Conversion of Bovine Pancreatic Phospholipase A2 at a Single Site into a Competitor of Neurotoxic Phospholipases A2 by Site-directed Mutagenesis*

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A 45-kDa polypeptide preferentially present in neuronal membranes was previously identified as a subunit of a binding (or receptor) protein for several phospholipase A2 variants with neurotoxicity, including crotoxin, by chemical cross-linking experiments (Yen, C.-H., and Tzeng, M.-C. (1991) *Biochemistry* 30, 11473–11477). The binding of crotoxin to this receptor protein was completely suppressed by sufficient F22Y, a mutated bovine pancreatic phospholipase A2 generated by site-directed mutagenesis of Phe^{22} of the wild-type enzyme to Tyr. The IC$_{50}$ of this inhibition was estimated to be 1 μM. In sharp contrast, the wild-type enzyme gave no effect even at 50 μM. This mutation resulted in only minor and localized structural perturbations with little effect on enzymatic activity. Other phospholipase A2 molecules capable of competing with crotoxin for this binding invariably have Tyr at this position. It was concluded that this Tyr residue is an important determinant for the binding of a number of phospholipase A2 variants to the 45-kDa receptor.

Proteins with phospholipase A2 (PLA2)[1] (EC 3.1.1.4) activity can be found in extracellular secretion as well as inside the cells of many organisms. The extracellular (secreted) PLA2 variants exhibit a variety of biological effects, including phospholipid metabolism, host defense, signal transduction, neurotoxicity (presynaptic and/or postsynaptic), myotoxicity, and alteration of coagulation, which may or may not be related to hydrolysis of phospholipids. Despite large differences in biological actions, the secreted PLA2 chains from most sources show high degrees of homology in the primary, secondary, and possibly tertiary structures. A small number of these proteins, including crotoxin from the South American rattlesnake *Crotalus durissus terrificus*, act primarily at the presynaptic level to cause synaptic blockade by inhibiting the release of neurotransmitters, though most of them also produce postsynaptic toxicity and other effects. To exert neurotoxicity, these PLA2 neurotoxins appear to bind to the synaptic membranes strongly, followed by hydrolysis of the membrane phospholipids by the PLA2 activity. Lack of such binding is apparently the reason why a larger number of PLA2 variants, such as pancreatic PLA2, are not neurotoxic despite high degrees of enzymatic activity. Strong binding to plasma membranes of other tissues may also be essential for the actions of many other PLA2s on these tissues (see Refs. 1–9 for recent reviews).

By the use of photoaffinity labeling and chemical cross-linking techniques, a few binding proteins have been identified for some of these presynaptic toxins (10–21). One subunit of the binding proteins for crotoxin and several other neurotoxic PLA2s is, as observed by us, a 45-kDa polypeptide preferentially present in the neuronal membrane (13). As one of our approaches to understanding the structural basis for this binding, we converted bovine pancreatic PLA2, which showed no detectable binding to the synaptic membrane, into a mutant capable of competing for the binding of crotoxin to this receptor by site-directed mutagenesis. This report is the first, to our knowledge, to convert pancreatic PLA2 into a competitor of any neurotoxic PLA2. This new way of using site-directed mutagenesis will be useful for the study of other toxic proteins as well.

EXPERIMENTAL PROCEDURES

Materials—Disuccinimidyl suberate and disuccinimidyl dithiobis-(propionate) were obtained from Pierce. Na[^{125}I] and the kit for site-directed mutagenesis were supplied by Amersham Corp. Bovine pancreatic PLA2 and the venom of *C. durissus terrificus*, from which crotoxin was purified as described (22), were purchased from Sigma. Purification of rat PLA2 from the pancreas was carried out according to published procedures (23). All other chemicals were of reagent grade.

Generation and Purification of Mutant PLA2—Mutations of bovine pancreatic PLA2 were generated by site-directed mutagenesis of a chemically synthesized gene for the wild-type protein (25) with an Amersham kit making use of the phosphorothioate method (24). The procedures in the manufacturer's manual were followed. To construct F22Y and F22A mutants, oligonucleotides with the underscored bases in CAT AAT TGA AAA TAT CAA replaced by ATA and AGC, respectively, were used as primers. The mutated gene was subcloned into the expression vector pTO-N, and the resulting plasmid was transfected into a competent strain of *Escherichia coli*, BL21[pLysS]. The desired protein was purified after lysis of the bacteria by sonication (25–27).

