JOURNAL OF MOLECULAR RECOGNITION, VOL. 9, 639–643 (1996)

Discriminatory Recognition of Membrane Phospholipids by Lysine-49-phospholipases A_2 from *Trimeresurus flavoviridis* Venom

Yasuyuki Shimohigashi*, Ayako Tani, Yoko Yamaguchi, Tomohisa Ogawa and Motonori Ohno
Laboratory of Biochemistry, Department of Chemistry, Faculty of Science, Kyushu University, Fukuoka 812–81, Japan

Basic proteins I and II (BP-I and BP-II) isolated from *Trimeresurus flavoviridis* venom, which are classified into a group of lysine-49-phospholipases A_2 (Lys-49-PLA_2), exhibited only limited lipolytic activity for the mixed micelles of various phospholipids. Based on the finding that BP-II elicits a strong contraction of guinea pig ileum due to the release of arachidonic acid, BP-II together with BP-I has been tested for their interaction with artificial phospholipid bilayer membranes. The dye leakage experiments indicated that BP-II interacts strongly with liposomes of β-arachidonoyl-γ-stearoyl-L-α-phosphatidylcholine. The perturbation of liposomes was observed only in the Ca^{2+}-containing buffer, and as demonstrated by HPLC analyses, accompanied by the release of arachidonic acid. The concentration of Ca^{2+} which gave a half maximal activity of BP-II was 3.0×10^{-4} M, suggesting that the affinity of BP-II for Ca^{2+} is more than 10 times stronger than that of BP-II without liposomes. These observations clearly show that Lys-49-PLA_2 of BP-II is the enzyme responsible for the hydrolysis of membrane phospholipids and that Ca^{2+} is essential for such enzymatic activity. The interaction of BP-I with liposomes was much weaker than BP-II. BP-I and BP-II share a common sequence except for Asp-67 (BP-I) and Asn-67 (BP-II) in the aligned sequences. This implies that the amino acid at position 67 of Lys-49-PLA_2s is the residue required for discriminatory recognition of phospholipid membranes.

Keywords: **Lys-49-phospholipases A_2; snake venom; phospholipid recognition; membrane recognition**

Introduction

Phospholipases A_2 (PLA_2s) [EC 3.1.1.4] are esterolytic hydrolases catalysing the hydrolysis of 1,2-diacyl-3-*sn*-phosphoglycerides at the C2-position. They are distributed abundantly in mammalian pancreas and also in reptile and insect venoms. Snake venom PLA_2s exhibit various physiological activities (Huang, 1984; Verheij *et al.*, 1980), some of which are based on their lipolytic enzyme activity. The authors have found recently that Asp-49-PLA_2 isolated from the venom of *Trimeresurus flavoviridis* (Habu snake) induces strong contraction of the longitudinal muscle of guinea pig ileum (Matsumoto *et al.*, 1991). This contractile activity is caused by arachidonic acid liberated by Asp-49-PLA_2 from phospholipid biomembranes of guinea pig ileum. Basic proteins I and II (designated as BP-I and BP-II, respectively) isolated from the same venom are the isozymes of Asp-49-PLA_2 and classified into a group of Lys-49-PLA_2 (Liu *et al.*, 1990; Yoshizumi *et al.*, 1990). As expected for Lys-49-PLA_2s, BP-I and BP-II exhibited only limited lipolytic activities (a few per cent of Asp-49-PLA_2) against the micelles of egg yolk and some synthetic phospholipids. However, BP-II elicited a contractile activity as strong as Asp-49-PLA_2 in the guinea pig ileum (unpublished data). The obtained results indicated that the contraction is due to the hydrolytic action of BP-II against phospholipid membranes, liberating arachidonic acid.

