Biochemical Pharmacology, Vol. 36, No. 13, pp. 2079–2086, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

INACTIVATION OF BEE VENOM PHOSPHOLIPASE A_2 BY MANOALIDE

A MODEL BASED ON THE REACTIVITY OF MANOALIDE WITH AMINO ACIDS AND PEPTIDE SEQUENCES*

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(*Received* 28 *July* 1986; *accepted* 3 *November* 1986)

Abstract—The marine natural product manoalide (MLD), a potent irreversible inhibitor of bee venom phospholipase A_2 (PLA_2), was shown to produce a chromophore (λ_{max} = 437 nm) during incubation with the enzyme. MLD also developed an identical chromophore when incubated with free lysine (Lys), cysteine (Cys) or tryptophan (Trp) but not with their *N*-α-amino-blocked analogs. These results suggest that the chromophore product was dependent on the presence of two nucleophilic groups which react by an ordered mechanism rather than by simple random collision. Lys polymers prevented MLD from inhibiting PLA_2, whereas monomeric Lys did not. The optimal active polymer of Lys appeared to be a tetralysine (L_4) peptide, and a degree of selectivity was obtained when the Lys residues were in a 1,4-Lys arrangement. The rate of chromophore development with PLA_2 and the rate of inactivation of PLA_2 by MLD appear to be independent processes. Based on these data, it is possible that the irreversible inactivation of PLA_2 may involve an ordered reaction with a peptide sequence in PLA_2 containing a 1,4-Lys arrangement.

The marine natural product manoalide, a sesterterpenoid isolated from the sponge *Luffariella variabilis* [1], antagonizes phorbol-induced local inflammation of murine epidermis [2] but not local inflammation induced by the topical application of arachidonic acid [2]. On this basis it was postulated that the potent anti-inflammatory activity of MLD could be associated with the direct inactivation of the rate-limiting enzyme of eicosanoid production, phospholipase A_2 (PLA_2‡) (EC 3.1.1.4).§ Topical application of the phorbol esters has been shown to increase, or activate, calcium-dependent membrane phospholipase A_2 [3], which contributes to increased levels of the proinflammatory eicosanoids in murine epidermis [4].

Direct inactivation of purified PLA_2 by MLD has been demonstrated for bee venom [5, 6], cobra venom [7], β-bungarotoxin neurotoxicity [5], and porcine pancreatic PLA_2 [8]. The inactivation of bee venom PLA_2 by MLD has been shown to be irreversible [6] and extremely potent, IC_{50} = 0.05 μM for approximately 25 nM bee venom PLA_2 [6]. Inactivation kinetics suggest a complex mechanism, i.e. pseudo first-order kinetics were not obtained even at the solubility limit of MLD [6]. The inactivation is also pH dependent, reaching maximum at pH 8.0, and calcium independent [6]. Recently, it was observed that the inactivation of cobra venom PLA_2 by MLD results in the modification of only lysine residues on that enzyme [7]. In this laboratory we observed that the reaction of MLD with bee venom PLA_2 results in the production of a chromophore (λ_{max} = 437 nm), and a similar chromophore is also produced by the reaction of MLD with free Lys [9]. It was also shown that MLD Lys conjugates are without effect on the ability of MLD to inactivate PLA_2, whereas Lys peptides ≥ 4 Lys residues result in the complete inactivation of MLD [9].

The present study investigates the structural (or spatial) requirements for the reactivity of MLD with free amino acids, amino acid derivatives, Lys peptides and PLA_2 inactivation. The purpose of these investigations was designed to explain on a molecular basis factors that may contribute to the complex kinetics observed during the inactivation of bee venom PLA.‖

* This research was sponsored in part by NOAA, National Sea Grant College Program, Department of Commerce, under Grant NA80AA-D00120, through the California Sea Grant College Program, and in part by the California State Resources Agency, Project No. R/MP-21. The U.S. Government is authorized to reproduce this paper and distribute it for governmental purposes. Keith B. Glaser is a Sea Grant Trainee.

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‡ Abbreviations: PLA_2, phospholipase A_2; MLD, manoalide; Lys, lysine; Cys, cysteine; Trp, tryptophan; $A_{437\ nm}$, absorbance at 437 nm; *N-t*-BOC, *N-tert*-butoxycarbonyl; L_2, L_3 and L_4, the di-, tri- and tetrapeptides of lysine respectively; 1,4 Lys, Lys-Trp-Gly-Lys; and 1,3-Lys, Lys-Tyr-Lys.

§ R. S. Jacobs, E. L. Clason, E. S. Burley, J. E. Hochlowski and D. J. Faulkner, manuscript submitted for publication.

‖ A preliminary report of this work was presented in *Fedn Proc.* **45**, 580 (1986).

MATERIALS AND METHODS

Materials. Bee venom phospholipase A_2 (1350 units/mg protein), L-α-phosphatidylcholine dipalmitoyl, glutathione (reduced and oxidized forms), L-amino acids, *N*-α-acetyl amino acids, *N*-*t*-BOC amino acids, and bovine serum albumin were purchased from the Sigma Chemical Co. (St. Louis, MO). Lysine peptides were purchased from Research Plus, Inc. (Bayonne, NJ). Phosphatidylcholine L-α-dipalmitoyl[2-*palmitoyl*-9,10-^{3}H) (sp. act. 57.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Phosphatidylcholine 1-palmitoyl-2[1-^{14}C]palmitoyl and [1-^{14}C]palmitic acid (sp. act. 56 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). Manoalide was isolated and supplied by Dr. D. J. Faulkner, Scripps Institute of Oceanography (La Jolla, CA).

