Expression and Activity of Mutants of Fasciculin, a Peptidic Acetylcholinesterase Inhibitor from Mamba Venom*

Pascale Marchot‡‡, Claudine N. Prowse‡, Joan Kanter‡, Shelley Camp‡, Elizabeth J. Ackermann‡, Zoran Radić‡, Pierre E. Bougis§, and Palmer Taylor‡‡

From the ‡Department of Pharmacology, University of California at San Diego, La Jolla, California 92093-0636 and §CNRS, Unité de Recherche Associée 1455, Institut Fédéral de Recherche Jean Roche, Université de la Méditerranée, Faculté de Médecine Secteur Nord, 13916 Marseille Cedex 20, France

Fasciculin, a selective peptidic inhibitor of acetylcholinesterase, is a member of the three-fingered peptide toxin superfamily isolated from snake venoms. The availability of a crystal structure of a fasciculin 2 (Fas2)-acetylcholinesterase complex affords an opportunity to examine in detail the interaction of this toxin with its target site. To this end, we constructed a synthetic fasciculin gene with an appropriate leader peptide for expression and secretion from mammalian cells. Recombinant wild-type Fas2, expressed and amplified in Chinese hamster ovary cells, was purified to homogeneity and found to be identical in composition and biological activities to the venom-derived toxin. Sixteen mutations at positions where the crystal structure of the complex indicates a significant interfacial contact point or determinant of conformation were generated. Two mutants of loop I, T8A/T9A and R11Q, ten mutants of the longest loop II, R24T, K25L, R27W, R28D, H29D, ΔPro30, P31R, K32G, M33A, and V34A/L35A, and two mutants of loop III, D45K and K51S, were expressed transiently in human embryonic kidney cells. Inhibitory potencies of the Fas2 mutants toward mouse AChE were established, based on titration of the mutants with a polyclonal anti-Fas2 serum. The Arg27, Pro30, and Pro31 mutants each lost two or more orders of magnitude in Fas2 activity, suggesting that this subset of three residues, at the tip of loop II, dominates the loop conformation and interaction of Fas2 with the enzyme. The Arg24, Lys32, and Met33 mutants lost about one order of magnitude, suggesting that these residues make moderate contributions to the strength of the complex, whereas the Lys35, Arg28, Val34, Leu35, Asp36, and Lys31 mutants appeared as active as Fas2. The Thr8-Thr9, Arg11, and His29 mutants showed greater ratios of inhibitory activity to immunochemical titers than Fas2. This may reflect immunodominant determinants in these regions or intramolecular rearrangements in conformation that enhance the interaction. Of the many Fas2 residues that lie at the interface with acetylcholinesterase, only a few appear to provide substantial energetic contributions to the high affinity of the complex.

Fasciculins, selective inhibitors of acetylcholinesterase

*This work was supported by CNRS and NATO (to P. M.) and by United States Public Health Service Grant GM18360 and DAMD Grant 17–95–1–9027 (to P. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 619-534-1366; Fax: 619-534-8248.

© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 6, Issue of February 7, pp. 3502–3510, 1997

Printed in U.S.A.

This paper is available on line at http://www-jbc.stanford.edu/jbc/

Fax: 619-534-8248.

The abbreviations used are: AChE, acetylcholinesterase; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CPK, Corey-Pauling-Koltun; Fas2, natural venom-derived fasciculin 2; FPLC, fast pressure liquid chromatography; HEK, human embryonic kidney; hAChE, recombinant acetylcholinesterase from mouse; NH4Ac, ammonium acetate; PAGE, polyacrylamide gel electrophoresis; rFas2, recombinant wild-type fasciculin 2; RIA, radioimmunoassay.
occlusion of substrate entry at the mouth of the active-site gorge. To reconcile the disparity in the kinetic and structural data, either a second portal for entry of substrate and catalytic site inhibitors in the complex or a conformational change in the enzyme, not obvious in the crystal structure, opening a gap between the gorge wall and the bound peptide, have been proposed (30).

Complete understanding of the chemistry of the Fas2-AChE association requires a functional map of the binding surfaces. By site-directed mutagenesis of a synthetic Fas2 gene, we have generated new probes aimed at analyzing the individual contributions of the fasciculin residues to complex formation and conformation. In a mammalian system, we expressed a fully processed recombinant fasciculin, rFas2, that is indistinguishable from the natural, venom-derived Fas2. Fourteen mutants, encompassing 16 amino acid residues distributed among the three loops (fingers) of Fas2, were designed based on both the kinetic and structural data. We show that common determinants are identified by the structural and the mutagenesis approaches, but only a few of the many Fas2 residues residing at the binding interface provide the critical contacts required for enzyme inhibition.