In addition to BP-I and BP-II, a number of Lys-49-PLA_2s have been isolated in recent years from the venom of snakes such as *Bothrops atrox* (Maraganore *et al.*, 1984), *Agkistrodon piscivorus piscivorus* (Maraganore *et al.*, 1984; Maraganore and Heinrikson, 1986) and *Bothrops asper* (Francis *et al.*, 1991). Despite the fact that Lys-49-PLA_2s contain His-48 and Asp-99, an essential catalytic diad specific for Asp-49-PLA_2s, they exhibit extremely low lipolytic activities. Enzymatic inactivity of Lys-49-PLA_2 from *A. piscivorus piscivorus* was repeatedly emphasized as a characteristic of Lys-49-PLA_2s (Condrea, 1989; van den Bergh *et al.*, 1988, 1989). Since the activities of BP-II for guinea pig ileum suggested that Lys-49-PLA_2s are the membrane-acting PLA_2 enzymes, the interactions of PLA_2 isozymes isolated from *T. flavoviridis* with artificial phospholipid bilayer vesicles were studied. It is reported that here Lys-49-PLA_2s are membrane-acting PLA_2 enzymes with a key amino acid at position 67 for discriminatory recognition of membrane phospholipids.

* Author to whom correspondence should be addressed.

Abbreviations used: ASPC, β-arachidonoyl-γ-stearoyl-L-α-phosphatidylcholine; BP-I, basic protein I; BP-II, basic protein II; CF, 5,6-carboxyfluorescein; DPLC, β,αγ-dilauroyl-L-α-phosphatidylcholine; DLPE, β,γ-dilauroyl-L-α-phosphatidylethanolamine; DLPG, β,γ-dilauroyl-L-α-phosphatidyl-DL-glycerol; PLA_2, phospholipase A_2.

CCC 0952–3499/96/060639–05

Received 26 May 1995
Accepted (revised) 21 July 1995

Experimental

Materials

T. flavoviridis Asp-49-PLA_2, BP-I and BP-II were purified from the lyophilized crude venom as reported previously (Ishimaru *et al.*, 1980; Liu *et al.*, 1990). Especially to avoid any contaminations of Asp-49-PLA_2, BP-I and BP-II were further purified by HPLC using a Resource RPC column (3 ml; Pharmacia, Uppsala, Sweden). The protein concentration was determined by utilizing the value of $E^{1\%}_{1cm}$ at 280 nm for each protein. Egg yolks and phospholipids such as β-arachidonoyl-γ-stearoyl-L-α-phosphatidylcholine (ASPC), namely 1-octadecanoyl-2-[(cis,cis,cis,cis)-5,8,11,14 - eicosatetraenoyl] - *sn* - glycero - 3 - phosphocholine, was obtained from Sigma (St Louis, MO, USA). β,γ-Dilauroyl-L-α-phosphatidylcholine (DPLC) and β,γ-dilauroyl-L-α-phosphatidylethanolamine (DLPE) were also obtained from Sigma, and β,γ-dilauroyl-L-α-phosphatidyl-DL-glycerol (DLPG) from Funakoshi (Tokyo, Japan). 5,6-Carboxyfluorescein (CF) purchased from Eastman Kodak Co. (Rochester, NY, USA) was recrystallized from ethanol–water. All other reagents were of analytical grade.

Determination of PLA_2 activity

The PLA_2 activity was determined utilizing egg yolk, ASPC, DLPC, DLPE and DLPG as substrates on a Radiometer RST-5 titration assembly (pH 8.0 and 37°C) as described previously (Liu *et al.*, 1990). Substrates were prepared as micelles mixed with deoxycholate for egg yolk and Triton X-100 for ASPC, DLPC, DLPE and DLPG. Enzymatically released fatty acids were titrated with 10 mM NaOH, and the unit of enzyme was defined in terms of the uptake of alkali in mmol/min. Specific activity was calculated as units per μmol protein.