PLA_2 radiometric assay. Bee venom PLA_2 (mol. wt 15,800) [10] activity was determined using mixed micelles of 1.36 mM dipalmitoyl phosphatidylcholine, 2.76 mM Triton X-100, 0.5 μCi of labeled dipalmitoyl phosphatidylcholine prepared in 10 mM 4-(2-hydropyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 1.0 mM $CaCl_2$, pH 7.4, at 41°. Approximately 25 nM enzyme, 0.2 μg protein added to 500 μl substrate, was incubated at 41° for 15 sec and released [^{3}H]palmitic acid extracted as described previously [6]. At 15 sec, the initial rate of hydrolysis was linear and $\leq$7% of the substrate present was hydrolyzed, ensuring initial rate kinetics.

Pancreatic PLA_2 was assayed at 1 unit/ml (one unit will hydrolyze 1 μmol/min at 37°, pH 8.0) using micelles of dipalmitoyl phosphatidylcholine in 10 mM piperazine-*N*,*N*'-bis[2-ethanesulfonic acid] (PIPES), 10 mM $CaCl_2$, pH 6.0, at 37° and 0.2 μCi phosphatidylcholine 1-palmitoyl-2[1-^{14}C]palmitoyl in a 500 μl final volume. Released [^{14}C]palmitic acid was extracted as described for bee venom PLA_2 [6].

Ultraviolet-VIS scanning spectrometry. All scanning spectroscopy was carried out in an IBM Instruments, Inc., 9430 scanning spectrophotometer. Chromophoric reactions of MLD were determined by incubation of MLD (vehicle spectral grade MeOH) with amino acids, derivative or Lys peptides for 120 min at 41° in 10 mM HEPES, 1.0 mM $CaCl_2$ (pH 7.4) before spectrum scanning at 200 nm/min. Reference cells contained all reagents less MLD.

MLD inactivation studies. MLD was preincubated with the respective compound (pH 7.4 at 41°) at 200× the desired final concentration. A 10-fold molar excess of amino acids or amino acid derivatives and equimolar Lys peptides were used to determine reduction of MLD activity. Aliquots (250 μl) of the MLD mixtures were removed at prescribed times and added to 250 μl of bee venom PLA_2 (5 μM) and incubated for 60 min at 41° to achieve maximal inactivation of PLA_2 by "free" MLD. After incubation, an aliquot (5 μl) was removed and assayed for PLA_2 activity as described above (final PLA_2 concentration assayed 25 nM, MLD 0.5 μM with 5 μM amino acid or derivative or 0.5 μM Lys peptide). Controls contained respective amino acid, derivative or Lys peptide and were treated in the same manner as MLD-treated samples. Reduction of MLD activity was assessed as the inability of the MLD-mixture to inhibit PLA_2 activity after 60 min preincubation.

Difference spectra. Difference spectra were generated in a 9430 scanning spectrophotometer (IBM Instruments, Inc.). The source spectra was stored in the spectrophotometer memory and automatically subtracted from each subsequent spectrum generated. Reference cell contained either buffer (10 mM HEPES, 1.0 mM $CaCl_2$, pH 7.4, at 41°) and vehicle (MeOH) or buffer and MLD (in MeOH). Sample cell contained PLA_2 (0.5 mg/ml). MLD (200 μM) was added to the sample cell after the source spectrum was determined, and difference spectra were generated at 2-min intervals for a 10-min period. Spectra were scanned at 400 nm/min. The reaction mixture was maintained at 41° in an IBM Instruments thermostated turet cell holder.

RESULTS

Chromophore reaction of manoalide with lysine. The chromophore reaction of MLD with Lys was observed in methanol (spectral grade) and, as shown in Fig. 1, the spectra illustrate that the reaction product at 437 nm developed slowly over 2 hr and that a complex reaction is occurring with respect to the mixture such that another maximum develops at 511 nm after 7 hr of incubation. The spectral data shown suggest that the reaction of MLD with Lys is not a simple one-step mechanism. Of twenty amino acids tested, MLD developed a chromophore with only three: Lys, Trp, and Cys. These amino acids demonstrated essentially identical spectral changes when reacted with MLD as those observed in the reaction of MLD with Lys. The difference spectra of the reaction of MLD with PLA_2 demonstrated three wavelengths at which positive peaks developed when MLD (200 μM) was reacted with PLA_2 (0.5 mg/ml) (data not shown). These peaks were essentially identical to the major peaks formed by the reaction of MLD with monomeric Lys (Fig. 1) at 432, 315 and 234 nm. Kinetically, the rate of peak development at 315 nm was slow as compared to the peak development at 432 and 234 nm. The rate of peak development at 432 and 234 nm was rapid, but no simple correlation is apparent with the rate of catalytic inactivation of PLA_2 by MLD (see Fig. 6).

