Chemical modification and inactivation of phospholipases A₂ by a manoalide analogue

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Chemical modification and inactivation of bovine pancreatic, porcine pancreatic, *Naja naja atra* and *Pseudechis australis* phospholipases A₂ (PLA₂s), belonging to Group I, and of *Trimeresurus flavoviridis*, *Vipera russelli russelli* and *Agkistrodon halys blomhoffii* PLA₂s, belonging to Group II, were investigated by the use of a manoalide (MLD)-analogue, 1-(2,5-dihydroxy-5-oxo-3-furanyl)-8,12-dimethyl-4-formyl-3,7,11-tridecatrienol. At appropriate time intervals, residual PLA₂ activities towards monodispersed, anionic mixed micellar and non-ionic mixed micellar substrates were measured. We tested the protective effect of micellar *n*-dodecylphosphocholine (*n*-C₁₂PC) on enzyme inactivation. Inactivation of pancreatic PLA₂s (Group I) was only observed towards anionic mixed micellar substrates. This inactivation was completely prevented by the presence of micellar *n*-C₁₂PC. From a fragmentation study of modified bovine pancreatic PLA₂ using lysyl endopeptidase, we speculated that Lys-56 of this enzyme was modified by MLD-analogue and that this modification was responsible for enzyme

inactivation. Inactivation of non-pancreatic PLA₂s was observed towards all types of substrate, except that no significant inactivation of *N. naja atra* PLA₂ (Group I) towards monodispersed substrate was noted. Micellar *n*-C₁₂PC protected *N. naja atra* PLA₂ (Group I) completely from inactivation by MLD-analogue, but had lesser protective effects on *P. australis* PLA₂ (Group I), *T. flavoviridis* and *V. russelli russelli* PLA₂s (Group II). However, no significant protection of *A. halys blomhoffii* PLA₂ (Group II) activity was observed. These results indicate that the inactivation of pancreatic and *N. naja atra* PLA₂s originates from the modification of Lys residues at the interfacial recognition site, and that inactivation of *P. australis*, *T. flavoviridis* and *V. russelli* PLA₂s arises from the modification of Lys residues at the catalytic site, interfacial recognition site and regions outside both sites. The inactivation of *A. halys blomhoffii* PLA₂ was assumed to be due to the modification of Lys residues outside the two sites described above.

INTRODUCTION

Manoalide (MLD) is a non-steroidal sesterterpenoid isolated from the sponge *Luffariella variabilis* [1]. The pharmacological properties of MLD include analgesic activity and inhibition of phorbol ester-induced inflammation, but not that induced by arachidonic acid, in the mouse ear [2]. This suggests that MLD inactivates phospholipase A₂ (PLA₂) directly by an anti-inflammatory mechanism. Subsequently MLD was also shown to inactivate the extracellular PLA₂s of bee [3] and cobra [4] venoms and bovine pancreas PLA₂ [5] irreversibly in a time-dependent manner. Amino acid analysis of the MLD-inactivated PLA₂s [4,6,7] and a protection experiment investigating enzyme inactivation in the presence of exogenous Lys [8] suggested that Lys residues in the PLA₂ molecule were modified.

PLA₂s are classified into two groups, I and II, according to differences in their primary structure [9]. Both types of PLA₂s have only a low activity towards substrates that exist in the monomeric state. When the substrates are in aggregated forms, however, such as in micelles, the enzymes show greater activity (by up to four orders of magnitude) [10]. It has been established

by numerous approaches that PLA₂s bind to the substrate interface via an interfacial recognition site, a site that is topologically and functionally distinct from the catalytic site of the enzyme where esterolysis occurs [11].

Previously Lombardo and Dennis [4] showed that MLD inhibited the hydrolysis of phosphatidylcholine by cobra (*Naja naja naja*) venom PLA₂ (Group I), but failed to inhibit the hydrolysis of phosphatidylethanolamine. This observation suggests that MLD does not inhibit PLA₂ by modification at the catalytic site of the enzyme. Reynolds et al. [12] showed that manoalide, a synthetic dehydroxy-MLD-analogue, modified three or four Lys residue of the *N. naja naja* enzyme and that one of these was probably Lys-6. Furthermore, Glaser et al. [6] reported that the inactivation by MLD of bee venom PLA₂, the primary structure of which differs significantly from that of Group I and II enzymes, resulted in the loss of three Lys residues one of which was probably Lys-94. These two Lys residues at positions 6 and 94 in the respective enzymes were thought to be involved in the interfacial recognition site.

Recently we synthesized a simple MLD-analogue, 1-(2,5-dihydro-2-hydroxy-5-oxo-3-furanyl)-8,12-dimethyl-4-formyl-

Abbreviations used: diC₆PC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; diC₁₂PC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; *n*-C₁₂PC, *n*-dodecylphosphocholine; MLD, manoalide; MLD-analogue, 1-(2,5-dihydro-2-hydroxy-5-oxo-3-furanyl)-8,12-dimethyl-4-formyl-3,7,11-tridecatrienol; PLA₂, phospholipase A₂; PE-PLA₂, S-pyridylethylated PLA₂.

