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Role of the *N*-terminal region of phospholipase A_2 subunit of β_1-bungarotoxin in the toxin–Ca^{2+} complex-formation

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β_1-Bungarotoxin consists of a phospholipase A_2 subunit and a non-phospholipase A_2 subunit. Modification of β_1-bungarotoxin with CNBr resulted in cleavage at Met-6 and Met-8 of its phospholipase A_2 subunit. Analysis of the fluorescence data of both the toxin–Ca^{2+} complex at 300–350 nm and the toxin–Tb^{3+} complex at 450–650 nm showed the existence of two binding sites for both metal ions on the different domains of the toxin molecule. At pH 7.6 the association constants for the high-affinity and low-affinity sites of the toxin–Ca^{2+} complex were determined to be $2.79 \times 10^3 \pm 0.21 \times 10^3$ M^{-1} and $0.47 \times 10^3 \pm 0.06 \times 10^3$ M^{-1} respectively. For the toxin–Tb^{3+} complex the association constant for the high-affinity site was $2.95 \times 10^3 \pm 0.43 \times 10^3$ M^{-1} and that for the low-affinity site was $0.11 \times 10^3 \pm 0.03 \times 10^3$ M^{-1}. Removal of the *N*-terminal octapeptide of the phospholipase A_2 subunit from the toxin molecule caused disintegration of the low-affinity site but did not disrupt the high-affinity site. This might accompany a change in the configuration around His-48 of the phospholipase A_2 subunit. Between pH 6 and 8 the binding of metal ions to the high-affinity site increased but that to the low-affinity site did not change with increasing pH. The neurotoxicity and enzymic activity of the toxin were lost on removal of the low-affinity site.

INTRODUCTION

The presynaptically active toxins in the venoms of snakes belonging to Elapidae, Crotalidae and Viperidae are very potent in blocking neuromuscular transmission (Chang, 1985). β_1-Bungarotoxin (β_1-BuTX), the main component of β-bungarotoxins in the venom of the Taiwan banded krait (*Bungarus multicinctus*) (Chen *et al.*, 1982), is a representative of this kind of toxin. It induces firstly facilitation and irreversible disruption of acetylcholine release from the cholinergic synapses before the blockade of neuromuscular transmission. Since this action is very specific and unique, the toxin has been exploited as an important tool or probe in physiological, biochemical and pharmacological studies.

β_1-BuTX consists of two subunits linked by a disulphide bond (Kondo *et al.*, 1978). One is a phospholipase A_2 (PLA_2) (EC 3.1.1.4) subunit having 120 amino acid residues, which shows a remarkable degree of structural similarity to other vertebrate PLA_2 enzymes (Kini & Evans, 1987). The other is a rather small subunit with 60 amino acid residues. Its role in the neurotoxic effect is currently unclear, despite the fact that it shows sequence similarity to dendrotoxin and toxins I and K from the venom of mambas, which are representative of Kunitz proteinase inhibitors (Dufton, 1985) and themselves show facilitation of transmitter release, interference with the binding of native β_1-BuTX in chick muscle and enhancement of the presynaptic effect of crotoxin and notexin (Harvey & Karlsson, 1982).

β_1-BuTX has an absolute requirement for Ca^{2+} as a cofactor for PLA_2 activity, which is believed to play an essential role in the neurotoxicity (Kelly *et al.*, 1976) in spite of the theory that the lethal toxicity of β_1-BuTX is not necessarily associated with its enzymic activity (Yang & Lee, 1986). Establishing the nature of the Ca^{2+}-binding domains in the β_1-BuTX molecule is important in elucidating the structure–function relationship of the protein molecule. The main goal of the present work was to establish the role of the *N*-terminal region of the PLA_2 subunit in the toxin–Ca^{2+} complex-formation.

EXPERIMENTAL

Materials

Crude venom of the Taiwan banded krait was supplied by Chen Hsin Tong Chemical Co., Taipei, Taiwan. CM-Sephadex C-25 and Sephadex G-50 were obtained from Pharmacia, Uppsala, Sweden. $TbCl_3$ was purchased from Alfa Products, Beverley, MA, U.S.A. All other chemicals were of reagent grade.

Preparation of β_1-BuTX and modification with CNBr

β_1-BuTX was isolated from the crude venom on a CM-Sephadex C-25 column (Chen *et al.*, 1982) and purified further on a Sephadex G-50 column (Lin *et al.*, 1984). It was cleaved with CNBr according to the method of Gross & Witkop (1962). The reaction mixture was dialysed against water to remove reagent and the small peptide fragment.