Amino acid reactivity with manoalide. The possible correlation between chromophore production and the specific amino acid residue modified on PLA_2 by MLD was investigated by observing the reaction of MLD with a 10-fold molar excess of free or *N*-α-amino modified amino acids. MLD reactivity was expressed as relative reactivity ($A_{437\ nm}$ sample/$A_{437\ nm}$ Lys) (Table 1). Of twenty amino acids tested only three produced a significant chromophore, Lys, Cys and Trp (Table 1). *N*-α-Amino blocked amino acids (*N*-α-acetyl or *N*-*t*-BOC) did not produce equivalent chromophore reactions. Under conditions of equivalent nucleophile concentrations, the modified amino acids did not produce equivalent chromophore production (Table 1). The nonequivalent chromophore that developed following reaction of MLD with amino blocked Lys was independent of the specific amino group (α or ε) blocked. Under conditions of equal amino group concentrations

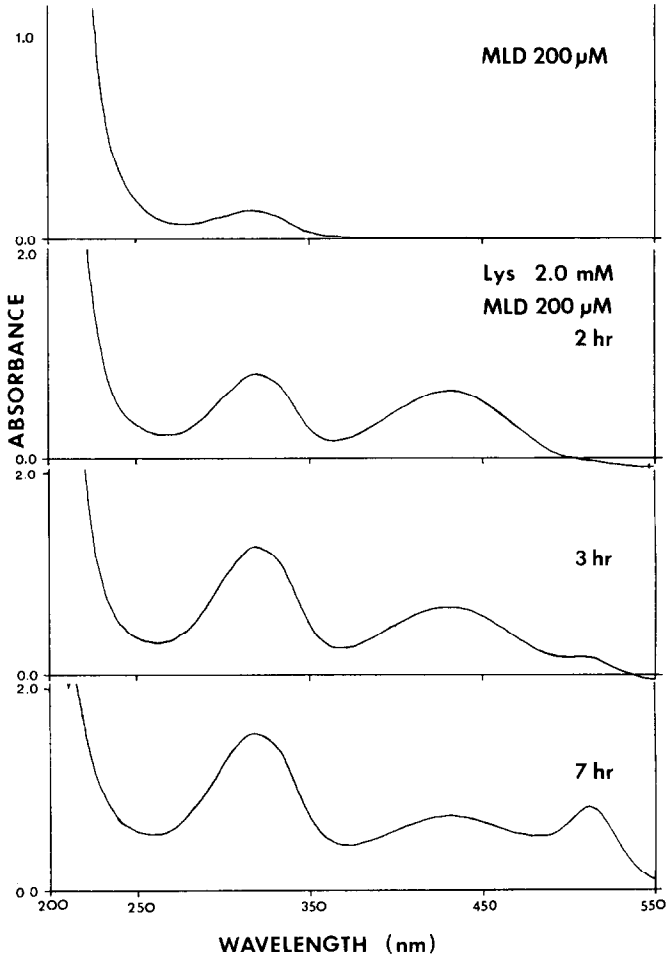

Fig. 1. Scanning spectrophotometry of the MLD–Lys reaction. MLD (200 μM) was incubated with Lys (2.0 mM) in MeOH (spectral grade reagent) at 41°. Spectral scans were performed at t = 0, 2, 3 and 7 hr at a scanning speed of 200 nm/min. Reference cells contained Lys (2.0 mM) in MeOH.

Table 1. Manoalide reactivity with free amino acids

Amino acid	Concn (mM)	Reactivity ratio
Lys	2.0	1.00
Gly	4.0	0.17
Cys	2.0	3.00
Trp	2.0	0.58
Orn	2.0	1.41
N-α-Acetyl-Lys	2.0	0.08
N-α-Acetyl-Lys	4.0	0.00
N-ε-Acetyl-Lys	2.0	0.20
N-ε-Acetyl-Lys	4.0	0.00
N-α-Acetyl-Cys	2.0	0.20
N-α-Acetyl-Cys	4.0	0.01
N-*t*-BOC-*S*-benzyl-Cys	2.0	0.20
N-*t*-BOC-*S*-benzyl-Cys	4.0	0.13
N-*t*-BOC-Trp	2.0	0.26
Glutathione		
Reduced	2.0	6.97
Oxidized	2.0	0.49

MLD (200 μM) was preincubated for 120 min at 41°, in 10 mM HEPES, 1 mM $CaCl_2$ at pH 7.4, with 2.0 mM or 4.0 mM free amino acid. Reactivity ratio is the absorbance of the amino acid/absorbance of Lys at 437 nm.

(4.0 mM Gly = 2.0 mM Lys), Gly also did not produce an equivalent chromophore. These results suggested that generation of the chromophore was not entirely dependent on the mass concentration of nucleophilic groups present. The inability of the *N*-α-acetyl blocked amino acids to produce an equivalent chromophore, even under conditions of equal nucleophilic group concentration (amino or sulfhydryl), suggests that the reaction producing the chromophore requires the interaction of MLD with two nucleophilic groups and is governed by a mechanism which is ordered rather than by simple random collision. The possibility that the distance between these nucleophilic groups may also affect chromophore production was demonstrated with ornithine (Orn) which has one less carbon than Lys in the side chain. Orn produced a slightly greater reactivity with MLD than did Lys, suggesting that the intramolecular distance between nucleophilic groups (—NH_3) may also govern the extent of the chromogenic reaction.