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Figure 1 Chemical structure of MLD-analogue

3,7,11-tridecatrienol (Figure 1) [5]. Although this MLD-analogue modified fewer lysine residues of bovine pancreatic PLA₂ (Group I) than did MLD, the degree of inactivation by the two compounds was practically the same [7].

In the present study we investigated the inactivation of various Group I and Group II PLA₂s by this MLD-analogue towards substrates in various physical states, and studied the effects of the presence of micellar *n*-dodecylphosphocholine (*n*-C₁₂PC) in the reaction mixtures. The results are discussed on the basis of amino acid sequence analysis. We have also identified and modified lysine residues responsible for the inactivation.

MATERIALS AND METHODS

Materials

The PLA₂s from the venoms of *N. naja atra*, *A. halys blomhoffii* and *T. flavoviridis* were prepared by methods similar to those of Lo and Chang [13,14], Kawauchi et al. [15] and Miyake et al. [16] respectively. The enzyme (Pa-12A) from *P. australis* venom and that (PLA₂-III) from *V. russelli russelli* venom were prepared by procedures similar to those described by Takasaki et al. [17] and S. Inoue, E.-P. Mo, K. Ikeda and K. Hayashi (unpublished work; details available on application to author) respectively. Bovine pancreatic PLA₂ was purified according to Nieuwenhuizen et al. [18] and Dutilh et al. [19], as described previously [10]. Porcine pancreatic PLA₂ was purchased from Boehringer Mannheim and further purified by Mono Q column chromatography. The concentrations of PLA₂s from *N. naja atra*, *A. halys blomhoffii*, *T. flavoviridis*, bovine pancreas and porcine pancreas were determined spectrophotometrically using the molar absorption coefficients $2.93 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 278 nm [20], $2.36 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 279 nm [21], $3.52 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm [16], $1.70 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 278 nm [19,22] and $1.82 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm [23] respectively. The concentrations of *P. australis* PLA₂ (Pa-12A) and *V. russelli russelli* PLA₂ (PLA₂-III) were determined spectrophotometrically using the molar absorption coefficients 2.01×10^4 and $1.72 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (at 280 nm) respectively, calculated based on the contents of Tyr and Trp residues ([24]; S. Inoue et al., unpublished work) by taking the respective molar absorption coefficients of these residues as 1.4×10^3 and $5.5 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm [25]. MLD-analogue was synthesized as described previously [5]. 1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine (diC₆PC) and *n*-C₁₂PC were synthesized and purified as described previously [10,14]. 1,2-Dilauroyl-*sn*-glycero-3-phosphocholine (diC₁₂PC), Triton X-100 and cholic acid were obtained from Sigma.

Inactivation of PLA₂ by MLD-analogue

The inactivation of PLA₂s by MLD-analogue was studied by incubation with each enzyme at 40 °C in 50 mM Tris/HCl buffer

(pH 8.0). The ionic strengths of incubation mixtures were adjusted to 0.2 with NaCl. At appropriate time intervals, aliquots were taken from the incubation mixture and immediately diluted into solutions for a pH-stat assay to determine residual activities. Similarly, aliquots were taken from the incubation mixture prepared under the same conditions and immediately desalted using NAP-5 Sephadex G-25 columns (Pharmacia LKB Biotechnology) equilibrated with 1% acetic acid to prepare the samples for determination of the number of modified Lys residues by amino acid analysis. Modification was initiated by addition to MLD-analogue dissolved in dioxane. For the control experiments, the same volume of dioxane was added instead of the MLD-analogue solution. The final concentrations of PLA₂s, MLD-analogue and dioxane in the incubation mixtures were 4 μM, 0.4 mM, and 8.0% respectively. To test the effect of micellar *n*-C₁₂PC on enzyme inactivation, 10 mM *n*-C₁₂PC and 10 mM Ca²⁺ were added to the incubation mixtures.

Enzyme assays

The three types of substrates (monodispersed diC₆PC and mixed micelles of diC₁₂PC with cholic acid or Triton X-100) were hydrolysed by incubation with the enzymes at 25 °C and pH 7.0 in the presence of 10 mM CaCl₂, and activity was measured by the pH-stat assay method using a system consisting of a Radiometer PHM 84 standard pH meter, a TTT 80 titrator and an ABU 80 autoburette, as described previously [10]. The ionic strengths of the assay solutions were adjusted to 0.2 with NaCl. Volumes of 1 ml of the substrate solutions consisting of 4 mM diC₆PC, 1 mM diC₁₂PC plus 5 mM cholic acid or 1 mM diC₁₂PC plus 5 mM Triton X-100 and 10 mM CaCl₂ (Merck) with an ionic strength of 0.2 were transferred into cells, and the pHs of the solutions were adjusted to 7.0 by addition of small volumes of a 30 mM NaOH solution. To these solutions, 5–100 μl of the PLA₂/MLD-analogue incubation mixtures were added, and the fatty acids released were titrated with 30 mM NaOH under a nitrogen stream to keep the pH at the initial value. The titration volumes recorded during the enzyme hydrolysis of the substrates were corrected by subtracting the volume used due to the spontaneous reactions without enzymes. All enzyme assays were conducted in triplicate and the data agreed with an error of < 5%.

