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INACTIVATION OF PHOSPHOLIPASE A_2 BY MANOALIDE

LOCALIZATION OF THE MANOALIDE BINDING SITE ON BEE VENOM PHOSPHOLIPASE A_2*

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Abstract—The marine natural product manoalide (MLD), a potent inhibitor of phospholipases, completely inactivates bee venom phospholipase A_2 (PLA_2) by an irreversible mechanism. It has been proposed [K. B. Glaser and R. S. Jacobs, *Biochem. Pharmac.* **36**, 2079 (1987)] that the reaction of MLD with PLA_2 may involve the selective reactivity of MLD to a peptide sequence, possibly a Lys-X-X-Lys peptide. Localization of the MLD binding site on bee venom PLA_2 demonstrated that upon MLD modification of bee venom PLA_2 the only change in amino acid content was an apparent loss of Lys, corresponding to approximately three of the eleven Lys residues present. Selective chemical modification of Lys residues with [^{14}C]maleic anhydride demonstrated that all eleven Lys residues on bee venom PLA_2 were accessible to this reagent (11.6 mol maleyl group incorporated/mol of PLA_2). Pretreatment of PLA_2 with MLD (<0.7% residual activity) resulted in a molar ratio of 8.7, also consistent with the loss of three Lys residues upon modification by MLD. Reverse phase high performance liquid chromatography (RP-HPLC) of the cyanogen bromide (CNBr) digestion product of MLD-treated PLA_2 produced three peaks (A_{280}). The second peak showed the most intense absorbance at 434 nm. This material corresponded to residues 81–128, as determined by gas-phase microsequence analysis. Sequencing failure was observed at Lys-88 in the MLD-treated fragment. The control carboxymethylated-PLA_2 fragment corresponding to residues 81–128 sequenced beyond Lys-88 without significant change in the expected yield. These data suggest that Lys-88 may correspond to one of the three MLD-modified Lys residues. The minor absorbance at 434 nm of the CNBr fragments containing residues 42–80 and 1–36 as compared to the fragment of residues 81–128 suggests that the major MLD binding fragment resides in residues 81–128.

Manoalide (MLD)‖, a marine natural product that has been shown to possess potent anti-inflammatory activity [1], has been demonstrated to be a direct and irreversible inactivator of phospholipases A_2 and C [2–5]. Inactivation of bee [3] and cobra [4] venom PLA_2 has been shown to be time-dependent and irreversible and may involve the selective modification of Lys residues [4] on these enzymes.

In recent studies, MLD has been shown to inhibit a PLA_2 activity in the cellular homogenate of polymorphonuclear leucocytes (PMN) [6]. Using prelabeled PMN stimulated by the calcium ionophore A23187, MLD was also shown to inhibit the release of arachidonic acid from both phosphatidylcholine (PC) and phosphatidylinositol (PI). There appeared to be a general correlation between the IC_{50} for the homogenate activity (1–7 μM) and the release of arachidonic acid (2 μM) without any significant effect on cellular phospholipase C activity [6]. Bennett *et al.*[7] have demonstrated in a comparative study of the PLA_2 activities of smooth muscle cellular homogenates and venom PLA_2s that MLD has differential inhibitory activities. Cellular homogenate activities appeared to be less sensitive than the venom PLA_2 activities to inhibition by MLD. Investigations into the effects of MLD on the substrate specificity of PLA_2 by Lombardo and Dennis [4] and others [8] demonstrated that inhibition of cobra venom PLA_2 was observed for the hydrolysis of phosphatidylcholine substrates without affecting and even enhancing the hydrolysis of phosphatidylethanolamine substrates [4]. The observations of (i) differential inhibition of different cellular and venom sources of PLA_2 [7], (ii) the differential inhibition of PC versus PE hydrolysis by MLD-inactivated cobra venom PLA_2 [4] and (iii) the selective modification

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‖ Abbreviations: MLD, manoalide; PLA_2, phospholipase A_2; CM-PLA_2, carboxymethylated PLA_2; Lys, lysine; PC, phosphatidylcholine; PI, phosphatidylinositol; CNBr, cyanogen bromide; RP-HPLC, reverse phase high performance liquid chromatography; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

of only Lys residues on the cobra venom enzyme [4] suggest that the differential inhibition of PLA_2s by MLD may be associated with the presence or absence of a Lys residue(s) located in the active pocket (catalytic site and/or substrate binding site). These facets of MLD inhibition of PLA_2 enzymes led to the investigation of the reactivity of MLD with the protein structure and subsequently to the present studies on the localization of the MLD binding site on bee venom PLA_2.