Preparation of phospholipid liposomes

Phospholipid (8 mg) dissolved in chloroform (2.0 ml) was exposed to nitrogen gas blow in a conical glass vessel. Dried lipid was allowed to stand under vacuum overnight and then vortex mixed for 30 min at 0°C with a mixture (1.5 ml) of 10 mM Tris-HCl buffer (pH 8.0) containing 100 mM CF. The resulting suspension was sonicated for 10 min at 0°C using a Tomy Seiko Model UR-200P ultrasonic disrupter and allowed to stand for 5 min at 0°C. This was repeated six times, and the resulting mixture of uni- and multilamellar vesicles was subjected to gel filtration on a column (0.8×15 cm) of Sepharose 4B. Elution was carried out with 10 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl. Small unilamellar vesicles entrapping CF were collected and utilized for dye-leakage and hydrolysis experiments as described later. The phospholipid concentration was determined by using Phospholipid Test-Wako (Wako, Osaka, Japan) principally according to the procedure by Allen (1940).

Fluorometric analysis of leaked CF

To a mixture of 10 mM Tris-HCl (pH 8.0) containing 100 mM NaCl in a quartz cuvette was added liposomes (4.4×10^{-5} M of lipid concentration) encapsulating 100 mM CF. To avoid effects by spurious Ca^{2+}, 100 mM EDTA was added to the stock solution of PLA_2s. EDTA was finally removed by Centricon (W.R. Grace, MA, USA). Leakage was induced by adding aliquots of PLA_2s (10 μl) dissolved in 10 mM Tris-HCl buffer (pH 8.0) to this vesicle suspension (2 ml) directly in the cuvette. Excitation was set at 470 nm and emission was recorded at 515 nm on a JASCO FP-550A spectrofluorometer. The fluorescence intensity was traced in the presence and absence of Ca^{2+}, and the complete dye release was achieved by disrupting the vesicles with 20 per cent Triton X-100 solution (10 μl; final concentration 0.1 per cent v/v). The results are expressed as

$$\%\ \text{CF leakage} = 100(F-F_0)/(F_t-F_0)$$

where F_0 is the initial fluorescence intensity before adding PLA_2s, F the fluorescence induced by PLA_2s at different times, and F_t the total fluorescence after addition of Triton X-100.

Hydrolysis of β-arachidonoyl-γ-stearoyl-L-α-phosphatidylcholine

Enzyme solution (10 μM final concentration) was added to a suspension of ASPC vesicles in 10 mM Tris-HCl (pH 8.0) buffer with 10 mM $CaCl_2$ and 100 mM NaCl. The reaction mixture was incubated at 37°C for 30 min and then evaporated. The residue was dissolved in ethanol (150 μl), and an aliquot (100 μl) was analysed by HPLC to detect liberated arachidonic acid. The retention time was determined using authentic arachidonic acid (Sigma). HPLC was carried out on a Hitachi 655A-11 liquid chromatograph with a reversed-phase TSK-gel ODS-120T column (4.6×250 mm) (Tohso, Tokyo). In order to test PLA_2s for ASPC monomers, to a solution of ASPC (260 μg) in a mixture of diethyl ether and ethanol (95:5 v/v) (0.2 ml) was added a solution (20 μl) of PLA_2s (150 μg/1.0 ml) dissolved in 10 mM Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$.

Results

PLA_2 activity against mixed micelles of various phospholipids

Three PLA_2 isozymes from *T. flavoviridis*, Asp-49-PLA_2, BP-I and BP-II, were tested for their lipolytic activity using egg yolk and four different synthetic phospholipids such as ASPC, DLPC, DLPE and DLPG. Each lipid was prepared as micelles mixed with deoxycholate or Triton X-100, and the activity was determined from the rate of alkaline uptake against liberated fatty acids. Despite the high activities of Asp-49-PLA_2, Lys-49-PLA_2s of BP-I and BP-II were very weak (0.2–3 per cent) as shown in Table 1, in which the activity of BP-I and BP-II is expressed as a percentage of Asp-49-PLA_2 against the same substrate. Against DLPG, BP-II (11.3 per cent) and BP-I (5.6 per cent) were relatively potent, but this was obviously due to the decreased activity

Table 1. Specific PLA_2 activity of *Trimeresurus flavoviridis* Asp-49-PLA_2, Lys-49-PLA_2s BP-I and BP-II against mixed micelles of various phospholipids