Assay of PLA_2 activity and toxicity

β_1-BuTX released the fatty acid from 10 μmol of egg phosphatidylcholine suspended in 4 ml of 5 mM-deoxycholate at 37 °C. The amount of fatty acid was measured by H^+ titration, which was carried out with a Radiometer pHM8 standard pH-meter attached to a T80 titrator and an ABU80 autoburette. One unit of PLA_2 activity was defined as the release of 1 μequiv. of fatty acid from phospholipid/min.

Eight albino mice weighing 15–20 g were injected intraperitoneally with β_1-BuTX or CNBr-β_1-BuTX. The 50% lethal dosage (LD_{50}) was determined according to a previously described method (Litchfield & Wilcoxon, 1949).

Measurement of c.d. and fluorescence

The concentration of β_1-BuTX was determined from the absorbance at 280 nm, by using $A^{1\%}_{1\,cm} = 12.5$ (Lin *et al.*, 1984).

Abbreviations used: β_1-BuTX, β_1-bungarotoxin; CNBr-β_1-BuTX, the CNBr-modified β_1-bungarotoxin; PLA_2, phospholipase A_2.

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The c.d. was measured with a Jasco J-20 spectropolarimeter under constant flushing with N_2 at room temperature. The mean residue elipticity, $[\theta]$, was estimated from the mean residue weight, which was calculated from the amino acid composition. The fluorescence intensity, expressed in arbitrary units, was measured at room temperature with a Hitachi F-4000 fluorescence spectrophotometer. Both the excitation and the emission slit width were 10 nm. Raman emission due to the scattering of solvent was minimized by adjusting the intensity scale. In the scanning of an emission spectrum of Tb^{3+} in the presence of protein in 0.02 M-Pipes buffer, the solution was freshly prepared. It took no more than 5 min to scan a spectrum to prevent precipitation. $F_{\lambda_1}^{\lambda_2}$ represented the fluorescence intensity at wavelength λ_2 (nm) when the fluor was excited at wavelength λ_1 (nm).

Analyses of fluorescence data

The modified Scatchard plot (Epstein *et al.*, 1974) was constructed to analyse the fluorescence data of a complex formed by a metal ion and β_1-BuTX or CNBr-β_1-BuTX:

$$|\Delta F|/[L]_{free} = (K_L \cdot F_\infty) - (K_L \cdot |\Delta F|) \quad (1)$$

where ΔF is the change in protein fluorescence on adding metal ion, L, and F_∞ the protein fluorescence in the absence of metal ion. In the analysis of Tb^{3+} fluorescence, ΔF is the change in Tb^{3+} fluorescence in the presence of protein, and F_∞ the fluorescence at infinite Tb^{3+} concentration. K_L is the association constant of the complex concerned. Throughout the titration, $|\Delta F|/[L]_{total}$ was plotted against $|\Delta F|$, since $[L]_{free}$ was close to $[L]_{total}$.

RESULTS

Modification of β_1-BuTX with CNBr

There are two methionine residues (Met-6 and Met-8 of the PLA_2 subunit) in the β_1-BuTX molecule. Cleavage of the toxin with CNBr yielded a modified toxin, CNBr-β_1-BuTX, which was devoid of methionine or homoserine and its lactone derivative, as shown in the analysis of amino acid composition given in Table 1. A comparison of the amount of each amino acid in the modified toxin with the corresponding amount in the native toxin revealed the removal of a peptide fragment comprising $Asx_2Glx_1Phe_1Ile_1Leu_1Met_2$ from β_1-BuTX by the chemical modification. The octapeptide turns out to be the rather hydrophobic NLINFMEM of the *N*-terminal region of the PLA_2 subunit. Apparently, all of 14 tyrosine and one tryptophan (Trp-19 of PLA_2 subunit) residues of β_1-BuTX remained in CNBr-β_1-BuTX.

β_1-BuTX showed weak PLA_2 activity but very potent neurotoxicity. Its enzymic activity in 10 mM-$CaCl_2$ at 37 °C was 115 units/mg of toxin and its LD_{50} was assayed to be 0.017 μg/g body wt. of mice. On the other hand, CNBr-β_1-BuTX showed no enzymic activity and caused no lethality even at a dose as high as 2 μg/g body wt. of mice.

Intrinsic fluorescences of the two proteins

The effect of $CaCl_2$ on the characteristics of intrinsic fluorescence of the aromatic residues in the two protein molecules in 0.02 M-Pipes buffer at pH 7.6 was compared.