**EXPERIMENTAL PROCEDURES**

**Materials**—HEK-293 and CHO-K1 cells were obtained from American Type Culture Collection. Ultraculture and serum-free Ultra-CCHO cell culture media were from BioWhittaker, and serum-free Dulbecco’s modified Eagle’s cell culture medium from Life Technologies, Inc. 1-Methionine sulfoximine, polyethylene glycol 8000, protease-free BSA, 5,5'-dithiobis-(2-nitrobenzoic acid), acetylthiocholine iodide, and the gel-filtration molecular weight markers (MW-GF-70 Kit) were products of Sigma. The prepacked FPLC columns, Mono S HR 5/5 and Superose-12 HR 10/30, were from Pharmacia Biotech Inc. Dialysis tubing (Spectra/Por6) was from Spectra Medical Industries. The BCA kit for protein assays was from Pierce. Prestained protein molecular weight standards for SDS-PAGE were from Life Technologies, Inc. 125I:Na (2100 Ci mmol⁻¹) was from Amersham. Complete and incomplete Freund’s adjuvants were from Difco Laboratories. Normal rabbit serum and goat anti-rabbit whole serum were from Jackson ImmunoResearch Laboratories. All buffers were made with deionized water from a Millipore MicroQ/Millipore system.

**Biological Materials**—The pGS expression vector was a gift from SciOs Nova Inc. (Mountain View, CA). Purification of Fas2 from *Denudospriasis angusticeps* venom has been detailed previously (32). Concentrations of stock solutions were determined from their UV spectra (ε²₅₀ = 4900 M⁻¹ cm⁻¹). Wild-type AChE from mouse recombinant DNA was expressed, concentrated, and titrated as described previously (33). The polyvalent anti-AChE serum was obtained by immunization of a Blanc du Bouc Exiv rabbit with subcutaneous multi-site injections of purified Fas2 (100–150 μg). Primary and booster injections were made with complete and incomplete Freund’s adjuvants, respectively (35).

**Design of Synthetic Genes Encoding Wild-type and Mutant Fas2**—A cDNA encoding rFas2 and the leader peptide from erabutoxin a (36) was synthesized as two sets of complementary oligonucleotides of ~130 base pairs in length, that were annealed, ligated together, and cloned into the expression vector pGS. rFas2 was expressed from the cytomegalovirus promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant promoter while glutamine synthetase under the control of the *Rous sarcoma virus-long terminal repeat was used as a dominant.

**pcDNA-3 expression vector containing the *Escherichia coli lacZ* gene were cotransfected (38). The cells were rinsed and placed in serum-free medium 24 h after transfection. Secreted fasciculin activity was examined 48 to 72 h later.

**Chromatography**—Ultraculture medium containing the expressed rFas2 (10 ml/10-cm plate) was harvested every 3–4 days, centrifuged (4 °C, 10 min) to remove cell debris, extensively dialyzed against 50 mM NH₄Ac, pH 7.5, 0.01% (v/v) NaN₃, and filtered through 0.22-μm cellulose acetate filters. rFas2 was purified by cation-exchange and size-exclusion FPLC (Pharmacia) performed at 4 °C in NH₄Ac, pH 7.5, with flow rates of 0.5 ml min⁻¹. The dialyzed and filtered cell culture medium (up to 500 ml) was loaded on a Mono S column previously equilibrated with 50 mM NH₄Ac, then the column was washed extensively with the same buffer. Elution of the rFas2 fraction was performed with a 50–200 mM NH₄Ac gradient over 60 min, followed by an isocratic step at 200 mM NH₄Ac. The eluted rFas2 fraction was lyophilized, redisolved in 100 mM NH₄Ac, and loaded as a 200-μl sample on a Superose-12 column equilibrated in 100 mM NH₄Ac. For the final cation-exchange step, the pooled rFas2-containing fractions emerging from successive gel filtrations were diluted twice and loaded on the Mono S column equilibrated in 50 mM NH₄Ac. Elution of rFas2 was performed under isocratic conditions with 100 mM NH₄Ac.

All Fas2 mutants were concentrated and partially purified from the culture medium through the first ion-exchange step, then further concentrated by ultrafiltration. To prevent cross-contamination, separate Mono S columns were used for rFas2 and the Fas2 mutants, and the columns were subsequently used.

**Electrophoresis—SDS-PAGE under reducing conditions used the discontinuous system of Laemmli (39) with a 20% resolving/5% stacking gel. Samples were denatured in 500 mM Tris-HCl, pH 6.8, in the presence of 5% (v/v) β-mercaptoethanol and 4% SDS, at 90 °C for 2 min, then loaded onto the gel in the presence of 10% (v/v) glycerol and 0.01% bromophenol blue. Isoelectric focusing was performed with pH 3–10 precast gels (Novex) as specified by the manufacturer. Staining was by silver nitrate.

**Peptide Analyses**—Amino acid analysis of rFas2 (5 nmol), previously hydrolyzed with 6 M HCl and 1% (w/v) phenol for 20 h in vacuo, was carried out on a CBS Auto Analyzer. Automated Edman analysis of rFas2 (250 pmol) was conducted on a CBS sequenator. Equivalent samples of venom-derived Fas2 were analyzed in parallel.

**Liquid-phase Radioimmunoassay (RIA)**—Lactoperoxidase-catalyzed radiiodination of Fas2 to a specific radioactivity of ~1200 Ci mmol⁻¹ has been detailed elsewhere (20). The titer of the anti-Fas2 serum, defined as the serum dilution which binds 50% of the 125I-Fas2 added to the immunoassay, was determined as a 315,000-fold dilution. A standard RIA, 125I-Fas2 (10,000 cpm) was incubated at 37 °C for 90 min with the anti-Fas2 serum at its titer in 200 μl of 50 mM NaH₂PO₄/Na₂HPO₄, pH 7.5, 0.1 mM NaCl, 1% BSA, and then the samples were diluted with buffer to 500 μl and incubated overnight at 4 °C. Double immunoprecipitation of the antigen-antibody complexes was performed at 4 °C with successive additions of normal rabbit serum (50 μl of a 1/50 dilution), goat anti-rabbit serum (50 μl of a 1/20 dilution), and polyethylene glycol (400 μl of a 20% (w/v) solution). Samples were centrifuged (10,000 × g, 25 min, 4 °C), and the radioactivity of the pellets was determined. Titration of the unlabeled rFas2 and Fas2 mutants by RIA was based on competition with 125I-Fas2 for complexation with anti-Fas2 serum and comparison to a Fas2 standard curve.