Substrates	% Relative activity[a] Asp-49-PLA_2	BP-I	BP-II
Egg yolk[b]	100 (179±17)[c]	0.25	0.21
ASPC	100 (11.0±0.90)	0.66	0.88
DLPC	100 (36.3±1.0)	0.32	0.55
DLPE	100 (31.0±4.3)	1.31	2.72
DLPG	100 (2.73±0.54)	5.57	11.3

[a] The activity of BP-I and BP-II is expressed as a percentage of Asp-49-PLA_2against the same substrate.
[b] Mixed micelles were prepared with Triton X-100 (deoxycholate for egg yolk) in a 3:2 molar ratio in 50 mM Tris-HCl (pH 8.0) containing 10 mM Ca^{2+}.
[c] Specific activity (mmol•min^{-1} protein). Results are means ±SE.

of Asp-49-PLA_2 (Table 1). It is evident hereto that, so far as they were tested for micelles, Lys-49-PLA_2s are much weaker than Asp-49-PLA_2s.

Leakage of liposome-encapsulated CF by PLA_2 isozymes

In order to evaluate the effects of PLA_2 isozymes on phospholipid membranes, model experiments were carried out based on the method to measure the fluorescent probe leaked from phosphatidylcholine liposomes (Weinstein *et al.*, 1977). The authors selected ASPC, which contains β-arachidonoyl ester at the C2-position of phosphoglycerides, to keep up the comparability between assays using biomembranes (guinea pig ileum) and artificial lipid membranes. For monitoring the interaction of PLA_2s with membranes, CF was encapsulated in ASPC liposomes and small unilamellar vesicles were collected. No more than 0.3 mM concentration of PLA_2s solution (10 μM final) was tested in this study.

Fig. 1 shows the typical time courses of CF leakage (%) induced by BP-II, monitored by the relief in CF fluorescent self-quenching. When ASPC liposomes were incubated with 10 μM BP-II, CF leakage occurred almost instantaneously. After the initial intense elevation, the fluorescence level increased gradually. CF leakage went up to about 60 per cent after 20 min and became almost constant at the 60–70 per cent level after prolonged incubation. The levels of CF leakage were dependent upon the concentrations of BP-II incubated.

Fig. 2 shows the CF leakage profiles of BP-I. BP-I was considerably weaker than BP-II. CF leakage by 10 μM BP-I did not reach a fluorescence level of 40 per cent even after prolonged incubation. On the other hand, Asp-49-PLA_2 showed a profile with leakage considerably more enhanced than BP-II (data not shown). When CF leakage was expressed as a function of concentration of PLA_2s, the difference in activities of BP-II and BP-I was prominent as shown in Fig. 3. BP-II was much more potent (more than

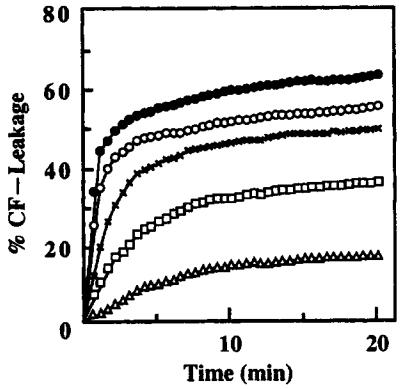

Figure 1. Time-dependent CF leakage from ASPC liposomes by *T. flavoviridis* Lys-49-PLA_2 BP-II. Leakage experiments were carried out by using 4.4×10^{-5} M ASPC liposomes encapsulating CF: λ_{ex}=470 nm and λ_{em}=515 nm. Incubation was carried out in 10 mM Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$ and 100 mM NaCl; at 37°C. Even after prolonged incubation (for example, after 24 h), the fluorescent levels remained almost constant at each concentration: (●), 10 μM; (○), 5.0 μM; (X), 2.0 μM; (□), 1.0 μM; and (△), 0.35 μM.

double) than BP-I in the whole concentration range tested.