Fig. 1(*a*) shows the emission spectra when the proteins were excited at 275 nm. Both β_1-BuTX and CNBr-β_1-BuTX exhibited a shoulder at 300–310 nm in addition to a main peak at 339 nm, but CNBr-β_1-BuTX produced less fluorescence intensity (Fig. 1*a*). The shoulder is contributed by tyrosine residues that do not transfer their resonance energy to Trp-19, and the peak arises from Trp-19, which is excited directly with absorption energy or indirectly through resonance energy transferred from the proximate tyrosine residue(s). Apparently, in both protein molecules the tyrosine residues contributing the shoulder are probably in the domains separable from Trp-19 and its neighbouring tyrosine residue(s) for the peak. Addition of $CaCl_2$ to the β_1-BuTX solution caused a considerable decrease in both F_{275}^{305} and F_{275}^{339} (Fig. 1*a*). The Ca^{2+}-induced change in F_{275}^{305} might reflect the characteristic of one Ca^{2+}-binding site and that of F_{275}^{339} the

Table 1. Amino acid composition of CNBr-β_1-BuTX

All values are expressed as molar ratios with respect to leucine. The values in parentheses give the amino acid composition from the primary structure of β_1-BuTX.

Amino acid	Composition
Asx*	20.5 (22)
Thr	11.9 (12)
Ser	4.3 (4)
Glx†	11.3 (12)
Pro	6.9 (7)
Gly	16.7 (17)
Ala	11.2 (11)
Cys	– (20)
Val	3.8 (4)
Met	0.2 (2)
Ile	8.1 (9)
Leu	6.0 (7)
Tyr	14.2 (14)
Phe	4.9 (6)
Lys	12.7 (13)
His	4.9 (5)
Arg	13.9 (14)
Trp‡	0.9 (1)
Homoserine + homoserine lactone	0.0

* Fifteen aspartic acid and seven asparagine residues from the primary structure of β_1-BuTX.

† Seven glutamic acid and five glutamine residues from the primary structure of β_1-BuTX.

‡ Tryptophan content was determined by a u.v.-absorption method (Scoffone & Fontana, 1975).

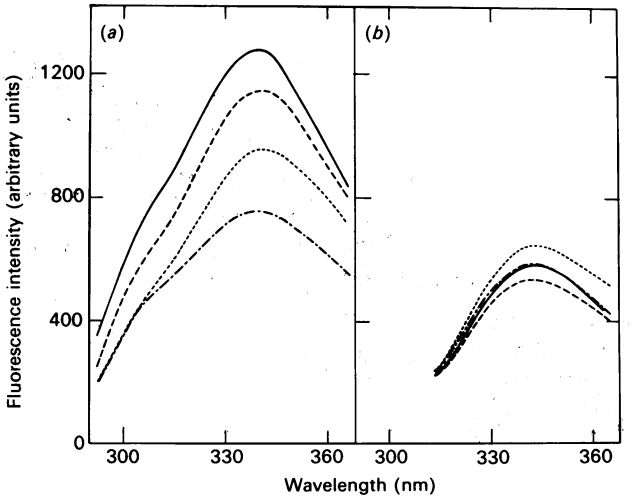

Fig. 1. Fluorescence emission spectra of β_1-BuTX and CNBr-β_1-BuTX in 0.02 M-Pipes at pH 7.6

The emission spectra were scanned with the excitation wavelength at 275 nm (*a*) or 295 nm (*b*). Both proteins were at 5 μM: ——, β_1-BuTX alone; – – –, β_1-BuTX and 10 mM-$CaCl_2$; ------, CNBr-β_1-BuTX alone; – – –, CNBr-β_1-BuTX and 10 mM-$CaCl_2$.

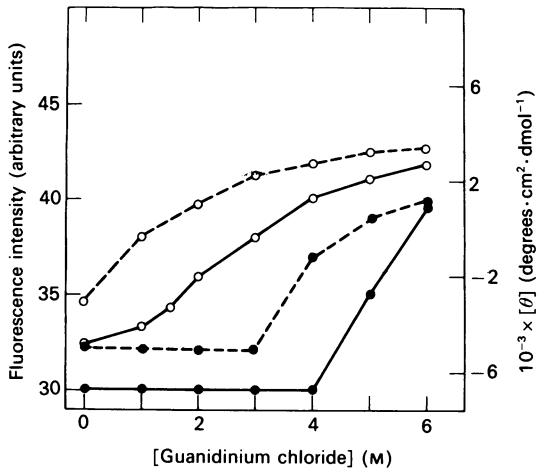

Fig. 2. Flexibility of tryptophan configuration and polypeptide backbone folding of β_1-BuTX and CNBr-β_1-BuTX in guanidinium chloride solution

Both proteins at 5 μM were used in the fluorescence study. Fluorescence was measured at 339 nm (○) and c.d. was measured at 222 nm (●): ——, β_1-BuTX; ----- CNBr-β_1-BuTX.