Glaser and Jacobs [9,10] have demonstrated recently that the inhibitory activity of MLD is reduced by preincubation of MLD with polymeric forms of Lys but not with monomeric Lys. The reaction of MLD with amino acids to produce a chromophore ($\lambda_{max} = 434$ nm) also requires more than one free nucleophile on the same molecule. This relationship, the requirement of both the free sulfhydryl and amino groups to be present on the same molecule, was also expressed in the ability of the amino acid cysteine to reduce the ability of MLD to inactivate PLA_2. There also appeared to be a selective reactivity of MLD with tetrapeptides of Lys, Lys-Lys-Lys-Lys and Lys-Trp-Gly-Lys, suggesting that a Lys-X-X-Lys peptide sequence arrangement may present MLD with an optimal sequence for reactivity. Therefore, the differential effects of MLD on PLA_2 may be associated with the presence of not only a Lys residue but also a suitable arrangement of Lys residues (nucleophiles) on these enzymes at or near the active site region.

The present study was designed to elucidate the binding site of MLD on bee venom PLA_2. Determination of the amino acids modified and their extent of modification on bee venom PLA_2 are also presented. The isolation and characterization of the MLD binding site were accomplished by CNBr fragmentation of bee venom PLA_2 followed by isolation of the peptide containing the chromophore [10] using reverse phase high performance liquid chromatography (RP-HPLC). CNBr peptides were subjected to automated Edman sequence analysis using a gas-phase microsequence analyzer to identify the positions of MLD binding in the CNBr fragments.

MATERIALS AND METHODS

Materials. Bee venom phospholipase A_2 (1500 units/mg protein), L-α-phosphatidylcholine dipalmitoyl, iodoacetic acid, dithioerythritol and maleic anhydride were purchased from the Sigma Chemical Co. (St. Louis, MO). Cyanogen bromide and constant boiling (6 N) HCl (sequenal grade) were purchased from Pierce (Rockford, IL). L-3-Phosphatidylcholine,1 - palmitoyl - 2 - [9,10(n) - ^{3}H]-palmitoyl (sp. act. 35 Ci/mmol) was purchased from New England Nuclear (Boston, MA). [^{14}C]Palmitic acid (sp. act. 25 mCi/mmol) and [2,3-^{14}C]maleic anhydride (sp. act. 25 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). HPLC solvents were purchased from Fisher Scientific (Tustin, CA). An Altex Ultrasphere ODS reverse phase HPLC column was purchased from Beckman Instruments (Fullerton, CA).

Radioassay of PLA_2 activity. Bee venom PLA_2 (mol. wt 15,800) [11, 12] activity was determined using mixed micelles of 1.36 mM dipalmitoyl phosphatidylcholine, 2.76 mM Triton X-100 and 0.5 μCi of labeled dipalmitoyl phosphatidylcholine prepared in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1.0 mM $CaCl_2$, pH 7.4, at 41° as previously described [3]. PLA_2, 0.2 μg, was added to 500 μl of mixed micelles and incubated at 41° for 15 sec, and released [^{3}H]palmitic acid was extracted as described previously [3]. At 15 sec the initial rate of hydrolysis was linear and $\leq$7% of the substrate present was hydrolyzed, ensuring initial rate kinetics.

Treatment of PLA_2 with manoalide. PLA_2 (1.0 mg/ml; 63 μM) was treated with MLD (400 μM) for 60 min at 41° in 10 mM HEPES, 1.0 mM $CaCl_2$ at pH 7.4 [10]. Less than 0.7% of the PLA_2 activity remained after this treatment. Excess MLD was removed by passage of the mixture through a column (1 × 40 cm) of Bio Gel P-2. Protein fractions were pooled and lyophilized. This preparation is referred to as MLD-treated PLA_2. For CNBr digestion and amino acid analysis, the MLD-treated PLA_2 was reduced and carboxymethylated as described below. This preparation is referred to as MLD-treated CM-PLA_2.