All experiments described earlier were performed in the buffer containing 10 mM Ca^{2+} using EDTA-treated Ca^{2+}-

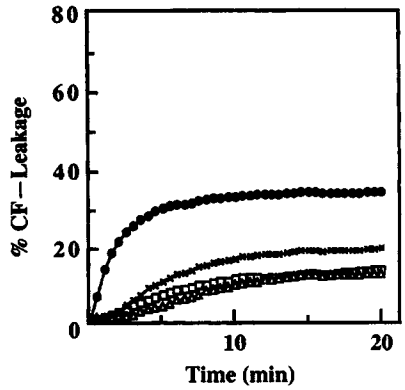

Figure 2. Time-dependent CF leakage from ASPC liposomes by *T. flavoviridis* Lys-49-PLA_2 BP-I. Leakage experiments were carried out essential as described for BP-II in Fig. 1. (●), 10 μM; (X), 2.0 μM; (□), 1.0 μM; and (△), 0.35 μM.

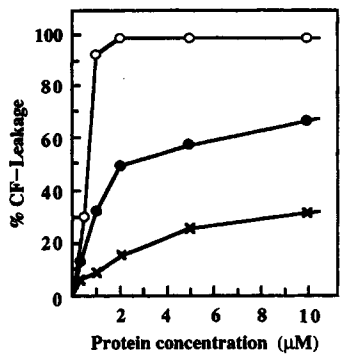

Figure 3. Comparison of concentration-dependent dye leakage activity between *T. flavoviridis* Lys-49-PLA_2, BP-I and BP-II. Leakage experiments were carried out using 4.4×10^{-5} M ASPC liposomes, and other conditions were as described in the legend for Fig. 1. (○), Asp-49-PLA_2; (●), BP-II; and (X), BP-I.

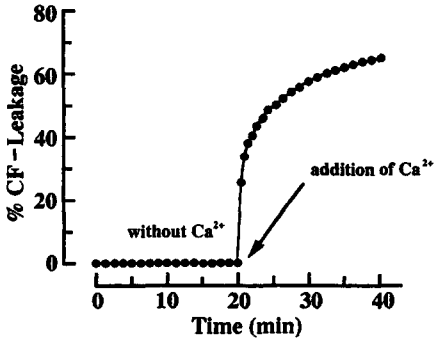

Figure 4. Influence of calcium ion on dye leakage activity of *T. flavoviridis* Lys-49-PLA_2 BP-II. Leakage experiments were initiated by using 4.4×10^{-5} M ASPC liposomes in the absence of Ca^{2+}. Other conditions were as described in the legend for Fig. 1. After 20-min incubation without calcium ion, 100 mM $CaCl_2$ (20 μl) was added to the reaction mixture (final concentration of Ca^{2+} = 1.0 mM).

free PLA_2 samples. When these leakage experiments were carried out in the Ca^{2+}-free buffer, BP-II induced no release of CF (Fig. 4), the fluorescence line remaining at the basal level even at a concentration of 10 μM of BP-II. On the other hand, when Ca^{2+} was added to the buffer on the way of this assay, instantaneous strong leakage occurred, reaching almost the same leakage level in the presence of Ca^{2+} (Fig. 4). This CF leakage was observed only when the liposomes had been incubated with BP-II. With no BP-II, Ca^{2+} did not in the least affect basal fluorescence level. A similar observation was also obtained for Asp-49-PLA_2 and BP-I. The maximal activation was achieved by 1.0–10 mM Ca^{2+}. Below 1.0 mM concentration of Ca^{2+}, the activation dropped sharply in a concentration-dependent manner (Fig. 5). These results suggested that the productive conformation of BP-II is constructed with essential Ca^{2+}. From the curve of leakage activity *versus* Ca^{2+} concentration of BP-II (Fig. 5), the effective concentration (EC_{50}) of Ca^{2+} which gives a half maximal activity of BP-II was roughly estimated to be 3.0×10^{-4} M.

Release of arachidonic acid from ASPC liposomes

In order to examine whether or not PLA_2s can release arachidonic acid from liposomes, PLA_2 isozymes were incubated with ASPC liposomes and liberated arachidonic acid was monitored by HPLC. After incubation of ASPC liposomes with BP-II for 30 min, about 75 per cent liberation of arachidonic acid was detected. At this condition, Asp-49-PLA_2 showed almost complete release of arachidonic acid, while BP-I liberated only about 20 per cent.

