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Synthesis and Characterization of a Chimeric Peptide Derived from Fasciculin that Inhibits Acetylcholinesterase

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Abstract: Fasciculins are peptides isolated from mamba (*Dendroaspis*) venoms which exert their toxic action by inhibiting acetylcholinesterase (AChE). They contain a characteristic triple stranded antiparallel β-sheet formed by residues 22–27, 34–39 and 48–53. A chimeric peptide named Fas-C, encompassing most of these sequences was synthesized using SPPS/Boc-chemistry and characterized chemically, structurally and functionally. Fas-C has two disulfide bridges, formed sequentially using dual cysteine protection.

SDS-PAGE patterns, HPLC profiles and MS proved the peptide identity. Circular dichroism indicated the presence of 13.6% and 41.6% of β-sheet and β-turn, respectively, comparable to values observed in the native toxin. An inhibitory effect on eel AChE was displayed by the peptide (K_i71.6 $\pm$ 18.3 μM), although not reaching the affinity level of the parent native toxin (K_i 0.3 nM). It is confirmed that the principal binding region of fasciculin to AChE resides within loop II. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: fasciculin; acetylcholinesterase; anticholinesterase activity; AChE

INTRODUCTION

Fasciculins are closely related peptides isolated from the venom of mambas (genus *Dendroaspis*), with a selective and potent inhibitory activity towards acetylcholinesterase (AChE) [1]. They interact with a peripheral site of AChE, at the rim of the gorge leading to the enzyme active site [2,3] being the only known peptidic AChE inhibitor with dissociation constants ranging 1–10 pM.

These peptides are members of a large family of toxins which includes α and κ-neurotoxins, cardiotoxins, muscarinic toxins, calcium channel blocking toxins and other polypeptides directed to diverse specific targets [4–6]. Nevertheless, all these toxins share a common feature: three loops protruding from a central core, like the central fingers of a hand [7]. Two fasciculins, differing by only one residue, have been purified from *Dendroaspis angusticeps* and their crystallographic analyses have indicated that loop I is formed by residues 5–15, the central loop II by residues 24–38 and loop III by residues 43–50 [7]. Molecular modelling of the fasciculin–AChE complex has shown that the central loop II plays a leading role in toxin binding [8]. Site-directed mutagenesis of the toxin [9] and crystal structures of fasciculin–AChE complexes [10,11] also disclosed that a cluster of hydrophobic residues from loop II, as well as core Tyr^4 and Tyr^{61} and certain residues from loop I, make crucial contacts with the enzyme.

Our laboratory has reported previously that synthetic peptides spanning the central loop sequence displayed inhibitory activity in the μmolar range,

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some orders of magnitude lower than the original toxin [12,13]. On the other hand, it has been suggested that residues from loop III play no critical functional role other than internal stabilization of loop II conformation [9–11].

One of the most characteristic structural features present in fasciculin and other related snake toxins is a triple stranded antiparallel β-sheet formed between residues 22–27, 34–39 and 48–53 [7]. In order to evaluate the contribution of this structural motif in the interaction of the toxin with AChE, a chimeric peptide named as Fas-C spanning the region was designed and has been synthesized. It contains fasciculin sequences 22–38 and 44–52 linked by the dipeptide Pro-Gly and two disulfide bridges not present in the native toxin mimic the native fasciculin triple β-sheet (Figure 1).