Large-scale preparation of bovine pancreatic PLA₂ modified by MLD-analogue

Bovine pancreatic PLA₂ (about 1.5 mg) was dissolved in 2.5 ml of 50 mM Tris/HCl buffer (pH 8.0), and a stock solution of MLD-analogue in dioxane was added to give a final concentration of 0.4 mM. The mixture was incubated at 40 °C for 20 min and then immediately passed through a Sephadex G-25 column (1.6 cm × 23 cm) pre-equilibrated with 50 mM acetate buffer (pH 5.0). The eluted protein fraction was dialysed for 24 h against 50 mM acetate buffer (pH 5.0) containing 8 M urea. The sample was fractionated on a Mono-S column (0.5 cm × 5 cm) equilibrated with the buffer used for dialysis described above. The adsorbed proteins were eluted with the same buffer containing a linear concentration gradient of NaCl from 0 to 0.1 M. The modified and unmodified enzymes could be separated by this method.

Fragmentation of MLD-analogue-modified and unmodified bovine pancreatic PLA₂s

S-Pyridylethylated (PE-) derivatives of bovine pancreatic PLA₂ modified or not by MLD-analogue were prepared essentially

Figure 2 Inactivation of PLA₂s towards non-ionic mixed micellar substrate by MLD-analogue

Bovine (●), *N. naja atra* (▲) and *P. australis* (■) PLA₂s, which belong to Group I (a), and *A. halys blomhoffii* (○), *T. flavoviridis* (△) and *V. russelli russelli* (□) PLA₂s, which belong to Group II (b), were incubated with MLD-analogue in 50 mM Tris/HCl buffer at 40 °C and pH 8.0. At appropriate time intervals, PLA₂ activity towards mixed micelles of diC₁₂PC with Triton X-100 was measured by the pH-stat method at 25 °C and pH 7.0. The initial velocities for the respective enzymes in the absence of MLD-analogue under the above conditions were 2.13, 307, 523, 213, 286 and 180 μmol/min per mg.

according to the method of Cavins and Friedman [26]. Approx. 0.2 mg of both forms of the enzyme were dissolved in 0.3 ml of 0.5 M Tris/HCl buffer (pH 8.0) containing 6 M guanidine/HCl and 10 mM EDTA, and reduced at 60 °C for 2 h in the presence of 0.09 mg of dithiothreitol. To this solution, 2.0 μl of 4-vinylpyridine was added. After standing for 3 h at room temperature, the solution was desalted by dialysis against 1 % acetic acid, and then lyophilized. The PE-enzymes were cleaved with *Achromobacter lyticus* lysyl endopeptidase (Wako). Digestion was carried out in 50 mM Tris/HCl buffer (pH 9.0) containing 4 M urea at 37 °C for 15 h with an enzyme/substrate ratio of 1:100 (w/w). The resulting peptides were separated by reversed-phase HPLC on a Capcell Pak C18 (Shiseido) column with 0.1 % trifluoroacetic acid containing a linear concentration gradient of acetonitrile from 0 to 80 %.

Amino acid analysis

Proteins and peptides were hydrolysed in a mixture of 5.7 M HCl and 0.2 % phenol vapour in sealed tubes under vacuum at 110 °C for 24 h. After evaporation of the solvent, the hydrolysates were analysed using a Hitachi L-8500 amino acid analyser.

Amino acid sequence analysis

Amino acid sequences of the PE-PLA₂ fragments were determined using an Applied Biosystems model 477A protein sequencer equipped with a 120A phenylthiohydantoin analyser.

RESULTS

Studies of PLA₂ inactivation using different kinds of substrate

Figure 2 shows the time courses of inactivation of several PLA₂s by MLD-analogue, when the mixed micelle of diC₁₂PC with a neutral detergent, Triton X-100, was used as the substrate. The residual activities of *T. flavoviridis* and *V. russelli russelli* PLA₂s decreased rapidly with time and reached about 10 % after 90 min. The residual activity of the *P. australis* PLA₂ decayed more

Figure 3 Inactivation of PLA₂s towards anionic mixed micellar substrate by MLD-analogue

Bovine (●), porcine (▼), *N. naja atra* (▲) and *P. australis* (■) PLA₂s, which belong to Group I (a), and *A. halys blomhoffii* (○), *T. flavoviridis* (△) and *V. russelli russelli* (□) PLA₂s, which belong to Group II (b), were incubated with MLD-analogue in 50 mM Tris/HCl buffer at 40 °C and pH 8.0. At appropriate time intervals, PLA₂ activity towards mixed micelles of diC₁₂PC with cholic acid was measured by the pH-stat method at 25 °C and pH 7.0. The initial velocities for the respective enzymes in the absence of MLD-analogue under the above conditions were 25.1, 92.8, 107, 248, 117, 223 and 172 μmol/min per mg.

slowly to about 20 % after 90 min. MLD-analogue also caused time-dependent inactivation of *N. naja atra* and *A. halys blomhoffii* PLA₂s; the residual activities amounted to about 60 and 40 % respectively after 90 min. However, no significant inactivation was observed of bovine pancreatic PLA₂ when neutral mixed micelle preparations were used as the substrate.

