Studies on the status of lysine residues in phospholipase A₂ from *Naja naja atra* (Taiwan cobra) snake venom

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Phospholipase A₂ (PLA₂) from *Naja naja atra* (Taiwan cobra) snake venom was subjected to lysine modification with trinitrobenzene sulphonic acid (TNBS), and two major trinitrophenylated (TNP) derivatives, TNP-1 and TNP-2, were separated by h.p.l.c. TNP-1 contained only one TNP group on Lys-6 and showed a marked decrease in enzymic activity, but still retained 45 % of the lethal toxicity. Both Lys-6 and Lys-65 were modified in TNP-2, and modification of Lys-65 caused a further reduction of the lethal toxicity to 12.6 %. However, the antigenicity of both TNP-1 and TNP-2 remained unchanged. The reactivity of Lys-6 and Lys-65 toward TNBS was greatly enhanced by Ca²⁺ and dihexanoyl-lecithin, suggesting that the two Lys residues are not directly involved in the binding of Ca²⁺ and substrate. The modified derivatives retained their affinity for Ca²⁺, indicating that Lys-6 and Lys-65 did not participate in the Ca²⁺ binding. The TNP derivatives could be regenerated with hydrazine hydrochloride. The biological activities of the regenerated PLA₂ are almost the same as those of native PLA₂. These results indicate that Lys-6 and Lys-65 are important for the biological activities of PLA₂, and incorporation of a bulky TNP group on Lys-6 and Lys-65 might give rise to a distortion of the active conformation of PLA₂.

INTRODUCTION

Snake venom phospholipases A₂ (PLA₂; EC 3.1.1.4) show a remarkable degree of structural homology but differ greatly in enzymic activity, lethal toxicity and pharmacological properties (Harris, 1985; Rosenberg, 1986). However, the relationship of the lethal toxicity and pharmacological effects to the enzymic activity of PLA₂ is poorly understood.

Studies on PLA₂ enzymes from *Naja naja atra* and *Naja nigricollis* venoms revealed that their lethal potency was not related to their enzymic activity, and measurements of actual phospholipid hydrolysis in various organs *in vivo* did not correlate with the lethal potencies and other pharmacological actions of PLA₂ enzymes (Condrea *et al.*, 1980, 1981*a,b*; Fletcher *et al.*, 1980, 1981, 1982). Zan *et al.* (1983) and Barrington *et al.* (1984) also found that the pharmacological effects are not due to the lysophospholipids or free fatty acids liberated in the tissues as a result of PLA₂ enzymic action.

Yang *et al.* (1981, 1982), Condrea *et al.* (1981*c*) and Rosenberg *et al.* (1983) have demonstrated that the lethal toxicity and pharmacological effects of PLA₂ from *Naja naja atra* and *Naja nigricollis* snake venoms can be dissociated from the accompanying phospholipid hydrolysis. Carbamylation of an increasing number of lysine residues caused a marked decrease in lethal toxicity, but the enzymic activity was little affected. In contrast, modification of carboxylate groups of PLA₂ caused a loss of enzymic activity with less effect on pharmacological actions. These results suggest that there may be two distinct active sites in snake venom PLA₂, one primarily responsible for enzymic activity and the other associated with lethal toxicity and pharmacological effects. Modification of tyrosine residues in snake venom

PLA₂ (Yang *et al.*, 1985; Soons *et al.*, 1986) is also in good agreement with the above suggestion. However, which lysine residues are essential for the biological activities of snake venom PLA₂ has not been determined.

In this study, the Lys residues of *N. naja atra* PLA₂ were selectively modified with trinitrobenzene sulphonic acid (TNBS), and the modified derivatives were separated by h.p.l.c. Based on changes in physicochemical and biological properties after Lys modification, the possible role played by Lys residues in *N. naja atra* PLA₂ is discussed.

MATERIALS AND METHODS

PLA₂ from *Naja naja atra* (Taiwan cobra) venom was isolated and purified as previously described (Yang *et al.*, 1981). TNBS was purchased from Pierce Chemical Co. and hydrazine hydrochloride from Sigma Chemical Co. L- α -Dihexanoyl-lecithin (diC₆PC) was obtained from Applied Science Lab.; acetonitrile, trifluoroacetic acid (TFA), CaCl₂ and LiChrospher 100 RP-18 column (4 mm $\times$ 25 cm) were from E. Merck. SynChropak RP-P columns (4.6 mm $\times$ 25 cm and 21.5 mm $\times$ 25 cm) were purchased from SynChrom Inc. (Lafayette, IN, U.S.A.). All other reagents were of analytical grade.