For ASPC monomers, only Asp-49-PLA_2 showed a peak at the retention time corresponding to arachidonic acid and with an expected amount (data not shown). BP-I and BP-II exhibited only traces of arachidonic acid. These results indicated that Asp-49-PLA_2 is able to hydrolyse monomers of ASPC, but both BP-I and BP-II were almost inactive.

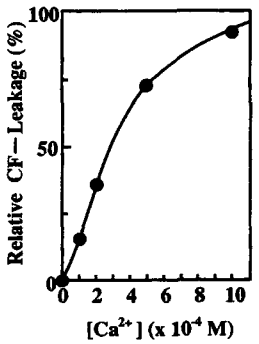

Figure 5. Concentration-dependent effect of calcium ion on dye leakage activity of *T. flavoviridis* Lys-49-PLA_2 PLA_2 BP-II. Leakage experiments were carried out essencially as in Fig. 4 with various concentration of Ca^{2+} (0.1–1.0 mM).

Discussion

It was demonstrated in the present study that Lys-49-PLA_2s isolated from *T. flavoviridis* are enzymatically active for phospholipid bilayer membranes. BP-I and BP-II cleaved arachidonic acid from ASPC liposomes, and as a consequence of this enzymatic activity they leaked CF entrapped in liposomes. Although enzymatic inactivity of Lys-49-PLA_2 from *A. piscivorus piscivorus* was repeatedly emphasized as a characteristic of Lys-49-PLA_2s, this inactivity is true only for the substrates of mixed micelles (Condrea, 1989; van den Bergh *et al.*, 1988 and 1989). The present results strongly suggest that Lys-49-PLA_2s are the enzymes with PLA_2 activity for phospholipid bilayer membranes. Indeed, in the authors' preliminary experiment using myotoxin II, a Lys-49-PLA_2 isolated form *Bothrops asper* was also highly active in the hydrolysis of ASPC liposomes. Even Lys-49-PLA_2 from *A. piscivorus piscivorus*, which the authors purified, was active (unpublished data). These results indicate that Lys-49-PLA_2s from snake venoms are generally active for phospholipid membranes.

The role of Asp-49 in Asp-49-PLA_2s is to control the binding of the calcium ion, which is necessary for stabilization of a tetrahedral intermediate formed between the enzyme and substrate (Dijkstra *et al.*, 1978; Fleer *et al.*, 1981; Kihara *et al.*, 1992; Renetseder *et al.*, 1985). It has been suggested that the ε-amino group of Lys-49 in Lys-49-PLA_2s may replace the position and the charge of this calcium ion (Maraganore *et al.*, 1987). Recent crystallographic studies on Lys-49-PLA_2 from *A. piscivorus piscivorus* by two different groups clearly demonstrated this hypothesis, showing that the site occupied by the bound calcium ion in the bovine pancreatic structure is filled instead by the ε-amino group of Lys-49 (Holland *et al.*, 1990; Scott *et al.*, 1992). However, in spite of such an exclusion of calcium ion from the calcium binding-loop, Lys-49-PLA_2s have still been found to exhibit a dependence on calcium ion for their enzymatic activity (Dijkstra *et al.*, 1981a; Liu *et al.*, 1990; Yoshizumi *et al.*, 1990). In the present study, this calcium dependency of Lys-49-PLA_2s, BP-II and BP-I was clearly shown for their activity for liposomes (Figs 4 and 5). The EC_{50} value of Ca^{2+}, which gives a half maximal activity of BP-II, was about 3.0×10^{-4} M. This can be assessed as a kind of dissociation constant of the BP-II–Ca^{2+} complex. It is apparent that the value was much smaller than that (4.2×10^{-3} M) without liposomes (Liu *et al.*, 1990), indicating that the affinity of BP-II for Ca^{2+} is more than 10 times stronger under conditions with liposomes than without liposomes. This

clearly showed that Lys-49-PLA_2s are the enzymes requiring the essential Ca^{2+} for their enzymatic activity.