Since the hydrolytic activity of pancreatic PLA₂s is known to be significantly enhanced by the presence of anionic surface-active agents [27], the time-dependence of the residual activity of the PLA₂s, including pancreatic PLA₂, was measured using as substrate micelles of diC₁₂PC mixed with an anionic detergent, cholic acid (Figure 3). Similar time-dependence curves as those seen with non-ionic micelles were obtained for most of the enzymes. However, the PLA₂s from bovine and porcine pancreas were found to be inactivated as rapidly as those from *T. flavoviridis* and *V. russelli russelli* venoms.

PLA₂s can generally hydrolyse micellar or aggregated substrates at much greater rates than monodispersed substrates [10]. This is thought to arise from the additional binding of substrate molecules to the interfacial recognition site, which is distinct from the catalytic site, of the enzyme molecule. Figure 4 shows the results obtained when monodispersed diC₆PC was used as the substrate. Thus the inactivation of PLA₂s by MLD-analogue as tested with the monodispersed substrate could be restricted to modification of the catalytic site of the enzyme molecule. The Group II PLA₂s and one of the Group I PLA₂s (*P. australis* PLA₂) gave similar time-dependent inactivation curves towards mixed micelles of diC₁₂PC with Triton X-100 or cholic acid. On the other hand, *N. naja atra*, bovine and porcine PLA₂s (Group I) were not inactivated.

Modification of Lys residues of PLA₂s

As MLD was shown to inhibit the enzymic activities of PLA₂s by irreversible modification of Lys residues [4,6–8], the numbers of Lys residues modified by MLD-analogue were determined by amino acid analysis.

Figure 4 Inactivation of PLA₂s towards monodispersed substrate by MLD-analogue

Bovine (●), porcine (▼), *N. naja atra* (▲) and *P. australis* (■) PLA₂s, which belong to Group I (a), and *A. halys blomhoffii* (○), *T. flavoviridis* (△) and *V. russelli russelli* (□) PLA₂s, which belong to Group II (b), were incubated with MLD-analogue in 50 mM Tris/HCl buffer at 40 °C and pH 8.0. At appropriate time intervals, the PLA₂ activity towards monodispersed diC₆PC was measured by the pH-stat method at 25 °C and pH 7.0. The initial velocities for the respective enzymes in the absence of MLD-analogue under the above conditions were 0.730, 1.20, 56.1, 82.0, 61.1, 114 and 44.1 μmol/min per mg.

Figure 5 Correlation between the residual activity of PLA₂s and the number of lysine residues modified by MLD-analogue

Residual activities of the MLD-analogue-modified bovine (●), porcine (▼), *N. naja atra* (▲) and *P. australis* (■) PLA₂s, which belong to Group I (a), and *A. halys blomhoffii* (○), *T. flavoviridis* (△) and *V. russelli russelli* (□) PLA₂s, which belong to Group II (b), towards mixed micelles of diC₁₂PC and cholic acid are plotted as a function of the number of lysine residues modified.

Figure 5 shows the plots of the residual PLA₂ activities towards mixed micelles of diC₁₂PC with cholic acid as a function of the number of modified Lys residues. Native PLA₂s from bovine pancreas, porcine pancreas, *A. halys blomhoffii*, *N. naja atra*, *P. australis*, *T. flavoviridis*, and *V. russelli russelli* contain 11, 9, 8, 5, 14, 10 and 11 Lys residues respectively. Losses of about 1, 1, 3, 3.5, 4, 4 and 6 lysine residues in the respective enzymes were shown by amino acid analysis when the residual activities were reduced to 50%. Marked decreases in the residual activities of bovine and porcine pancreatic enzymes were observed when just one or two Lys residues were modified.

Table 1 Amino acid composition of unmodified and MLD-analogue-modified bovine pancreatic PLA₂

Figures in parentheses are based on the amino acid sequence determined by Dijkstra et al. [28].

Amino acid	Intact PLA ₂	Modified PLA ₂
Asp	26.1 (25)	25.8
Thr	4.1 (4)	4.0
Ser	9.9 (10)	9.7
Glu	8.6 (8)	8.5
Pro	5.3 (5)	5.2
Gly	6.6 (6)	6.5
Ala	6.4 (6)	6.3
Val	4.1 (4)	4.2
Met	1.0 (1)	1.0
Ile	4.6 (5)	4.6
Leu	8.2 (8)	8.2
Tyr	6.8 (7)	6.8
Phe	3.9 (4)	3.9
Lys	11.0 (11)	10.0
His	2.1 (2)	2.1
Arg	2.0 (2)	2.0
Cys*	— (14)	—
Trp*	— (1)	—

* Cys and Trp were not determined.