Modification of amino groups with TNBS

PLA₂ was modified with TNBS according to the procedure described by Yang & Chang (1988). PLA₂ (3 μ mol) in 9 ml of 0.05 M-borate buffer (pH 8.6) was incubated with a 2-fold molar excess of TNBS. The reaction was allowed to proceed at 30 °C for 30 min and the mixture was desalted by passage through a column of Sephadex G-25 (2 cm $\times$ 100 cm) equilibrated with 0.2 M-

Abbreviations used: PLA₂, phospholipase A₂; TNBS, trinitrobenzenesulphonic acid; TNP, trinitrophenyl; diC₆PC, L- α -dihexanoyl-lecithin.

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acetic acid. The extent of trinitrophenylation was determined spectrophotometrically based on the molar absorption coefficient (ϵ) of $11\,500\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 345 nm in 0.1 M-HCl (Satake *et al.*, 1960). Modification of PLA₂ with TNBS was also carried out in the presence of 20 mM-Ca²⁺ and 1 mM-diC₆PC to study the influence of Ca²⁺ and substrate on trinitrophenylation.

The trinitrophenylated (TNP) derivatives were separated by h.p.l.c. on a SynChropak RP-P column (preparative, 21.5 mm × 25 cm) equilibrated with 0.1% TFA and eluted with a linear gradient of 30–50% acetonitrile for 80 min. The eluent was monitored at 280 nm.

Identification of the incorporated TNP groups

In order to determine the position(s) of the incorporated TNP group(s) in the sequence of PLA₂, the TNP derivatives were reduced and S-carboxymethylated (RCM) by the procedure described by Crestfield *et al.* (1963) followed by tryptic digestion. The RCM-protein was dissolved in 0.2 M-ammonium bicarbonate buffer (pH 7.8) and trypsin was added (40: 1, w/w). Digestion was carried out at 37 °C for 4 h and the hydrolysates were lyophilized.

The tryptic hydrolysates were separated by h.p.l.c. on a LiChrospher 100 RP-18 column (4 mm × 25 cm) equilibrated with 0.1% TFA and eluted with a linear gradient of 0–40% acetonitrile for 100 min followed by 40–50% acetonitrile for 10 min. Flow rate was 0.8 ml/min and the eluent was monitored at 345 nm to facilitate identification of TNP peptides. TNP peptide fractions were lyophilized for amino acid analysis and sequence determination.

Regeneration of TNP amino groups

Regeneration of the TNP amino groups was performed according to the procedure described by Takahashi *et al.* (1984). TNP derivatives (6 mg) were treated with 2.5 ml of 0.2 M-hydrazine hydrochloride (pH 9.0) at 35 °C. At time intervals of 1, 2, 4, 6 and 8 h, aliquots (0.5 ml) were removed. After desalting and lyophilization, appropriate amounts of each sample were taken for determination of enzymic activity, and also for monitoring the regeneration of TNP proteins with time by h.p.l.c.

Amino acid sequence analysis

TNP peptides were subjected to automated cycles of Edman degradation on an Applied Biosystems gas/liquid phase Model 470A sequencer equipped with an on-line Model 120A phenylthiohydantoin-amino acid analyser.

Other methods

Determination of PLA₂ activity, lethal toxicity and antigenicity, polyacrylamide-gel electrophoresis, amino acid analysis and Ca²⁺-induced difference spectroscopy were performed in essentially the same manner as previously described (Yang & Chang, 1984, 1988; Chang & Yang, 1988).

RESULTS

Trinitrophenylation of PLA₂ with TNBS

PLA₂ from *N. naja atra* snake venom was incubated with TNBS at pH 8.6 for 30 min. As shown in Fig. 1, two major TNP derivatives, TNP-1 and TNP-2, were separated by h.p.l.c. Polyacrylamide-gel electrophoresis revealed that both TNP-1 and TNP-2 are homogeneous (results not shown). Spectral determination of the extent of modification based on a molar absorption coefficient of 11 500 at 345 nm in 0.1 M-HCl indicated that one and two TNP groups had been incorporated into TNP-1 and TNP-2, respectively (Table 1).