Reduction and carboxymethylation of PLA_2. PLA_2 or MLD-treated PLA_2 (10 mg) was dissolved in 0.2 M Tris–HCl (pH 8.0) containing urea (8 M) and EDTA (20 mM). Dithioerythritol was added to a final concentration of 10 mM, an excess over cysteine residues present, and stored at room temperature under nitrogen for 4 hr. To this enzyme solution iodoacetic acid dissolved in 0.2 M NaOH was added to a final concentration of 40 mM and stored under nitrogen in the dark at room temperature for 30 min. The reduced carboxymethylated enzyme was placed in Spectra/Por (MWCO 12-14000) dialysis tubing and dialyzed for 8 hr against 5% acetic acid and for 8 hr against nanopure water and then lyophilized.

Amino acid analysis. Amino acid analyses were carried out with a Varian model 5560 high performance liquid chromatograph equipped with a 0.4 × 15 cm MicroPak "hydrolysate" column and a ninhydrin-based, post-column reaction system. Data collection and analysis were accomplished by the Chromatochart program of Interactive Microware run in an Apple model IIe microcomputer which was interfaced with the Varian chromatograph. CM-PLA_2 and MLD-treated CM-PLA_2 sample preparation and hydrolysis were carried out according to Spackman *et al.* [13]. Corrections for losses during hydrolysis were applied for serine and theonine [14].

Cyanogen bromide fragmentation of PLA_2. CNBr fragmentation of bee venom PLA_2 was performed as described by Gross [15]. Briefly, 10 mg (0.63 μmol) of CM-PLA_2 or MLD-treated CM-PLA_2 was dissolved in 70% formic acid, and CNBr dissolved in 70% formic acid was added to a final 300-fold molar excess over methionine residues (3) present in the enzyme (0.5 mmol CNBr final). The mixture was allowed to react for 72 hr at room temperature (20°) in the dark under a nitrogen barrier. The reaction was terminated by addition of 10 vol. of distilled water. CNBr was removed by lyophilization, and the remaining cleavage products were isolated by RP-HPLC.

^{14}C-Maleylation of PLA_2. Maleylation of Lys residues on PLA_2 was performed according to the method of Butler *et al.* [16]. PLA_2 at 1.0 mg/ml (63.3 μM enzyme; 692 μM Lys residues) was reacted with MLD as described above and then dissolved in 0.2 M sodium borate (pH 9.0). PLA_2 (1.0 mg/ml) and MLD-treated PLA_2 (1.0 mg/ml) were then treated with a 30–100 molar excess of [2,3-^{14}C]maleic anhydride solution ([^{14}C]maleic anhydride was diluted to a sp. act. of 49 μCi/mmol with cold maleic anhydride in dioxane). Maleylation was allowed to continue for 90 min at 2°; samples were then placed in Spectra/Por MWCO 12-14000) dialysis tubing and dialyzed against 0.1 M NH_4HCO_3 with several buffer changes for 16 hr or until the diffusate dpm were equal to background ^{14}C dpm. Aliquots of 100 μl of the maleylated PLA_2 were counted in triplicate in an LKB Rackbeta 1219 liquid scintillation spectrometer in 3.0 ml of Ecoscint (National Diagnostics) scintillation fluid.

Sodium boro[^{3}H]hydride reduction of MLD-treated PLA_2. Sodium boro[^{3}H]hydride was diluted with cold sodium borohydride to a specific activity of 5 mCi/mmol in 0.1 M NaOH. CM-PLA_2 and MLD-treated CM-PLA_2 were then reacted for 120 min at 2° with 20 mCi of (4.0 mM) sodium borohydride in 0.1 M sodium borate buffer (pH 9.0). After 120 min, excess sodium boro[^{3}H]hydride was removed by dialysis for 24 hr against nanopure water or until a constant radioactivity of the sample was obtained. Aliquots of 100 μl were counted in triplicate in 3.0 ml Ecosint (National Diagnostics) liquid scintillation fluid in an LKB Rackbeta 1219 liquid scintillation spectrometer. After dialysis samples were lyophilized, resuspended at 10 mg/ml, and digested with CNBr as described above. Peptide fragments were isolated by RP-HPLC to identify the labeled peptide fragments.

