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# Binding of $^{125}$I-Fasciculin to Rat Brain Acetylcholinesterase

## THE COMPLEX STILL BINDS DIISOPROPYL FLUOROPHOSPHATE*

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**Iodination of fasciculin 3 (FAS3) from *Dendroaspis viridis* venom provided us with a fully active specific probe of fasciculin binding sites on rat brain acetylcholinesterase (AChE). Binding and inhibition are concomitant, as association and inhibition rate constants $k_1$ and $k_i$ are identical. The $^{125}$I-FAS3·AChE complex dissociates very slowly ($t_{1/2}$ = 48 h) and is characterized by a dissociation constant, $K_d$, of 0.4 pM. All the specific binding of $^{125}$I-FAS3 to AChE is prevented by FAS3 as well as by the two other fasciculins, FAS1 and FAS2, from *D. angusticeps* venom ($K_d$ = 0.4, 14, and 25 pM, respectively). It is also prevented by propidium iodide, BW284C51, and *d*-tubocurarine, which bind to peripheral anionic sites of AChE, by $Ca^{2+}$ and $Mg^{2+}$, known to enhance AChE activity through an allosteric phenomenon and by acetylthiocholine concentrations which lead to excess substrate inhibition of the enzyme. Diisopropyl fluorophosphate and paraoxon, which inhibit AChE by phosphorylating the catalytic serine, have no effect on either the binding rate or the number of binding sites of $^{125}$I-FAS3. *O*-Ethyl-*S*$^2$-diisopropylaminoethyl methylphosphonothionate, however, which binds irreversibly to the AChE catalytic site but reversibly to a peripheral site, induces a 130% increase in the binding rate of $^{125}$I-FAS3, without changing the total number of $^{125}$I-FAS3 binding sites. Our results demonstrate that fasciculins bind on a peripheral site of AChE, distinct from the catalytic site and, at least partly, common with the sites on which some cationic inhibitors and the substrate in excess bind. Since phosphorylation of the catalytic serine (esteratic subsite) by [1,3-$^3$H]diisopropyl fluorophosphate can still occur on the FAS3·AChE complex, the structural modification induced by fasciculins may affect the anionic subsite of AChE catalytic site.**

Acetylcholinesterase (AChE)[1] of nerve and muscle tissues is a polymorphic glycoprotein whose catalytic properties have been extensively studied (for a review see Rosenberry, 1975; Massoulié and Bon, 1982; Quinn, 1987). The crystal structure of *Torpedo californica* AChE, which has recently been solved (Sussman *et al.*, 1991), provides new evidence to explain the high catalytic efficiency of AChE, as well as the existence of multiple AChE inhibitors with multiple inhibition pathways. The AChE catalytic site was already known to be composed of an esteratic and an anionic subsite (or acetyl- and choline-binding sites, respectively). The site is now revealed to lie at the bottom of a deep and narrow gorge, which is lined by a high number of aromatic residues probably involved in a guidance mechanism facilitating two-dimensional diffusion of ACh from the gorge entrance to the buried active site (Sussman *et al.*, 1991). Additional binding sites for cationic ligands, remote from the active center, have been evidenced in biochemical studies and proposed to act as allosteric sites (Changeux, 1966; Taylor and Lappi, 1975; Epstein *et al.*, 1979). These peripheral anionic sites, which are now thought to be located at the rim area surrounding the gorge, would induce their long-range effects on active center reactivity through a relay mechanism along the aromatic surface of the gorge (Shafferman *et al.*, 1992).

A large number of organic compounds reversibly or irreversibly inhibit AChE (Long, 1963), which bind either to the esteratic or the anionic subsite of AChE catalytic site or to the peripheral site of the enzyme. Most of them are synthetic substances, sometimes bearing insecticidal properties (Murphy *et al.*, 1984). Few natural inhibitors of AChE are known and, to date, fasciculins are the only known proteinic AChE inhibitors. They are components of mamba snake venoms and have been shown to display a powerful inhibitory activity toward mammalian AChE. Up to now, three iso-fasciculins have been characterized: fasciculin 1 (FAS1) and fasciculin 2 (FAS2) from the venom of *Dendroaspis angusticeps* (green mamba) (Rodriguez-Ithurralde *et al.*, 1983), and toxin C from the venom of *Dendroaspis polylepis* (black mamba) (Joubert and Taljaard, 1978). The pharmacological and biochemical activities of FAS2 have been studied both *in vivo* and *in vitro* on various AChE-containing tissues (Karlsson *et al.*, 1984; Lin *et al.*, 1987). FAS2 inhibits several (but not all) AChEs from different sources with $K_i$ values of about 0.1 nM and has been shown to displace propidium, which is known to bind specifically to a peripheral anionic site of AChE (for a review see Harvey *et al.*, 1984; Cervenansky *et al.* 1991a).

In this study, we isolated from a *Dendroaspis viridis* venom a fasciculin (FAS3) which was found identical to toxin C from *D. polylepis* venom. To further define the relationships between binding site occupancy by fasciculins and rat brain AChE inhibition, we iodinated FAS3 and examined the $^{125}$I-FAS3 binding properties. With respect to other AChE inhibitors, $^{125}$I-FAS3 proved to be a new highly specific probe of

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[1] The abbreviations used are: ACh, acetylcholine; AChE, acetylcholinesterase (EC 3.1.1.7); ATCh, acetylthiocholine; BSA, bovine serum albumin; BW284C51, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-1-dibromide; DFP, diisopropyl fluorophosphate; DTNB, dithiobis-2-nitrobenzoic acid; HPLC, high-performance liquid chromatography; MPT, *O*-ethyl-*S*[2-(diisopropylamino)ethyl] methylphosphonothionate; paraoxon, diethyl *p*-nitrophenyl phosphate.

AChE peripheral site. As AChE inhibitors could be used as therapeutic agents against some cholinergic deficiencies, the detailed knowledge of the molecular mechanism of action of proteinic and peripheral-specific AChE ligands such as fasciculins is of particular interest.

## MATERIALS AND METHODS

*Venom and Reagents*—Crystallized *D. viridis* venom, batch 1963 no. 3, was from the Institut Pasteur (Paris, France). Sephadex G50 superfine was from Pharmacia (Uppsala, Sweden), and CM52 was from Whatman Biosystems (Maidstone (Kent), United Kingdom). The following items: HPLC-grade formic acid, TLC plastic sheets of precoated silica gel 60, analytical-grade chemicals and reagents were from Merck (Darmstadt, Federal Republic of Germany). Micropolyacrylamide A1700 TLC foils were from Schleicher and Schuell (Dassel, Federal Republic of Germany). Fluorescamine was from Hoffmann Laroche (Basel, Switzerland). HPLC-grade ammonium acetate was from Baker (Deventer, Holland), and HPLC-grade acetonitrile was from Touzard et Matignon (Vitry-sur-Seine, France). Enzymes were from the following sources: endoproteinase Arg-C, Boehringer (Mannheim, Federal Republic of Germany); *Staphylococcus aureus* $V_8$, Miles Laboratories (Elkhart, IN). Lactoperoxidase, carboxypeptidase A, BSA fraction IV, DTNB, propidium iodide, paraoxon, BW284C51, *d*-tubocurarine, and DFP were from Sigma. ATCh was from Serva (Heideberg, Federal Republic of Germany), and $CaCl_2$ and $MgCl_2$ were from Merck. MPT was a gift from Dr. J. Massoulié (Ecole Normale Supérieure, Paris, France). $^{125}$I (2100 Ci mmol$^{-1}$) was from Amersham Corp. (Amersham, United Kingdom) and [1,3-$^3$H] DFP (10 Ci mmol$^{-1}$) from New England Nuclear (Du Pont de Nemours, Les Ulis, France). Scintillation solution Pico-Fluor™15 was from Packard (Downers Grove, IL). Water was issued from a Millipore MilliRO/MilliQ system (Millipore, Bedford, MA).

*Purification of Fasciculins*—FAS1 and FAS2 from *D. angusticeps* venom were purified in the laboratory as described previously (Le Du *et al.*, 1989). FAS3 was purified as follows.

*D. viridis* venom, 5.3 g in 60 ml of water, was first dialyzed against water with spectrapore 3 (Spectrum Industries, Los Angeles, CA) for 4 h at 4 °C. After centrifugation at 10,000 × *g* for 35 min at 4 °C, the supernatant was made 0.1 M ammonium acetate, pH 8.5, and immediately gel-filtered through a set of four (5 × 100 cm) columns of Sephadex G50. Elution was performed with 0.1 M ammonium acetate, pH 8.5, at a flow rate of 60 ml h$^{-1}$. The fraction displaying the strongest AChE inhibitory activity was made 0.05 M ammonium acetate, pH 7.2, and loaded on a CM52 (2.5 × 45 cm) column equilibrated in the same solvent. Elution was performed with ammonium acetate, pH 7.2, as follows: 0.05 M for 22 h, a linear gradient from 0.05 to 0.5 M in 18 h, 0.5 M for 5 h, a linear gradient from 0.5 to 2 M in 5 h, and a 2 M final isocratic step. The flow rate was 60 ml h$^{-1}$. An additional chromatography of the AChE inhibitory fraction was carried out on CM52 in 0.2 M ammonium acetate, pH 7.2, at a flow rate of 20 ml h$^{-1}$. Finally, two HPLC procedures were successively performed on a Waters system (Millipore): a reverse-phase HPLC on a semipreparative (10 × 250 mm) column prepacked with 5-μm ultrasphere-octyl (Beckman) with a linear gradient from 8 to 45% of acetonitrile in 0.15 M ammonium formate, pH 2.7, in 82 min at a flow rate of 5 ml min$^{-1}$ and an ion-exchange HPLC on a (6 × 150 mm) column prepacked with IEX-535-K carboxyspherogel-TSK (Beckman) in isocratic conditions with 0.3 M ammonium acetate, pH 5.0, containing 15% (v/v) acetonitrile, at a flow rate of 1 ml min$^{-1}$. A microtitration assay was set up to monitor the AChE inhibitory activity of the fractions obtained through the successive chromatographic steps. Each well of a Limbro 96 flat bottom well plate (Flow Laboratories, McLean, VA) contained, to a final volume of 150 μl, 50 mM $NaH_2PO_4/Na_2HPO_4$, pH 8.0, 0.1 mg ml$^{-1}$ BSA, 320 μg ml$^{-1}$ $P_2$, 0.6 mM DTNB, 0.3 mM ATCh (2 × $K_m$) and the sample. After stirring the microtitration plate for 15 min at 37 °C, absorbance at 414 nm was determined using a Titertek Multiscan MC spectrophotometer (Flow Laboratories).