Fig. 1. Separation of TNP-PLA₂ on a SynChropak RP-P column

The column (preparative, 21.5 mm × 25 cm) equilibrated with 0.1% TFA was eluted with a linear gradient of 30–50% acetonitrile for 80 min. The flow rate was 4 ml/min, and the eluent was monitored at 280 nm.

Table 1. Properties of modified PLA₂

	Incorporated TNP group(s)		Enzymic activity (%)	LD ₅₀ (% of native) (reciprocal)	Antigenic activity* (%)
	Number	Position			
Native PLA ₂	0	—	100	100	100
TNP-1	1	Lys-6	7.2	45	101
TNP-2	2	Lys-6 and Lys-65	4.0	12.6	99
Regenerated PLA ₂ †	0	—	98	100	100

* Antigenic activity was determined by a quantitative precipitin reaction as described by Yang & Chang (1984).

† The regenerated PLA₂ was purified by h.p.l.c. on a SynChropak RP-P column (4.6 mm × 25 cm) as shown in Fig. 5(b).

Identification of the positions of the incorporated TNP groups

Chromatographic analysis of the tryptic hydrolysates of RCM-TNP derivatives (Fig. 2) indicated that one (1a) and two (2a and 2b) TNP peptides were obtained from

Fig. 2. Separation of the tryptic TNP peptides from RCM-TNP-PLA₂.

Tryptic hydrolysates of RCM-TNP-PLA₂ were applied to a LiChrospher RP-18 column (4 mm × 25 cm) equilibrated with 0.1% TFA and eluted with a linear gradient of 10–40% acetonitrile for 100 min followed by 40–50% acetonitrile for 10 min. Flow rate was 0.8 ml/min and the eluent was monitored at 345 nm to facilitate identification of the TNP peptides.

TNP-1 and TNP-2 respectively. The results of amino acid analysis and sequence determination revealed that the tryptic peptides 1a and 2b have an identical sequence of Asn¹-Leu-Tyr-Gln-Phe-Lys(TNP)-Asn⁷ and 2a has a sequence of the Phe⁶⁴-Lys(TNP)-Thr-Tyr⁶⁷. Thus it is apparent that Lys-6 was modified in TNP-1, and both Lys-6 and Lys-65 were modified in TNP-2.

Influence of Ca²⁺ and substrate on modification of TNBS

Spectrophotometric monitoring of the modification of PLA₂ with TNBS indicated that the rate of trinitrophenylation increased markedly in the presence of Ca²⁺, and further accelerated with the addition of diC₆PC (Fig. 3a). H.p.l.c. profiles (Fig. 3b) also revealed that the reactivity of Lys-6 and Lys-65 toward TNBS was enhanced by the Ca²⁺ and substrate.

Properties of Lys-modified PLA₂

As seen in Table 1, trinitrophenylation of Lys-6 caused a loss of enzymic activity to 7.2% of that of the native PLA₂, but the modified derivative still retained 45% of the lethal toxicity. Modification of Lys-65 further decreased the lethal toxicity to 12.6%. However, the antigenicity of both TNP-1 and TNP-2 remained unchanged. This implies that Lys-6 is closely related to the enzymic activity, and that both Lys-6 and Lys-65 are important for the lethal toxicity of PLA₂.

As illustrated in Fig. 4, a red shift of the Ca²⁺-induced difference spectra with a peak at 240 nm was noted for PLA₂. The spectra of Lys-modified derivatives differ from those of the native enzyme in the presence of two new positive perturbations with maxima at 325 nm and 420 nm, and two troughs with minima at 365 nm and 465 nm. Since the TNP group manifests a characteristic spectrum between 250 and 500 nm with a maximum at 345 nm (Satake *et al.*, 1960), these new perturbations should result from the newly added chromophore. The perturbations of TNP groups on TNP-2 were more pronounced than those on TNP-1, reflecting the fact that both incorporated TNP groups in TNP-2 should be perturbed by Ca²⁺.