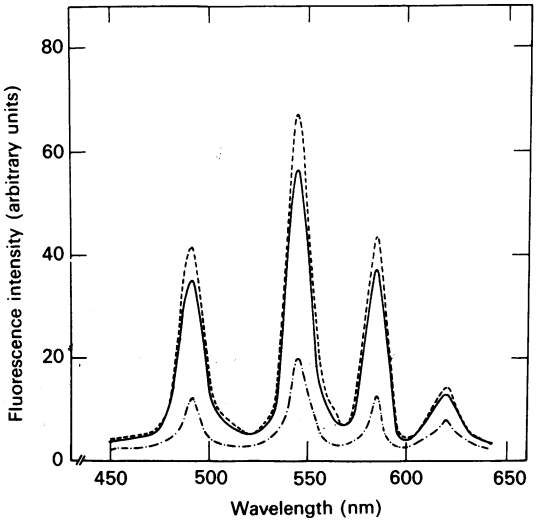

Fig. 3. Emission spectra of Tb^{3+} in the presence of β_1-BuTX or CNBr-β_1-BuTX in 0.02 M-Pipes at pH 7.6

The spectra were scanned with the excitation wavelength at 295 nm. Tb^{3+} was at 1 mM and each protein was at 5 μM: ·–·–·, Tb^{3+} alone; ——, Tb^{3+} and β_1-BuTX; ------, Tb^{3+} and CNBr-β_1-BuTX.

characteristic of the other Ca^{2+}-binding site. The former was lost but the latter remained when the octapeptide from the *N*-terminus of the PLA_2 subunit was removed from the toxin molecule, as shown by the fact that addition of $CaCl_2$ to the CNBr-β_1-BuTX solution diminished F_{275}^{339} considerably but had no effect on F_{275}^{305} (Fig. 1*a*).

The two proteins showed similar emission spectra arising from excitation of tryptophan at 295 nm (Fig. 1*b*). Both proteins exhibited a peak at 339 nm with a slight difference in fluorescence intensity at each wavelength. The peak shifted to 346 nm when β_1-BuTX was in 6 M-guanidinium chloride and to 349 nm when the protein disulphide bonds were broken with dithiotheritol (results not shown). Similar behaviour occurred with the peak of CNBr-β_1-BuTX. Apparently, the sole tryptophan residue of both proteins is restricted into a configuration that differs from

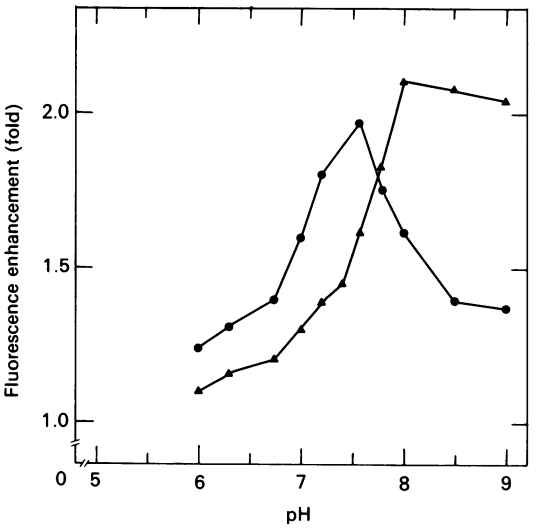

Fig. 4. Enhancement of Tb^{3+} fluorescence induced by either β_1-BuTX or CNBr-β_1-BuTX in 0.02 M Pipes buffer at pH 6–9

Fluorescence enhancement is represented by a ratio of Tb^{3+} (1 mM) fluorescence in the presence of each protein (5 μM) to the fluorescence in the absence of protein: ▲, β_1-BuTX; ●, CNBr-β_1-BuTX.

that of free tryptophan in aqueous solution. The F_{295}^{339} of both proteins was diminished by addition of $CaCl_2$ to the protein solution.

We compared further the change in both tryptophan fluorescence and $[\theta]_{222}$ of the two proteins in 0–6 M-guanidinium chloride (Fig. 2). The β_1-BuTX fluorescence changed slightly at at concentrations less than 1.5 M-guanidinium chloride but increased markedly from 2 M- to 4 M-guanidinium chloride and levelled off at 5–6 M-guanidinium chloride. In contrast, the CNBr-β_1-BuTX fluorescence increased gradually in 0–3 M-guanidinium chloride and levelled off at the concentrations higher than 3 M-guanidinium chloride.

Both proteins gave double minima at 220–222 and 208–210 nm in the c.d. between 200 and 250 nm, indicating the existence of a helical structure in the protein molecules. The magnitudes of $[\theta]_{222}$ and $[\theta]_{209}$ of β_1-BuTX were -6700 and -8400 degrees·cm²·dmol^{-1} respectively, and those of CNBr-β_1-BuTX were -5200 and -6200 degrees·cm²·dmol^{-1} respectively. The helix–coil transition as represented by the change in $[\theta]_{222}$ occurred in 4–6 M- and 3–6 M-guanidinium chloride for β_1-BuTX and CNBr-β_1-BuTX respectively. The structure of the two proteins in 6 M-guanidinium chloride became an unordered form that gave an average mean residue ellipiticity of 1000 degrees·cm²·dmol^{-1}. On the basis of a simple method for calculating the helical content of a protein from its c.d. spectra (Chen *et al.*, 1972, 1974), the difference between the helical contents of the two proteins could be implied in a ratio of $([\theta]_{222}-1000)_{\text{CNBr-}\beta_1\text{-BuTX}}$ to $([\theta]_{222}-1000)_{\beta_1\text{-BuTX}}$, where $[\theta]_{222}$ is measured in the absence of guanidinium chloride. The ratio was estimated to be 0.8, revealing that 80% of the helical structure of β_1-BuTX remains in CNBr-β_1-BuTX.