Cross-linking of ^{125}I-Crotoxin to the Synaptic Membrane—Synaptic membrane fraction was purified from guinea pig brain by established methods (28, 29). Crotoxin was labeled as previously described (13) with Na[^{125}I] using the chloramine-T method (30) to a specific activity of about 100 Ci/g protein (~1 atom of iodine/molecule of crotoxin). ^{125}I-Crotoxin was mixed with synaptic membranes (0.25 mg of protein/ml), with or without the presence of unlabeled pancreatic PLA2, in 10 mM Tris-HCl buffer, pH 7.4, containing 0.5% bovine serum albumin, 150 mM NaCl, 10 mM SrCl2, and 0.5 mM EGTA at 25 °C for 2.5 h. After

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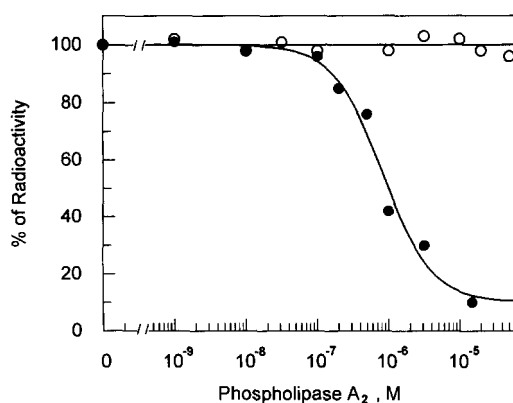
[1] The abbreviation used is: PLA2, phospholipase A2.

FIG. 1. **Effects of bovine pancreatic PLA₂ and the F22Y mutant on the cross-linking of ^{125}I-crotoxin to synaptic membranes from the brain.** Various amounts of the F22Y mutant (●) or the wild-type bovine pancreatic PLA₂ (○) together with ^{125}I-crotoxin (7 ng) were incubated with the synaptic membrane fraction (50 μg) from the guinea pig brain. After cross-linking, the counts of radioactivity in the 60-kDa conjugate were measured. The data are expressed as percentages of the counts in the absence of unlabeled PLA₂.
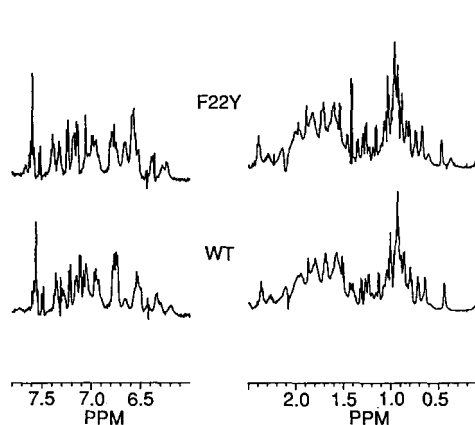

FIG. 2. **One-dimensional proton NMR spectra of pancreatic PLA₂ and its F22Y mutant in D₂O.** Spectra of samples of 1.5 mM protein in 300 mM NaCl and 50 mM CaCl₂, pH 4.0, were recorded at 500 MHz and 37 °C. Free induction decays from 200 scans were processed with gaussian multiplications (line broadening, −5; gaussian broadening, 0.1). *WT*, wild type.

dilution with 10 mM Tris-HCl, pH 7.4, the mixture was centrifuged at 8,000 × *g* for 10 min. The pellet was then washed and resuspended in phosphate-buffered saline. Disuccinimidyl suberate or disuccinimidyl dithiobis(propionate) in dimethyl sulfoxide was added to the resuspended membrane, and the mixture was incubated for 4 min at 25 °C. The reaction was stopped by adding 0.1 ml of 1 N glycine to each vial of the reaction mixture. After centrifugation at 15,000 × *g* for 15 min, the membrane pellet was solubilized with 0.1 M Tris-HCl buffer, pH 6.8, containing 5% glycerol and 2% SDS, and analyzed by SDS-polyacrylamide gel electrophoresis (31). Precolored proteins (32) were used as molecular weight markers. After electrophoresis the position of the 60-kDa conjugate band was revealed by autoradiography. The corresponding area in the gel was cut off and counted by a γ-counter.