**Assay for AChE Inhibition**—Relative AChE activities (40) were recorded at room temperature by microtitration on a Vmax kinetic microplate reader (Molecular Devices Corp.) at 405 nm, in the presence of 0.5 mM acetylthiocholine iodide and 0.3 mM 5,5'-dithiobis-(2-nitrobenzoic acid), in 50 mM NaPO₄ buffer, pH 8.0, 0.1 mM mg⁻¹ BSA (20). The activities of purified rFas2 and of the Fas2 mutants were monitored by inhibition of 5–10 μM AChE after a 1-h incubation at 37 °C and 14–18 h at room temperature.

**Characterization of the Mutants**—Culture media, 72 h after transfection, were screened for immunoreactivity and inhibitory activity directly or after 10–20-fold concentration by ultrafiltration. The Fas2 mutants were titrated by RIA, and their inhibitory activities were quantitated by AChE inhibition assay after partial purification and concentration by cation exchange of the media (100–200 ml) and ultrafiltration of the chromatographic fractions. The Fas2 mutants were submitted to at least two independent transfection-chromatography-assay sequences.
Synthesis of cDNA encoding rFas2. The nucleotide sequence was designed from frequent mammalian codon usage and flanked on the 5’, 3’ ends by the signal peptide of erabutoxin a (36). Positive numbering of the encoding amino acid sequence starts at the first residue of the processed rFas2. Initiation and termination codons are underlined, and relevant restriction sites are indicated.

RESULTS

Expression of rFas2 in CHO Cells and Purification—Synthesis of the oligonucleotides encoding rFas2 enabled us to use codons of high usage frequency for mammalian cells and place restriction sites at convenient locations within the open reading frame and flanking regions. To ensure secretion and processing, a sequence encoding the leader peptide of a structurally related three-fingered toxin, erabutoxin a (36), was joined at the 5’ end (Fig. 1). A Scal site was generated at the linkage between the two sequences by replacing Thr at position 41 by Ser.

Selected clonal cells at confluence secreted up to 1.7 μg ml⁻¹ (0.25 μM) of rFas2 in the 3-day intervals between medium changes; this rate continued for up to 10 weeks. Purification employed three steps on 500-mL batches of media (Fig. 2). Initial cation exchange led to removal of most medium proteins with a 440-fold enrichment (in pmol of Fas2 per mg of total protein) in rFas2, with more than 90% of recovery (Fig. 2A). A gel-filtration step purified rFas2 1500-fold (Fig. 2B). Final removal of trace contaminants was achieved by a second cation-exchange chromatography performed in isocratic conditions, yielding purification of rFas2 from trace contaminants was achieved by cation exchange under isotropic conditions, which accounts for the skewed peak.

Physical and Biological Characterization of rFas2—SDS-PAGE in reducing conditions (Fig. 3, inset) and isoelectric focusing analysis (not shown) each revealed a single, sharp band that migrated at a position identical to the venom-purified Fas2. Parallel runs on the amino acid analyzer yielded identical compositions for rFas2 and Fas2. Amino-terminal sequencing of native rFas2 yielded a single sequence, TMXY-SHTTTS, identical to that of Fas2, with a blank third step (noted as X) for non-reduced Cys. UV absorbance of natural Fas2 was used to estimate concentration of rFas2 stocks. Radioimmuno- and AChE-inhibition assays showed equivalent potencies for rFas2 and venom-derived Fas2 for inhibiting mouse AChE and competing with 125I-Fas2 for binding to a polyclonal anti-Fas2 serum (Fig. 3 and Table I). Hence fully processed and correctly folded rFas2 is directly produced in mammalian cells.

Mutant Fasciculins

Selection of Positions for Mutation of Fas2—The overall net positive charge (+4) at neutral pH of Fas2, coupled with its association at a peripheral anionic site on AChE, suggested an electrostatic attractive component with the cationic amino acids on Fas2 (Fig. 4). Cationic positions unique to the fasciculins were considered first. Hence, Arg¹¹, Arg²⁴, Lys²⁵, Arg²⁷, Arg²⁸, Lys³², and Lys⁵¹ were mutated, but Arg³⁷ and Lys⁵⁸, being
Loop III mutations

Residues at the binding interface of the Fas2-AChE complex were expressed relative to the monomer with 5 or 10 pM AChE, were calculated from ratios of dilutions for 50% inhibition and competition in the inhibition and immunomunoassay titrations, respectively (cf. Fig. 6); the values were normalized to ratios with the venom-derived Fas2. Apparent \( K_i \) values are expressed relative to the \( K_i \) value of Fas2 for mouse AChE (2.5 pm) (21).