*Protein Analysis*—Protein samples were hydrolyzed under vacuum for 20 or 70 h in the presence of 6 N HCl containing 1% (w/w) phenol, with a Waters Pico-Tag work station (Millipore). Amino acid analyses were carried out on a Beckman 6300 auto analyzer. Reduction and *S*-carboxymethylation of FAS3 (RCM-FAS3) were performed as described previously (Kopeyan *et al.*, 1975). CNBr cleavage of RCM-FAS3 at Met bonds was performed according to Gross (1967) by using 70% formic acid and adding solid CNBr with a molar excess of 70 over Met. Incubation was performed at room temperature in the dark for 24 h. Endoproteinase Arg-C (2% w/w) cleavage of RCM-FAS3 was performed in 0.05 M *N*-ethylmorpholine, pH 8.0, at 37 °C, for 5.5 h. *S. aureus* $V_8$ (10%, w/w) cleavage of RCM-FAS3 was performed in ammonium acetate, pH 4.0, at 37 °C for 24 h (Houmard and Drapeau, 1972). Peptides issued from both chemical and enzymatic cleavages were separated by TLC on silica gel 60 plastic sheets. For both CNBr and endoproteinase Arg-C peptides, the first dimension chromatography was performed by using an ammonia 25%/ethanol (40/60, v/v) solvent and the second dimension chromatography by using an ethanol/water/acetic acid (51/40/9, v/v) solvent. Only the first dimension chromatography was performed for $V_8$-peptides. After TLC, fluorescamine staining of peptides was performed according to Fishbein *et al.* (1980) and visualized at 366 nm. Peptides were extracted from the gel by 50% acetic acid or by the first dimension solvent. The extract was centrifuged at 10,000 × *g* for 10 min, filtered on glass wool then on 0.45 μm HV Millipore filters, and dried before being submitted to amino acid analysis and sequencing. Edman automated sequencing was performed on a Beckman system 890 M sequencer as described (Gatineau *et al.*, 1990; Bechis *et al.*, 1984). Manual dimethylaminoazobenzene-4′-isothiocyanate/phenylisothiocyanate method (Chang, 1983) was also used to determine peptide N-terminal sequence. The C-terminal sequence was obtained by hydrolysis of endoproteinase Arg-C-peptide$_{28-61}$ with carboxypeptidase A (10%, w/w) in 0.1 M *N*-ethylmorpholine, pH 8.0, at 37 °C. Aliquots were removed at defined intervals, dried, and submitted to amino acid analysis.

*Iodination of FAS3*—FAS3 (5 μg) was enzymatically iodinated in the presence of 1 mCi of Na$^{125}$I with lactoperoxidase, then iodine in excess was removed by adsorption on Dowex Ac 1X10 (Bio-Rad) as already described (Martin *et al.*, 1987). A Crystal II Multidetector RIA system (Packard) was used for γ-radioactivity quantification. A specific activity of at least 1500 Ci mmol$^{-1}$ was routinely achieved. $^{125}$I-FAS3 was stored 1 μM in 50 mM $NaH_2PO_4/Na_2HPO_4$, pH 7.5, 1 mg ml$^{-1}$ BSA, at 4 °C and used within 3 weeks.

The homogeneity of $^{125}$I-FAS3 was attested by HPLC on a reverse-phase ultrasphere-octyl (4.5 × 250 mm) column (Beckman) by using a linear gradient from 10 to 50% of acetonitrile in 0.15 M ammonium formate, pH 2.7, in 40 min, at a flow rate of 1.5 ml min$^{-1}$ (Bougis *et al.*, 1986). The AChE inhibitory activity of the HPLC fractions was determined by using the microtitration assay above described. After multi-enzymatic digestion of $^{125}$I-FAS3 (Marchot *et al.*, 1988), the respective proportions of $^{125}$I-Tyr and $^{125}I_2$-Tyr were further determined by HPLC on the same column by using a linear gradient from 5 to 25% of acetonitrile in 0.15 M ammonium formate, pH 2.7, in 20 min and at a flow rate of 1.5 ml min$^{-1}$. Carboxypeptidase A hydrolysis of $^{125}$I-FAS3 was performed in 0.2 M *N*-ethylmorpholine, pH 8.5, at 37 °C, with an enzyme to $^{125}$I-fasciculin ratio of 20 (w/w). Aliquots of 100 μl (2 × 10$^5$ cpm) were taken at defined intervals, immediately mixed with 100 μl of 0.15 M ammonium formate, pH 2.7, containing 100 nmol of each Tyr, I-Tyr, and $I_2$-Tyr standards, then loaded on the reverse-phase ultrasphere-octyl (4.5 × 250 mm) column. Elution was performed with two successive linear gradients, from 5 to 25% and from 25 to 75% of acetonitrile in 0.15 M ammonium formate, pH 2.7, in 20 min and 25 min, respectively, at a flow rate of 1.5 ml min$^{-1}$. Fractions were collected and counted for radioactivity.

*Rat Brain AChE*—The crude synaptosomal fraction ($P_2$) of Wistar rat brain was prepared as described by Gray and Whittaker (1962). The protein content was determined using the Bio-Rad protein assay with BSA as standard. The specific AChE activity was 12.5 ± 3.0 μmol of ATCh h$^{-1}$ mg$^{-1}$ $P_2$ proteins (slightly depending on the preparation in use) and was stable for at least 80 h at 25 °C. Until use, $P_2$ was stored in liquid nitrogen without loss of AChE activity.

*AChE Activity Assays*—The AChE activity was determined at 25 °C by following the method of Ellman *et al.* (1961) with 1.5 mM ATCh (10 × $K_m$) and 0.32 mM DTNB in 100 mM $NaH_2PO_4/Na_2HPO_4$, pH 8.0, 0.1 mg ml$^{-1}$BSA (buffer I) to a final volume of 1.5 ml. Two successive determinations of $\Delta A$ min$^{-1}$ (interval time, 40 s) were performed with a Unicam 8700 spectrophotometer (Cambridge, United Kingdom) equipped with the optional kinetic program. Data points correspond to duplicates that differed by less than 10%. For inhibition kinetic assays, FAS3 was incubated with $P_2$ (0.1 mg ml$^{-1}$) in buffer I. At defined intervals, two aliquots of 250 μl were taken and immediately assayed for residual AChE activity.

*$^{125}$I-FAS3 Binding Kinetic Assays*—Binding assays were performed at 25 °C in rhesus tubes containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mg ml$^{-1}$ BSA (buffer II), by adding successively $^{125}$I-FAS3 and $P_2$, to a final volume of 250 μl. For nonspecific binding determi-

nation, a 1000-fold excess of FAS3 over the $^{125}$I-FAS3 concentration used was also added. After incubation, the tube was centrifuged at 11,000 × *g* for 5 min. The supernatant was reserved for determination of the free $^{125}$I-FAS3 concentration. The pellet was washed twice with 1 ml of ice-cold buffer II and counted for radioactivity. Data points correspond to duplicates that differed by less than 10% and from which nonspecific binding had been subtracted. For the competition binding assays, the competitor was first allowed to react during 30 min with $P_2$, then the initial binding kinetics of $^{125}$I-FAS3 were determined and compared with that of a control experiment performed in the absence of competitor. Because of the high rate of substrate hydrolysis by AChE, ATCh was not preincubated with $P_2$ but added together with $^{125}$I-FAS3.

*Titration of AChE and Turnover Number Determination*—The total number of $^{125}$I-FAS3 binding sites present on $P_2$ was determined from the plateau value of the titration curve (Weber and Changeux, 1974a) obtained after incubation, for 4 h at 25 °C, of a fixed binding site concentration (0.1 mg ml$^{-1}$ $P_2$ proteins) with increasing amounts of $^{125}$I-FAS3 (from 25 pM to 1 nM) for a final volume of 500 μl in buffer II. Nonspecific binding was determined in a parallel assay by adding a 1000-fold excess of FAS3 over the highest $^{125}$I-FAS3 concentration used. After incubation, the tubes were treated as above described for $^{125}$I-FAS3 binding assays.

The total number of [1,3-$^3$H]DFP binding sites on $P_2$ was determined with increasing amounts of [1,3-$^3$H]DFP (from 25 pM to 1 nM). In that case however, the pellets of centrifugation were dissolved in 20 mM EDTA, pH 8.75, SDS, 1% (w/w) (100 μl), vortexed vigorously, and then transferred into scintillation vials in the presence of Pico-Fluor™15 (5 ml). A LS3801 detector (Beckman) was used for β-radioactivity quantification. In both titration assays ($^{125}$I-FAS3 and [1,3-$^3$H]DFP), the turnover number of the membrane preparation was calculated by taking into account its initial specific AChE activity.

$P_2$ was also titrated by recording the variation of its residual AChE activity as a function of increasing amounts of FAS3 or of MPT (from 20 to 400 pM) (Vigny *et al.*, 1978) after a 4-h or an overnight incubation (0.1 mg ml$^{-1}$ $P_2$ proteins, final volume of incubation 500 μl, 25 °C). Other conditions were the same as in inhibition kinetic assays. All the above described titration experiments were performed on the same $P_2$ preparation, the specific activity of which was 15.5 μmol of ATCh h$^{-1}$ mg$^{-1}$.