Fig. 3. TNBS modification of PLA₂ in the absence and presence of Ca²⁺ and diC₆PC

PLA₂ (1 mg) in 3 ml of 0.05 M-borate buffer (pH 8.6) was incubated with a 4-fold molar ratio of TNBS in the absence and presence of 20 mM-Ca²⁺ and 1 mM-diC₆PC. The reaction was allowed to proceed at 30 °C for 30 min, and followed by desalting and lyophilization. (a) Trinitrophenylation of PLA₂ was monitored at 345 nm. The absorbance of TNBS was subtracted from initial absorbance at zero time. (b) Reverse-phase h.p.l.c. profiles, showing the TNP derivatives appearing in the absence and presence of Ca²⁺ or diC₆PC. The column (SynChropak RP-P, 4.6 mm × 25 cm) equilibrated with 0.1% TFA was eluted with a linear gradient of 25–50% acetonitrile for 70 min. The flow rate was 0.8 ml/min and the eluent was monitored at 280 nm.

Fig. 4. Spectral changes of native and TNP-PLA₂ induced by Ca²⁺ in 0.025 M-Tris/0.1 M-NaCl buffer (pH 8.0) at 25 °C

Sample cuvettes contained 0.45 mg of native or TNP-PLA₂/ml in the presence of Ca²⁺ (mM) as indicated. The reference cell contained 0.45 mg of the respective protein/ml, and spectra were recorded with 0–0.2 absorbance scale. Scatchard plots of data obtained by Ca²⁺-induced difference absorbance were monitored at 240 nm (inserts). Solid lines were obtained by a linear regression method, and the different symbols refer to two separate experiments.

As seen from the inserts in Fig. 4, native PLA₂ and TNP derivatives exhibit a linear relationship between $(\Delta A/\Delta A_{\max})/[\text{Ca}^{2+}]$ and $\Delta A/\Delta A_{\max}$. The linearity indicates that native and Lys-modified PLA₂ possess one type of identical, independent Ca²⁺-binding site. However, the x -intercept of $\Delta A/\Delta A_{\max}$ is only about 0.75. Since measurements of ligand–protein interaction by spectroscopic techniques are based on the assumption that the fractional change in the absorbance, $\Delta A/\Delta A_{\max}$, is to be linearly proportional to the fractional saturation of binding sites (Oberfelder & Lee, 1985), this deviation might be due to non-proportionality between ligand binding and optical response. The Ca²⁺-binding affinities of PLA₂, TNP-1 and TNP-2 are 0.50 mM, 0.42 mM and 0.34 mM respectively, revealing that the binding ability for Ca²⁺ is not impaired after Lys modification.

Regeneration of TNP-Lys residues

Treatment of TNP-1 and TNP-2 with hydrazine hydrochloride progressively restored their enzymic activity, which reached a maximum of about 40% of that of native PLA₂ at 8 h (Fig. 5a). H.p.l.c. profiles (Fig. 5b) showed that the regenerated PLA₂ increased with time, which was accompanied by the decrease in the amounts of TNP derivatives. Some side-reaction products appeared in the regeneration process, causing the restoration of the enzymic activity to reach only 40%. However, the purified regeneration product possesses almost the same biological activities as those of native PLA₂ (Table 1).

DISCUSSION

Results of this study suggest that Lys-6 is important for the enzymic activity, and that both Lys-6 and Lys-65 are associated with the lethal toxicity of PLA₂. The biological activities of the regenerated PLA₂ are almost the same as those of native PLA₂, indicating that the intact Lys-6 and Lys-65 are essential for the biological activities of PLA₂.

Ca²⁺ and diC₈PC greatly enhance the reactivity of Lys-6 and Lys-65 toward TNBS rather than providing a protective effect, suggesting that the two Lys residues are not directly involved in the binding of either Ca²⁺ or substrate. Inspection of the tertiary structure of bovine pancreatic PLA₂ shows that the identical positions of Lys-6 and Lys-65 of *N. naja atra* PLA₂ are located at the area nearby the Ca²⁺-binding site (Fig. 6). The incorporated TNP groups on the side chains of Lys-6 and Lys-65 might be near the Ca²⁺-binding site, and thus may be perturbed by the binding of Ca²⁺. However, the K_d value of Ca²⁺ for TNP enzymes is similar to that of native enzyme, indicating that Lys-6 and Lys-65 do not participate in Ca²⁺ binding.