Data are from the major peak when more than a single peak appeared on the cation-exchange resin (cf. Fig. 5); \( n \), number of independent experiments.

### Table I

| Fasciculin | Relative inhibitory activity (normalized to Fas2) | Apparent \( K_i^a \) |
|------------|-----------------------------------------------|------------------|
| Wild-type  |                                              |                  |
| rFas2 (purified) | 1.00 ± 0.05 (n = 4) | 2.3 ± 0.1        |
| rFas2 (transient) | 0.98 ± 0.026 (n = 5) | 2.3 ± 0.6       |
| Loop I mutations |                  |                  |
| TS\&TA9 | 18 ± 13 (n = 3) | ≤2.3           |
| R11Q | 6.3 ± 3.1 (n = 2) | ≤2.3           |
| Loop II mutations |            |                  |
| R24T | 0.080 ± 0.003 (n = 2) | ≤21          |
| R25L | 1.08 ± 0.06 (n = 5) | 2.1 ± 0.1      |
| R27W | 0.021 ± 0.017 (n = 3) | 112 ± 91      |
| R28D | 1.10 ± 0.05 (n = 3) | 2.1 ± 0.9      |
| R29D | 7.3 ± 7.1 (n = 3) | ≤2.3           |
| ΔP50 | 0.0052 ± 0.0065 (n = 4) | 440 ± 550     |
| P31R | 0.0016 ± 0.0002 (n = 2) | 1440 ± 180   |
| K32G | 0.32 ± 0.12 (n = 3) | 7.2 ± 2.7      |
| M33A | 0.13 ± 0.05 (n = 2) | 17.7 ± 6.8    |
| V34A/L35A | 0.93 ± 0.60 (n = 2) | 2.5 ± 1.6     |
| Loop III mutations |          |                  |
| D45K | 0.7 ± 0.1 (n = 3) | ≥3.3 ± 0.5    |
| K51S | 1.08 ± 0.01 (n = 2) | 2.1 ± 0.02    |

\( a \) Values are shown as average ± the variation (n = 2) and means ± S.D. (n > 2).

### Transient Expression of Fas2 Mutants in HEK Cells—Expression from transiently transfected cells enabled detailed analysis of activity and/or peptide production in the cell culture media.

Analysis of AChE inhibition and RIA titers allowed an initial classification of the Fas2 mutants. Class I comprised the TS\&TA9, R25L, R28D, H29D, D45K, V34A/L35A, and K51S mutants, which displayed significant AChE inhibition and immunoreactivity. Class II comprised the R24T, R27W, ΔP30, P31R, K32G, and M33A mutants, which displayed significant immunoreactivity but less AChE inhibition. Class III comprised only the R11Q mutant, which displayed AChE inhibition but in complete curves immunoreactivity curves. Production yields, determined by RIA titration of rFas2 and the Fas2 mutants secreted into the media, ranged between 0.5 and 5 pmol ml\(^{-1}\) in a 3-day period.

### Chromatography of the Fas2 Mutants—Elution positions of the Fas2 mutants on the cation-exchange resin were monitored by RIA and AChE inhibition assay for the class I and class II mutants. Representative profiles are shown in Fig. 5. Compared to rFas2 (top panels), 10 of the 14 mutants showed elution positions consistent with their net charge. Mutants TS\&TA9, ΔP30, M33A, and V34A/L35A, all possessing the same net charge as rFas2, eluted at the same ionic strength as rFas2. Mutants K25L, R27W, K32G, and K51S, which lost one net positive charge, eluted at lower ionic strength than rFas2. Mutants P31R and D45K, which gained one and two net positive charges(s), eluted at higher ionic strengths than rFas2. In contrast, mutants R11Q, R24T, R28D, and H29D, which lost either one or two positive charge(s), eluted at a similar ionic strength as rFas2, suggesting that the initial and newly introduced side chains at positions 11, 24, 28, and 29 are not solvent-accessible and/or do not interact with the anionic matrix of the column. Two mutants, R24T and R27W, eluted as several peaks, but calculation of concentrations based on a simple reversible equilibrium for ligand binding revealed that the additional peaks are minor components.

Whether these peaks reflect incomplete processing or folding of the peptides, or slow conformational equilibria, has not been ascertained.