Identification of the Lys residues of modified bovine pancreatic PLA₂

As shown in Figure 5, when only one or two lysine residues of bovine pancreatic PLA₂ were modified by MLD-analogue, the residual activity fell to about 10%. To identify the Lys residues modified, a large amount of the enzyme treated with MLD-analogue was prepared. Since this sample contained unmodified enzyme, the modified molecules were purified by Mono S column chromatography.

Table 1 shows the amino acid composition of the intact and modified PLA₂ compared with that of the enzyme based on amino acid sequence analysis [28]. The amino acid composition of the modified enzyme differed from that of the intact enzyme by the apparent loss of one of the eleven Lys residues.

The modified and intact PLA₂s were *S*-pyridylethylated and digested with lysyl endopeptidase under the same conditions. The peptides obtained were separated by reversed-phase HPLC (Figure 6). By amino acid analysis, the isolated HPLC peaks K-1, K-2, K-3, K-5, K-6, K-7, K-9 and K-10 were found to correspond to fragments consisting of residues 1–10, 11–12, 13–53, 57–62, 63–108, 109–115, 119–122 and 123–126 respectively. Two fragments, representing residues 54–56 and 116–118, were not found. Comparison of the HPLC patterns of the modified and intact enzymes showed that the height of the K-5 peak of the modified enzyme was smaller than that of the intact enzyme and that a new peak appeared at a retention time of about 25 min for the modified enzyme. Gas-phase microsequence analysis indicated that this new peak corresponded to the peptide comprising residues 58–62 (Leu-Asn-Ser-Cys-Lys), which lacked one Lys residue at position 57, contained in peptide K-5 (residues 57–62).

Protection by micellar *n*-C₁₂PC against chemical modification

n-C₁₂PC, an analogue of lysolecithin, forms well characterized uniform micelles [29]. Micellar *n*-C₁₂PC is known to bind to the interfacial recognition site of PLA₂s [21]. *n*-C₁₂PC was shown by

Figure 6 Separation of the lysyl endopeptidase digests of MLD-modified (A) and unmodified (B) bovine pancreatic PLA₂

The peptides were fractionated on a Capcell Pak C18 column with a linear concentration gradient of acetonitrile from 0 to 80% in 0.1% trifluoroacetic acid.

Table 2 Effects of *n*-C₁₂PC on the inactivation and modification of PLA₂s by MLD-analogue

PLA₂ (4 μM) were incubated with MLD-analogue (0.4 mM) in 50 mM Tris/HCl buffer (pH 8.0) in the presence or absence of 10 mM *n*-C₁₂PC and 10 mM Ca²⁺ at 40 °C and ionic strength 0.2 for 30 and 90 min. Aliquots were removed from the incubation mixture, and the residual activities towards three types of substrate and the number of modified Lys residues were measured by the pH-stat method and amino acid analysis respectively.

		Residual activity (%)							
PLA ₂	Incubation time (min)	Mixed micelles of diC ₁₂ PC and Triton X-100 (1:5)		Mixed micelles of diC ₁₂ PC and cholate (1:5)		Monodispersed diC ₆ PC		Number of modified Lys residues	
		— <i>n</i> -C ₁₂ PC	+ <i>n</i> -C ₁₂ PC	— <i>n</i> -C ₁₂ PC	+ <i>n</i> -C ₁₂ PC	— <i>n</i> -C ₁₂ PC	+ <i>n</i> -C ₁₂ PC	— <i>n</i> -C ₁₂ PC	+ <i>n</i> -C ₁₂ PC
Group I									
Bovine pancreas	30	95.1	—	19.6	86.2	100.0	—	0.9	0.4
	90	96.6	—	8.9	77.2	100.0	—	1.8	1.0
Porcine pancreas	30	—	—	26.2	94.7	106.8	—	1.4	0.2
	90	—	—	18.9	93.3	102.7	—	2.1	0.5
<i>N. naja atra</i>	30	79.0	96.9	69.3	—	98.5	—	3.3	0.2
	90	63.2	92.6	50.3	—	107.0	—	3.6	0.4
<i>P. australis</i>	30	45.6	67.4	50.2	—	44.3	73.1	4.3	0.7
	90	19.4	50.6	27.3	—	17.1	40.0	7.1	2.1
Group II									
<i>T. flavoviridis</i>	30	28.3	60.0	20.0	—	26.8	64.4	4.6	1.0
	90	7.4	45.0	6.0	—	11.6	49.2	5.3	1.7
<i>V. russelli russelli</i>	30	11.9	65.0	6.2	—	13.0	73.4	6.4	1.5
	90	10.9	50.8	4.6	—	12.3	49.5	6.7	2.1
<i>A. halys blomhoffii</i>	30	38.9	67.1	77.4	—	60.0	55.9	1.9	0.9
	90	38.8	32.2	64.0	—	35.5	30.5	3.8	1.3

X-ray crystallography to bind also to the catalytic site of bovine pancreatic PLA₂ [30]. Therefore the presence of *n*-C₁₂PC at concentrations above its critical micellar concentration would be expected to protect PLA₂ from modification to both its catalytic site and its interfacial recognition site.