Inactivation of manoalide by free Lys and Cys. The reaction of MLD with monomeric Lys or Cys producing the chromophore might also prevent MLD from inhibiting bee venom PLA_2. This hypothesis

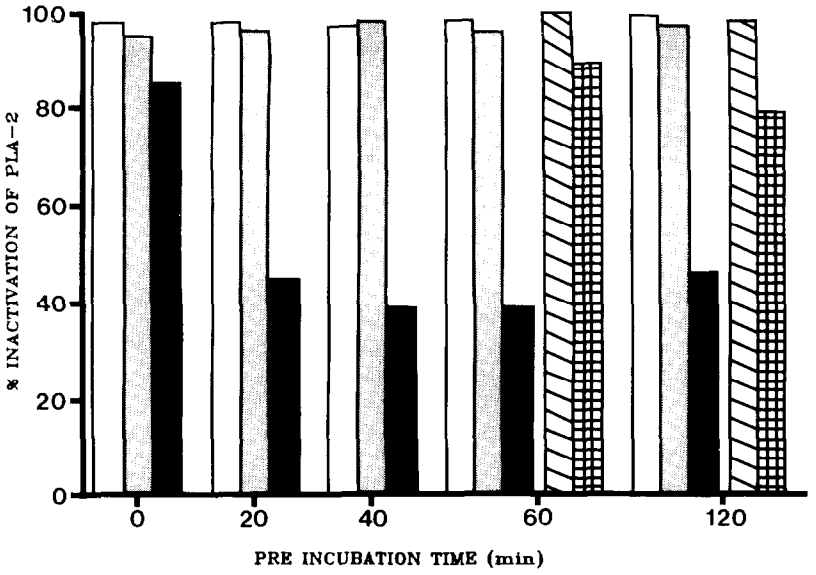

Fig. 2. Effects of Lys and Cys on the ability of MLD to inactivate bee venom PLA_2. MLD (100 μM) was preincubated with buffer (□), 1.0 mM of Lys (▩), Cys (■), *N*-α-acetyl-Lys (▨) or *N*-α-acetyl-Cys (▦) at 41° (pH 7.4); aliquots were removed at the indicated times, added to an equal volume of PLA_2 (5.0 μM), incubated for 60 min at 41° (pH 7.4), and assayed for PLA_2 activity. Final concentrations assayed were; MLD, 0.5 μM; Lys, Cys and *N*-α-amino modified analogs, 5.0 μM; and PLA_2, 25 nM. The standard error of the mean was 10% or less of the mean for each data point (N = 4).

was investigated by reacting MLD (100 μM) with Lys, Cys or their *N*-α-acetyl analogs (1.0 mM) and subsequently assaying for the ability of the MLD–amino acid mixture to inhibit PLA_2 at different times of preincubation. Lys, Cys or their *N*-α-acetyl analogs alone did not affect PLA_2 activity. As shown in Fig. 2, Lys did not prevent MLD from inhibiting PLA_2 after 120 min of preincubation, nor did *N*-α-acetyl-Lys. In contrast, reaction of MLD with Cys produced a time-dependent loss of MLD activity, reaching a maximum of 62% after 1 hr. *N*-α-Acetyl-Cys produced only 20% reduction of MLD activity following 2 hr of incubation suggesting that, as with chromophore production, both nucleophilic groups on the cysteine are necessary for the observed reduction of MLD activity. Thus, under these experimental conditions, the chromophore resulting from the MLD–lysine conjugate appears independent of