In contrast to the findings of Yang *et al.* (1981, 1982) that even after carbamylation of all five Lys residues in *N. naja atra* PLA₂, the modified enzyme still possessed 59% of the enzymic activity, trinitrophenylation of Lys-6 caused a precipitous drop in the enzymic activity. Similar results were also observed by Maraganore & Heinrikson (1985) in their study on the role of Lys residues

Fig. 5. Regeneration of TNP-1 and TNP-2 with hydrazine hydrochloride

Details of regeneration of TNP-PLA₂ with hydrazine hydrochloride are described in the Materials and methods section. (a) Restoration of the enzymic activity of TNP-1 (○) and TNP-2 (●) with time. (b) H.p.l.c. profiles showing the regenerated products of TNP-1 and TNP-2 at time intervals of 1, 4 and 8 h. The conditions are the same as those of Fig. 3(b).

Fig. 6. Tertiary structure of bovine pancreatic PLA₂ showing the relative locations of Ca²⁺-binding site and Lys residues

The model is based on that of Dijkstra *et al.* (1981) and the numbering system is according to Kini & Iwanaga (1986).

in PLA₂ from *Agkistrodon piscivorus piscivorus* snake venom. X-ray crystallographic analysis of PLA₂ enzymes (Dijkstra *et al.*, 1981, 1983; Brunie *et al.*, 1985; Renetseder *et al.*, 1985) showed that a hydrogen bonding network connects the 'catalytic network' formed by the side chains of His-48, Tyr-52, Tyr-73 and Asp-102 (Fig. 6) to the *N*-terminal region. Brunie *et al.* (1985) suggested that the hydrogen bonding network is an 'activation network' which could stabilize the productive conformation of PLA₂ enzymes for aggregated substrate. The results of our recent study on the *N*-terminal region of *N. naja atra* PLA₂ (Yang & Chang, 1988) are in good agreement with the above suggestion. It is likely that incorporation of a bulky, hydrophobic TNP group rather than a small carbamyl group on Lys-6 might result in a specific perturbation of the hydrogen bonding network, thereby causing PLA₂ to lose its enzymic activity.

Yang *et al.* (1985) and Soons *et al.* (1986) found that modification of Tyr-63 resulted in a marked decrease in enzymic activity and lethal toxicity of PLA₂ from *N. naja atra* and *N. nigricollis* snake venoms. In sharp contrast

The carbonyl oxygen of Tyr-28, Gly-30 and Gly-32, the carboxylate oxygens of Asp-49 and two water molecules form an oxygen cage that sequesters a calcium ion (Dijkstra *et al.*, 1981). The amino acid residues composing the 'catalytic network' are also indicated by (●). Broken lines indicate the deleted segment or residues in *N. naja atra* PLA₂. Numbers in parentheses indicate the positions of Lys-6 and Lys-65 in *N. naja atra* PLA₂.

with the loss of enzymic activity, modification of Tyr-3 in *N. naja atra* PLA₂ could cause a more than 6-fold increase in lethal potency. Alternatively, the PLA₂ of *N. nigricollis* still retained 50 % of its lethal toxicity after modification of Tyr-3. Together with the results of this study, this suggests that the neighbouring region surrounding Tyr-63 and Lys-65 might be related to the lethal toxicity of PLA₂. The X-ray model of PLA₂ (Dijkstra *et al.*, 1981, 1983; Brunie *et al.*, 1985; Renetseder *et al.*, 1985) indicates that the segment of the polypeptide carrying Tyr-73 (which corresponds to Tyr-67 in *N. naja atra* PLA₂) could be stabilized by the hydrogen bonding network that involves the *N*-terminal α -amino group and the invariant Gln-4 (Achari *et al.*, 1987). Tsai *et al.* (1987) proposed that the *N*-terminal region of presynaptic PLA₂ neurotoxins could enhance the cationic nature of their toxicity domain at positions 59–73. It is therefore probable that modification of Lys-6 at the *N*-terminal region would affect the molecular feature responsible for the lethal toxicity of PLA₂.

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