Enhancement of Tb^{3+} fluorescence by the two proteins

The emission spectrum of Tb^{3+} at pH 7.6 showed a distinct quartet between 450 and 650 nm (Fig. 3). Both β_1-BuTX and CNBr-β_1-BuTX were able to enhance the Tb^{3+} fluroescence (Fig. 3).

The enhancement of Tb^{3+} fluorescence by the two proteins at pH 6–9 was compared (Fig. 4). The fluorescence enhancement was represented in terms of the ratio of F_{295}^{545} in the presence of

Table 2. Association constants for the formation of the complex between β_1-BuTX and metal ion

The association constants were determined by the two methods discussed in the text. K_{a1} and K_{a2} are the association constants for the high-affinity and low-affinity sites respectively.

	$10^{-3} \times Ca^{2+}$ binding K_a (M)			$10^{-3} \times Tb^{3+}$ binding K_a (M)		
	β_1-BuTX		CNBr-β_1-BuTX	β_1-BuTX		CNBr-β_1-BuTX
pH	K_{a1}*	K_{a2}	K_a	K_{a1}	K_{a2}	K_a
8.0	3.60±0.49	0.45±0.07				
7.6	2.79±0.21	0.47±0.06	0.56±0.06	2.95±0.43	0.11±0.03	0.67±0.07
6.8	0.63±0.05	0.46±0.06				
6.5	0.26±0.05	0.49±0.06	0.12±0.04	0.30±0.04	N.D.†	0.21±0.05

* The average value determined from the two modified Scatchard plots shown in Fig. 5.
† It could not be determined precisely.

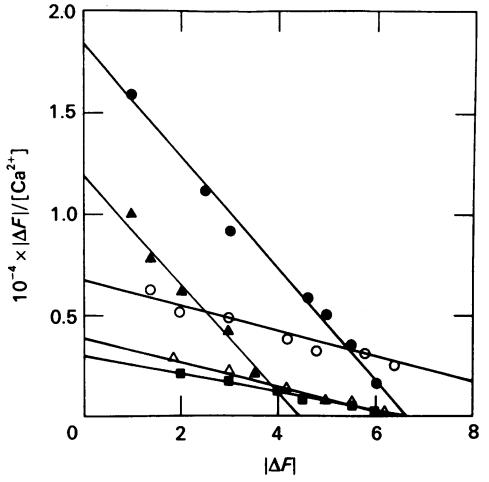

Fig. 5. Modified Scatchard plot for the binding of Ca^{2+} to either β_1-BuTX or CNBr-β_1-BuTX

At pH 7.6 and room temperature, each protein at 5 μM was titrated with the same solution in the presence of 0.1 M-$CaCl_2$. The fluorescence data were analysed by using eqn. (1) with linear-regression fitting. The correlation coefficient was calculated to be more than 0.95 for each curve. ■, F_{275}^{305} for β_1-BuTX; ●, F_{275}^{339} for β_1-BuTX; ▲, F_{295}^{339} for β_1-BuTX; ○, F_{275}^{339} for CNBr-β_1-BuTX; △, F_{295}^{339} for CNBr-β_1-BuTX.

protein to that in the absence of protein. β_1-BuTX enhanced Tb^{3+} fluorescence slightly at pH 6–7; this was followed by a marked increased at pH 7–8 and then a levelling off at pH 8–9 (Fig. 4). The midpoint of the change was at pH 7.5. CNBr-β_1-BuTX increased the Tb^{3+} fluorescence gradually below pH 6.7 and enhanced the fluorescence markedly from pH 6.7 to pH 7.6, giving the midpoint at pH 7.0, but the enhancement diminished gradually from pH 7.6 to 9.0 showing the midpoint at pH 7.9 (Fig. 4).