Reverse phase HPLC of peptides. RP-HPLC of tryptic and CNBr fragments of PLA_2 was performed on a Waters model M-6000A dual pump system equipped with a Rheodyne model 7125 sample injector with a 20 μl sample loop, a Waters model 450 variable wavelength detector, a Waters model 660 solvent programmer, and a Pharmacia strip chart recorder. The analyses were performed on an Altex Ultrasphere ODS (4.6 × 250 mm), 5 μm, column (Beckman Instruments).

Starting solvent (A) was 0.1% trifluoroacetic acid with 2% 2-propanol and the gradient solvent (B) was 100% 2-propanol. The organic phase was chosen as 2-propanol for the effective separation of large denatured peptide fragments [17]. Samples were prepared in solvent A, and 5 min after injection a gradient of 0–60% of solvent B was carried out over 45 min at a flow rate of 1.5 ml/min.

Amino acid sequence analysis. Analysis of the CNBr peptides was performed by gas-phase automated Edman degradation sequence analysis using an Applied Biosystems Inc. 470 Protein Sequencer with an Applied Biosystems model 120 PHT Analyzer equipped with a reverse phase PHT-C_{18} cartridge (2.1 × 200 mm) [18].

RESULTS

Maleylation of PLA_2 and MLD-treated PLA_2. The possibility that MLD is modifying Lys residues on bee venom PLA_2, as demonstrated for cobra venom PLA_2 [4], was investigated by quantitation of the number of free amino (—NH_2) groups protected by MLD from [^{14}C]maleic anhydride labeling [16].

Table 1. Effect of MLD on [^{14}C]maleylation of bee venom PLA_2

	Maleic anhydride (mM)	Extent of reaction (mol of maleyl group/mol of PLA_2)
PLA_2 (μM)		
63.3	20	11.1
63.3	20	11.3
63.3	20	10.6
63.3	40	13.3
6.3	4	11.7
		Mean = 11.6 ± 0.5 (±SE)
MLD-PLA_2 (μM)		
63.3	20	8.7
63.3	20	7.8
63.3	20	8.7
63.3	20	9.5
		Mean = 8.7 ± 0.3 (±SE)

PLA_2 or MLD-treated PLA_2 (1.0 mg/ml, 63 μM) was reacted with a 30-to-60-fold molar excess of [^{14}C]maleic anhydride (sp. act. = 49 μCi/mmol) for 90 min at 2° in 0.1 M sodium borate buffer, pH 9.0. Excess maleic anhydride was removed by dialysis against 0.1 M ammonium bicarbonate buffer, pH 8.5, at 4° until diffusate dpm were at background level. For control PLA_2, N = 5 and for MLD-treated PLA_2, N = 4.

When native PLA_2 was treated with a 30- to 60-fold molar excess of [^{14}C]maleic anhydride, 11.6 ± 0.5 (N = 5) mol of maleyl group were incorporated per mol of PLA_2 (Table 1). The theoretical number of free amino groups in bee venom PLA_2 is twelve, eleven Lys residues and the amino terminus; the incorporation of 11.6 maleyl groups suggests that all the Lys residues are accessible to this reagent, none being "buried" in the folded protein [19]. When [^{14}C]maleic anhydride was reacted with MLD-treated PLA_2, which retained <0.7% residual catalytic activity (see Materials and Methods), there were 8.7 ± 0.3 mol of maleyl group incorporated per mol of PLA_2 (Table 1). The difference between [^{14}C]maleyl group incorporation in native PLA_2 and MLD-treated PLA_2 is 2.9, indicating that approximately three amino groups out of a possible twelve on bee venom PLA_2 are modified by MLD. These results suggest that MLD is not acting as a general amino modifying reagent but may be selectively reactive with only a fraction of the Lys residues on PLA_2 enzymes.

Amino acid composition studies. The amino acid composition of both CM-PLA_2 and MLD-treated CM-PLA_2 were determined from 24 hr, 6 N HCl hydrolysates. Table 2 shows the theoretical and experimentally determined amino acid compositions of bee venom PLA_2. CM-PLA_2 values are in good agreement with the theoretical values based on the primary sequence [11]. The composition of the MLD-treated CM-PLA_2 only differed by the apparent loss of three out of eleven residues. The values from amino acid analysis and [^{14}C]maleic anhydride labeling of MLD-treated CM-PLA_2 are in good agreement, indicating that approximately three Lys residues on bee venom PLA_2 are modified by MLD, and MLD selectively reacts with a preferential number of the total Lys residues on the protein.