Table 2 summarizes the effect of *n*-C₁₂PC on the chemical modification and inactivation of several PLA₂s by MLD-analogue. Modification of lysine residues of all PLA₂s was generally suppressed by the presence of micellar *n*-C₁₂PC in the incubation mixtures. In the case of the Group I PLA₂s, except for that of *P. australis*, micellar *n*-C₁₂PC completely protected the enzymes

from inactivation. Inactivation of the *P. australis* enzyme and of the Group II PLA₂s, except for that of *A. halys blomhoffii*, was suppressed by the presence of micellar *n*-C₁₂PC, but the extent of suppression was less for these enzymes. In contrast, no significant protective effect was observed for *A. halys blomhoffii* PLA₂.

DISCUSSION

As can be seen from Figure 7, PLA₂s are classified into two groups, I and II [9]. The enzymes from bovine [28] and porcine [31] pancreas and from the venoms of *N. naja atra* [32] and *P. australis*

Group I	10	20	30	40	50	60	
1. Bovine pancreas	ALWQFNGMIK	CKIPSSPEPL	DFNNYGCYCG	LGGSGTPVDD	LDRCCQTHDN	CYQAKKLDS	
2. Porcine pancreas	ALWQFRSMIK	CAIPGSHPLM	DFNNYGCYCG	LGGSGTPVDE	LDRCCETHDN	CYRDAKNLDS	
3. <i>N. naja atra</i>	NLYQFKNMIQ	CTVP-SRSWW	DFADYGCYCG	RGSGTPVDD	LDRCCQVHDN	CYNEAEKISG	
4. <i>P. australis</i>	NLIQFGNMIQ	CANKGSRPSL	NYADYGCYCG	WGGSGTPVDE	LDRCCQVHDN	CYEQAGK-KG	
Group II							
5. <i>T. flavoviridis</i>	GLWQFENMII	-KVKKSGIL	SYSAYGCYCG	WGGRGKPKDA	TDRCCFVHDC	CYG---KVTG	
6. <i>V. russelli russelli</i>	NLFQFAEMIV	-KMTGKNPLS	SYSYDGCYCG	WGGKGPQDA	TDRCCFVHDC	CY-E-GKKS-	
7. <i>A. halys blomhoffii</i>	SLMQFETLIM	-KIAGRSGIW	YYGSYGCYCG	AGGQGRPQDA	SDRCCFVHDC	CYG---KVTG	
	70	80	90	100	110	120	130
1.	CKVLVDNPLYT	NNYSYSCSNN	EITCSSENNA	CEAFICNCDR	NAAICFSKV-	P-YNKEHKNL	DKK-NC
2.	CKFLVDNPLYT	ESYSYSCSNT	EITCSKNNA	CEAFICNCDR	NAAICFSKA-	P-YNKEHKNL	DTKKYC
3.	C-W----PYF	KTYSYECSQG	TLTCKGGNNA	CAAAVCDCCR	LAAICFAGA-	P-YNDNDYNI	NLKARC
4.	C-F----PKL	TLYSWKCTGN	VPTCNSK-TG	CKSFVCACDA	AAAKCFAKA-	P-YKKENYNI	DTKKRCK
5.	C-----NPKL	GKYTYSWNNG	DIVC-EGDGP	CKE-VCECDR	AAAICFRDNL	DTYDRNKYWR	YPASNCQEDS EPC
6.	CK-----PKL	SLYSYSFQNG	GIVC-GDHNS	CKRAVCECDR	VAATCFRDNL	NTYD-KKYHN	YPPSQCTGT- EQC
7.	C-----D-PKL	DVYTYTEENG	AIVC-GGDDP	CKKQICECDK	DAAICFRDNI	DTYD-NKYWF	FPAKNCQEES EPC

Figure 7 Comparison of the amino acid sequences of seven PLA₂s

Sequence data are compared for bovine pancreas [28], porcine pancreas [31], *N. naja atra* [32], and *P. australis* [24] PLA₂s, which belong to Group I, and *T. flavoviridis* [33], *V. russelli russelli* (S. Inoue, E.-P. Mo, K. Ikeda and K. Hayashi, unpublished work) and *A. halys blomhoffii* [34] enzymes, which belong to Group II. Gaps (-) were introduced for maximal alignment of cysteine residues and maximal sequence identity. The shading indicates Lys residues.