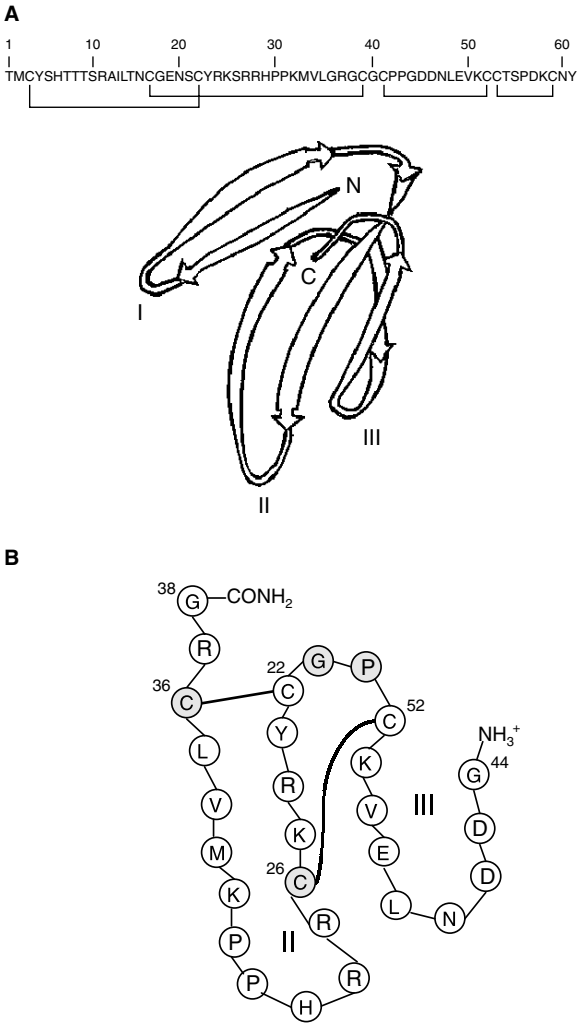

Figure 1 (**A**) Amino acid sequence and ribbon representation of fasciculin 2. Labels I, II and III refer to fasciculin looped sequences 5–15, 24–38 and 43–50, respectively. (**B**) Sequence of the synthetic fragment Fas-C. Thick solid lines indicate disulfide bridges and numbers denote amino acid position as found in fasciculin sequence.

MATERIALS AND METHODS

Materials

Boc amino acid derivatives, AChE from electric eel (type Vs), 5, 5′ dithiobis-2-nitrobenzoic acid (DTNB),Triton X-100 and acetylthiocholine iodide were obtained from Sigma-Aldrich (St Louis, MO, USA). Benzhydrylamine resin was purchased from Peninsula Laboratories Inc. (Belmont, CA, USA). All reagents for peptide synthesis were obtained from Applied Biosystems Inc. (Foster City, CA, USA).

Peptide Synthesis

Fas-C synthesis was carried out by a stepwise manual solid phase method, using Boc chemistry and benzhydrylamine resin (0.6 mmole/g) as support on a 0.15 mmole scale. Reactive side chains were protected as follows: Tos for Arg and His; Bzl(Me) for $Cys^{22,36}$; Acm for $Cys^{26,52}$; Z(Cl) for Lys; diClBzl for Tyr; Bzl for Glu and Asp. The synthesis protocol employed 25% TFA in DCM for Boc-deprotection and 10% Et_3N in DCM for neutralization. Activation was accomplished using 3 eq. of Boc-amino acid and 3 eq. of *N*, *N*′-dicyclohexylcarbodiimide in DCM. The coupling efficiency at each step was monitored by the ninhydrin test [14]; when positive, double coupling was performed. Peptide deprotection and cleavage from the resin were achieved by HF treatment (45 min; 0 °C; 10% anisole). The peptide was extracted with 10% acetic acid and lyophilized to give 0.95 g of crude Fas-C.

Disulfide Bond Formation

Approximately 100 mg of crude peptide with Acm-protected Cys was reduced in 400 ml of 50 mM ammonium acetate buffer at pH 8.0, containing 1.4 μM β-mercaptoethanol. After standing 1 h at room temperature in N_2 atmosphere, the solution was acidified to pH 3.0 with acetic acid and lyophilized. The reduced peptide was redissolved in the same buffer under highly diluted conditions (0.10 mg/ml) and stirred for 10–12 h at room temperature to allow disulfide pairing between Cys^{22} and Cys^{36}. The solution was concentrated to a final

volume of 60 ml and loaded in aliquots of 12 ml onto a Sephadex G-25 fine column (1.6 × 60 cm), equilibrated and eluted with 0.1 M acetic acid.