Cyanogen bromide fragmentation of PLA_2 and sequence analysis. CNBr fragmentation of CM-PLA_2 generated the expected three peptides absorbing at 280 nm on RP-HPLC analysis (Fig. 1). The first peptide eluted at 20 min, the second peptide at 26 min, and the third peptide at 28 min into the 2-propanol gradient. Gas-phase microsequence analysis of the isolated HPLC peaks indicated that the first peak corresponded to the CNBr alpha fragment residues 1–36, the third peak corresponded to the CNBr beta fragment residues 42–80, and the second peak corresponded to the CNBr gamma fragment residues 81–128. Identification of CNBr fragments was based on the primary sequence published by Shipolini *et al.* [11, 12].

Fragmentation of the MLD-treated CM-PLA_2 apparently generated the three expected peaks absorbing at 280 nm (Fig. 2), of which the second peak eluting at 26 min into the 2-propanol gradient had the greatest absorbance at 434 nm (Fig. 2, inset), which would correspond to the presence of the MLD-adduct chromophore [10]. Other peaks with absorbance at 434 nm were all minor compared to the peak which eluted at 26 min.

Sodium boro[^{3}H]hydride reduction of the MLD-treated PLA_2 would be expected to incorporate tritium (i) if the MLD-PLA_2 adduct was formed in part by a Schiff base at either or both the hydroxy-

Table 2. Amino acid composition of CM-PLA_2 and MLD-treated CM-PLA_2

Residue	Theoretical (number of residues/molecule PLA_2)	Experimental (number of residues/molecule PLA_2)	
		CM-PLA_2	MLD-treated
Lys	11	11.4	8.4
His	6	6.8	6.8
Arg	6	5.9	6.2
CM-Cys	8	8.0	5.5
Asn/Asp	16	15.9	16.6
Thr	10	11.1	11.3
Ser	10	10.8	12.1
Gln/Glu	6	6.2	6.8
Pro	4	4.0	5.2
Gly	11	10.9	12.0
Ala	4	4.5	4.8
Cys	8	0.0	0.0
Val	5	5.7	5.3
Met	3	2.4	2.7
Ile	4	2.7	2.7
Leu	8	8.5	9.1
Tyr	8	7.4	7.5
Phe	5	4.9	4.9
Trp	2	ND	ND

Residue numbers were determined from the residue percent values generated through the Chromatochart program of Interactive Microware and the primary sequence data given by Shipolini *et al.* [11]. The data given were determined from reduced and carboxymethylated PLA_2 (CM-PLA_2) and MLD-treated PLA_2 (MLD-treated CM-PLA_2) and are representative of other experimentally determined amino acid compositions of native and MLD-treated PLA_2 which had not been carboxymethylated (N = 2). The error in this method of analysis was ±1 residue percent for each value. ND = not determined.

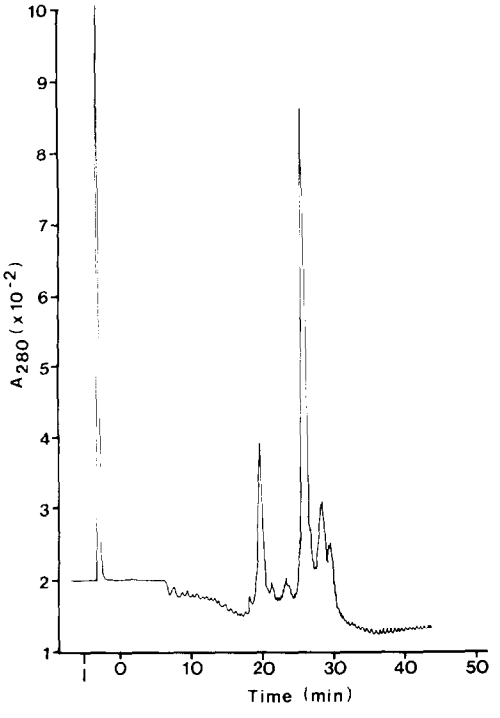

Fig. 1. RP-HPLC of CM-PLA_2 CNBr fragments. Fifty micrograms of digest was dissolved in 0.1% trifluoroacetic acid, 2% 2-propanol (20 μl); temperature, 22°; flow, 1.5 ml/min. Starting solvent (A) was 0.1% trifluoroacetic acid, 2% 2-propanol in water and a gradient of 2-propanol was employed 5 min after injection of digest from 0–60% 2-propanol in 45 min.