### Analysis of the Apparent Dissociation Constants (\( K_f \)) of the Fas2 Mutants—The Fas2 mutants, enriched in purity and concentrated by cationic exchange and ultrafiltration, were titrated by RIA and analyzed for inhibitory activity (Fig. 6). The RIA profiles were generally consistent with the amounts of peptide expected based on transfection efficiency and purification yields. In contrast, the concentration dependences of the AChE inhibition curves, relative to those of immunoreactivity, varied widely, illustrating significant differences in the affinities of the Fas2 mutants for AChE. The transiently expressed rFas2 sample, enriched and concentrated, yielded the same AChE inhibition potency as the stably expressed and fully purified rFas2 (cf. Fig. 3). Compared to rFas2, no significant differences in inhibition potency were found for mutants K25L, R28D, K32G, V34A/L35A, D45K, and K51S. In contrast, significant differences were found for mutants TS\&TA9, R11Q, R24T, R27W, H29D, ΔP30, P31R, and M33A. The inhibitory activities of the Fas2 mutants toward mouse AChE, calculated from the dilutions for 50% inhibition in the inhibition assays and 50% competition in the RIA titrations, and normalized to Fas2, are reported in Table I. The apparent \( K_f \) values that are also reported are expressed relative to a \( K_f \) value of 2.3 pm for Fas2 (21). Because of the limitations that may arise from slow equilibration, from the disparity between added and free ligand, and from using immunoreactivity to reflect peptide concentration, the mutants with low dissociation constants are specified only by an upper limit.
Based on the relative inhibitory activities of the rFas2 mutants (Table I), the mutations can be classified in four categories: (i) those which cause little or no apparent effect on Fas2 activity, as seen for mutants K25L, R28D, V34A/L35A, D45K, K51S (no change), or K32G (3-fold decrease), (ii) those which cause a decrease by about one order of magnitude in the Fas2 activity, as seen for mutants R24T (13-fold) and M33A (8-fold), (iii) those which cause a decrease by two or more orders of magnitude, as seen for mutants R27W (49-fold), DP30 (192-fold), and P31R (625-fold), and (iv) those which cause an apparent increase in the Fas2 inhibitory activity, as seen for mutants T8A/T9A, R11Q, and H29D (18-, 6-, and 73-fold, respectively).

The contribution of the mutated residues to activity, relative to their location in the three-dimensional structure of Fas2 bound to mouse AChE (30), is shown in Fig. 7. The three residues that dominate the AChE-inhibitory activity of Fas2, Arg27, Pro30, and Pro31, form a subset located at the tip of loop II. The surface area of this subset, 261 Å², represents only 6% of the total accessible surface area of Fas2 but 25% of the Fas2 area buried in the Fas2-mAChE complex. Fas2 residues Thr8, Thr9, and Arg11, mutation of which resulted in an apparent increase in the Fas2 inhibitory activity, form a second subset located at the tip of loop I. The surface area of this subset, 300 Å², represents 7% of the total accessible surface area of Fas2 but 29% of the Fas2 area buried in the complex. Hence, a small fraction of the many Fas2 residues which make up the overall functional site provide the critical contacts required for the high affinity interaction.

DISCUSSION

Several groups have produced recombinant three-fingered snake toxins, α- and κ-neurotoxins, as fusion proteins in heterologous bacterial expression systems, and some have reported site mutagenesis studies on these toxins (43, 44, 46–52). The lower activity of the fusion proteins, compared to the natural toxins, however, precludes direct analysis of the mutants before in vitro cleavage of the hybrid by chemical or enzymatic means. More recently, a recombinant κ-neurotoxin was produced in yeast with full activity, although possessing a short amino-terminal extension due to altered signal peptide cleavage (53). In our study, we used a mammalian system to express secreted and fully processed rFas2 and 14 Fas2 mutants. Direct expression of rFas2 in its functional conformation demonstrates that the use of the signal peptide sequence of erabutoxin A and the change at position 21 were not critical for post-translational events such as folding, cleavage of signal peptide, and disulfide bond formation of the rFas2 molecule in mammalian cells.

Owing to the use of complementary assays, RIA titration and AChE inhibition, 14 transiently expressed Fas2 mutants could be quantitated and functionally characterized without extensive purification. The polyclonal anti-Fas2 serum is characterized by a high titer in antibodies, likely to be mostly IgGs (35). The shapes of the RIA curves, that generally extend over a concentration range wider than expected for interaction of 125I-Fas2 with a single site (Figs. 3 and 6), suggest that the serum contains at least two IgG populations presumably directed...
toward distinct epitopes on Fas2. The existence of several antigenic determinants on the fasciculin molecule is in accord with previous reports of three to four distinct epitopes simultaneously accessible to specific antibodies (Fab fragments) directed against α-neurotoxins and of two monoclonal antibodies directed toward distinct areas of a cardiotoxin (cf. Ref. 54). Because of the heterogeneity of polyclonal antisera, as well as the multiplicity of residues involved in the contact area between each IgG and its specific epitope, single residue modifications of the three-fingered toxins usually have little influence on polyclonal antibody titers. Antigenic diversity proved to be generally useful for quantifying the loss in the inhibitory activity of the Fas2 mutants based on immunotitration of enriched samples.

Mutants T8A/T9A, R11Q, and H29D, however, displayed greater ratios of inhibitory activity to immunochemical titer than did wild-type Fas2. While this may reflect enhanced inhibitory affinity for AChE, several caveats preclude a precise determination of a dissociation constant. First, with a fasciculin dissociation rate constant of $4.0 \times 10^{-3}$ min$^{-1}$ (21), extended equilibration times are required for fractional AChE occupation. Excessive dilution of AChE to satisfy the condition of $K_i$ to total enzyme concentration ratio that assures a reasonable fraction of free ligand (55, 56) would have required unrealistic equilibration times for the putative high affinity...
Arg24, Lys25, and all residues from Arg27 through Leu35, were Pro30 not only removes the cyclic side chain, but also alters Gly342, and Ile365 is likely to contain bound water molecules in both the length of loop II and the \( \text{cis} \) conformation of its tip. As a result, the cavity formed by mAChE residues Glu292, Ser293, and Tyr341, as well as Coulombic repulsion with Fas2-Lys32 and/or mAChE-Arg296 (30). To resolve these possible interactions, residue substitutions at positions 27, 30, and 31 with progressive changes in side chain properties may help delineate the respective contributions of the charge, volume, and stereochemical characteristics of these three residues to conformation and stability of the complex.