Characterization of the Ca^{2+}-binding sites on β_1-BuTX

The association constants of the complex-formation are summarized in Table 2 for comparison. The fluorescence data obtained from adding Ca^{2+} to the β_1-BuTX or the CNBr-β_1-BuTX solution were analysed using eqn. (1). At pH 7.6 a linear curve was obtained in the modified Scatchard plot for each of the five cases shown in Fig. 5. In the β_1-BuTX solution, the linear curve for the effect of Ca^{2+} on F_{275}^{339} was nearly parallel with that for the effect of Ca^{2+} on F_{295}^{339} (Fig. 5). These two curves appeared to characterize the same Ca^{2+}-binding site. The association constant for this affinity site was estimated to be $2.79 \times 10^3 \pm 0.21 \times 10^3$ M^{-1}. Another Ca^{2+}-binding site was characterized by the effect of Ca^{2+} on F_{275}^{305} (Fig. 5). The association constant for this affinity site was calculated to be $0.47 \times 10^3 \pm 0.06 \times 10^3$ M^{-1}. Apparently, β_1-BuTX has two distinct Ca^{2+}-binding sites. At pH 6–8 the binding of Ca^{2+} to the low-affinity site was pH-independent whereas that to the high-affinity site was pH-dependent: the lower the pH value, the weaker the affinity (Table 2).

In the CNBr-β_1-BuTX solution, only one Ca^{2+}-binding site could be determined from the effect of Ca^{2+} on F_{275}^{339} or F_{295}^{339} (Fig. 5), but no Ca^{2+}-binding site could be detected by the effect of Ca^{2+} on F_{275}^{305}. As for the high-affinity site of β_1-BuTX, the binding of Ca^{2+} to CNBr-β_1-BuTX was weaker at the lower pH value. The association constant for the CNBr-β_1-BuTX–Ca^{2+} complex was determined to be $0.56 \times 10^3 \pm 0.06 \times 10^3$ M^{-1} at pH 7.6 and $0.12 \times 10^3 \pm 0.04 \times 10^3$ M^{-1} at pH 6.5 from the modified Scatchard plot.

Tb^{3+}, a tervalent lanthanide ion, has been demonstrated to be an excellent probe for studying the binding domain of Ca^{2+} in protein molecules (Epstein *et al.*, 1974; Wang *et al.*, 1981). Our previous work showed that the fluorescence studies on the binding of Tb^{3+} to β_1-BuTX are relevant to Ca^{2+} binding (Chu & Chen, 1989). At pH 7.6 β_1-BuTX or CNBr-β_1-BuTX, both at 5.0 μM, was titrated with the same protein concentration in the presence of $TbCl_3$. The fluorescence data were analysed by using eqn. (1). A concave curve was obtained for β_1-BuTX in the modified Scatchard plot (Fig. 6), revealing the existence of multiple binding sites of Tb^{3+} on the β_1-BuTX molecule. Calculation on the basis of the existence of two distinct Ca^{2+}-binding sites on β_1-BuTX as proved above gave the association constant of the high-affinity site to be $2.95 \times 10^3 \pm 0.43 \times 10^3$ M^{-1} and that of the low-affinity site to be $0.11 \times 10^3 \pm 0.03 \times 10^3$ M^{-1}. These two values had the same order of magnitude as those determined for the β_1-BuTX–Ca^{2+} complex. Binding of Tb^{3+} to the low-affinity site was prominent only at high molar ratio of Tb^{3+} to β_1-BuTX, under which condition the enhancement of Tb^{3+} fluorescence was so small that the determination for the association constant was less accurate. On the other hand, a linear curve was obtained for CNBr-β_1-BuTX in the modified Scatchard plot, indicating a single binding site of Tb^{2+} on the CNBr-β_1-BuTX molecule (Fig. 6). The association constant was estimated to be $0.67 \times 10^3 \pm 0.07 \times 10^3$ M^{-1}. At pH 6.5, the binding strength of Tb^{3+} to the high-affinity sites of both β_1-BuTX and CNBr-β_1-BuTX decreased to a great extent. The association constant for the high-affinity site on β_1-BuTX decreased to $0.30 \times 10^3 \pm 0.04 \times 10^3$ M^{-1} and that for the CNBr-β_1-BuTX–Tb^{3+} complex was down to $0.21 \times 10^3 \pm 0.05 \times 10^3$ M^{-1}. Estimation of the association

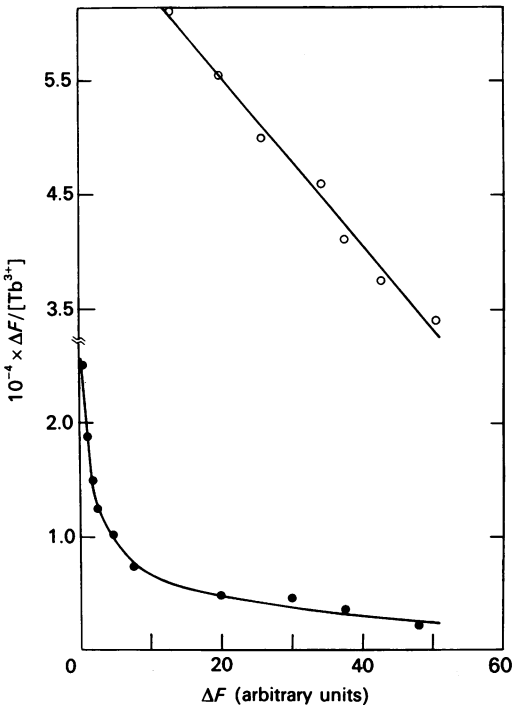

Fig. 6. Modified Scatchard plot for the binding of Tb^{3+} to either β_1-BuTX or CNBr-β_1-BuTX