*Data Analysis*—Nonlinear least square fit of the association, inhibition, and dissociation data to the following equations was performed with the Enzfitter program (Elsevier Biosoft, Cambridge, United Kingdom).

Association data were analyzed as a single exponential according to Equation 1,

$$B = B_e\,[1 - exp\,(-k_{obs/bind}\,t)] \qquad \text{(Eq. 1)}$$

where $B$ and $B_e$ are the amounts of $^{125}$I-FAS3 specifically bound at all times and at equilibrium (limit), respectively, and $k_{obs/bind}$ is the pseudo-first-order rate constant of the association process. Validity of the chosen model equation was attested by transforming the data according to the semilogarithmic form of Equation 1, *i.e.* $\ln(B_e - B) = -k_{obs/bind}\,t + \ln B_e$.

Inhibition data were analyzed as a single exponential decay according to Equation 2,

$$v = v_0\,[exp\,(-k_{obs/inh}\,t)] + \text{offset} \qquad \text{(Eq. 2)}$$

where $v_0$ and $v$ are AChE activity ($\Delta A$ min$^{-1}$, within the linear part of the Ellman reaction) for initial time (total activity) and for all times (residual activity) of incubation in the presence of FAS3, respectively, and $k_{obs/inh}$ is the pseudo-first-order rate constant of the inhibition process. The offset value, which represents AChE activity remaining as a constant value even for the longest incubation times, was calculated by the program. Validity of the chosen model equation was attested by transforming the data according to the semilogarithmic form of Equation 2, *i.e.* $\ln(v - \text{offset}) = -k_{obs/inh}\,t + \ln(v_0 - \text{offset})$.

Second-order rate constants $k_1$ (association process) and $k_i$ (inhibition process) were determined according to Equations 3a and 3b, respectively,

$$k_{obs/bind} = k_1\,[I^*] + k_{-1} \qquad \text{(Eq. 3a)}$$

$$k_{obs/inh} = k_i\,[I] + k_{-i} \qquad \text{(Eq. 3b)}$$

where $[I^*]$ and $[I]$ are the concentration of free $^{125}$I-FAS3 and of FAS3, respectively.

Dissociation data were analyzed as a single exponential decay according to Equation 4,

$$B = B_e\,[exp\,(-k_{-1}\,t)] \qquad \text{(Eq. 4)}$$

where $B_e$ and $B$ are the amounts of $^{125}$I-FAS3 specifically bound at equilibrium (initial time) and at all times, respectively. Validity of the chosen model equation was attested by transforming the data according to the semilogarithmic form of Equation 4, *i.e.* $\ln(B) = -k_{-1}\,t + \ln B_e$.

Competition experiments were performed by quantifying, with the use of $^{125}$I-FAS3, the amount of binding sites which are *not* occupied by the competitor. Assuming that the time required for the determination of the initial binding rate of $^{125}$I-FAS3 was short enough (<10 min) not to disturb the competitor equilibrium binding to a mutually exclusive site on AChE, the competitor concentration yielding the half-effect ($K_{0.5}$, determined graphically) is its actual dissociation constant ($K_d$) from this site in the absence of $^{125}$I-FAS3 (Weber and Changeux, 1974b).

Titration data were analyzed graphically as an equilateral hyperbola, the horizontal asymptote of which is the number of binding sites, $B_{max}$ (Boeynaems and Dumont, 1980).

## RESULTS

*Purification of Fasciculin FAS3*—After dialysis and centrifugation, *D. viridis* venom was, without prior lyophilization, filtered through Sephadex G50 (Fig. 1*A*). The strongest AChE inhibitory activity was found for the last third of fraction 4. Successive steps of ion-exchange chromatography and reverse-phase HPLC allowed FAS3 to be purified to homogeneity (Fig. 1, *B–E*). Further details are given in the legend of Fig. 1.

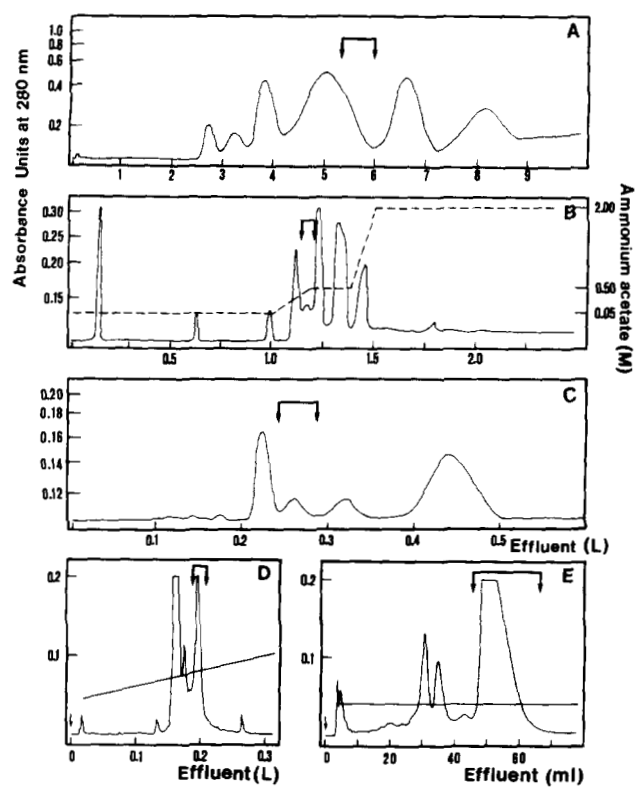


FIG. 1. **Purification of FAS3 from *D. viridis* venom.** *A*, gel-filtration on Sephadex G50 of the dialyzed venom. The fractioning with AChE inhibitory activity is *overlined*. *B*, ion-exchange chromatography on CM52 of the fraction obtained in *A*; *C*, isocratic ion-exchange chromatography on CM52 of the fraction obtained in *B*; *D*, reverse-phase HPLC on 5-μm ultrasphere-octyl of the fraction obtained in *C*; *E*, isocratic ion-exchange HPLC on IEX-535K carboxyspherogel-TSK of the fraction obtained in *D*.

*Chemical Characterization of FAS3*—The first 25 residues (except residues 5, 7–10, and 20–22), and residue in position 32, were determined on RCM-FAS3 (Fig. 2*a*). The last 3 residues were obtained by the action of carboxypeptidase A on endoproteinase Arg-C-peptide$_{28-61}$ (Fig. 2*g*). The CNBr-, endoproteinase Arg-C- and $V_8$-peptides were purified by TLC. Amino acid analysis and/or sequencing of these peptides yielded the necessary overlaps and allowed the complete amino acid sequence of FAS3 to be obtained (Fig. 2, *b–h*).

*Characterization of $^{125}$I-FAS3*—With reference to the specific radioactivity obtained (1500 Ci mmol$^{-1}$), the iodine incorporation was 0.7 atom of iodine/molecule of FAS3. $^{125}$I-FAS3 eluted from reverse-phase HPLC as a single peak with a 5-min delay with regard to the retention time of FAS3; AChE inhibitory activity co-eluted with the radioactivity. After multi-enzymatic digestion of $^{125}$I-FAS3 and subsequent analysis using reverse-phase HPLC (Fig. 3*A*), at least 95% of the radioactivity was recovered as $^{125}$I-Tyr (data not shown).

FAS3 has 4 Tyr residues at positions 4, 23, 47, and C-terminal 61 (Fig. 2). Thus, the location of $^{125}$I-Tyr was investigated by reverse-phase HPLC after carboxypeptidase A hydrolysis of $^{125}$I-FAS3. Fig. 3*B* shows the kinetics of the radioactivity released as $^{125}$I-Tyr. After 60 min of hydrolysis, 50% of the radioactivity of the sample submitted to chromatography was released as $^{125}$I-Tyr, whereas the remaining radioactivity still eluted as $^{125}$I-FAS3. Therefore, one-half of the radioactivity was incorporated on Tyr$^{61}$. When compared with FAS3, FAS2 has only 3 Tyr residues at positions 4, 23, and C-terminal 61 (Tyr$^{47}$ is replaced by Asn$^{47}$ in FAS2). Then, FAS2 was iodinated in the same experimental conditions as FAS3 (specific radioactivity achieved: 1500 Ci mmol$^{-1}$). When $^{125}$I-FAS2 was submitted to carboxypeptidase A analysis, 100% of the radioactivity of the sample was recovered as $^{125}$I-Tyr after as little as 30 min of incubation. FAS2 was thus iodinated on Tyr$^{61}$ only, Tyr$^{4}$ and Tyr$^{23}$ being not reactive. As both Tyr$^{4}$ and Tyr$^{23}$ in FAS3 were likely not to be reactive either, the half of the radioactivity remaining after carboxypeptidase A hydrolysis of $^{125}$I-FAS3 should be incorporated on Tyr$^{47}$, *i.e.* the only additional residue which may be iodinated. Two hypotheses arise from these results: (i) one-half of FAS3 was iodinated on both Tyr$^{47}$ and Tyr$^{61}$, the second half being not iodinated; ii) one-half of FAS3 was iodinated on Tyr$^{47}$ and the second half on Tyr$^{61}$. Since the AChE inhibitory activity co-eluted with the radioactivity only, the first hypothesis was not valid. Finally, to compare the reactivity of Tyr$^{47}$ and Tyr$^{61}$, FAS3 was iodinated in the presence of 10 times less Na$^{125}$I (specific radioactivity achieved: 140 Ci mmol$^{-1}$). Again, carboxypeptidase A hydrolysis revealed that 50% of the radioactivity was incorporated on Tyr$^{61}$, thus suggesting that Tyr$^{47}$ and Tyr$^{61}$ were equally reactive regardless of the ratio of $^{125}$I/FAS3 used. It was not possible to differentiate $^{125}$I-Tyr$^{47}$-FAS3 from $^{125}$I-Tyr$^{61}$-FAS3 with reverse-phase HPLC; the mixture was therefore used all through the study.