Both the substitutions of Thr for Arg24 and Ala for Met33 resulted in an order of magnitude decrease in Fas2 activity (Table I). Arg24, located at the base of loop II (Fig. 7), is a structurally important residue that stabilizes the carboxyl-terminal and core regions of Fas2 through hydrogen bonding to the carbonyl atom of Tyr61 and stacking interaction with Arg37 (30, 41). None of these interactions should be retained by the shorter, uncharged Thr side chain. Thus, a less stable complex forms with mAChE because of indirect destabilization of the mutated peptide molecule. The Met33 \( \rightarrow \) Ala mutation should have a more direct influence. The loss of the sulfur atom and shortening of the side chain preclude stacking interactions with Trp286 and van der Waals contacts with Tyr341, two key mAChE residues located at the peripheral anionic site (21, 58, 59). In addition, the smaller Ala side chain cannot retain secondary hydrophobic interactions with mAChE residues Tyr72, Tyr124, and Tyr341 (30). In the Fas2-mAChE complex, most of the solvent-accessible surface area of Met33 is lost; in fact, this residue contributes about 10% of the Fas2 surface area buried at the interface (30), a value slightly greater than the individual contributions of neighboring critical Fas2 residues Arg27, Pro30, and Pro31. On the other hand, of the several peripheral anionic site residues that interact with Met33, a single one, Tyr124, does not establish any other contact with Fas2 (30). A decrease in apparent affinity for Fas2 of two orders of magnitude was observed for mAChE mutant Y124Q (21). More than a single order of magnitude in the decrease in Fas2 activity could therefore have been expected upon mutation of Met33; however, the minimal change in the side chain volume with an Ala substitution likely preserves the tight fit of the other loop II residues with the peripheral anionic site.

Substitutions of Leu for Lys35, of Asp for Arg28, and of an Ala doublet for Val34-Leu35, resulted in unaltered Fas2 activity (Table I). These results are in accord with the crystallographic observations that show that the Lys35 \( \rightarrow \) Arg28 side chains point away from the Fas2-mAChE interface, and that cavities exist between the van der Waals surfaces defined by the side chains of Fas2 residues Val34 and Leu35 and mAChE residues Leu76, Tyr72, and His297, respectively (30). Replacement of Arg28 by Asp probably induces structural rearrangements leading to a different network of internal charge compensation; surprisingly, this does not perturb the activity of the mutant toward the enzyme.

Substitution of Asp for His29 resulted in an apparent increase of two orders of magnitude in the Fas2 activity (Figs. 6 and 7 and Table I). In the crystalline complex, the side chain of His29 participates in van der Waals interactions with mAChE-Glu292 that the newly introduced Asp side chain, negatively charged, cannot establish because of charge repulsion (30). However, the Asp side chain may be sufficiently flexible to rotate toward the side chain of either Arg27 or Arg28 and thereby participate in van der Waals interactions with mAChE-Glu292.

FIG. 7. Positions of the functionally important residues of Fas2. The three-dimensional structure of Fas2 complexed with mouse AChE (30) is viewed looking toward the concave side of the molecule; the molecular surface of mAChE buried in the complex is displayed (dote). The side chains of the Fas2 residues whose mutation causes virtually no effect on Fas2 activity are displayed as light gray sticks (K25L, R28D, K32G, V34A/L35A, D45K, and K51S). Those whose mutation causes a decrease by about one order of magnitude in Fas2 activity are displayed as dark gray sticks (R24T and M33A). Those whose mutation causes a decrease by two or more orders of magnitude in Fas2 activity are displayed as dark gray CPK (R27W, ΔP30, and P31R). Those whose mutation causes an apparent increase in Fas2 activity are displayed as light gray CPK (TSA79A, R11Q, and H29D). The two subsets of functionally important residues (CPK mode) reside at the tips of loop I and loop II, respectively. N and C denote the amino and carboxyl termini of the Fas2 molecule, respectively. This figure was generated with program TURBO-FRODO (70).
undergo charge compensation. Also, an internal salt bridge could form with Arg9. Thus, an improved fit of the more rigid tip of loop II with the mAChE peripheral anionic site residues might result from stabilization of internal structure. However, as discussed below, a change in structure might also affect the immunoreactivity of this mutant.