Toxicity Test—A test of the neurotoxicity of the PLA₂ mutants toward the central nervous system was performed by intracisternal injection of the proteins into mice weighing 22–25 g as described by Schanberg *et al.* (33) and Schweitz (34). Peripheral toxicity was determined by intraperitoneal injection. Three dosage groups of mice with at least two animals in each group were given 1, 9, or 18 mg, respectively, of protein/kg of body weight in 20 μl of phosphate-buffered saline.

NMR Analysis—The enzyme sample (10 mg) was dissolved in 0.5 ml of D₂O containing 300 mM NaCl and 50 mM CaCl₂ and then lyophilized. The residue was dissolved in 0.5 ml of 100% D₂O, and the pH was adjusted to 4.0 with dilute DCl. Spectra at 500 MHz were recorded at 37 °C. Chemical shifts are relative to internal sodium 3-trimethylsilyl-propionate-2,2,3,3-d_4. Other details are as described previously (35).

RESULTS AND DISCUSSION

We labeled crotoxin with ^{125}I without affecting its neurotoxicity. After ^{125}I-crotoxin had been incubated with the synaptic membrane fraction from the brain to reach maximal binding, disuccinimidyl suberate or disuccinimidyl dithiobis(propionate) was employed to cross-link the binding complexes. Similar to results reported previously (13), cross-linking with the above cross-linkers resulted in a 60-kDa conjugate when analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (data not shown). Crotoxin is a complex of subunit A of 9,000 Da and subunit B of 14,400 Da in noncovalent association. The subunit B is an active PLA₂ (36, 37).

Utilizing the phosphorothioate method (24), we produced mutants of bovine pancreatic PLA₂ by site-directed mutagenesis of a chemically synthesized gene for the wild-type protein (25). When the bovine pancreatic PLA₂ mutant F22Y, in which the Phe[22] of the wild type is replaced by Tyr (35), was present during the binding period, the subsequent formation of the 60-kDa radioactive conjugate was suppressed with an IC₅₀ of 1 ± 0.1 μM as estimated from the curve in Fig. 1 and three other separate experiments. In sharp contrast, the wild-type PLA₂

purified from the bovine pancreas gave no effect even at a concentration as high as 50 μM (Fig. 1). The wild-type pancreatic enzyme produced by cloning techniques and another mutant F22A, which has Ala at residue 22, were also without effect at the highest concentration used.

The one- and two-dimensional NMR spectra of F22Y and the wild type are almost identical except for the obvious changes arising from the new phenolic OH group and a 0.19 ppm change in one of the three chemical shifts of Phe[106], which is in close proximity to residue 22, forming the second half of the Phe[22]-Phe[106] aromatic sandwich (Figs. 2 and 3 and Table I). Similarly, except right around the mutated residue, the NMR spectra of F22A are perturbed only slightly (35). The enzymatic activity of the two mutants is also comparable with that of the wild-type enzyme (35). Among the many mutants we have analyzed, F22Y is most similar to the wild type structurally and kinetically. Hence it is unlikely that the inhibitory effect of the F22Y mutant is due to the hydrolysis of membrane phospholipids. In addition, we have chosen the assay condition that minimizes the enzymatic activity by using a solution containing 10 mM Sr²⁺, 0.5 mM EGTA, and no Ca²⁺, as it has been shown that Sr²⁺ is antagonistic to Ca²⁺ (see Refs. 1–4 for reviews), which is required for the enzymatic activity of secreted PLA₂. Moreover, F22Y was equally effective in suppressing the formation of the 60-kDa conjugate when the experiments were performed at 4 °C to completely arrest the enzymatic activity. We thus conclude that the F22Y mutant blocked the formation of the radioactive conjugate by competing the binding of ^{125}I-crotoxin to the binding protein.

However, because the binding affinity of the F22Y mutant was not high relative to that of crotoxin (<10 nM), there must be other residues also involved in binding and thereby in neurotoxicity. Judging from the affinity of F22Y, we would not expect it to be neurotoxic. All mice injected with the F22Y mutant, either intraperitoneally or intracisternally, lived and behaved normally even at a dose of 18 mg of protein/kg of body weight. We have iodinated the F22Y mutant and attempted to demonstrate its binding to the synaptic membrane directly. Specific binding was not evident, apparently because the affinity is too low.