*Kinetic Experiments*—The rate of binding of $^{125}$I-FAS3 to AChE was studied for concentrations ranging from 45 pM to 1 nM. Fig. 4 shows the results obtained for 45 pM (Fig. 4*A*) and 445 pM (Fig. 4*B*). A single association process was observed regardless of the $^{125}$I-FAS3 concentration used. Plotting the data according to Equation 1 allowed the pseudo-first-order kinetic constant, $k_{obs/bind}$, to be determined. Plotting $k_{obs/bind}$ against free $^{125}$I-FAS3 concentrations according to Equation 3a yielded the second-order kinetic constant of the association process, $k_{+1} = (9 \pm 1) \times 10^6$ M$^{-1}$ s$^{-1}$ (Fig. 4*C*). Theoretically, the first-order kinetic constant $k_{-1}$ should be determined from the intercept on the $y$ axis of the plot, according to Equation 3a. In the present case, however, the intercept was so close to zero ($(1 \pm 3) \times 10^{-4}$ s$^{-1}$) that $k_{-1}$ could only be roughly estimated as $<10^{-4}$ s$^{-1}$.

To verify if $^{125}$I-FAS3 is as a fully active AChE ligand as FAS3, we studied the rate of inhibition of AChE activity by FAS3 for concentrations ranging from 0.1 to 1.5 nM. Fig. 5 shows the results obtained for 0.1 nM (Fig. 5*A*) and 1.25 nM (Fig. 5*B*). A single inhibition process was observed regardless of the FAS3 concentration used. About 10% of the initial AChE activity remained not inhibited, even for the highest FAS3 concentrations. Taking into account this value as an offset value, plotting the data according to Equation 2 allowed the pseudo-first-order kinetic constant, $k_{obs/inh}$, to be determined. Plotting $k_{obs/inh}$ against FAS3 concentrations according to Equation 3b yielded the second-order kinetic constant of the inhibition process $k_i = (10.9 \pm 0.7) \times 10^6$ M$^{-1}$ s$^{-1}$ (Fig. 5*C*). In that case, $k_{-i}$ could only be roughly estimated as $<10^{-4}$ s$^{-1}$ ($(3 \pm 6) \times 10^{-4}$ s$^{-1}$).

The dissociation process of $^{125}$I-FAS3 initially bound to AChE was visualized after the addition of a 1000-fold excess of FAS3. Plotting the data according to Equation 4 yielded the first-order kinetic constant $k_{-1} = (4 \pm 1) \times 10^{-6}$ s$^{-1}$, *i.e.* a

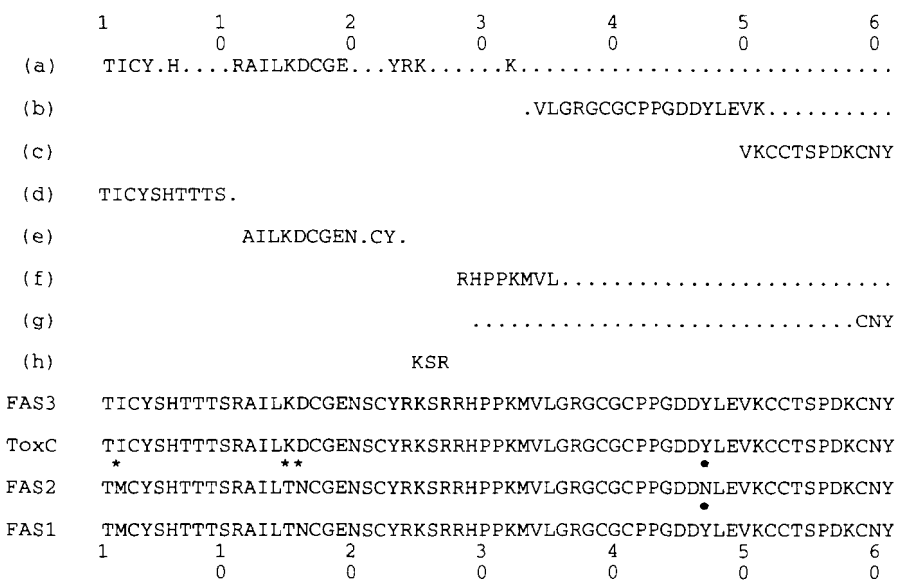


FIG. 2. **Determination of the amino acid sequence of FAS3 and comparison with the known sequences of toxin C (Joubert and Taljaard, 1978), FAS2 (Viljoen and Botes, 1973), and FAS1 (Ducancel *et al.*, 1991).** *a*, Edman automated sequencing performed on RCM-FAS3; *b*, on CNBr-peptide$_{34-61}$; *c*, on *S. aureus* $V_8$-peptide$_{50-61}$; *d*, on endoproteinase Arg-C-peptide$_{1-11}$; *e*, on endoproteinase Arg-C-peptide$_{12-24}$; *f*, on endoproteinase Arg-C-peptide$_{28-61}$; *g*, carboxypeptidase A hydrolysis of endoproteinase Arg-C-peptide$_{28-61}$; *h*, sequence of endoproteinase Arg-C-peptide$_{25-27}$ as determined from amino acid composition. *, indicates differences between the sequences.

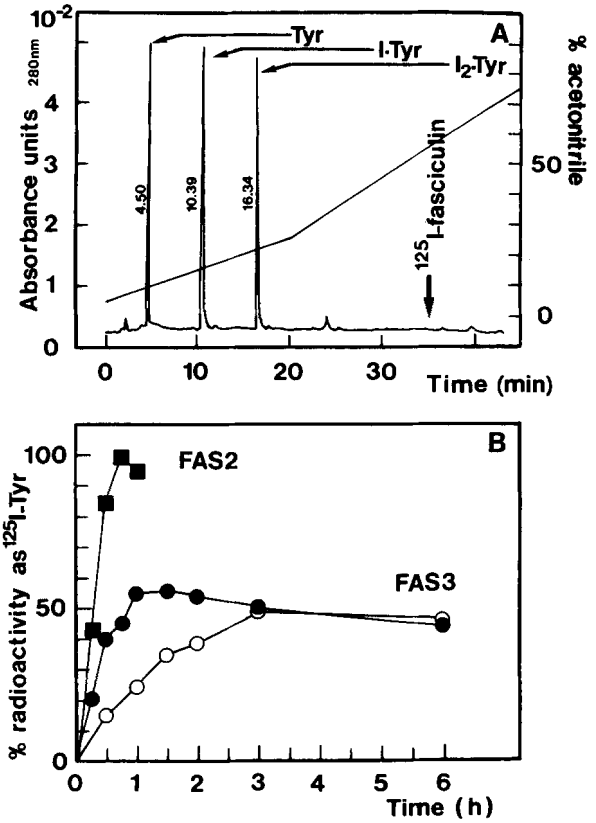


FIG. 3. **Characterization of the products obtained after carboxypeptidase A hydrolysis of $^{125}$I-FAS3.** *A*, reverse-phase HPLC elution diagram of a sample constituted of Tyr, I-Tyr, $I_2$-Tyr and, as located by radioactivity, $^{125}$I-FAS3; *B*, kinetics of $^{125}$I-Tyr release upon incubation of $^{125}$I-FAS3 (●) (1500 Ci mmol$^{-1}$), $^{125}$I-FAS3 (○) (140 Ci mmol$^{-1}$), and $^{125}$I-FAS2 (■) (1500 Ci mmol$^{-1}$) with carboxypeptidase A. 100% of radioactivity (*y* axis) corresponds to the radioactivity of the sample submitted to chromatography ($2 \times 10^5$ cpm).

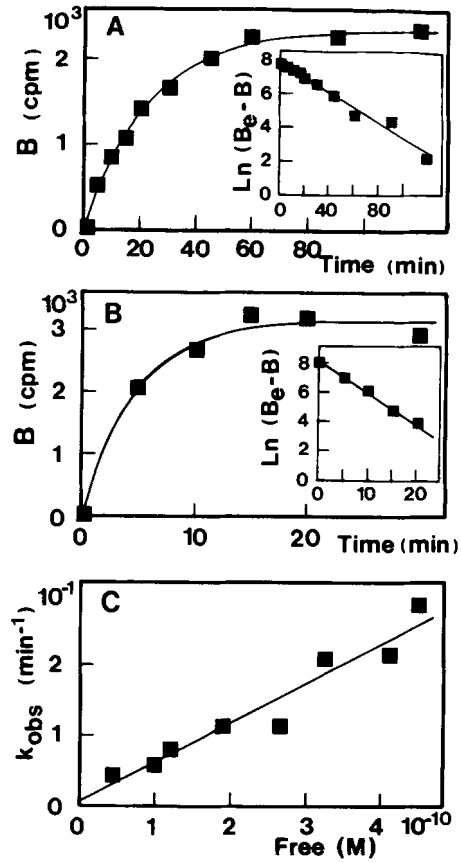


FIG. 4. **Kinetics of formation of the $^{125}$I-FAS3·AChE complex.** *A*, association kinetics performed in the presence of 45 pM of $^{125}$I-FAS3 and 0.1 mg ml$^{-1}$ $P_2$ proteins; the data were fitted to Equation 1, yielding $k_{obs/bind} = (7.2 \pm 0.3) \times 10^{-4}$ s$^{-1}$; *inset*, semilogarithmic plot of the data; *B*, association kinetics performed in the presence of 445 pM of $^{125}$I-FAS3 and 0.1 mg ml$^{-1}$ $P_2$ proteins; the data were fitted to Equation 1, yielding $k_{obs/bind} = (4.8 \pm 0.3) \times 10^{-3}$ s$^{-1}$; *inset*, semilogarithmic plot of the data; *C*, $k_{obs/bind}$ values were plotted against the $^{125}$I-FAS3 free concentrations according to Equation 3a, yielding the second-order rate constant $k_i = (9 \pm 1.0) \times 10^6$ M$^{-1}$ s$^{-1}$.

