the chimera a toxin a fold. Finally, loop 3 has a toxin a sequence but its conformation is probably triggered by the loop 2 orientation. It therefore appears that the nature of the sequence of loop 2 affects the overall conformation of the second b-sheet. Interestingly, the part of loop 3 that corresponds to a fasciculin 2 fold is surrounded by two glycine residues at positions 39 and 48, respectively. This confirms the assumptions made concerning the need to preserve both the consensus sequences, which characterized the three-fingered fold, together with the secondary structure and loop organization typical of fasciculins. It also, emphasizes the great structural flexibility of that fold and how neatly the host toxin a can structurally accommodate the imposed modifications. The detailed structural analysis of recombinant chimera II is presented and discussed in Le Du et al. (53). The coordinates of the rChII model have been deposited to the Protein Data Bank with entry code 1qm7.

FIG. 5. Dixon plots with data from E. electricus AChE inhibition by native fasciculin 2 and recombinant chimera II. Activities were measured in 1-ml reaction mixtures containing 50 nM phosphate buffer, pH 7.5, 0.1 mg/ml BSA, and 0.32 mM 5,5′-dithiobis(2-nitrobenzoic acid), after preincubation with the inhibitor for 60 min at 37 °C. Hydrolysis of 1 mM acetylthiocholine was followed spectrophotometrically as described under “Experimental Procedures.” Data points correspond to duplicates that differed by less than 5%. V/2, V/3, etc. indicate the points in the curve corresponding to the inhibitor concentration giving one-half, one-third, etc. of the enzyme activity measured without inhibitor. Dixon plots of rChII (A) and nFas2 (B) are shown.

FIG. 6. Lineweaver-Burk plots of E. electricus AChE at different concentrations of recombinant chimera II (A) and I (B), as indicated with acetylthiocholine. Data points represent the average value of two to three measurements differing by less than 5%.

DISCUSSION

Structural Deviations versus Functional Differences—The aim of this paper was to explore the possibility that within a structurally related family of proteins, i.e. three-fingered toxins from snakes, subtle structural deviations could reflect the localization of a particular functional topographies. Clearly, our results strongly support that idea. Indeed, the fasciculin regions initially identified as differing most of toxin a (r.m.s. deviations comprised between 2 and 13 Å) have now been neatly superimposed within rChII with an r.m.s. deviation less than 0.5 Å when compared with fasciculin 2 (53). Thus, loop 1 in its entirety now adopts the fold it displays in native fasciculins characterized in particular by longer b-sheets and great mobility, because it occupies within rChII the conformation described for free fasciculin 2, which is also that of the bound state. Similarly, transferred residues 27–37 (toxin a numbering) from loop 2 of fasciculins not only superimposed on the structure they have in native fasciculins but also constrained the conformation of adjacent residues. Finally, the typical inverse orientation of the tip of the central loop in fasciculins is found in rChII, which displays an opposite concavity to toxin a. These data are in total agreement with the recent elucidation of the structure of the complex formed between AChE showing that: i) AChE and Fas conformations in the complex are very similar to those of their isolated structures; ii) the high affinity of Fas for AChE is because of a remarkable surface complementarity involving many residues specific to fasciculins; iii) the first loop is responsible for the particular capacity of fasciculins to bind at the peripheral anionic site onto the surface of AChE; iv) loop 2 enters the AChE gorge because of its particular shape and orientation, allowing in particular an unusual stacking interaction between Met-33 (Fas) and Trp-279 (AChE), and finally that the C-terminal residues makes contact with the enzyme (21, 22).

Major structural deviations between short-chain neurotoxins
and fasciculins clearly reflect their difference of biological activities. It has been recently shown that a more limited transfer of 7 of the 11 residues forming the curaremimetic site of toxin $\alpha$ within a similar $\beta$-sheet of charybdotoxin failed to generate a potent curaremitic agonist, mostly for conformational reasons (54). Therefore, to respect the structural integrity of the functional regions, we transferred 42.6% of specific residues (26 of 61 amino acid residues) from fasciculins into toxin $\alpha$ mostly spread over two homogenous areas, i.e. loop 1 and half of the central loop. Thus, our results demonstrate that the structural deviations between short-chain neurotoxins and fasciculins and the location of their functional sites are intimately related. Indeed, substitution of structurally different regions actually confers on the host scaffold the expected biological activity, these heterologous segments containing most of the functionally important residues as experimentally identified, and transferred stretches keeping their native fold. This constitutes with the $\alpha/\beta$ scorpion motif (55) a second example of the stability, structural flexibility, and functional adaptability of animal toxins, which are promising host scaffolds for protein engineering. Finally, we propose the identification of structure-based features as a convenient alternative way of guiding systematic mutations in the mapping of a functional epitope at the surface of biologically unrelated toxins adopting the same fold.