At pH 7.6 and room temperature, each protein at 5 μM was titrated with the same solution in the presence of 0.1 M-$TbCl_3$. The fluorescence data were fitted to eqn. (1) with linear regression. The correlation coefficients of the curves for the high-affinity site and the low-affinity site on β_1-BuTX (●) were 0.97 and 0.94 respectively. The correlation coefficient of the curve for the site on CNBr-β_1-BuTX (○) was 0.98.

constants at pH 6.5 was less accurate, since the Tb^{3+} fluorescence enhanced by the proteins at pH 6.5 was less than that at pH 7.6. Under the circumstances, a precise determination of the association constant for the low-affinity site was not feasible.

DISCUSSION

Previously, the multiple Ca^{2+}-binding sites on the β_1-BuTX molecule were independently revealed by a Scatchard plot obtained by a binding assay with the use of radioactive Ca^{2+} (Abe *et al.*, 1977) or by the effect of Ca^{2+} on the absorption spectrum of β_1-BuTX (Yang & Lee, 1986). However, these two approaches are limited to exploring the configuration around the Ca^{2+}-binding domain. For one thing, Ca^{2+} lacks useful spectroscopic properties that can be employed in probing the nature of its binding site. For another, the Scatchard plot for each of the two approaches gives no information about each Ca^{2+}-binding domain. These two problems are overcome to a certain extent by the analytical methods used in the present work, which enables us to define the two distinct Ca^{2+}-binding sites on the different domains of the β_1-BuTX molecule. This is useful in elucidating the structural features of the β_1-BuTX–Ca^{2+} complex-formation. Our results indicate clearly that: (1) there are two distinct Ca^{2+}-binding sites on the different domains of β_1-BuTX; (2) the *N*-terminal region of the PLA_2 subunit plays an essential role in maintaining the integrity of the low-affinity site, which seems to be necessary for the neurotoxicity and enzymic activity towards the aggregated substrate.

A comparison of the tertiary structures of bovine pancreatic PLA_2 and *Crotalus atrox* PLA_2 shows that the main-chain conformations of the two molecules are very similar (Dijkstra *et al.*, 1978, 1983; Keith *et al.*, 1981). One Ca^{2+}-binding loop and six segments in both proteins, which comprises more than 83% of the total amino acid residues of the molecules, have a nearly superimposable backbone (Renetseder *et al.*, 1985). These six similar segments may be taken as a single rigid unit to represent the 'homologous core' structure that may be globally preserved in vertebrate PLA_2 enzymes. The PLA_2 subunit of β_1-BuTX contains highly conserved amino acid residues and sequences characteristic of all PLA_2 molecules sequenced to date (van Scharrenberg *et al.*, 1982; Dijkstra *et al.*, 1984; Achari *et al.*, 1987). In the absence of an X-ray structure for β_1-BuTX, we have superimposed the six segments of similar sequence and the Ca^{2+}-binding loop predicted in β_1-BuTX on to the main-chain conformation of bovine pancreatic PLA_2 and *C. atrox* venom PLA_2 and made some educated guesses. The plausible three-dimensional network (Fig. 7) together with the spectral properties shown in this work shed some light on several important structural features.

The c.d. characteristics shown in Fig. 2 suggest a stable tertiary structure of β_1-BuTX in a segmental sense. The *N*-terminal region (residues 1–12) of the PLA_2 family is a segment of similar sequence in helical conformation (Dijkstra *et al.*, 1978), which is believed to be part of the so-called 'interface recognition site' that requires the presence of Ca^{2+} in the interaction process with the micellar lipid/water interface (van Dam-Mieras *et al.*, 1975). The active conformation of PLA_2 for the aggregated substrate is preserved in a hydrogen-bonded network (Renetseder *et al.*, 1985), which in the β_1-BuTX PLA_2 subunit may involve invariant His-48, Tyr-52, Pro-63, Tyr-68, Asp-94 and the *N*-terminal region (Fig. 8). Either Tyr-52 or Tyr-68 is distant from Trp-19 (Fig. 7). Considering the fluorescence characteristics shown in this work, we suspect the participation of the phenolic group of Tyr-52 and/or Tyr-68 in the low-affinity site. His-48 is restricted and its imidazole group may have an abnormal pK_a at 7.5 (Fig.