$t_{1/2}$ value of 48 h (Fig. 6). The true dissociation constant of the $^{125}$I-FAS3·AChE complex was derived from the dissociation and association kinetic constants, *i.e.* $K_d = k_{-1}/k_1 = 0.4$ pM.

*Competition Binding Experiments*—The effect of fasciculins FAS1, FAS2, and FAS3, as well as of the anti-cholinesterase agents BW284C51, propidium iodide, DFP, paraoxon, *d*-tubocurarine, and MPT, on the binding of $^{125}$I-FAS3 to AChE after their preincubation with $P_2$ was studied on the basis of the decrease in the initial binding rate of $^{125}$I-FAS3 (Fig. 7). Whereas 1 mM of either DFP or paraoxon totally inhibits AChE, increasing concentrations of DFP or paraoxon, up to 1 mM, had no significant effect on the initial binding rate of $^{125}$I-FAS3 (data not shown). On the contrary, preincubation of $P_2$ in the presence of increasing concentrations of FAS3, FAS2, and FAS1, as well as of BW284C51, propidium iodide, and *d*-tubocurarine, provoked a significant decrease in the initial binding rate of $^{125}$I-FAS3. In each case, the inhibition of $^{125}$I-FAS3 binding was complete and occurred within 2 orders of magnitude of the anti-cholinesterase agent concentration. The half-effect ($K_{0.5} = K_d$, see "Materials and Methods") was obtained for 0.45 ± 0.05 pM of FAS3, 14 ± 2 pM of FAS1, 25 ± 3 pM of FAS2 (each value being the average of three independent experiments) (Fig. 7*A*), 3.2 nM of BW284C51, 0.1 μM of *d*-tubocurarine, and 5.6 μM of propidium iodide (Fig. 7*B*).

The effect of $CaCl_2$ and $MgCl_2$, as well as of ATCh, on $^{125}$I-FAS3 binding is also reported on Fig. 7*B*. The rate was found to decrease within 2 orders of magnitude of the salt concentration, up to almost total inhibition of $^{125}$I-FAS3 binding ($v/v_0$ <10% for 100 mM). Half-effect was obtained for 12.6 mM of both $CaCl_2$ and $MgCl_2$. As well, a significant decrease in the binding rate was observed for ATCh concentrations higher than 1.0 mM, up to a 40% decrease for 10 mM ATCh. In an attempt to correlate this phenomenon to the effect of excess substrate AChE inhibition, the $P_2$ enzymatic activity was assayed for ATCh concentrations ranging from 1.5 μM to 15.5 mM. As expected, the AChE specific activity significantly decreased for ATCh concentrations higher than 1.0 mM, up to a 45% decrease for the highest concentration 15.5 mM (data not shown).

The effect of MPT on the initial binding rate of $^{125}$I-FAS3 to AChE was also assayed (Fig. 7*B*, *inset*). The rate was found to *increase* up to 130% from 0.1 nM and within 2 orders of magnitude of the MPT concentration. For MPT concentrations higher than 10 nM, the rate drew nearer the 100% value but never joined it, even for the highest concentration tested (0.1 mM).

*Titration of AChE and Turnover Number Determination*—The number of $^{125}$I-FAS3 binding sites on $P_2$ was studied for $^{125}$I-FAS3 concentrations ranging from 25 pM to 1 nM (Fig. 8*A*). Nonspecific binding accounted for about 25% of total binding. The $^{125}$I-FAS3 specific binding was saturable and the plateau of the titration curve was used to estimate the number

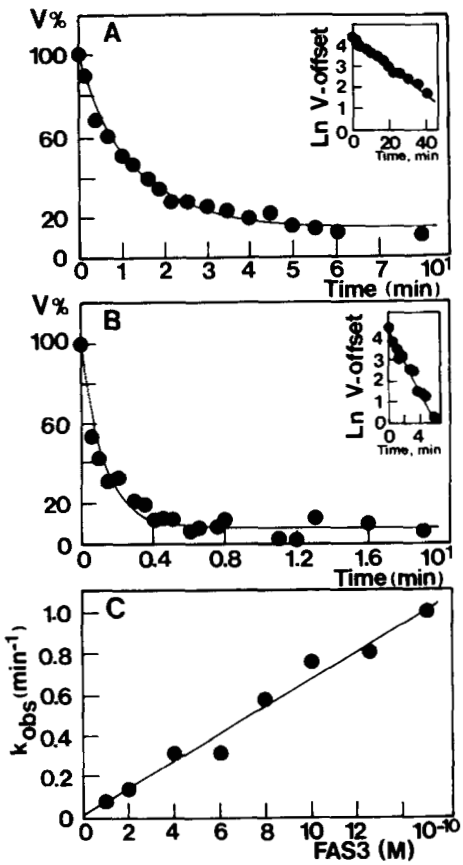


FIG. 5. **Kinetics of AChE inhibition by FAS3.** *A*, residual AChE activity of 250-$\mu$l aliquots of the incubation mixture containing 0.1 nM of FAS3 and 0.1 mg ml$^{-1}$ $P_2$ proteins; the data were fitted to Equation 2, yielding $k_{obs/inh} = (12.3 \pm 0.8) \times 10^{-4}$ s$^{-1}$; *inset*, semilogarithmic plot of the data; *B*, residual AChE activity of 250-$\mu$l aliquots of the incubation mixture containing 1.25 nM of FAS3 and 0.1 mg ml$^{-1}$ $P_2$ proteins; the data were fitted to Equation 2, yielding $k_{obs/inh} = (13 \pm 2) \times 10^{-3}$ s$^{-1}$; *inset*, semilogarithmic plot of the data; *C*, $k_{obs/inh}$ values were plotted against FAS3 initial concentrations according to Equation 3b, yielding the second-order rate constant $k_i = (10.9 \pm 0.7) \times 10^6$ M$^{-1}$ s$^{-1}$.

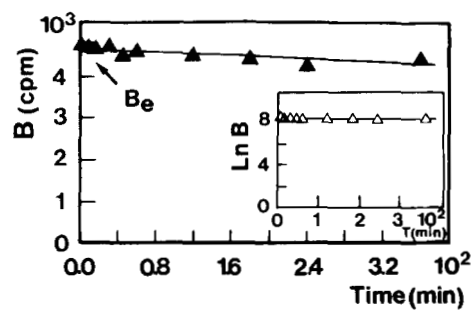


FIG. 6. **Kinetics of dissociation of the $^{125}$I-FAS3·AChE complex.** $^{125}$I-FAS3 was 300 pM and was initially allowed to bind to 0.1 mg ml$^{-1}$ $P_2$ proteins. A 1000-fold excess of FAS3 was added to the mixture to initiate the complex dissociation and then aliquots of 250 $\mu$l were taken at defined intervals and the amount of bound $^{125}$I-FAS3 determined. The data were fitted to Equation 4, yielding the first-order rate constant $k_{-1} = (4 \pm 1) \times 10^{-6}$ s$^{-1}$; *inset*, semilogarithmic plot of the data.

of binding sites $B_{max} = 0.42 \pm 0.01$ pmol mg$^{-1}$. Taking into account the specific AChE activity of the preparation (15.5 $\mu$mol of ATCh h$^{-1}$ mg$^{-1}$), the turnover number per site was $3.7 \times 10^7$ mol of ATCh hydrolyzed h$^{-1}$. The total number of $^{125}$I-FAS3 binding sites was found unchanged when determined in the presence of MPT in excess preincubated with $P_2$.

Titration of the number of [1,3-$^3$H]DFP binding sites on $P_2$ is reported on Fig. 8*B*. Nonspecific binding accounted for about 10% of total binding. The specific binding was saturable with $B_{max} = 0.52 \pm 0.01$ pmol mg$^{-1}$, and the turnover number per site was calculated to be $3.0 \times 10^7$ mol of ATCh h$^{-1}$. The total number of [1,3-$^3$H]DFP binding sites was not significantly changed when determined in the presence of FAS3 in excess preincubated with $P_2$. Moreover, the kinetic binding of [1,3-$^3$H]DFP (4 nM) to AChE was also found unchanged when assayed in the presence of FAS3 (4 nM) preincubated with $P_2$ for 30 min (data not shown).

The variation of the residual AChE activity of $P_2$ was assayed as a function of either FAS3 or MPT concentration (Fig. 8*C*). After 4 h of incubation, the plot obtained for FAS3 was a straight line which, when extrapolated, yielded $B_{max} = 0.4$ pmol mg$^{-1}$ as the number of FAS3 binding sites and a turnover number per site of $3.6 \times 10^7$ mol of ATCh h$^{-1}$. An identical result was obtained after an overnight incubation. In contrast, the plot obtained for MPT largely diverged from linearity in the case of 4 h of incubation (data not shown). When incubation was extended to overnight, the plot diverged as early as at 50% inhibition. Extrapolation to zero activity based on initial 50% inhibition data points yielded the number of MPT binding sites $B_{max} = 1.0$ pmol mg$^{-1}$ and a turnover number per site of $1.55 \times 10^7$ mol of ATCh h$^{-1}$. This result was found unchanged after longer incubation.

## DISCUSSION

When compared with the sequences of fasciculins FAS1 and FAS2 from *D. angusticeps* venom and toxin C from *D. polylepis* venom, the sequence of FAS3 is identical with that of toxin C (Fig. 2). The possibility for the presence of identical fasciculins in venoms of mambas of two different species has to be questioned. Moreover, it should be noted that the true origin of the venom that we used has been already questioned with regard to the isolation of $\alpha$-neurotoxins (Bechis *et al.*, 1976). *D. viridis* venom has been reported to contain several anti-cholinesterase toxins (Harvey *et al.*, 1984). A *D. viridis* venom of certified origin (Latoxan, Rosans, France), as well as several other individual *D. viridis* venoms, were also tested for AChE inhibitory activity. No such activity, however, was found in these venoms (Marchot, 1986). When venoms are collected, misidentification of snake species and subspecies should be considered as of outstanding importance.