butenolide or pyran moiety(s) or (ii) by reduction of either of the aldehydes generated in the open ring form of MLD [4] or (iii) by reduction of either the pyran or hydroxybutenolide moiety if the other moiety is participating in the MLD-PLA_2 adduct formation [20]. Reduction of the MLD-treated CM-PLA_2 resulted in a 10-fold greater incorporation of tritium (173 μCi/mg protein) as compared to control CM-PLA_2 (12 μCi/mg protein). CNBr digestion of the tritium-labeled CM-PLA_2 and MLD-treated CM-PLA_2 resulted in an identical pattern of peptide fragments on HPLC analysis as shown in Fig. 2 (data not shown). Table 3 shows the amino acid sequence analysis performed on the CNBr gamma fragment (residues 81–128), which contains the MLD-adduct chromophore [10]. A sequencing failure was observed at the cycle corresponding to Lys-88 with an increase in tritium incorporation, 760 cpm in cycle #7 (Thr-87) to 1030 cpm in cycle #8 (Lys-88). No further amino acids were detected in the MLD-treated CNBr gamma fragment until cycle #13 (Glu-93). The amino acid sequence analysis of the corresponding control CM-PLA_2 CNBr gamma fragment showed no sequence failure at Lys-88, no change in the expected recovery (pmol) for that cycle and only a slight change in tritium incorporation, 714 cpm in cycle #7 (Thr-87) to 876 cpm in cycle #8 (Lys-88). However, a significant increase in tritium incorporation was observed at cycle #11 in the control CM-PLA_2 CNBr gamma fragment, 270 cpm in cycle #10 (Tyr-91) to 1015 cpm in cycle #11 (Lys-92), but no sequence failure or change in expected recovery (pmol) was observed in this analysis.

The sequence analysis of the CNBr gamma fragments demonstrating a sequencing failure at Lys-88, an increase in tritium incorporation corresponding to Lys-88, and the detection of Lys-88 in the control CM-PLA_2 fragment suggests that Lys-88 may be one of the three Lys residues modified by MLD. In sequence analysis of non-radiolabeled MLD-treated CM-PLA_2 CNBr fragments, a sequencing failure was observed at approximately Lys-47. The sequences of both the CNBr alpha and beta fragments were not analyzed in the sodium boro[^{3}H]hydride reduced fragments because of their minor absorbance at 434 nm as compared to the CNBr gamma fragment.

DISCUSSION

The initial characterization of the MLD binding site involved definition of the residues on bee venom PLA_2 that were modified by MLD and the extent to which the residues were modified. On cobra venom PLA_2, amino acid analysis demonstrated that MLD selectively modifies four of six Lys residues, but two of the six residues are "buried" in the native protein and are inaccessible to reagents such as maleic anhydride [4]. In these studies, amino acid analysis of the MLD-treated bee venom CM-PLA_2 demonstrated that Lys was the only residue to show significant reduction, and this reduction corresponded to a loss of three of the eleven Lys residues. The modification of Lys residues on bee venom PLA_2 was also demonstrated with [^{14}C]maleic anhydride labeling of free

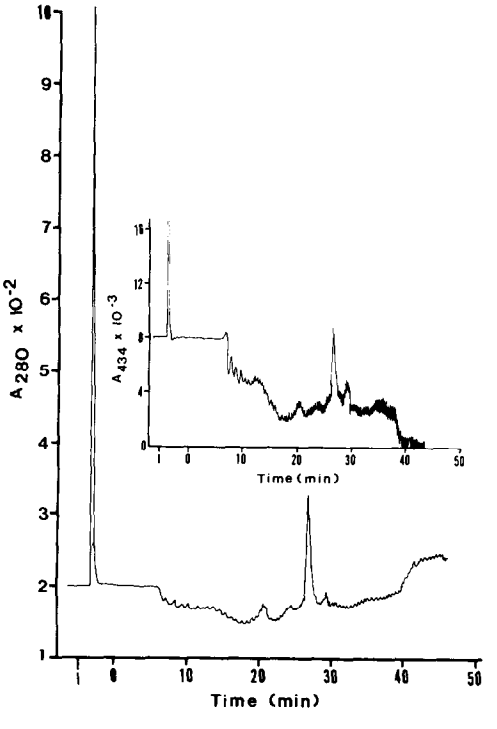

Fig. 2. RP-HPLC of MLD-treated CM-PLA_2 CNBr fragments. Conditions were identical to those in Fig. 1.