Deprotection of the Acm group from Cys^{26} and Cys^{52}, was carried out at a 3–4 mg/ml peptide concentration with 2 eq. of $Hg(OAc)_2$ in 30% (v/v) acetic acid. The mixture was stirred for 1 h and Hg^{2+} ions were precipitated with a 20-fold excess of β-mercaptoethanol. After 1 h the mercuric salt was separated by filtration through a Celite pad. The reduced peptide was desalted on the Sephadex G-25 fine column described above and lyophilized. Disulfide pairing of Cys^{26} and Cys^{52} was performed following the conditions already described for closing the disulfide bond Cys^{22}-Cys^{36}.

Chromatography

The fully oxidized peptide was purified by HPLC on a C_{18} Vydac semipreparative column (1 × 25 cm) eluted with a linear acetonitrile gradient (0–40%, 50 min) in 0.1% TFA at 0.5 ml/min. The main peak was collected and lyophilized.

Prolyl Endopeptidase Digestion

The purified peptide was dissolved in 50 mM sodium phosphate buffer, pH 7.0. Prolylendopeptidase (ICN Biomedicals) was added to this solution, at an enzyme/substrate ratio 1/50 w/w. Digestion was carried out for 2 h at 37 °C and the peptides were separated by RP-HPLC followed by MS.

Chymotryptic Digestion

The purified peptide was digested in 100 mM ammonium bicarbonate, pH 8.1 by porcine chymotrypsin and incubated at 37 °C for 4 h (enzyme/peptide ratio 1/40 w/w). Fragments were submitted to chemical characterization after chromatographic analysis.

Chemical Characterization

SDS-PAGE was carried out on a Bio-Rad mini gel using a Tris/Tricine buffer at pH 8.45 [15]. The separating gel contained 12% polyacrylamide plus 13% glycerol and the stacking gel contained 4% polyacrylamide. Ten 20 µg samples were boiled for 5 min at 100 °C with 4% SDS (without β-mercaptoethanol), run for 2–3 h at 100 V and stained with Coomassie blue R. To perform amino acid analyses, 50 µg of peptide was hydrolysed with 100 µl 6 N HCl at 110 °C for 20 h. Hydrolysates were dried and analysed on a Beckman automatic analyser (Fullerton,CA, USA). Cysteines were determined as cysteic acid in samples oxidized with performic acid. UV absorption spectra of purified peptides were run on a UV-160 spectrophotometer (Shimadzu Corp., Kyoto, Japan). Aliquots were hydrolysed and analysed for their amino acid composition, and the protein content was correlated with the recorded absorbance.

Ion spectra of the peptide were obtained with a Sciex Perkin Elmer (Shelton, CT, USA) tandem quadrupole API III electrospray mass spectrometer. The instrument m/z scale was calibrated with ammonium adduct ions of polypropylene glycol.

Circular Dichroism Studies

CD measurements were performed on a Jasco J20 spectropolarimeter flushed with N_2. Spectra were recorded at 20 °C using a 0.5 mm pathlength cell containing 0.085 mM Fas-C in distilled water. Data were collected using a 0.2 nm spectral bandwidth, a 4 s time constant and averaging data over three scans.

Assay of AChE Inhibition

AChE activity was measured with 0.47 mM acetylthiocholine as substrate according to Ellman *et al.* [16]. Standard assays were performed with approximately 0.056 IU of AChE in the presence of 0.3 mM DTNB, 0.001% Triton X-100 in 0.1 M sodium phosphate buffer pH 7.9. Assays were carried out at 25 °C for 5 min by monitoring the absorbance at 412 nm as a function of time. Fas-C was dissolved in 3 ml of sodium phosphate buffer and the percentage inhibition was obtained by comparison with control assays without peptides. Enzymatic hydrolyses of substrate concentrations over the 0.02–0.2 mM range were measured and the data were plotted according to the Lineweaver-Burk representation. An inhibition constant for Fas-C was obtained according to Berman and Leonard's analysis [17].