[24] belong to Group I; and those from the venoms of *T. flavoviridis* [33], *V. russelli russelli* (S. Inoue, E.-P. Mo, K. Ikeda and K. Hayashi, unpublished work) and *A. halys blomhoffii* [34] to Group II. The peptide chains of Group II PLA₂s are longer than those of Group I enzymes by about seven amino acid residues at their C-termini. Disulphide bonds between Cys-11 and Cys-77 and between Cys-50 and C-terminal Cys-133 are characteristic of Group I and II PLA₂s respectively.

As can be seen from Figures 2–4, the time courses of inactivation of various PLA₂s by MLD-analogue differed significantly from enzyme to enzyme. This variation was ascribable to the difference in distribution of Lys residues on the respective enzyme molecules, since Lys residues are hardly conserved among PLA₂s (Figure 7).

Inactivation of pancreatic and *N. naja atra* PLA₂s (Group I) by MLD-analogue

Figure 3 shows that the enzymic activities of all the PLA₂s towards the anionic micellar substrate decreased as a result of modification, although to differing extents from enzyme to enzyme. The enzymic activities of the pancreatic and *N. naja atra* PLA₂s (Group I) towards monodispersed substrate were not decreased significantly (Figure 4). As can be seen from Table 2, micellar *n*-C₁₂PC completely protected these enzymes from inactivation by MLD-analogue. These results indicate that the inactivation of Group I enzymes, except that from *P. australis*, i.e. pancreatic and *N. naja atra* PLA₂s, originates from modification of Lys residues at the interfacial recognition site of the enzymes but not at the catalytic site.

Previously, Lombardo and Dennis [4] demonstrated that MLD inhibits the hydrolysis of phosphatidylcholine by *N. naja naja* PLA₂ (Group I) but failed to inhibit hydrolysis of phosphatidylethanolamine, suggesting that the MLD-modified enzyme still contained a functional catalytic site. Reynolds et al. [12] showed that modification of the Lys residue at position 6,

one of the three modified lysine residues in *N. naja naja* PLA₂ (Group I), was responsible for the inactivation of this enzyme, since the N-terminal α -helix including Lys-6 is considered to be the putative interfacial recognition sites of this PLA₂.

Determination of Lys residues in bovine pancreatic PLA₂ modified by MLD-analogue

Comparison of the HPLC pattern of lysyl endopeptidase-generated fragments of modified bovine pancreatic PLA₂ with that for the intact enzyme showed that the height of peak K-5 decreased and a new peak (retention time about 25 min) appeared as the reaction proceeded (Figure 6). By microsequence analysis, this new peak was found to correspond to a peptide comprising residues 58–62, lacking the Lys residue at position 57 contained in the K-5 fragment (residues 57–62) of the intact enzyme. When a peptide with a continuous sequence of Lys residues such as Xaa-Lys-Lys-Yaa is digested by lysyl endopeptidase, the peptide bond between the two Lys residues is generally digested preferentially to that between Lys and Yaa. Since this enzyme contains Lys residues at positions 56 and 57, the C-terminal band of Lys-57 was therefore thought to become more sensitive to digestion following modification of Lys-56 by MLD-analogue.

Noel et al. [35] carried out site-directed mutagenesis of bovine pancreatic PLA₂ in which Lys-56 was replaced by a neutral or hydrophobic amino acid residue, and conducted an X-ray crystallographic study of the mutant enzyme containing Met-56 instead of Lys-56. The results showed that replacement of Lys-56 decreased the enzymic activity towards anionic micelles of phosphatidylglycerol, but increased the activity towards non-ionic micelles of phosphatidylcholine, and that this mutation caused conformational changes in the loop neighbouring residue 56.

As shown in Figures 2 and 3, the enzymic activity of bovine pancreatic PLA₂ towards the anionic micellar substrate decreased as the chemical modification proceeded, but that towards the

non-ionic micelles remained unchanged. If this enzyme inactivation originated predominantly from the modification of Lys-56, the findings of Noel et al. [35] regarding activity towards anionic micelles are consistent with our present results, but those regarding activity towards non-ionic micelles are not. This latter inconsistency can be interpreted as indicating that the putative adduct of Lys-56 and MLD-analogue, which has a bulky hydrophobic chain, might disturb the activation of the enzyme towards non-ionic micellar substrates that accompanies the disappearance of a positive charge at position 56.

Table 1 shows that the modified bovine pancreatic PLA₂ contained one fewer Lys residue than the intact PLA₂. However, the chromatogram of fragments of the modified enzyme (Figure 6) showed that the K-5 fragment did not disappear completely. This indicates that MLD-analogue can also modify Lys residues of bovine PLA₂ other than Lys-56. These additional modified Lys residues could not be identified in this study, but about two-thirds of our sample was estimated to have been modified as Lys-56, as judged from the peak areas in the HPLC chromatogram (Figure 6). The modification of Lys-56 seems, therefore, to be responsible for enzyme inactivation. Although Lys-56 is not currently thought to be included in the interfacial recognition site of the enzyme, our present results indicate that this residue participates in the interfacial binding between bovine pancreatic PLA₂ and micellar substrates.