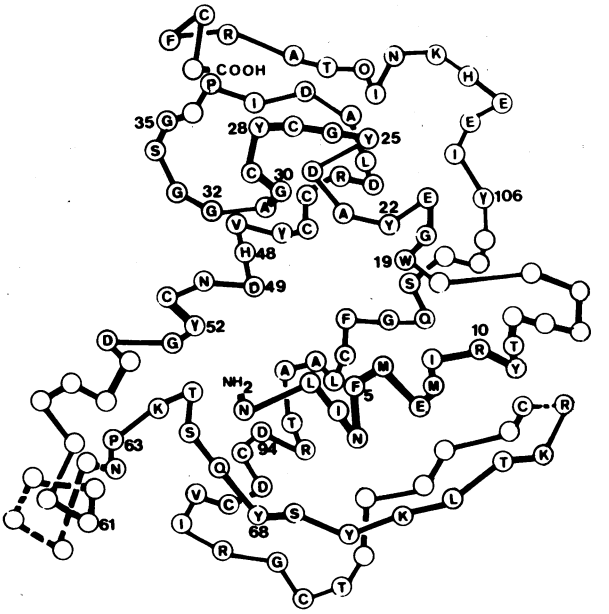

Fig. 7. Superimposition of the primary structure of PLA_2 subunit of β_1-BuTX on to the main conformation of bovine pancreatic PLA_2 (Dijkstra *et al.*, 1978)

The broken line indicates the main points of variance between pancreatic and *Crotalus atrox* PLA_2 enzymes. The amino acid residues predicted for each analogous segment and the Ca^{2+}-binding loop as described in the text are denoted.

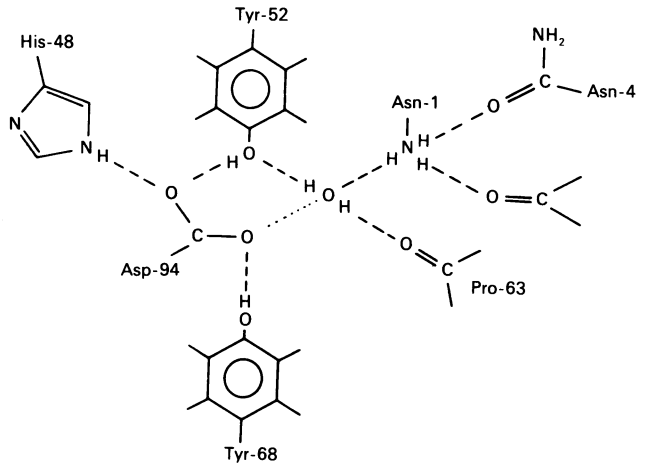

Fig. 8. Schematic representation of a proposed hydrogen-bonded network in the β_1-BuTX

The network is drawn according to that described by Dijkstra *et al.* (1981).

4). The pK_a shifts to a normal value at pH 7.0 (Fig. 4), as the restriction exerted on His-48 may be released by removal of the *N*-terminal region, which does not contact directly with His-48. This causes disintegration of the low-affinity site, which may be not affected by the charged status of His-48 in native toxin (Table 2).

The high-affinity site resides in a 'Ca^{2+}-binding loop' running from residue 25 and 42, which are highly conserved glycine-rich sequences characteristic of PLA_2 molecules (Dijkstra *et al.*, 1981). An oxygen cage that sequesters the metal ion is stabilized by this loop (Dijkstra *et al.*, 1981). In the β_1-BuTX molecule the oxygen cage may be formed in part from the three main-chain oxygen atoms at Tyr-28, Gly-30 and Gly-32 and the carboxylate group of Asp-49. The phenolic group of Tyr-28 is excluded as a Ca^{2+} ligand on the basis of the conclusion drawn from an n.m.r. study on pig and bovine PLA_2 molecules (Fisher *et al.*, 1989). This is reflected in the fact that the high-affinity site is not characterized by the effect of Ca^{2+} on F_{275}^{305}. In CNBr-β_1-BuTX only the high-affinity site is apparently stabilized by a group with pK_a 7.9, as suggested from the characteristics shown in Fig. 4. This charged group might be ascribed to the α-amino group of Arg-1 of the non-PLA_2 subunit, which might interact with the *N*-terminal region of the PLA_2 subunit. The Ca^{2+}-binding loop may not be so rigid that binding of Ca^{2+} to the high-affinity site causes a change in local conformation around Trp-19 (Fig. 1*b*). Tyr-22 and Tyr-106, both of which are close to Trp-19, are separated from the two Ca^{2+}-binding sites (Fig. 7). Resonance energy transfer from these two tyrosine residues to Trp-19 accounts partly for F_{275}^{339}.

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