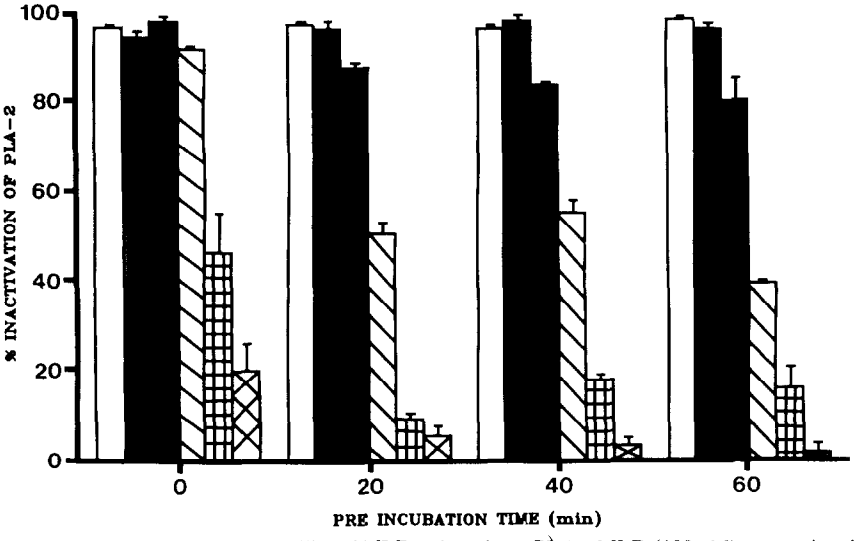

Fig. 3. Effects of Lys peptides on the ability of MLD to inactivate PLA_2. MLD (100 μM) was preincubated with buffer (□), 1.0 mM Lys (▩), or equimolar L_2 (■), L_3 (▨), L_4 (▦) and poly-L-Lys (▧) at 41° (pH 7.4); aliquots were removed at 20-min intervals, added to an equal volume of PLA_2 (5 μM), incubated for 60 min at 41° (pH 7.4), and assayed for PLA_2 activity. Final concentrations assayed were: MLD, 0.5 μM; Lys, 5.0 μM; Lys peptides, 0.5 μM; and PLA_2, 25 nM (N = 3).

enzyme inactivation, whereas formation of a manoalide–cysteine conjugate appears to partially reduce the activity of the drug.

Effects of glutathione (reduced and oxidized forms) on manoalide. Reduced glutathione contains essentially the same nucleophilic groups as Cys, except that the sulfhydryl of glutathione is a stronger nucleophile than that of Cys. Reduced but not oxidized glutathione produced an intense chromophore with MLD (7 times that of Lys and 2.5 times that of Cys) with a shift in the λ_{max} to 451 nm (Table 1). The inability of oxidized glutathione to produce an equivalent chromophore suggests that the sulfhydryl group must be in the reduced state (i.e. —SH) to react with MLD. Furthermore, if MLD acted as a reducing agent, it would be expected to produce a chromophore product equivalent to the reaction seen with reduced glutathione. Reduced glutathione produced only a 10% reduction in MLD activity following 120 min of preincubation at a 2-fold excess over MLD, and oxidized glutathione did not affect the activity of the drug (data not shown). It is important to note that there are no free cysteine residues on purified bee venom PLA_2 [11] thus eliminating the MLD–Cys reaction as a likely mechanism involved in inactivation of bee venom PLA_2. The lack of reactivity of MLD with oxidized glutathione suggests that protein denaturation due to reduction of disulfide bonds is also not involved in the enzyme inactivation mechanism.

Effects of lysine peptides on manoalide reactivity. In contrast to the observed inability of free Lys to reduce the activity of MLD we have found that a polymer of Lys appeared to have the necessary characteristics to reduce the activity of the drug. The distance between nucleophilic (ε amino) groups in peptide form was varied by use of lysine peptides (L_2-L_4 and poly-L-lysine). Figure 3 summarizes the effect of lysine peptides (L_2-L_4) and poly-L-lysine (mol. wt 14,000; 192 Lys residues) on the ability of MLD to inhibit PLA_2. As can be seen, as the lysine peptide length increased, the ability of that peptide to reduce MLD activity also increased. There was a rapid and almost complete loss of MLD activity by the L_4 peptide and poly-L-lysine. The L_2 and L_3 peptides reduced MLD activity at a slow rate and to a markedly lesser degree than the L_4 peptide. This is in contrast to what was observed using monomeric Lys (Fig. 2). Because of the potent effect of the L_3 peptide on MLD activity over the 60-min preincubation period, the rate of loss of MLD activity by the L_3 versus L_4 peptide was investigated. The rate at which the L_4 peptide reduced MLD activity was significantly greater than the reduction observed with the L_3 peptide over the entire 60-min preincubation period (Student's t-test; $P < 0.05$) (data not shown). The preferential reactivity of MLD with the L_4 peptide was best demonstrated at 10-min preincubation where there was an 8% loss of MLD activity with the L_3 peptide and with the L_4 peptide there was a 52% loss of MLD activity. The effect of an equivalent concentration of nucleophilic groups in peptide form (1x L_4 vs 2x L_2) to reduce the activity of MLD is shown in Fig. 4. The L_2 peptide at a 2-fold molar excess over the L_4 peptide did not reduce MLD activity significantly (<10% at 60-min preincubation), whereas the L_4 peptide produced a 70% reduction of MLD activity after a 60-min preincubation. These results suggest that the reactivity of MLD is dependent on an interaction with multiple nucleophilic groups, and a specific intramolecular distance (i.e. the L_4 peptide) between these nucleophilic groups may be required. The optimal distance between ε-amino groups in a synthetic peptide chain, Lys-Trp-Gly-Lys (1,4-Lys) and Lys-Tyr-Lys (1,3-Lys), was investigated and, as shown in Table 2, the 1,4-Lys was more reactive with MLD than the 1,3-Lys peptide ($P < 0.05$). The 1,4-Lys peptide reduced MLD activity by 16%, whereas the 1,3-Lys peptide produced only a 3% loss. The degree of reduction in MLD activity observed with the 1,4-Lys was significantly less than that obtained for the L_4 peptide (Lys-Lys-Lys-Lys), suggesting that substitution of Lys residues in the tetrapeptide with a Trp-Gly sequence reduces its ability to react with MLD. This is in contrast to the substitution of Lys in the tripeptide with a Tyr residue which caused an almost complete loss of reactivity with MLD. These data suggest that the 1,4-Lys arrangement may represent an optimal sequence for the reduction of MLD activity (inactivation of MLD) relative to either monomeric Lys, the L_2 peptide, or the 1,3-Lys peptide. The optimal amino acid sequence of the 1,4-Lys peptide is not known as yet.