Maraganore *et al.* (1987) have suggested that the Lys-49-ε-amino group first interacts with a negatively charged phosphorus group of the substrate in the region corresponding to the calcium binding loop in Asp-49-PLA_2s, and forms a stable enzyme-substrate complex. Calcium ion must bind to this Lys-49-PLA_2/phospholipid complex in order to form a catalytic complex. The present results indicate the crucial role of calcium ion in the catalytic action of BP-I and BP-II for phospholipid membranes. There should be a binding site of calcium ion in the enzyme–substrate complex, where calcium ion may convert the non-productive conformation to the productive one.

Another important feature elucidated in the structure–function relationships of Lys-49-PLA_2s is a big activity difference between BP-I and BP-II. BP-II was much more active than BP-I in both experiments using guinea pig ileum and ASPC liposomes. In the assays using ASPC liposomes, BP-II was more potent than BP-I in both CF leakage and liberation of arachidonic acid. It should be emphasized that their sequence difference occurs only at position 67, namely Asp-67 for BP-I and Asn-67 for BP-II (Liu *et al.*, 1990; Yoshizumi *et al.*, 1990). This Asp/Asn substitution was demonstrated to be due to independent mRNAs, but not to modification after translation or artefact during purification (Ogawa *et al.*, 1992). Residue 67 is involved in a lipid interface-recognition site (Dijkstra *et al.*, 1981b; Holland *et al.*, 1990; Scott *et al.*, 1992). It is thus likely that the activity difference between BP-I and BP-II is due to the different ability of residue 67 in these isozymes in interacting with phospholipid bilayers.

References

Allen, R. J. L. (1940). The estimation of phosphorus. *Biochem. J.* **34**, 858–865.

Condrea, E. (1989). Comparison of enzymatic and pharmacological activities of lysine-49 and aspartate-49 phospholipases A_2 from *Agkistrodon piscivorus piscivorus* snake venom. A reconsideration. *Toxicon* **27**, 705–706.

Dijkstra, B. W., Drenth, J., Kalk, K. H. and Vandermaelen, P. J. (1978). Three-dimensional structure and disulfide bond connections in bovine pancreatic phospholipase A_2. *J. Mol. Biol.* **124**, 53–60.

Dijkstra, B. W., Drenth, J. and Kalk, K. H. (1981a). Active site and catalytic mechanism of phospholipase A_2. *Nature* **289**, 604–606.

Dijkstra, B. W., Renetseder, R., Kalk, K. H., Hol, W. G. J. and Drenth, J. (1981b). Structure of porcine pancreatic phospholipase A_2 at 2.6Å resolution and comparison with bovine phospholipase A_2. *J. Mol. Biol.* **168**, 163–179.

Fleer, E. A. M., Verheij, H. M. and de Haas, G. H. (1981). Modification of carboxylate groups in bovine pancreatic phospholipase A_2. *Eur. J. Biochem.* **113**, 283–288.

Francis, B., Gutierrez, J. M., Lomonte, B. and Kaiser, I. I. (1991). Myotoxin II from *Bothrops asper* (Terciopelo) venom is a lysine-49 phospholipase A_2. *Arch. Biochem. Biophys.* **284**, 352–359.

Holland, D. R., Clancy, L. L., Muchmore, S. W., Ryde, T. J., Einspahr, H. M., Finzel, B. C., Heinrikson, R. L. and Watenpaugh, K. D. (1990). The crystal structure of a lysine 49 phospholipase A_2 from the venom of the Cottonmouth snake at 2.0-Å resolution. *J. Biol. Chem.* **265**, 17649–17656.

Huang, H. C. (1984). Release of slow reacting substance from the guinea-pig lung by phospholipases A_2 of *Vipera russelli* snake venom. *Toxicon* **22**, 359–372.