RESULTS

The chimeric Fas-C peptide contains the 22–38 sequence of fasciculin loop II linked by a Pro-Gly turn to the third strand of fasciculin β-sheet which runs from residue 44 to 52 (Figure 1). Ser^{26} and Gly^{36} in the toxin sequence were replaced by cysteine residues in order to establish disulfide linkages with Cys^{52} and Cys^{22} (according to fasciculin sequence

numbering), respectively. The disulfide bridge Cys^{22}-Cys^{36} cyclizes the sequence corresponding to loop II while Cys^{26}-Cys^{52} linkage folds residues 44–52 from loop III close to loop II. Dual cysteine protections were employed in our approach to achieve a selective disulfide formation, Bzl(Me) groups were removed during the HF cleavage step from Cys^{22} and Cys^{36} and oxidation of the — SH free peptide yielded the required Cys^{22}-Cys^{36} linkage. Subsequent removal of Acm groups from Cys^{26} and Cys^{52} and oxidation allowed the second disulfide formation. No free — SH group was detected with Ellman's reagent in the crude disulfide-bridged peptide (data not shown), indicating that, most likely, all cysteine residues in Fas-C are involved in disulfide bonds.

The lyophilized crude peptide was purified using semipreparative HPLC on a C_{18} column (Figure 2). The fraction collected between 34 and 36 min was lyophilized. A single peak was obtained after rechromatography of the purified material on the same column; it had the correct amino acid composition: Asp 2.9 (3), Glu 1.2 (1), Gly 2.9 (3), His 0.9 (1), Arg 4.05 (4), Pro 2.8 (3), Tyr 0.8 (1), Val 1.9 (2), Met 1.0 (1), Leu 1.8 (2), Lys 2.7 (3), Cys 3.8 (4). The yield of purified disulfide-bridged material was 18–25%.

SDS-PAGE of crude Fas-C showed several bands (Figure 3, lane 1) indicating oligomeric species. After disulfide bridge formation the band corresponded mostly to the monomer (Figure 3, lane 2) and the purified material showed a single band corresponding to the MW of the monomer (Figure 3, lane 3). Analysis of Fas-C by ESI-MS confirmed its identity, showing predominant molecular ions with a mass value in coincidence with the theoretical value: 3220.5 Da.

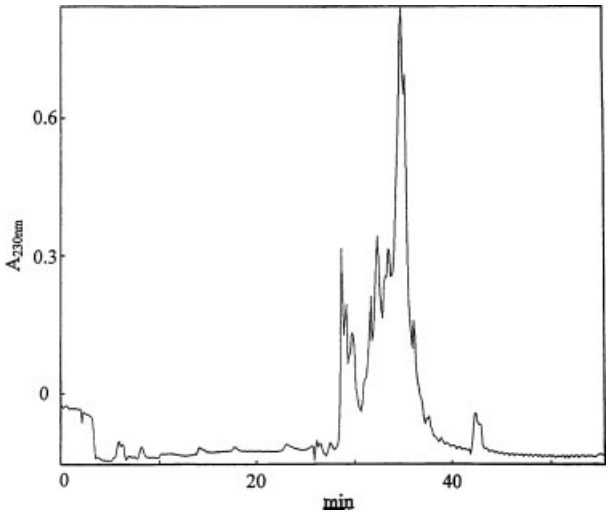

Figure 2 Purification of Fas-C by RP- HPLC on a C_{18} Vydac semipreparative column (1 × 25 cm); flow rate 0.5 ml/min; solvent A, 0.1% TFA; solvent B, 0.1% TFA/80% acetonitrile. The gradient was 0–40% B in 50 min.