We were also aware that residue 22 of the pancreatic PLA₂ of the rat is Tyr. If the rat enzyme blocked ^{125}I-crotoxin from forming the radioactive conjugate, our conclusion would be further substantiated. We therefore purified the PLA₂ from the rat pancreas and then investigated its effect on the conjuga-

FIG. 3. Phase-sensitive nuclear Overhauser effect spectroscopy proton NMR spectra in D_2O at 500 MHz. Samples are as described in the legend of Fig. 2. The mixing time was 200 ms. A 4096 × 512 matrix in the time domain was recorded and zero-filled to a 4096 × 2048 matrix prior to multiplication by a gaussian function (line broadening, −3; Gaussian broadening, 0.1). Chemical shifts for the indicated spin systems are given and assigned in Table I.
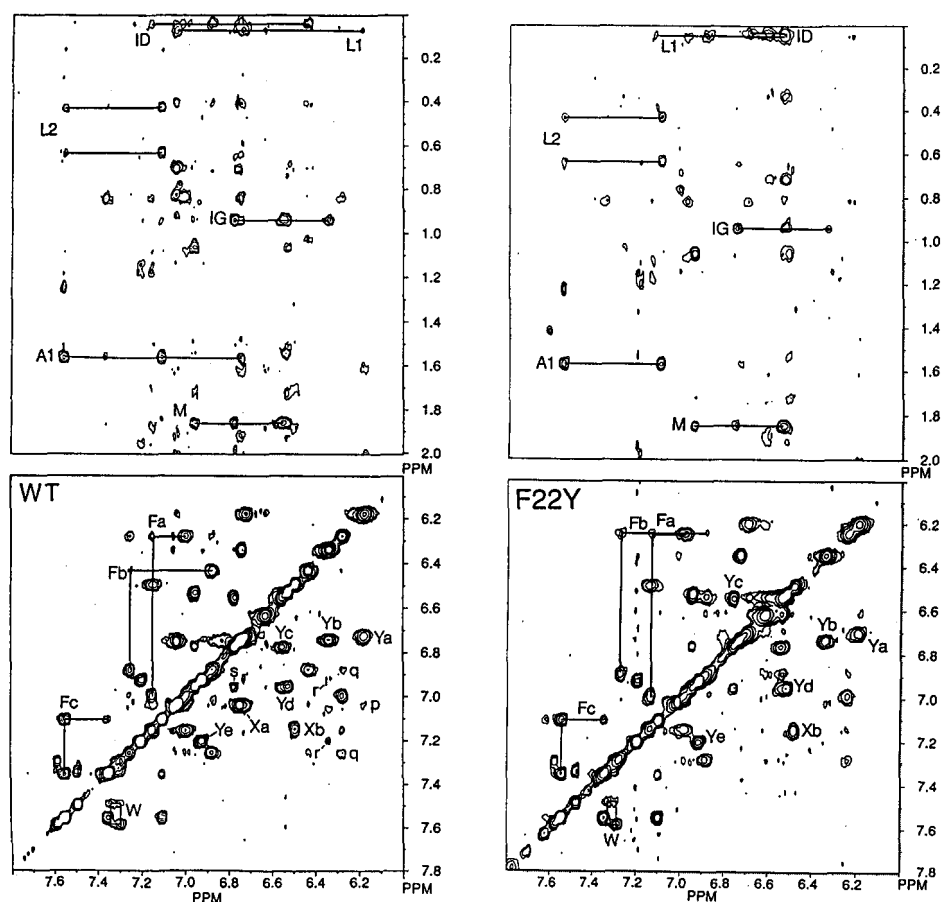
TABLE I
Chemical shifts of the assigned spin systems for wild type and F22Y

Resonances that differ by >0.03 ppm between wild type and mutant are underlined. Parenthetical values are tentatively assigned.