Effects of pH on MLD reactivity with the tetrapeptide of Lys. The effects of preincubation pH on the inactivation of MLD by the L_4 peptide is shown in Fig. 5. As the pH of the preincubation mixture increased, the ability of the L_4 peptide to inactivate MLD increased. At pH 5.5, there was no reduction

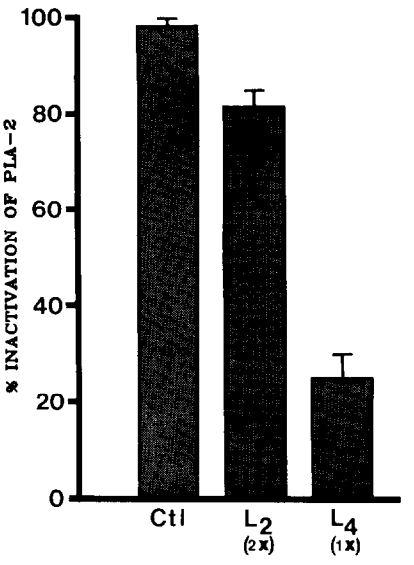

Fig. 4. Effect of equal ε-amino concentration, 2x L_2 versus 1x L_4, on the ability of MLD to inactivate PLA_2. MLD (100 μM) was preincubated for 60 min at 41° (pH 7.4) with buffer (Ctl), L_2 (200 μM) or L_4 (100 μM); aliquots were added to an equal volume of PLA_2 (5.0 μM), incubated for 60 min at 41° (pH 7.4), and assayed for PLA_2 activity. Final concentrations assayed were: MLD, 0.5 μM; L_2, 1.0 μM; L_4, 0.5 μM; and PLA_2, 25 nM (N = 5).

Table 2. Effect of preincubation of MLD with equimolar Lys-Trp-Gly-Lys (LTGL) or Lys-Tyr-Lys (LTL) on the ability of MLD to inactivate PLA_2

Time (min)	% Inactivation of PLA_2		
	MLD alone	LTGL	LTL
0	98.90 ± 0.16	97.67 ± 0.40*	99.42 ± 0.24
60	99.08 ± 0.15	84.09 ± 2.34*	96.89 ± 0.39*

MLD was preincubated with the respective peptide, and aliquots were removed at 0 and 60 min, added to an equal volume of bee venom PLA_2 (5.0 μM) and incubated for 60 min to achieve maximal inactivation of the enzyme before assay. Final concentrations assayed were: MLD = 0.5 μM; peptides, 0.5 μM; and PLA_2, 25 nM (N = 5).

* Significantly different from MLD alone (Student's t-test, $P < 0.05$).

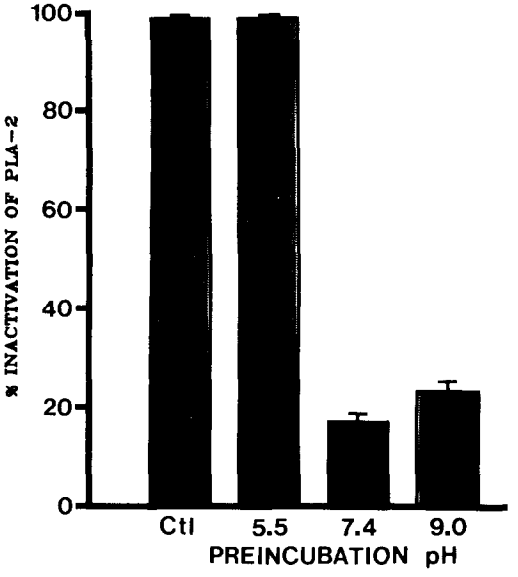

Fig. 5. Effect of pH on the inactivation of MLD by the L_4 peptide. MLD (100 μM) was preincubated with buffer (Ctl) or the L_4 peptide (100 μM) at pH 5.5 (10 mM [*N*-morpholino]ethanesulfonic acid 1 mM $CaCl_2$), pH 7.4 (10 mM HEPES, 1 mM $CaCl_2$) or pH 9.0 (10 mM Trizma base, 1 mM $CaCl_2$) at 41° for 60 min; aliquots were removed, added to an equal volume of PLA_2 (5.0 μM) in 100 mM HEPES (pH 7.4 at 41°), incubated for 60 min at 41°, and assayed for PLA_2 activity. Final concentrations assayed were: MLD, 0.5 μM; L_4, 0.5 μM; and PLA_2, 25 nM (N = 3).

of MLD activity by the L_4 peptide, whereas at pH 7.4 to 9.0 there was a 79.7% reduction in MLD activity (values at pH 7.4 and 9.0 were not significantly different; Student's t-test, $P < 0.05$). These results qualitatively correlate with the pH dependence of the irreversible inactivation of bee venom PLA_2 by MLD [6] in which there is a rapid change in the inhibition profile between pH 6.0 and 7.0 and maximum inactivation is achieved by pH 8.0.