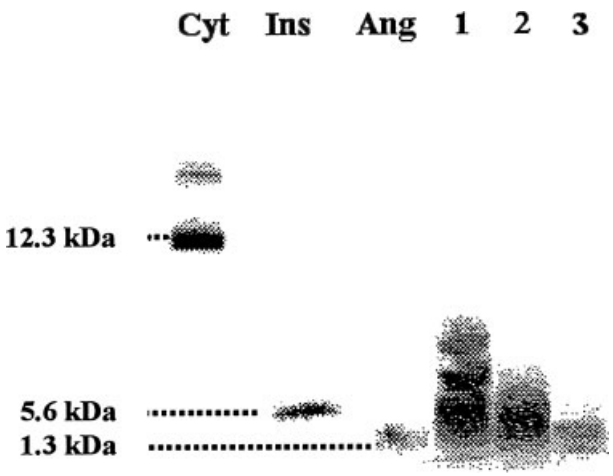

Figure 3 SDS-PAGE of synthetic Fas-C. Samples were boiled for 5 min at 100 °C after addition of 4% SDS and run on 12% polyacrylamide and 13% glycerol in the presence of 0.1% SDS using Tris-Tricine buffer, pH 8.45. Lanes correspond to the following samples: (1) crude Acm-$Cys^{26,52}$ Fas-C, (2) Fas-C after formation of disulfide linkages Cys^{22}-Cys^{36} and Cys^{26}-Cys^{52}, (3) purified Fas-C. Standards of cytochrome c (Cyt), insulin (Ins) and angiotensin I (Ang) are also shown.

Disulfide pairings were mapped as Cys^{22}-Cys^{36} and Cys^{26}-Cys^{52} as determined from enzymatic cleavage of oxidized Fas-C with chymotrypsin or proline specific endopeptidase. Peptide enzymatic digests were analysed by gel chromatography, SDS-PAGE, HPLC and MS. As positive controls of proteolytic activity, simultaneous digests of similar amounts of reduced Fas-C showed fragmentation of the peptide either by chymotrypsin or proline endopeptidase (data not shown). A schematic representation of protease treatments and assignment of disulfide linkages of Fas-C is shown in Figure 4. Analytical Sephadex G-25 chromatography and SDS-PAGE proved the existence in chymotryptic digests of one predominant fragment with a molecular size similar to intact Fas-C. MS of this fragment purified by HPLC indicated an experimental mass of 3225 Da. Chymotryptic cleavage at the tyrosine residue of Fas-C should allow the detection of two lower molecular weight fragments if pairings Cys^{22}-Cys^{52} and Cys^{26}-Cys^{36} are present and

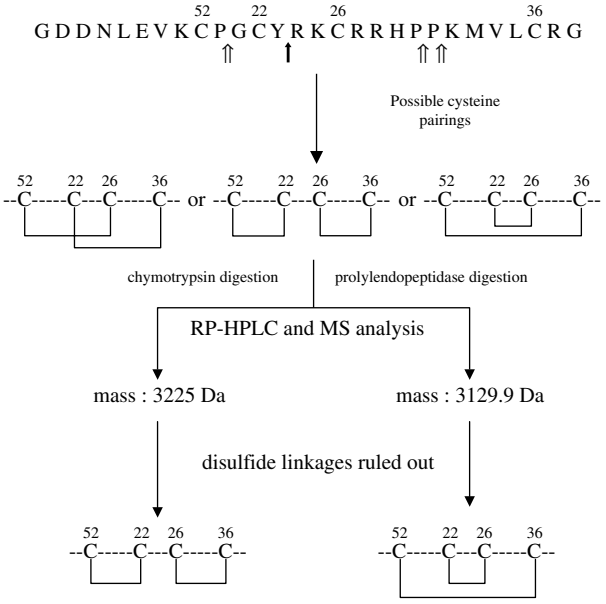

Figure 4 Schematic representation of protease treatment and assignment of disulfide bridges in Fas-C. Numbers indicate positions of cysteine residues in the fasciculin sequence and arrows indicate cleavage sites for chymotrypsin (⬆) or prolylendopeptidase (⇑).

since peptide fragmentation was not observed, those incorrect pairings were ruled out.

Three prolines in the Fas-C sequence are targets of prolylendopeptidase digestion, but the correct disulfide pairing would not allow the isolation of small fragments in such digests. In fact, no fragmentation was shown by analytical gel chromatography or SDS-PAGE after prolylendopeptidase treatment of Fas-C, whereas the reduced peptide yielded various fragments (data not shown). The digested fragment behaved very similarly to intact Fas-C; amino acid analysis and molecular mass of the obtained peak (3129.9) indicated the absence of one Pro and ruled out the incorrect pairing Cys^{22}-Cys^{26} and Cys^{36}-Cys^{52}.