Spin system		Wild type			F22Y		
Fa	(F5)	6.28	7.00	7.15	6.24	6.98	7.13
Fb	F106	6.43	6.88	7.26	6.24	6.90	7.28
Fc	F94	7.10	7.35	7.55	7.10	7.35	7.54
Ya	Y111	6.18	6.72		6.19	6.70	
Yb	Y52	6.34	6.74		6.34	6.74	
Yc	Y73	6.55	6.78		6.54	6.77	
Yd	Y75	6.52	6.95		6.52	6.95	
Ye	Y69	6.92	7.20		6.92	7.21	
W	W3	7.30	7.34	7.48	7.30	7.34	7.48
		7.58			7.58		
Xa	(F22)	6.75	7.04				
Xb	(Y28)	6.49	7.15		6.48	7.15	
ID	(I9)	0.05			0.03		
L1	(L41)	0.07			0.05		
L2	L58	0.63	0.44		0.62	0.43	
IG	I95	0.94			0.94		
A1	(A55)	1.56			1.56		
M	(M8)	1.86			1.85		

tion. As expected, the rat pancreatic PLA_2 could completely inhibit the generation of the 60-kDa band, although the potency ($IC_{50} = 10$ μM) was lower than that of the F22Y mutant of the bovine PLA_2. This may be due to the differences between the rat and the bovine enzymes at other areas. As would be expected, we found that the rat PLA_2 was nontoxic when tested as described above.

Although further work is needed to extend our findings for a generalization concerning the binding of the neurotoxic PLA_2s, it would be useful to put forward some suggestions for further testing. It has not been possible for us to study the binding of all variants of PLA_2, but available data seem to indicate that the 45-kDa polypeptide may be a common binding protein, or one of its subunits, for most neurotoxic PLA_2s, and strong binding to this polypeptide appears to be linked to neurotoxicity (see Ref. 1 for review; Refs. 11 and 13). Significantly, examination of the aligned sequences of the PLA_2 chains from various sources (38, 39) revealed that the active PLA_2 chain of each of the PLA_2 neurotoxins has Tyr at the position corresponding to Phe[22] of the bovine enzyme in their aligned sequences (it is position 21 for many PLA_2 chains, including subunit B of crotoxin), whereas those with Phe are nontoxic or marginally toxic. Our present results would suggest that Tyr[22] in these neurotoxic PLA_2 variants is also an important determinant for such binding and that other as yet undefined determinants are also involved. On such ground, one may resolve the seeming conflict that, though a small number of PLA_2s with Tyr[22] exhibit low toxicity, most of them are potent toxins. With Tyr[22] in its PLA_2 chain, β-bungarotoxin is to date the only neurotoxic PLA_2 that does not bind to the 45-kDa polypeptide. This can be explained by steric hindrance due to another polypeptide covalently bonded to its PLA_2 chain, an interpretation supported by the insusceptibility of this residue to chemical modification (40). As to the other determinants for binding, a definitive answer has not been obtained for any of the PLA_2 variants, but some information is available (see Ref. 41 for review). Based on comparisons of the amino acid sequences of the PLA_2 variants with presynaptic toxicity and those of the nontoxic ones, basic residues around position 59, at position 69, around position 76, and at position 93 (or 94) and the segments of residues 1–7, 68–85, and 80–110 (all numbered with respect to pancreatic PLA_2 according to Ref. 42) have been proposed to be involved in binding (43, 44). From the variations in amino acid sequences of the three ammodytoxins, Tyr[115], Arg[118], and Lys[128] have been suggested to participate in binding (45, 46).

Chemical modification and other studies indicate that Trp[118], Tyr[7], Tyr[76] and Tyr[83] of notexin, Tyr[73] of the PLA$_2$ chain of β-bungarotoxin, and Asn[1], Met[8], Lys[58], and Lys[92] of Pa-11 are probably needed for their neurotoxicity, but binding experiments have not been performed except for some modified Pa-11 (40, 47–50). Extension of the site-directed mutagenesis studies that we have reported here will hopefully provide a clear-cut answer.

In summary, we have identified Tyr[22] as one of the important determinants for the neurotoxicity of PLA$_2$ proteins. The Phe[22] of bovine pancreatic PLA$_2$ is located at the B-helix, away from the active site and exposed to solvent. It does not play an important structural role because mutations at this position cause only minor and localized structural perturbations. The F22Y is a silent mutation catalytically, but it acquires the ability to compete with crotoxin (a neurotoxic PLA$_2$) for binding to its receptor protein with an IC$_{50}$ value of 1 μM. Although such a binding affinity is still too low for *in vivo* neurotoxicity (the affinity of crotoxin for binding to the same receptor protein is <10 nM), the results have demonstrated a dramatic effect that can possibly be induced by a point mutation and have shed light on how the small PLA$_2$ protein (14 kDa for pancreatic enzymes) could give rise to hundreds of natural variants displaying a great variety of pharmacological actions.

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