**Mutations in Loop I**—Loop I of Fas2 encompasses 13 residues, located between Cys9 and Cys17 (Fig. 4). Both the mutations made on Thr8-Thr9 and Arg11 lead to apparent increases of one order of magnitude in the activities of the mutants toward mAChE (Fig. 6 and Table I). The Thr8-Thr9-Arg11 subset is fully exposed at the tip and external edge of loop I (Fig. 7), which fits in a crevice near the tip of the mAChE catalytic gorge and maximizes the surface area of contact of loop II at the gorge entry (30). In particular, the Thr8-Thr9 doublet potentially interacts with eight mAChE residues, Tyr70, Glu71, Tyr72, Val73, Leu92, Glu270, Val282, and Asp283 (30). Substitution of an Ala doublet eliminates hydrogen bonding of the Thr side chains with Glu270 and Asp283. The smaller size of the two Ala side chains might facilitate a rearrangement of the backbone at the tip of loop I, enabling it to come closer to loop II and establish a stabilizing internal interaction with Fas2-Leu92. In addition, the smaller side chain of Ala9 may establish more favorable interactions with mAChE residues Tyr70, Leu92, and Val282 and provide a tighter fit of the tip of loop I with the furrow that exists on the mAChE surface (30), possibly compensating for the loss of hydrogen bonding energy. On the other hand, Arg11 hydrogen bonds with mAChE-residues Glu84 and Asn87. Substitution of Gln for Arg11 likely preserves the capacity for hydrogen bonding, especially as the smaller size of the Gln side chain should favor a tighter fit between the tip of loop I and the enzyme. Internal stabilization of the Fas2 molecule and/or loop I anchorage at the enzyme surface could thus contribute to tighter occlusion, by the tip of loop II, of the catalytic site gorge of the enzyme. Incidentally, the apparent increase in Fas2 activity that results from the removal of the positive charge does not match with the 40–73% loss in the activity of Fas2 chemically modified at Arg11 (57). The modifying agent, however, produces a substantial enlargement in the effective side chain that is not reproduced by the Gln mutation.

Although infrequent, single residue substitutions have shown enhanced affinity with the three-fingered peptidic toxins. The 35-fold higher affinity of Fas3, compared to Fas1, for rat brain AChE likely results from the Thr15 → Lys substitution in loop I (20). Substitution of Arg for Ile36 in erabutoxin a loop II led to a 7-fold increase in this neurotoxin’s affinity for the nicotinic receptor (44). Two mutations of Bungarus AChE, near the presumed peripheral site, resulted in up to a 14-fold increase in affinity for Fas2 (22). Nevertheless, the uncertainties in the relative activities of the Fas2 mutants TSA/T9A, R11Q, and H29D preclude accurate determinations of specific activity without the availability of homogeneous peptides.

In several three-fingered toxins, the edge of the first loop and the tip and concave side of the second loop are domains immuno-reactive to monoclonal antibodies. In Fas2, the bulky side chains of Arg11 and His29 could be particularly immunogenic, as was found for Tyr1 of a cardiotoxin, toxin γ, and Lys2 of a neurotoxin, toxin α (54). Thus, apart from the structural arguments that may explain increased inhibitory activity, the Arg11 → Gln and His29 → Asp mutations may also have altered dominant epitopes located on loop I and loop II. Hence, a diminished, although not eliminated, recognition of the mutant by the anti-Fas2 serum (Fig. 6) may result in an overestimation of their relative inhibitory activities toward AChE. Similar considerations could influence our results for the double mutant TSA/T9A.

Despite the unique conservation of Arg11 in the fasciculins, the cationic side chain at this position does not appear to be a major contributor to the affinity nor to the inhibitory capacity of Fas2. In the crystalline complex, Arg11 associates with the region of Trp286, proposed as a second portal, or “back door,” for substrate entry into the enzyme (60, 61). Should translation of the choline cation through the putative back door region be rate-limiting in catalysis in the Fas2-AChE complex, a greater influence of Arg11 on AChE inhibition by Fas2 would have been expected. The side chain of Arg11, however, displays high temperature factors (B factors) not only in the structures of unbound Fas1 and Fas2 (41, 62), but also in those of Fas2 bound to mAChE and Torpedo AChE (30, 31). In general, the highly flexible loop I may adopt an ensemble of conformations (41, 62); yet, a single conformation is selected in the formation of the high affinity complex (30, 31).

**Mutations in Loop III**—Loop III of Fas2 encompasses 10 residues, located between Cys41 and Cys52 (Fig. 4). The unaltered Fas2 activities of the D45K and K51S mutants (Figs. 6 and 7 and Table I) are consistent with the lack of interaction of residues Asp45 and Lys51 with mAChE, as observed in the crystalline complex. Yet, in the fasciculins, compared to the other members of the three-fingered toxin family, the Asp45, Arg46 doublet confers to loop III an exclusive anionic locus which dictates the orientation of the dipole vector of the molecule (18, 42). An internal structural rearrangement in the D45K mutant molecule may occur, since the Lys side chain introduced at position 45 should repel Arg46, which stabilizes Asp45 in Fas2 (41, 42). In the mutant, however, it may be compensated by the neighboring side chain of Asp46.

Changes in the Fas2 structure have been proposed earlier to explain the 57% decrease in Fas2 activity upon acetylation of Lys51 (63). Two residues of loop III, Asn47 and Leu48, interact with mAChE-His287, but at distances of ~4 Å (30); however, Leu48 does not interact with Torpedo AChE (31). Loop III residues, therefore, likely play no critical functional role other than internal stabilization of loop II conformation.

Numerous aromatic residues reside at the Fas2-mAChE complex interface: Fas2-Tyr4, His6, His29, and Tyr61 and mAChE-Tyr70, Tyr72, Tyr77, Tyr124, Trp286, His287, Tyr341, as well as several nonaromatic residues which undergo hydrophobic interactions: Fas2-Pro30, Pro31, and Met33 and mAChE-Pro78 (30). Mutation of Fas2-Pro30 and Pro31, which are central to the interface, resulted in two to three orders of magnitude decreases in Fas2 activity. Single-site substitutions of mouse AChE peripheral anionic site residues Tyr72, Tyr124, and Trp286, also central to the interface, reduced the affinity of Fas2 by two to six orders of magnitude (27). Thus, patches of high hydrophobicity at the interface dominate the binding free energy of the Fas2-AChE complex, consistent not only with the tight intermolecular packing areas observed in the crystal structures (30, 31), but also with general structural patterns observed at interfaces of high affinity protein-protein complexes (64–69).