Iodination of FAS3 with mild procedure provided us with a fully active specific probe of exceptionally high affinity for an AChE peripheral binding site. This site is, at least partly, shared with the respective binding sites of some other inhibitors, as well as of substrate in excess. Characterization of $^{125}$I-FAS3 showed that, among the 4 Tyr residues of FAS3 (in positions 4, 23, 47, and 61), only Tyr$^{47}$ and Tyr$^{61}$ may be iodinated by using lactoperoxidase catalysis (Fig. 3). For the identically shaped elapid toxins, cardiotoxins and short $\alpha$-neurotoxins, it has been proved that Tyr$^{23}$, next to Cys$^{22}$ (fasciculin numbering), cannot be enzymatically iodinated because of its buried location within the molecule (Sato and Tamiya, 1970; Rochat *et al.*, 1977; Bougis *et al.*, 1983). For similar reasons, Tyr$^{23}$ and Tyr$^4$ (next to Cys$^3$) of fasciculins were likely not to be accessible to iodination, as evidenced from recent resolution of the three-dimensional x-ray structure of FAS1 (Le Du *et al.*, 1992). Characterization of $^{125}$I-FAS2, which we demonstrated to be iodinated only on Tyr$^{61}$, as Tyr$^{47}$ is replaced by Asn$^{47}$, confirms this assumption.

Kinetic studies conducted with $^{125}$I-FAS3 and FAS3 on

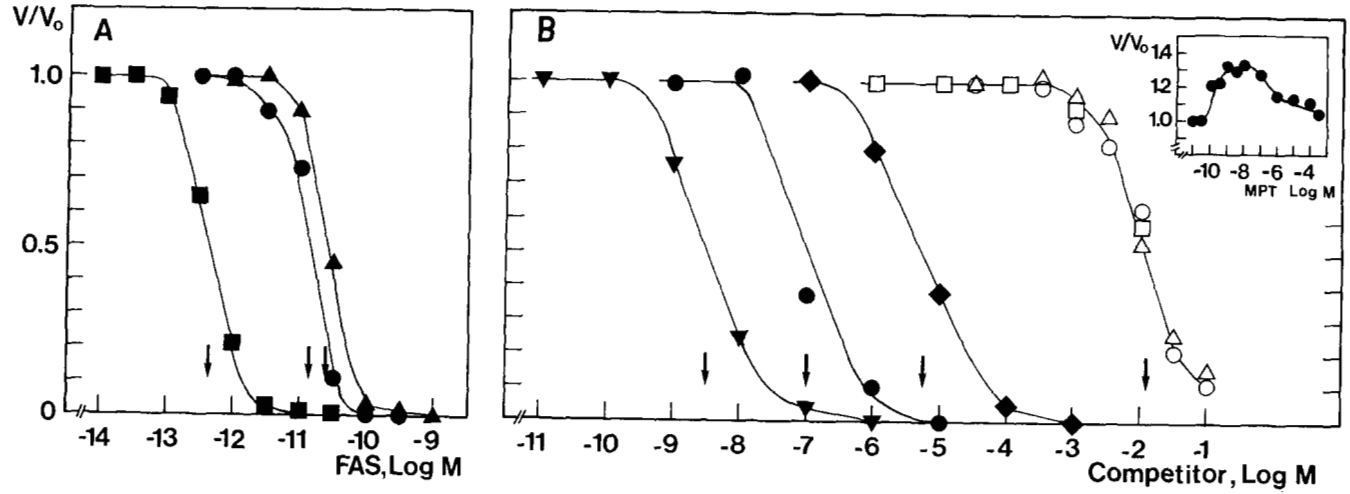


FIG. 7. **Competition binding experiments between $^{125}$I-FAS3 and either FAS3, FAS2, and FAS1, or various AChE effectors, or acetylthiocholine.** *A*, the initial binding rate of 100 pM $^{125}$I-FAS3 to 0.1 mg ml$^{-1}$ $P_2$ proteins was determined in the absence ($v_0$) and in the presence ($v$) of increasing concentrations of FAS3 (■), FAS1 (●), or FAS2 (▲), preincubated with $P_2$ during 30 min. *B*, the same experiment was performed with increasing concentrations of either BW284C51 (▼), *d*-tubocurarine (●), propidium iodide (♦), $CaCl_2$ (○), or $MgCl_2$ (△) preincubated with $P_2$ during 30 min or of ATCh (□) not preincubated; *inset*, the experiment was performed in the presence of increasing concentrations of MPT, preincubated with $P_2$ during 30 min.

binding to AChE and on AChE inhibition, respectively, were both well fitted by single exponential processes (Figs. 4 and 5). The great similarity of the two second-order kinetic constants, $k_1$ and $k_i$, means that the rate of FAS3 association to AChE is not modified upon iodination and that binding and inhibition are concomitant processes in the time scale of the respective experiments. The kinetics of $^{125}$I-FAS3 dissociation, the data of which were well fitted by a single exponential decay equation, clearly indicate an extremely slow process ($t_{1/2}$ = 48 h) (Fig. 6). This result is consistent with reports on the apparent irreversibility of the action of fasciculins on murine diaphragm preparations (Anderson *et al.*, 1985) and on red blood cell AChE (Lin *et al.*, 1987). The dissociation constant of the $^{125}$I-FAS3·AChE complex, which was determined from the kinetic constants (Figs. 4 and 6), is exceptionally low ($K_d$ = 0.4 pM). As the same $K_d$ value was determined for the FAS3·AChE complex by quantifying, with $^{125}$I-FAS3, the amount of binding sites not occupied by FAS3 (Fig. 7*A*), the affinity of FAS3 for AChE is not either changed upon iodination. The dissociation constants of complexes formed with FAS1 et FAS2 (14 and 25 pM, respectively) are significantly higher than that of the complex formed with FAS3 (Fig. 7*A*) and in agreement with the $K_i$ value of 16 pM kinetically determined for FAS1 on liposomal bovine brain AChE (Puu and Koch, 1990) and with the "apparent" $K_i$ value of 35 pM reported in an equilibrium study on rat brain AChE inhibition by FAS2 (Karlsson *et al.*, 1984). FAS1 and FAS2 differ by the single amino acid substitution Tyr/Asn at position 47, which may account for their 2-fold difference in affinity, the Tyr residue slightly enhancing the stability of the FAS·AChE complex. In contrast, FAS3 differs from both FAS1 and FAS2 by substitutions in positions 2 (Ile/Met), 15 (Lys/Thr) and 16 (Asp/Asn), respectively (Fig. 2). The second and the third substitutions, which bring not only local differences in charge, but also the long side chain of the Lys residue, are more likely to account for the 35-fold higher affinity of FAS3 as compared with FAS1. These results, together with the partial loss of activity occurring upon chemical modification of Arg and Lys residues of FAS2 (Cervenansky *et al.*, 1991b), support the hypothesis of multipoint attachment of fasciculins on AChE, according to which several residues of the fasciculin molecule are involved simultaneously in the interaction with the target enzyme. Such a mode of attachment, which has already been described for the interaction of snake venom α-neurotoxins with their binding sites on nicotinic ACh receptor (Faure *et al.*, 1983; Martin *et al.*, 1983), most probably explains the exceptionally high affinity of FAS3 for rat brain AChE. Such an affinity is, up to now, the highest reported for fasciculins.

Incidentally, the fact that all our data were well fitted by single exponential processes (kinetic data) or by single sigmoidal processes (competition data) strongly support the presence of a single AChE form in our membrane preparation. In rat brain, indeed, membrane-bound AChE has been reported to be made exclusively of the globular G4 form (Rieger and Vigny, 1976; Bisso *et al.*, 1991). The four G4 subunits, the catalytic properties of which are essentially identical (Vigny *et al.*, 1978; Massoulié and Bon, 1982), thus appear to be also equally susceptible to fasciculins.

DFP and paraoxon are organophosphorus compounds which irreversibly inhibit AChE by phosphorylating the esteratic serine in the catalytic site. On the contrary, propidium iodide, *d*-tubocurarine, and BW284C51 are known to reversibly inhibit AChE by acting through AChE anionic sites. Propidium iodide selectively binds to a peripheral anionic site (Taylor and Lappi, 1975) which is remote from the AChE catalytic site and has a regulatory role (allosteric site). *d*-Tubocurarine also binds to such a site, sometimes with complex behavior depending on the ionic strength of the medium (Changeux, 1966; Mooser and Sigman, 1974; Zorko and Pavlic, 1986; Friboulet *et al.*, 1990). BW284C51 has been thought to bind selectively to the anionic subsite of the catalytic site (Austin and Berry, 1953); however, an additional peripheral interaction between AChE and BW284C51 has been reported (Friboulet *et al.*, 1986). Cations, and particularly bivalent ions, have been shown to induce an increase in substrate affinity and reaction velocity of AChE (Changeux, 1966; Robaire and Kato, 1974), but a decrease both in the inhibition due to some competitive inhibitors and in excess substrate inhibition of AChE; interaction with peripheral anionic centers was suggested. The competition binding experiments performed with the AChE inhibitors or effectors above cited allowed us to