Relationship between chromophore development and inactivation of bee venom PLA_2. The correlation between chromophore development in a manoalide-bee venom PLA_2 complex and its rate of inactivation is shown in Fig. 6. Inactivation of 32 μM bee venom PLA_2 by 200 μM MLD was rapid with a maximum rate of inactivation occurring between 15–30 sec of incubation (inset Fig. 6). The chromophore development at 437 nm was slower and reached a maximum rate at approximately 1 min of incubation (inset Fig. 6). At 8 min the inactivation of PLA_2 was complete (100%) but the chromophore had only developed to 50% of its maximum. These data suggest that these two processes, chromophore production and rate of inactivation of PLA_2, may be independent. Depending on the concentrations used, pH, and incubation condition for the inactivation of bee venom PLA_2 by MLD, the chromophore development may be associated in some manner with the complex kinetics observed, perhaps coincident with the irreversibility of this drug.

DISCUSSION

The inactivation of PLA_2 enzymes by MLD appears to be essentially irreversible [6, 7] and may involve covalent modification of lysine residues [7]. Not all sources of PLA_2 are inactivated by MLD [8]. It is conceivable that this differential inhibition of PLA_2 may be related to the presence or absence of a lysine residue(s) associated with the catalytic mechanism [12], the substrate binding site [7], and/or the calcium binding site [13] unique to the individual enzyme. The irreversible inactivation of PLA_2 by MLD is a complex reaction exhibiting non-typical kinetics for both bee [6] and cobra [7] venom PLA_2s. The complexities of the inactivation mechanism are possibly due to multiple reactions between MLD and PLA_2 [6] or a sequential modification of the MLD molecule [7]. In the present study we demonstrate that the reactivity of MLD, to produce a chromophore (λ_{max} 437 nm) or inactivate PLA_2, is dependent on the presence of two nucleophilic groups, and that a specific intramolecular distance between these nucleophilic groups may be necessary.

The spectral data of the MLD–Lys reaction demonstrate a slow and sequential reaction that produced chromophores with λ_{max} at 437 and 511 nm. Lombardo and Dennis [7] demonstrated that MLD forms two carbonyl groups, the hydroxybutenolide and pyran moieties opening to form reactive aldehyde functions on MLD. Bennett *et al.* [8] have proposed a model whereby a nucleophile would undergo Michael addition at the hydroxybutenolide aldehyde of the open ring form to produce a dial that accounts for the chromophore production. On the other hand, our data suggest that the chromophore production is not entirely dependent on the mass concentration

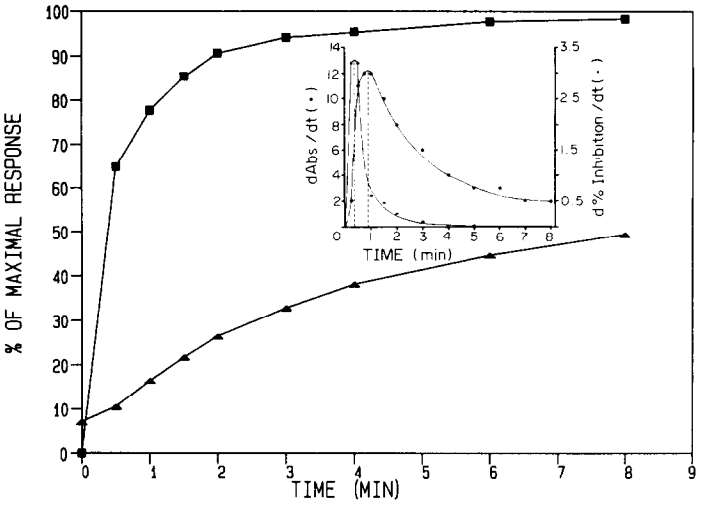

Fig. 6. Comparison of the rate of inactivation and rate of chromophore development of bee venom PLA_2 by MLD. PLA_2 (0.5 mg/ml, 32 μM) was preincubated (41° at pH 7.4) with MLD (200 μM) and assayed at the indicated time intervals for remaining PLA_2 activity (■) and also monitored at 437 nm for chromophore development (▲). Maximum values of inactivation and chromophore production were taken at 60 min of preincubation (99.64% inactivation and 0.387 ABS, respectively), and data points are expressed as percent of maximal response. Inset figure is the first derivative plot of inactivation (■) and chromophore (●) curves (N = 3).

of nucleophilic groups with which MLD may react but also on the presence of two nucleophilic groups that have an ordered mechanism for chromophore production rather than simple random collision with a single nucleophilic group as suggested by Bennett *et al.* [8]. The requirement for two separate nucleophilic groups for reactivity may also explain the observed biphasic nature of the percent inhibition vs pH profile of Lombardo and Dennis [7] which suggests that a sequential modification of MLD may occur. Therefore, the hydroxybutenolide and pyran moieties of MLD express different reactivity towards nucleophilic attack. (The hydroxybutenolide moiety has been observed to have a greater reactivity towards Lys, reacting with Lys before the pyran moiety in NMR studies.*) It is plausible that chromophore production, under conditions where MLD is able to inactivate bee venom PLA_2, may be associated with the reaction of MLD with a nucleophilic group at the hydroxybutenolide moiety, whereas the irreversible inactivation of bee venom PLA_2 may depend on a subsequent reaction with a second nucleophile at the pyran moiety. These possibilities are under investigation.