**Evolutionary and Structural Considerations**—The choice of a short-chain curaremimetic as host scaffold was not arbitrary. We focused our study on three-fingered toxins whose target is a macromolecule, which is naturally recognized by the neurotransmitter acetylcholine. These toxins include short- and long-chain curaremimetic toxins, which block muscular nicotinic acetylcholine receptors, neuronal or muscarinic toxins, which act on neuronal or muscarinic receptors, respectively, and fasciculins, which inhibit acetylcholinesterases. Various studies suggest that these toxins are evolutionarily related (56), and a number of observations support the idea that they are derived from a curaremimetic toxin ancestor (57). Therefore, curaremimetic toxins and especially short-chain neurotoxins appeared as most plausible hosts to design three-fingered toxins with functionally distinct behaviors. Recent experimental data show
that a long-chain neurotoxin, whose characteristic fifth disulfide bond was selectively reduced, behaves like a short-chain toxin in terms of specificity of receptor recognition (16). Furthermore, the addition of a fifth disulfide bond at the tip of the central loop of a short chain toxin increases its very low affinity for neuronal receptor (58). Similarly, our results demonstrate that a short-chain toxin can adopt mostly through “loop grafting,” a totally unrelated biological activity toward a distinct target, and that this functional acquisition is structurally and functionally compatible with a conserved core of a different origin. Taken together, these data lend further support to the idea of an ancestor role of short-chain toxins in the phylogeny of three-fingered proteins.

Functional Properties of Recombinant Chimera—Newly designed Tox α/Fas 2 recombinant chimeric molecules, and especially rChII, display biological properties and specificities that mimic almost completely those exhibited by native fasciculins. Recombinant chimeras I and II differ only by the point substitution: Asn or Tyr at position 41, respectively. However, despite this small difference, rChI is characterized by a 10 times lower inhibitory constant than rChII toward EeAChE. Our results confirm the importance played by the third point of contact between Fas and AChE, which involves a tyrosine cluster constituted of Tyr-4 and Tyr-61 side chains (21, 22) in fasciculins, but rare in other three-fingered toxins) is a structurational Tox R.
45. Vozza, L. A., Wittwer, L., Higgins, D. R., Purcell, T. J., Bergsheim, M., Collins-Racie, L. A., LaVallie, E. R., and Hoeffer, J. P. (1996) Bio/Technology 14, 77–81
46. Ruoppolo, M., Moutiez, M., Mazzeo, M. F., Pucci, P., Ménez, A., Marino, G., and Quéméneur, E. (1998) Biochemistry 37, 16060–16068
47. Weber, M., and Changeux, J. P. (1974) Mol. Pharmacol. 10, 1–14
48. Boulain, J. C., and Ménez, A. (1982) Science 217, 732–733
49. Tréméau, O., Boulain, J. C., Couderc, J., Fromageot, P., and Ménez, A. (1986) FEBS Lett. 208, 236–240
50. Zinn-Justin, S., Pillet, L., Ducancel, F., Thomas, A., Smith, J. C., Boulain, J. C., and Ménez, A. (1994) Protein Eng. 7, 917–923
51. Ducancel, F., Merienne, K., Fromen-Romano, C., Tréméau, O., Pillet, L., Drevet, P., Zinn-Justin, S., Boulain, J. C., and Ménez, A. (1996) J. Biol. Chem. 271, 31345–31353
52. Masson, P., Froment, M. T., Bartels, C. F., and Lockridge, O. (1996) Eur. J. Biochem. 235, 36–48
53. Le Du, M. H., Ricciardi, A., Khayati, M., Ménez, R., Boulain, J. C., Ménez, A., and Ducancel, F. (2000) J. Mol. Biol. 296, 1017–1026
54. Drakopoulou, E., Zinn-Justin, S., Guenneugues, M., Gilquin, B., Ménez, A., and Vita, C. (1996) J. Biol. Chem. 271, 11979–11987
55. Vita, C. (1997) Curr. Opin. Biotechnol. 8, 429–434
56. Strydom, D. J. (1979) in Snake venoms, Handbook of Experimental Pharmacology (Lee, C. Y., ed) Vol. 52, pp. 159–212, Springer-Verlag, Berlin
57. Dufton, M. J. (1984) J. Mol. Evol. 20, 128–134
58. Servent, D., Mourier, G., Antil, S., and Ménez, A. (1998) Toxicol. Lett. 28, 199–203
Do Structural Deviations between Toxins Adopting the Same Fold Reflect Functional Differences?
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