Ishimaru, K., Kihara, H. and Ohno, M. (1980). Purification and properties of phospholipase A_2 from venom of *Trimeresurus flavoviridis* (Habu Snake). *J. Biochem. (Tokyo)* **88**, 443–451.

Kihara, H., Uchikawa, R., Hattori, S. and Ohno, M. (1992). Myotoxicity and physiological effects of three *Trimeresurus flavoviridis* phospholipases A_2. *Biochem. Int.* **28**, 895–903.

Liu, S.-Y., Yoshizumi, K., Oda, N., Ohno, M., Tokunaga, F., Iwanaga, S. and Kihara, H. (1990). Purification and amino acid sequence of basic protein II, a lysine-49-phospholipase A_2 with low activity, from *Trimeresurus flavoviridis* venom. *J. Biochem., (Tokyo)* **107**, 400–408.

Maraganore, J. M. and Heinrikson, R. L. (1986). The lysine-49 phospholipase A_2 from the venom of *Agkistrodon piscivorus piscivorus*. *J. Biol. Chem.* **261**, 4797–4804.

Maraganore, J. M., Merutka, G., Cho, W., Welches, W., Kézdy, F. J. and Heinrikson, R. L. (1984). A new class of phospholipase A_2 with lysine in place of aspartate 49. *J. Biol. Chem.* **259**, 13839–13843.

Maraganore, J. M., Poorman, R. A. and Heinrikson, R. L. (1987). Phospholipases A_2: Variations on a structural theme and their implications as to mechanism. *J. Protein Chem.* **6**, 173–189.

Matsumoto, H., Shimohigashi, Y., Nonaka, S., Saito, R., Takano, Y., Kamiya, H. and Ohno, M. (1991). Contractile activity of *Trimeresurus flavoviridis* phospholipase A_2 on guinea pig ileum and artery. *Biochem. Int.* **24**, 181–186.

Ogawa, T., Oda, N., Nakashima, K., Sasaki, H., Hattori, M., Sakaki, Y., Kihgaha, H. and Ohno, M. (1992). Unusually high conservation of untranslated sequences in cDNAs for *Trimeresurus flavoviridis* phospholipase A_2 isozymes. *Proc. Natl. Acad. Sci. USA* **89**, 8557–8561.

Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J. and Sigler, P. B. (1985). A comparison of the crystal structures of phospholipase A_2 from bovine pancreas and Crotalus atrox venom. *J. Biol. Chem.* **260**, 11627–11634.

Scott, D. L., Achari, A., Vidal, J. C. and Sigler, P. B. (1992). Crystallographic and biochemical studies of the (Inactive) Lys-49 phospholipase A_2 from the venom of *Agkistrodon piscivorus piscivorus*. *J. Biol. Chem.* **267**, 22645–22657.

van den Bergh, C. J., Slotboom, A. J., Verheij, H. M. and de Haas, G. H. (1988). The role of aspartic acid-49 in the active site of phospholipase A_2. *Eur. J. Biochem.* **176**, 353–357.

van den Bergh, C. J., Slotboom, A. J., Verheij, H. M. and de Haas, G. H. (1989). The role of Asp-49 and other conserved amino acids in phospholipase A_2 and their importance for enzymatic activity. *J. Cell. Biochem.* **39**, 379–390.

Verheij, H. M., Boffa, M. C., Rothen, C., Bryckaert, M. C., Verger, R. and de Haas, G. H. (1980). Correlation of enzymatic activity and anticoagulant properties of phospholipase A_2. *Eur. J. Biochem.* **112**, 25–32.

Weinstein, J. N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W. A. (1977). Liposome-cell interaction: Transfer and intracellular release of a trapped fluorescent marker. *Science* **195**, 489–492.

Yoshizumi, K., Liu, S.-Y., Miyata, T., Saita, S., Ohno, M., Iwanaga, S. and Kihara, H. (1990). Purification and amino acid sequence of basic protein I, a lysine-49-phospholipase A_2 with low activity, from the venom of *Trimeresurus flavoviridis* (Habu Snake). *Toxicon* **28**, 43–54.