Site-directed mutagenesis of the Fas2 molecule and expression of the mutants from transiently transfected mammalian cells have provided substantial information on the respective contributions of 16 residues of Fas2 positioned for interaction with mouse AChE. The determinants identified by the structural and the functional approaches do coincide, but only a few of the many residues which make up the overall interactive site of the Fas2 molecule provide the strong interactions required for high affinity binding. Mutant cycle analyses, coupled with kinetic and crystallographic studies, should further delineate the contribution of individual residues to the affinity and inhibitory capacity of the fasciculin-AChE complexes.
Acknowledgments—We are grateful to Drs. Tyler White, John Lewicki, Barbara Cordell, and Andy Lin (Scios Nova, Inc.) for the gift of the pGEX vector, to Dr. Daniel Donoghue (UCSD) for synthesis of the 130-base pair oligonucleotides used in construction of the expression plasmid, to Siv Garod (UCSD) for amino-terminal sequencing of the toxins, and fruitful discussions. Assistance from Kael Duprey and Jonathan Eads (UCSD) in inhibition and radioimmunoassays and from Maryse Avitre (CNRS) in rabbit serum production is much appreciated.

REFERENCES

1. Lee, C. Y. & Chang, C. C. (1966) Mem. Inst. Butantan (Sao Paulo) 32, 555–572
2. Changeux, J. P., Kassai, M. & Lee, C. Y. (1970) Proc. Natl. Acad. Sci. U. S. A. 67, 1241–1247
3. Endo, T. & Tamiya, N. (1991) in Snake Toxins (Harvey, A. L., ed) pp. 165–222, Pergamon Press, New York
4. Chiapinelli, V. A. (1983) Brain Res. 277, 9–22
5. Oswald, R. E., Sutcliffe, M. J., Bamberger, M., Loring, R. H., Braswell, E. & Taborsky, G. (1993) Biochim. Biophys. Acta 11233–11239
6. Sutcliffe, M. J., Jaseja, M., Hyde, E. I., Lu, X. & Williams, J. A. (1994) J. Biol. Chem. 269, 909–916
7. Wright, C. S. (1987) in Snake Toxins (Harvey, A. L., ed) pp. 245–447, Pergamon Press, New York
8. McDowell, R. S., Dennis, M. S., Louie, A., Shuster, M., Mulkerrin, M. G. & Sutcliffe, M. J. (1994) Biochemistry 33, 1248–1250
9. Adem, A., Aslab, S., Johansson, G., Mbugua, P. M. & Karlsson, E. (1988) Biochim. Biophys. Acta 968, 340–345
10. Vellom, D. C., Radic, Z., Pickering, N. A., Camp, S. & Taylor, P. (1993) J. Biol. Chem. 263, 12074–12084
11. Sutcliffe, M. J., Jaseja, M., Hyde, E. I., Lu, X. & Williams, J. A. (1994) Structure 2, 226–232
12. Kr('\i)mmler, K., Roberts, J. D. & Zaks, B. A. (1987) Methods Enzymol. 154, 367–382
13. Lefkowitz, R. J., Kuhn, M. T., Barad, D. & Ariel, N. (1992) J. Biol. Chem. 267, 11233–11239
14. Sutcliffe, M. J., Jaseja, M., Hyde, E. I., Lu, X. & Williams, J. A. (1994) Structure 2, 226–232
15. Radic, Z., Pickering, N. A., Vellom, D. C., Camp, S. & Taylor, P. (1993) J. Biol. Chem. 268, 2437–2440
16. Harel, M., Kleywegt, G. J., Ravelli, R. B. G., Silman, I. & Sussman, J. L. (1995) Protein Sci. 4, 703–715
17. Padlan, E. A. (1990) in Protein Eng. 3, 139–143
18. Sutcliffe, M. J., Jaseja, M., Hyde, E. I., Lu, X. & Williams, J. A. (1994) Structure 2, 226–232
19. Rosenthal, J. A., Hsu, S. H., Schneider, D., Gentile, L. N., Messier, N. J., Vaslet, C. A. & Hawrot, E. (1994) J. Biol. Chem. 269, 11178–11185
20. Lu, X., Rahman, S., Kakkar, V. V. & Audhi, S. L. (1996) J. Biol. Chem. 271, 289–294
21. Sussman, J. L., Sussman, L. I. & Mezes, A. (1991) in Snake Toxins (Harvey, A. L., ed) pp. 35–90, Pergamon Press, New York
22. Padlan, E. A. (1990) in Protein Eng. 3, 213–224
23. Mezes, A. (1991) in Snake Toxins (Harvey, A. L., ed) pp. 35–90, Pergamon Press, New York
24. Sussman, J. L., Sussman, L. I. & Mezes, A. (1991) in Snake Toxins (Harvey, A. L., ed) pp. 35–90, Pergamon Press, New York
25. Sussman, J. L., Sussman, L. I. & Mezes, A. (1991) in Snake Toxins (Harvey, A. L., ed) pp. 35–90, Pergamon Press, New York