Inactivation of *P. australis* PLA₂ (Group I) and Group II PLA₂s by MLD-analogue

As seen in Figures 2–4, the activity of Group II PLA₂ and of *P. australis* PLA₂ (Group I) towards not only micellar substrates but also the monodispersed substrate decreased after modification. As can also be seen from Table 2, *P. australis* PLA₂ (Group I) and *T. flavoviridis* and *V. russelli russelli* PLA₂s (Group II) were not completely protected against inactivation by the presence of micellar *n*-C₁₂PC. The residual activities of these three enzymes decreased to about 10–20% after 90 min in the absence of micellar *n*-C₁₂PC, and to only about 40–50% in the presence of micellar *n*-C₁₂PC. No significant protective effect of micellar *n*-C₁₂PC was observed for *A. halys blomhoffii* PLA₂ (Group II). Previously we showed that the binding constants of *N. naja atra* [36], *T. flavoviridis* [37] and *A. halys blomhoffii* [21] PLA₂s to micellar *n*-hexadecylphosphorylcholine (*n*-C₁₆PC) were of the same order. This suggests that the difference in the degree of protection by micellar *n*-C₁₂PC among these PLA₂s originates from differences in the number and locations of Lys residues that are modified by MLD-analogue, but not from differences in the affinities of these enzymes for micellar *n*-C₁₂PC.

Our results suggest that, in addition to the chemical modification of Lys residues present in the catalytic site and/or interfacial recognition site, modification at additional sites is responsible for the inactivation of *P. australis*, *T. flavoviridis* and *V. russelli russelli* PLA₂s. Our results also suggest that Lys residues at the catalytic site and the interfacial recognition site of *A. halys blomhoffii* PLA₂ would not exceptionally be modified by MLD-analogue and that modification at some additional sites in this enzyme is responsible for enzyme inactivation.

Possible Lys residues of *P. australis* PLA₂ (Group I) and Group II PLA₂s modified by MLD-analogue

X-ray crystallographic studies of several complexes of PLA₂ with substrate analogues have been reported [32,38,39]. The results indicated that the hydroxy group of Tyr-69 makes a hydrogen bond with one of the oxygen atoms of the phosphate group in the

analogue molecule. As shown in Figure 7, group I PLA₂s (except for *P. australis* PLA₂), for which no significant inactivation was observed towards monodispersed substrate, have a Tyr residue at position 69, whereas Group II PLA₂s and *P. australis* PLA₂, for which enzyme inactivation was observed towards monodispersed substrate, have a Lys residue at this position. This suggests that the inactivation of *P. australis*, *T. flavoviridis* and *V. russelli russelli* PLA₂s was due to the modification of Lys-69.

The present study showed that these three PLA₂s may also be inactivated by the modification of Lys residues present at locations other than the catalytic site and the interfacial recognition site. Such inactivation would not be suppressed by the presence of micellar *n*-C₁₂PC. By comparison of the amino acid sequences (Figure 7), we can predict that the modification sites of these three enzymes are Lys residues located at the end of the N-terminal α -helix (position 15 and its vicinity), at the Ca²⁺-binding site (position 36) (*P. australis* PLA₂ does not have a Lys residue at this position) and at position 92, which is conserved among Group II and *P. australis* PLA₂s. Since these predicted sites are generally not considered to be part of the catalytic site or the interfacial recognition site, we have to assume that modification at these sites influences the enzymic activities through conformational changes. In the case of *A. halys blomhoffii* PLA₂, the inactivation was not prevented by the presence of micellar *n*-C₁₂PC, suggesting that inactivation is the result of modification of Lys residues other than those at the catalytic site and the interfacial recognition site. We can, therefore, predict that modification of Lys-100 and/or Lys-92 is responsible for inactivation of this enzyme, since Lys-100 is located on the opposite side of the interfacial recognition site and in close vicinity to Asp-99, which is in the hydrogen-bonded network of the enzyme catalytic site, and Lys-92 is conserved among Group II PLA₂s and *P. australis* PLA₂.

Thus the present study demonstrates that Lys-56 of bovine pancreatic PLA₂, Lys-69, Lys-92 and a Lys residue at around position 15 of *P. australis*, *T. flavoviridis* and *V. russelli russelli* PLA₂s, a Lys residue at around position 36 of *T. flavoviridis* and *V. russelli russelli* PLA₂s, and Lys-100 and Lys-92 of *A. halys blomhoffii* PLA₂ might be targets for modification by MLD-analogue.

In conclusion, we have shown that: (1) Lys residues of PLA₂s are generally located at the surface of the enzyme molecules, and a large number of Lys residues are modified by MLD-analogue; (2) Lys-56 of bovine pancreatic PLA₂ was modified comparatively specifically by the analogue; and (3) since putative adducts of Lys and MLD-analogue have a very bulky hydrophobic chain, this chain could disturb the binding of enzymes to monodispersed or micellar substrates and give rise to conformational changes leading to inactivation.

This work was supported by a Grant-in-Aid for Scientific Research (no. 05671853) from the Ministry of Education, Science and Culture of Japan.

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