Table 3. Protein sequence analysis of bee venom PLA_2: Effects of manoalide treatment on CNBr gamma fragment (residues 81–128)

Cycle No.	Published sequence*	Control fragment Amino acid	pmol	cpm	MLD-treated fragment Amino acid	pmol	cpm
1	Tyr	Tyr	34	557	Asp	32	1053
2	Phe	Phe	87	281	Phe	36	823
3	Asn	Asn	79	778	Asn	18	634
4	Leu	Leu	105	386	Leu	33	828
5	Ile	Ile	55	678	Ile	13	697
6	Asn	Asn	66	655	Asp	12	726
7	Thr	Thr	50	714	Thr	13	763
8	Lys	Lys	50	876	ND	ND	1030
9	CM-Cys	ND	ND	490	ND	ND	971
10	Tyr	Phe	21	270	ND	ND	745
11	Lys	Lys	15	1015	ND	ND	638
12	Leu	Leu	20	486	ND	ND	897
13	Glu	Glu	15	319	Glu	2	633
14	His	His	5	366	ND	ND	601
15	Pro	Pro	11	186	—	—	—

Amino acid sequence analysis was performed on an Applied Biosystems 470 Protein sequencer with an Applied Biosystems model 120 PHT Analyzer equipped with a reverse phase C_{18} cartridge (2.1 × 200 mm). The peptide fragment from HPLC analysis was taken to dryness and resuspended in 0.1% trifluoroacetic acid for loading into the protein sequence. ND = not detected.

* See Ref. 11.

amino groups. [^{14}C]Maleic anhydride specifically modifies Lys residues at alkaline pH [16], and a reduction in ^{14}C-incorporation of the MLD-treated PLA_2 may be interpreted as modification of ε amino groups of Lys by MLD. Labeling of MLD-treated PLA_2, which retained <0.7% residual activity, indicated that MLD had modified three of eleven Lys residues, in contrast to cobra venom PLA_2 in which MLD modifies four out of six Lys residues, with >20% residual activity [4]. The maleic anhydride labeling results are in good agreement with amino acid composition studies and suggests that there may be a selective binding site for MLD on bee venom PLA_2 which involves a preferential number of Lys residues.

Localization of the MLD binding site was attempted by CNBr digestion of the MLD modified enzyme using a similar procedure as that for localization of the atractyloside binding site on the ADP/ATP carrier protein [21]. The chromophore (λ_{max} = 434 nm) produced by MLD upon binding to PLA_2 [10] was used as a marker for the MLD binding site. Untreated CM-PLA_2 produced the expected three peptide fragments absorbing at 280 nm on RP-HPLC analysis of the CNBr digest. CNBr digestion of MLD-treated CM-PLA_2 demonstrated that the most intense absorbance at 434 nm was found in the second peak (retention time 26 min) corresponding to the CNBr gamma fragment (residues 81–128). This peak, absorbing at 434 nm, suggests that it does

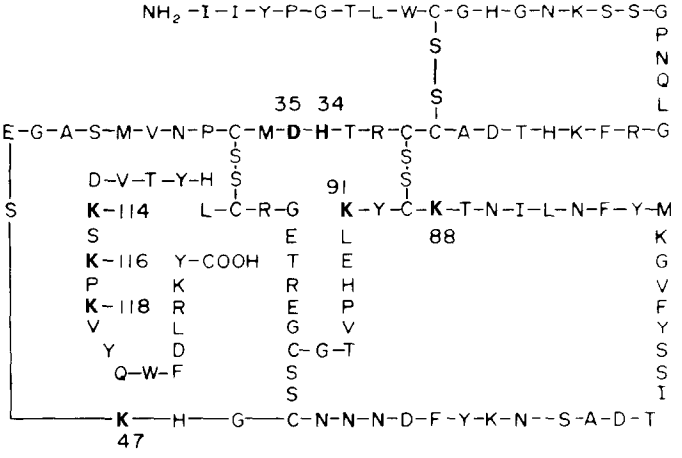

Fig. 3. Primary sequence of bee venom PLA_2 from Shipolini *et al.* [11, 12]. Possible Lys (K) binding sites are residues 47, 88, 91, 114, 116 and 118. The probable active site His (H) and Asp (D) pair is located at residues 34 and 35 respectively. All Cys (C) residues are in the form of disulfide bonds. All Lys (K) residues are available for reactions with such reagents as maleic anhydride.