The CD spectrum of Fas-C recorded in water in the far-UV region (201–250 nm) exhibited a minimum at 211 nm (Figure 5). Computation [18] indicated 13.6% of β-sheet content and 41.6% of β-turn. Nevertheless, the calculation also predicted 20.0% of α-helical and 18.4% of random conformation. The calculated contribution of disulfide bridges plus aromatic chromophores was 6.4%.

The synthetic peptide was tested for inhibitory activity on electric eel AChE and the data indicated that a 9.6 μM solution of Fas-C showed 29.4 ± 7.8% inhibition. Since several basic residues are present

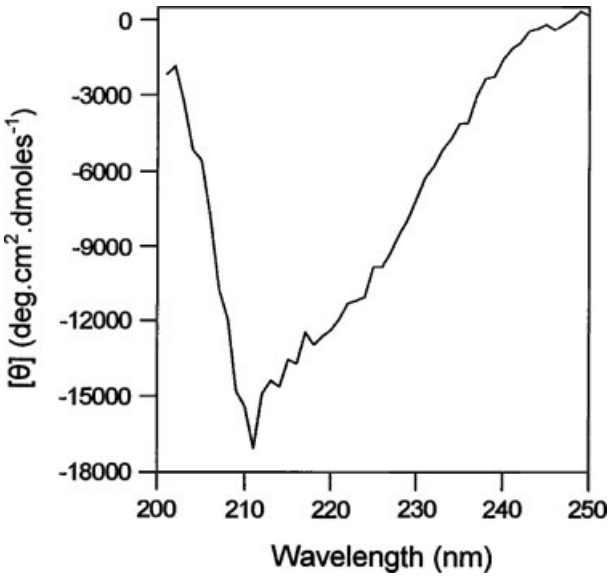

Figure 5 Far UV CD spectrum of Fas-C obtained in water at 20 °C with a 0.085 mM peptide concentration.

in the synthesized sequence, the inhibitory potencies of free arginine, lysine and histidine were evaluated as a control, but no inhibitory effect at mM concentrations was found. Other positively charged peptides, unrelated to the fasciculin sequence such as polylysines MW 4000 or 120 000, also proved inactive at 400 μM and 0.01 μM, respectively. Reciprocal plots for the inhibition of acetylthiocholine AChE hydrolysis by Fas-C yielded a K_i value of 71.6 ± 18.3 μM (Figure 6), 10^5 times lower than that reported for the natural toxin (K_i: 0.3 nM) [2].

DISCUSSION

The x-ray structure of the snake toxin fasciculin bound to mouse [10] or *Torpedo californica* AChE [11] disclosed that three toxin domains anchor it to the enzyme and delineate a large contact area consistent with the picomolar dissociation constant of the complex. The central loop of fasciculin 2 was found to fit appropriately at the gorge entrance and seems to preclude the entrance of other molecules, even as small as water, thus upon binding of the toxin the entry of the substrate to the catalytic site is blocked and by direct steric occlusion fasciculin 2 alters AChE conformation [10] acting as an allosteric modulator [19]. Molecular dynamics simulation showed that the toxin inhibits the enzyme by combined allosteric and dynamic means [20].

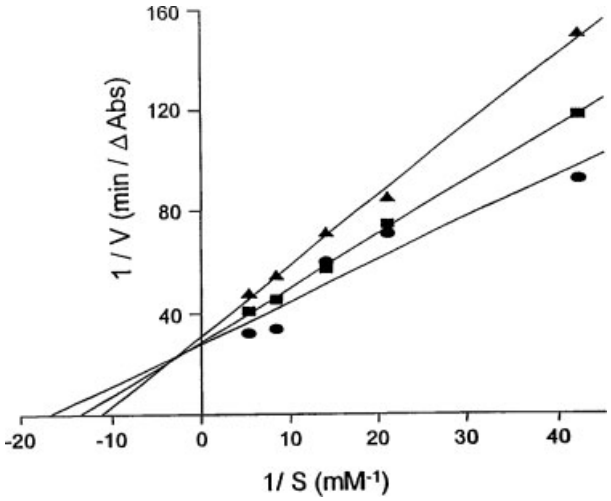

Figure 6 Analysis of AChE inhibition by Fas-C after 10 min incubation with the enzyme. Double reciprocal plots of reaction velocity versus acetylthiocholine concentration in the presence of different concentrations of Fas-C (● control, ■ 5 μM, ▲ 30 μM).