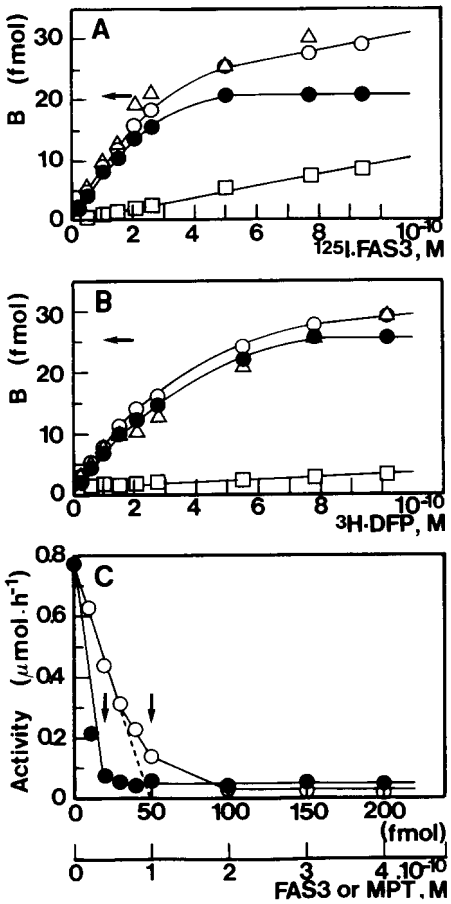


FIG. 8. **Titration of the binding sites present on $P_2$.** The $P_2$ preparation (0.1 mg ml$^{-1}$, 15.5 μmol of ATCh h$^{-1}$ mg$^{-1}$) was incubated during 4 h in the presence of increasing concentrations of inhibitor; *A*, determination of the number of $^{125}$I-FAS3 binding sites: total (○), nonspecific (□), and specific (●) binding; △ represents the total binding of $^{125}$I-FAS3 in the presence of MPT ($10^{-7}$ M) preincubated with $P_2$ for 2 h; *B*, determination of the number of [1,3-$^3$H]DFP binding sites: total (○), nonspecific (□), and specific (●) binding; △ represents the total binding of [1,3-$^3$H]DFP in the presence of FAS3 ($10^{-7}$ M) preincubated with $P_2$ for 2 h; *C*, residual AChE activity of $P_2$ as a function of either FAS3 (●) or MPT (○) concentration. *Dashed line* shows the extrapolation to zero activity based on initial 50% inhibition data points. The double scale (*x* axis) permits comparison with the *y* axis of the data plots reported in *A* and *B*.

examine the relationships between binding site occupancy by $^{125}$I-FAS3 and AChE inhibition. Although both DFP and paraoxon fail to prevent $^{125}$I-FAS3 from binding to its site, inhibitors acting through AChE peripheral anionic sites, *i.e.* BW284C51, *d*-tubocurarine, and propidium iodide, are able to compete with $^{125}$I-FAS3 (Fig. 7*B*). The $K_d$ values which we obtained are in agreement with previously reported $K_d$ or $K_i$ values: for BW284C51, 3.2 nM has to be compared with 1.2 nM reported as a maximal $K_i$ value (Austin and Berry, 1953); for *d*-tubocurarine, 0.1 μM has to be compared with 3.0 μM as determined by back-titration of propidium iodide fluorescence in the case of *T. californica* AChE (Taylor and Lappi, 1975) but in low ionic strength conditions in which *d*-tubocurarine is also reported to bind at the ionic subsite of the AChE catalytic site (Mooser and Sigman, 1974); for propidium iodide, 5.6 μM has to be compared with 3.0 μM, as determined in identical ionic strength conditions (Berman and Taylor, 1978), with 1.1 μM, as determined in low ionic strength conditions (Berman and Leonard, 1990), or with 0.3 μM (Taylor and Lappi, 1975) or 0.1 μM (Berman *et al.*, 1981). The extinction, by FAS2, of the fluorescence of propidium iodide bound to AChE has been reported (Karlsson *et al.*, 1984). However, extinction was not complete (about 20% of the signal remained) until $Ca^{2+}$ was added, indicating mutually exclusive binding sites for $Ca^{2+}$ and propidium. Fasciculins are also likely to share, at least partly, such sites, since $Ca^{2+}$ and $Mg^{2+}$ prevent more than 90% of $^{125}$I-FAS3 binding on rat brain AChE (Fig. 7*B*). Similar results were reported for inhibition of *Bungarus multicinctus* venom AChE by FAS2 and toxin C (Lin *et al.*, 1987); $Mg^{2+}$ was also reported to displace human erythrocyte AChE from a FAS2-cyanogen bromide-activated Sepharose 4B column (Sindhuphak *et al.*, 1988). Those results confirm that fasciculins bind on a peripheral site of the enzyme, distinct from the catalytic site and, at least partly, common with the sites on which some cationic inhibitors bind.

Among cholinesterases, only AChE presents an inhibition by excess of substrate with ACh as well as ATCh, for concentrations above 2 mM (Augustinsson, 1963; Cohen and Oosterbaan, 1963). Rat brain AChE displays such a bell-shaped activity curve, the AChE activity starting to decrease significantly for ATCh concentrations higher than 1.6 mM (data not shown). An inhibition of the initial rate of $^{125}$I-FAS3 binding to AChE was observed for ATCh concentrations higher than 1.5 mM (Fig. 7*B*). These results, which are in favor of a direct relation between the two phenomena, strongly support early proposals (Changeux, 1966; Aldridge and Reiner, 1969) as well as recent findings (Radic *et al.*, 1991) according to which a peripheral anionic center serves as supplementary binding site for ACh. Moreover, since the half-effect of the competition process was nearly obtained around 10–15 mM ATCh, the decreased $^{125}$I-FAS3 binding rate that we observed could not be generated by the product inhibitor thiocholine, the concentration of which reaches a maximal value of 0.2 mM in our experimental conditions. Therefore, the peripheral anionic site on which $^{125}$I-FAS3 binds would also be partly common with the additional substrate binding site responsible for the excess substrate inhibition of AChE. *The* AChE peripheral site, as it is usually referred to in the literature, is likely to consist of a matrix of partially overlapping loci corresponding to the diverse known peripheral ligands. For one of these loci to be entirely shared by fasciculins and peripheral ligands would be of interest, especially since fasciculins, because of their size, may be expected to cover a larger area of the AChE surface than low molecular weight ligands.

The number of $^{125}$I-FAS3 binding sites on $P_2$ (0.42 pmol mg$^{-1}$) yielded a turnover number of the membrane preparation of $3.7 \times 10^7$ mol of ATCh h$^{-1}$ (Fig. 8*A*). Both values were confirmed by titrating $P_2$ with FAS3 (Fig. 8*C*). However, the number of [1,3-$^3$H]DFP binding sites on $P_2$ was found to be 20% higher (0.52 pmol mg$^{-1}$) (Fig. 8*B*). As DFP is known as an active serine inhibitor (Neurath, 1984), the additional DFP binding sites are likely to belong to some serine hydrolases other than AChE. Incidently, such hydrolases may be responsible for the 10% ATCh hydrolysis that remains insensitive to high FAS3 concentrations (Fig. 5). Titration of the number of MPT binding sites (1.00 pmol mg$^{-1}$), however, led to a turnover number of $1.55 \times 10^7$ mol of ATCh h$^{-1}$ (Fig. 8*C*). Although very close to that of $1.32 \times 10^7$ mol of ATCh h$^{-1}$ reported by Vigny *et al.* (1978) for rat brain AChE, this value is 2-fold lower than that determined with [1,3-$^3$H]DFP, $^{125}$I-FAS3, or FAS3. MPT has been reported to interact not only with the catalytic site of AChE, as DFP does (Vigny *et al.*, 1978), but also with a second site, which may overlap or be linked to the peripheral anionic site of AChE, since it shares common features with the BW284C51 and propidium binding

sites (Friboulet *et al.*, 1986, 1990). The binding capacity which we obtained upon titration of AChE with MPT may thus account for both MPT binding sites, leading to a 2-fold underestimation of the turnover number. Furthermore, a decrease in the MPT effect at the active site has been reported to occur upon additional binding of MPT on its peripheral site. The curved shape of our MPT titration plot may therefore proceed from such a phenomenon, *i.e.* an increase in the MPT concentration needed for a given AChE inhibition, as occupancy of the peripheral site lowers the inhibition effect due to catalytic site occupancy. As already postulated (Friboulet *et al.*, 1990), this phenomenon may be explained by conformational changes in the AChE structure. In contrast to MPT, and more specifically than DFP, fasciculins form a 1:1 complex with the AChE catalytic monomer and, thus, prove to be more appropriate than organophosphorus compounds for specific titration of AChE.

In contrast with the other inhibitors and effectors above cited, MPT induces an *increase* in the $^{125}$I-FAS3 binding rate (*inset*, Fig. 7*B*). Since the total number of $^{125}$I-FAS3 binding sites was found unchanged after preincubation of $P_2$ with MPT (Fig. 8*A*), this increase must proceed from an increase in the value of second-order kinetic constant $k_1$ for $^{125}$I-FAS3. We can postulate that the accelerated binding of $^{125}$I-FAS3, in the presence of MPT, is due to a local change in the structure of the FAS3 binding site. This change may concern at least some AChE residues involved in the approach and docking of fasciculins. The fact that high concentrations in MPT cancel the accelerated binding phenomenon, but fail to prevent $^{125}$I-FAS3 from binding to its site, argues for allosteric changes rather than for overlapping binding sites.

Recent resolution of the x-ray structure of *Torpedo* AChE (Sussman *et al.*, 1991) evidenced a deep and narrow gorge, the bottom of which contains the catalytic triad ($Ser^{200}$, $His^{440}$, $Glu^{327}$). The gorge is lined with a high number of aromatic residues which may provide a "guidance mechanism" for ACh molecules. These residues, which make the ACh binding site hydrophobic rather than anionic, may also serve as a relay of molecular events from the peripheral binding sites, presumably located at the rim of the gorge, to the active site (Shafferman *et al.*, 1992). They are thus likely to be related to the multiple effects of the various peripheral ligands, including ACh in excess, on active center reactivity (Pattison and Bernhard, 1978; Tomlinson *et al.*, 1980; Berman and Leonard, 1990). Fasciculins, the three-dimensional structure of which is now solved (Le Du *et al.*, 1992), are too large to enter the gorge, except with severe writhing. Not to negate the hypothesis of steric hindrance, fasciculins should therefore mask the gorge entrance by binding to its rim. Thus, fasciculin occupation of its own peripheral binding site would preclude crossing of ACh, as well as of catalytic site-directed inhibitors, through the gorge. Alternatively, fasciculins may sufficiently perturb the structure of AChE to be able to inhibit the substrate catalysis allosterically. AChE inhibition would thus result from structural modification of the catalytic site or/and disturbance of the postulated guidance mechanism for ACh. Actually, bound fasciculin fails to prevent subsequent occupation of the esteratic subsite of AChE catalytic site, since the total number of [1,3-$^3$H]DFP binding sites, as well as the kinetics of [1,3-$^3$H]DFP association, was found unchanged after preincubation of $P_2$ with FAS3 (Fig. 8*B*). This result evidences that a ternary complex FAS3·AChE·DFP may form with a 1:1:1 ratio and, thus, invalidates the hypothesis of steric hindrance. Since phosphorylation, by DFP, of the AChE catalytic serine can still occur on the FAS3·AChE complex, the AChE inhibition due to fasciculin tight binding may be thought to proceed from structural modification, at a distance, of the anionic subsite of the AChE catalytic site. Whether this subsite is actually modified should be elucidated by resolution of the FAS·AChE-complex x-ray structure.