contain bound MLD, which would produce the chromophore. The amino acid sequence analysis of this peptide failed at residue Lys-88, indicating an alteration or incompatibility to sequence beyond this residue. Lys-88 is the first residue of the Lys-X-X-Lys arrangement on bee venom PLA_2, which could be a selective MLD binding site (Fig. 3) [10]. The peptide containing the Lys-88, the CNBr gamma fragment, also contains the Lys-X-Lys arrangements near the carboxy terminus. These possible MLD binding sites could not be determined due to the sequence failure at Glu-93. The third CNBr digest peak, CNBr beta fragment retention time 28 min, had only a minor absorbance at 434 nm but during gas-phase sequence analysis a failure was observed at Lys-47. Therefore, Lys-88 which is present in the most chromogenic fragment may correspond to one of the three MLD modified Lys residues (Fig. 3). However, Lys-47 may also be modified but the minor intensity of this fragment at 434 nm suggests that it is not a major MLD binding fragment.

The molecular pharmacology of MLD, as revealed by both mechanistic and localization studies, demonstrates that MLD has a selective binding site on bee venom PLA_2. This binding site(s) corresponds to the selective modification of three out of eleven Lys residues on PLA_2. The possibilities of multiple MLD binding sites may account for the complex inactivation kinetics observed [3, 4], especially if these binding sites do not have equivalent affinity for MLD. Without the tertiary structure of bee venom PLA_2 it would be difficult to assess which binding site is involved in the catalytic inactivation of PLA_2. However, the proximity of the Lys-X-X-Lys arrangement to the active site "core" (His^{34}-Asp^{35}, Fig. 3) [22] strongly implicates this peptide fragment as the likely candidate. Similar arrangements of two or more Lys residues between residues 88 and 92 are found in the PLA_2s of the venoms of *Enhydrina schistosa*, *Notechis scutatus*, *N. scutatus* fraction II-5, and *Bitis caudalis* [22]. It would be of interest to compare the sensitivity of these enzymes to inhibition by MLD with that of bee venom PLA_2.

As proposed by Glaser and Jacobs [10], reactions that prevent MLD from inactivating PLA_2 require at least two nucleophilic groups, e.g. peptides of Lys but not monomeric Lys, one reacting primarily at the hydroxybutenolide moiety with the subsequent involvement of the pyran moiety, carbonyl at C-24. The presence of the α,β-unsaturated aldehyde at C-24 (pyran moiety) has been shown to be essential for the irreversible binding of MLD to PLA_2 [10, 23, 24] and chromophore production [10].

Therefore, inactivation of various venom PLA_2 may be dependent on a selective binding site for MLD and these binding sites may correspond to portions of the amino acid sequence necessary for substrate binding but not necessarily directly with the catalytic residues, His or Asp. This hypothesis is supported by the results of Lombardo and Dennis [4] for cobra venom PLA_2 and the results presented here.

In summary, the binding of MLD to bee venom PLA_2 was associated with modification of three out of eleven possible Lys binding sites. The possible modification of Lys-47 by MLD may not account for the major MLD binding fragment (residues 81–128) but may explain complex inactivation kinetics observed with MLD due to multiple binding sites. The intense absorbance at 434 nm of the CNBr fragment residues 81–128 suggests that this is the major MLD binding fragment which contains a peptide sequence that may present MLD with a selective and highly reactive binding site, -Lys-X-X-Lys- [10]. The first residue of this Lys-X-X-Lys peptide, Lys-88, was not present in the amino acid sequence analysis of the MLD-treated PLA_2 and suggests that this may be one of the three Lys residues modified by MLD. Therefore, other sources of PLA_2, other phospholipases or lipases may or may not be inactivated by MLD due to the presence or absence of this selective MLD binding site near the active pocket of these enzymes.

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