All members of the three-fingered toxin superfamily present a characteristic β-sheet fold comprising loop II and residues from loop III. In our approach, a synthetic peptide containing most of such a sequence folded in a triple stranded β-sheet was synthesized using a suitable pairwise combination of —SH blocking groups (Bzl(Me)/Acm) in order to form sequentially the desired disulfide bridges between Cys^{22}-Cys^{36} and Cys^{26}-Cys^{52}.

Protease treatment after formation of disulfide bridges in Fas-C confirmed their correct pairings. Fas-C contains only one tyrosine susceptible to chymotryptic cleavage (Figure 4), thus two fragments should be obtained only if the incorrect Cys^{22}-Cys^{52} and Cys^{26}-Cys^{36} linkages were present. The isolation of a predominant fragment with a molecular size similar to intact Fas-C ruled out those incorrect pairings. Proline endopeptidase digestion of Fas-C indicated that the other incorrect pairings Cys^{22}-Cys^{26} and Cys^{36}-Cys^{52} were absent. Three proline residues in Fas-C are targets for this enzyme (Figure 4), but the correct disulfide pairing would not allow the isolation of small fragments in such digests. Again, from digested Fas-C only one fragment which behaved similarly to intact peptide was isolated, thus ruling out the incorrect pairings Cys^{22}-Cys^{26} and Cys^{36}-Cys^{52}.

CD spectra provided evidence for the existence of an ordered β-sheet structure in Fas-C and a high β-turn content, consistent with the existence of turns in the second and third loop of the natural toxin, allowing the formation of the triple stranded β-sheet. Although the minimum at 217 nm is indicative of β-sheet structure [21], the predicted α-helix content was not shown in our results, since minima at 222 and 208 nm, reported as characteristic for such structure, were not observed. Certain types of β-turns could explain the displacement of the minimum at 211 nm [18].

Fas-C exerted incipient activity in the micromolar range not attributable to nonspecific interactions of positive charge, since fasciculin interacts with the well described peripheral anionic site [22–24], but free basic amino acids or unrelated positively charged peptides were inactive. Lineweaver-Burk plots suggested a mixed type inhibition, different from the non-competitive behaviour observed in fasciculin [2,3]. In accordance with site-directed mutagenesis studies [9] and our previously reported results [12, 13, 25] the key role for residues from loop II in binding to the enzyme is confirmed.

The higher peptide concentration required to achieve comparable binding to that shown by the native toxin may be attributed to conformational differences in the fasciculin chimera and/or the lack of residues from other regions known to interact with AChE. Furthermore, no evidence of an additional contribution of residues 44–52 from loop III, which complete the triple stranded antiparallel β-sheet, to binding affinity towards AChE was obtained. This finding is in agreement with studies of the crystal structure of the fasciculin–AChE complex showing that fasciculin makes contact with the peripheral anionic site of the enzyme through its first two loops, with loop III pointing away from AChE [11]. Recently, a chimeric protein was crystallized resulting from the transfer of partial fasciculin 2 sequences, corresponding to loop I, half of loop II and its *C*-terminal residue to toxin α, a curaremimetic short-chain toxin belonging to the same structural family but differing in amino acid sequence and target [26]. The chimera retains the general structural characteristics of a three fingered toxin and with only 41% of fasciculin sequence displays the functional specificity of the transferred sequence, showing an inhibition constant on AChE only 15-fold lower than that of fasciculin [26]. Loop III sequence contributes weakly to the complex interface, as only residues Asn^{47} and Leu^{48} located at the tip of the loop are in van der Waals interactions with AChE, the other residues face the solvent and may help to maintain loop II specific conformation [10].

CONCLUSION

Our results confirm, by application of a synthetic peptide strategy, the key role of the fasciculin central loop and the weak contribution of residues from loop III to the binding of the toxin to AChE. It is likely that an extensive set of stabilizing interactions with additional residues, present outside this region, is required to reach high affinity for the enzyme.

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