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REFERENCES

Aldridge, W. N., and Reiner, E. (1969) *Biochem. J.* **115,** 147–162
Anderson, A. J., Harvey, A. L., and Mbugua, P. M. (1985) *Neurosci. Lett.* **54,** 123–128
Augustinsson, K. B. (1963) in *Cholinesterases and Anticholinesterase Agents, Handbook of Experimental Pharmacology.* (Koelle, G. B., ed) Vol. 15, pp. 89–128, Springer-Verlag, Berlin
Austin, L., and Berry, W. K. (1953) *Biochem. J.* **54,** 695–700
Bechis, G., Granier, C., Van Rietschoten, J., Jover, E., Rochat, H., and Miranda, F. (1976) *Eur. J. Biochem.* **68,** 445–456
Bechis, G., Sampieri, F., Yuan, P.-M., Brando, T., Martin, M. F., Diniz, C. R., and Rochat, H. (1984) *Biochem. Biophys. Res. Commun.* **122,** 1146–1153
Berman, H. A., and Leonard, K. (1990) *Biochemistry* **29,** 10640- 10649
Berman, H. A., and Taylor, P. (1978) *Biochemistry* **17,** 1704–1713
Berman, H. A., Becktel, W., and Taylor, P. (1981) *Biochemistry* **20,** 4803–4810
Bisso, G. M., Biancesco, R., and Michaelek, H. (1991) *Neurochem. Res.* **16,** 571–575
Boeynaems, J. M., and Dumont, J. E. (1980) *Outlines of Receptor Theory* (Boeynaems, J. M., and Dumont, J. E., eds) pp. 5–14, Elsevier/North-Holland Medical Press, Amsterdam
Bougis, P. E., Tessier, M., Van Rietschoten, J., Rochat, H., Faucon, J. F., and Dufourcq, J. (1983) *Mol. Cell. Biochem.* **55,** 49–64
Bougis, P. E., Marchot, P., and Rochat, H. (1986) *Biochemistry* **25,** 7235–7243
Cervenansky, C., Dajas, F., Harvey, A. L., and Karlsson, E. (1991a) in *Snake Toxins* (Harvey, A. L., ed) pp. 303–321, Pergamon Press, Inc., New York
Cervenansky, C., Engström, A., and Karlsson, E. (1991b) *Toxicon* **29,** 1163–1164
Chang, J. Y. (1983) *Methods Enzymol.* **91,** 455–466
Changeux, J. P. (1966) *Mol. Pharmacol.* **2,** 369–392
Cohen, J. A., and Oosterbaan, R. A. (1963) in *Cholinesterases and Anticholinesterase Agents Handbook of Experimental Pharmacology* (Koelle, G. B., ed) Vol. 15, pp. 299–373, Springer-Verlag, Berlin
Ducancel, F., Bouchier, C., Tamiya, T., Boulain, J. C., and Ménez, A. (1991) in *Snake Toxins* (Harvey, A. L., ed) pp. 395–414, Pergamon Press, Inc., New York
Ellman, G. L., Courtney, K. D., Andres, Jr. V., and Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7,** 88–95
Epstein, D. J., Berman, H. A., and Taylor, P. (1979) *Biochemistry* **18,** 4749–4754
Faure, G., Boulain, J. C., Bouet, F., Montenay-Garestier, T., Fromageot, P., and Ménez, A. (1983) *Biochemistry* **22,** 2068- 2076
Fishbein, J. C., Place, A. R., Ropson, I., Powers, D. A., and Sofer, W. (1980) *Anal. Biochem.* **108,** 193–201
Friboulet, A., Goudou, D., and Rieger, F. (1986) *Neurochem. Int.* **9,** 323–328
Friboulet, A., Rieger, F., Goudou, D., Amitai, G., and Taylor, P. (1990) *Biochemistry* **29,** 914–920
Gatineau, E., Takechi, M., Bouet, F., Mansuelle, P., Rochat, H., Harvey, A. L., Montenay-Garestier, Th., and Menez, A. (1990) *Biochemistry* **29,** 6480–6489
Gray, E. G., and Whittaker, V. P. (1962) *J. Anat.* **96,** 79–88
Gross, E. (1967) *Methods Enzymol.* **11,** 238–241
Harvey, A. L., Anderson, A. J., Mbugua, P. M., and Karlsson, E. (1984) *J. Toxicol. Toxin Rev.* **3,** 91–137
Houmard, J., and Drapeau, G. R. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 3506–3509
Joubert, F. J., and Taljaard, N. (1978) *South Afr. J. Chem.* **31,** 107–110
Karlsson, E., Mbugua, P. M., and Rodriguez-Ithurralde, D. (1984) *J. Physiol. (Paris)* **79,** 232–240
Kopeyan, C., Miranda, F., and Rochat, H. (1975) *Eur. J. Biochem.* **58,** 117–122
Le Du, M. H., Marchot, P., Bougis, P. E., and Fontecilla-Camps, J. C. (1989) *J. Biol. Chem.* **264,** 21401–21402
Le Du, M. H., Marchot, P., Bougis, P. E., and Fontecilla-Camps, J. C. (1992) *J. Biol. Chem.* **267,** 22122–22130
Lin, W. W., Lee, C. Y., Carlsson, F. H. H., and Joubert, F. J. (1987) *Asia Pac. J. Pharmacol.* **2,** 79–85
Long, J. P. (1963) in *Cholinesterases and Anticholinesterase Agents Handbook of Experimental Pharmacology* (Koelle, G. B., ed) Vol. 15, pp. 374–427, Springer-Verlag, Berlin
Marchot, P. (1986) *Contribution à l'Etude de la Purification et du Mode d'Action des Toxines des Venins de Serpents Elapidae.* Thèse de Doctorat de l'Université d'Aix-Marseille II (Neurosciences)
Marchot, P., Frachon, P., and Bougis, P. E. (1988) *Eur. J. Biochem.* **174,** 537–542
Martin, B. M., Chibber, B. A., and Maelicke, A. (1983) *J. Biol. Chem.* **258,** 8714–8722
Martin, M. F., Garcia y Perez, L. G., El Ayeb, M., Kopeyan, C., Bechis, G., Jover, M., and Rochat, H. (1987) *J. Biol. Chem.* **262,** 4452–4459
Massoulié, J., and Bon, S. (1982) *Annu. Rev. Neurosci.* **5,** 57–106
Mooser, G., and Sigman, D. S. (1974) *Biochemistry* **13,** 2299–2307
Murphy, S. D., Coster, L. G., and Wang, C. (1984) *Cell. Mol. Neurotoxicol.* 165–176
Neurath, H. (1984) *Science* **224,** 350–357
Pattison, S., and Bernhard, S. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 3613–3617

Puu, G., and Koch, M. (1990) *Biochem. Pharmacol.* **40,** 2209–2214
Quinn, D. M. (1987) *Chem. Rev.* **87,** 955–979
Radic, Z., Reiner, E., and Taylor, P. (1991) *Mol. Pharmacol.* **39,** 98–104
Rieger, F., and Vigny, M. (1976) *J. Neurochem.* **27,** 121–129
Robaire, B., and Kato, G. (1974) *Biochem. Pharmacol.* **23,** 2476- 2480
Rochat, H., Tessier, M., Miranda, F., and Lissitzky, S. (1977) *Anal. Biochem.* **82,** 532–548
Rodríguez-Ithurralde, D., Silveira, R., Barbeito, L., and Dajas, F. (1983) *Neurochem. Int.* **5,** 267–274
Rosenberry, T. L. (1975) in *Advances in Enzymology* (Meister, A., ed) Vol. 43, pp. 103–218, Interscience, New York
Sato, S., and Tamiya, N. (1970) *J. Biochem. (Tokyo)* **68,** 867–872
Shafferman, A., Velan, B., Ordentlich, A., Kronman, C., Grosfeld, H., Leitner, M., Flashner, Y., Cohen, S., Barak, D., and Ariel, N. (1992) *EMBO J.* **11,** 3561–3568
Sindhuphak, R., Karlsson, E., Conradi, S., and Ronnevi, L. O. (1988) *J. Neurol. Sci.* **86,** 195–202
Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., and Silman, I. (1991) *Science* **253,** 872–879
Taylor, P., and Lappi, S. (1975) *Biochemistry* **14,** 1989–1997
Tomlinson, G., Mutus, B., and McLennan, I. (1980) *Mol. Pharmacol.* **18,** 33–39
Vigny, M., Bon, S., Massoulié, J., and Leterrier, F. (1978) *Eur. J. Biochem.* **85,** 317–323
Viljoen, C. C., and Botes, D. P. (1973) *J. Biol. Chem.* **248,** 4915- 4919
Weber, M., and Changeux, J. P. (1974a) *Mol. Pharmacol.* **10,** 1–14
Weber, M., and Changeux, J. P. (1974b) *Mol. Pharmacol.* **10,** 15–34
Zorko, M., and Pavlic, M. (1986) *Biochem. Pharmacol.* **35,** 2287- 2296