Recently, a new marine natural product was discovered, Luffariellolide, which lacks the pyran moiety in MLD.† This compound inactivated bee venom PLA_2 (IC_{50} = 0.23 μM), was partially reversible by dialysis, did not produce a chromophore, and was not inactivated by the L_4 peptide.† These results suggest that the initial inactivation of bee venom PLA_2 by MLD and its irreversible effect could be associated with the reactivity at the hydroxybutenolide of MLD with one nucleophilic group, and the irreversible character of the inhibition may be associated with reactivity of a second nucleophilic group at the pyran moiety.

The absence of free Cys residues on bee venom PLA_2 [11] eliminates the MLD–Cys reaction as a likely participant in the inactivation mechanism. However, the observed reduction in MLD activity by sulfhydryl groups (which may also require the presence of a second nucleophile) may become significant if the target enzyme and a protein with free Cys residues are in competition and generally express the same "affinity" for MLD. This phenomenon has been demonstrated by Bennett *et al.* [8] using bovine serum albumin (BSA) and poly-L-lysine in a 10,000-fold molar excess over PLA_2. We have also observed a reduction in MLD activity by BSA (1 mg protein/ml) and cell culture medium containing 5% fetal calf serum (1.5 mg protein/ml), both of which reduced the ability of MLD to inhibit PLA_2 by 50% after a 60-min preincubation (data not shown). The reactions of MLD with proteins and Cys, causing a reduction of MLD activity, were much slower than the observed inactivation of bee venom PLA_2 by MLD [6], but they may account for some of the observed differences in IC_{50} values between different sources of purified enzymes [6–8] and crude enzyme preparations containing other intracellular proteins [8, 14], although Bennett *et al.* [8] have demonstrated with preparations of intracellular PLA_2s that sensitivity to inactivation by MLD does not correlate with the amount of total protein present. However, they also demonstrated, using a purified venom PLA_2, that the addition of exogenous

* M. Kernan and D. J. Faulkner (Scripps Institute of Oceanography, La Jolla, CA), personal communication, cited with permission.

† K. F. Albizati, T. Holman, D. J. Faulkner, K. B. Glaser and R. S. Jacobs, Experientia; in press.

proteins does shift the dose–response curve to the right (increasing the apparent IC_{50}). It is apparent that there may be other factors related to the primary and tertiary structure of these PLA_2s that govern sensitivity to inactivation by MLD.

The structural requirements for chromophore production and reduction of MLD activity by free amino acids and analogs, along with the data of Lombardo and Dennis [7] suggest that the two nucleophilic groups required for optimal MLD reactivity (inactivation of PLA_2) may be the ε-amino groups of Lys residues that are located a suitable distance from one another, in the primary or tertiary structure of the enzyme. Our data collectively indicate that this optimal distance for MLD reactivity may be a 1,4-Lys arrangement as the L_4 peptide and Lys-Trp-Gly-Lys peptide expressed a preferential reactivity towards MLD as compared to the L_2, L_3, 1,3-Lys peptides or free Lys. The greater reactivity of the L_4 peptide was not due to mass concentration of ε-amino groups in peptide form as a 2-fold molar excess of the L_2 peptide did not reduce MLD activity significantly. Poly-L-lysine (~192 Lys residues) provides MLD with a multiple number of reactive sites, and complete inactivation of MLD was obtainable almost immediately in our system. A 1,4-Lys arrangement is present in bee venom PLA_2 [11] and is adjacent to the hypothesized active site His residue [15].

We have also demonstrated that the inactivation of bee venom PLA_2 and chromophore production by MLD appear to be distinct processes. There was no direct correlation between the inactivation rate which is very rapid and the chromophore development which is a much slower process. It may be that the binding of MLD to certain residues (e.g. Lys or 1,3-Lys arrangements) will produce a chromophore reaction that develops after the enzyme is inactivated, suggesting more than one binding site for MLD on PLA_2. Therefore, it is plausible that the rapid irreversible inactivation of PLA_2 may be associated with the binding of MLD to a specific, "high affinity", peptide sequence on the enzyme. Using the casual relationship between inactivation of PLA_2 and generation of the chromophore, elucidation of the MLD binding site using the chromophore as a marker is currently under investigation.

Another degree of specificity for MLD may be dependent on the optimal environmental pH for that enzyme. This selectivity has been observed for inhibition of pancreatic PLA_2 where preincubation at pH 6.0 (enzyme optimum) did not inactivate the enzyme but preincubation at pH 7.4 and assay at pH 6.0 resulted in inactivation (unpublished observations). It should be noted that reduction in MLD activity by the L_4 peptide is also pH dependent, that is, acidic pH prevented the L_4 peptide–MLD conjugate from forming.

In summary, the following conclusions can be drawn: (1) The reaction producing the chromophore (λ_{max} = 437 nm) of MLD with amino acids, and possibly bee venom PLA_2, requires two nucleophilic groups that are presented in an ordered mechanism rather than by simple random collision. (2) The MLD–Cys reaction, which partially reduces MLD activity, can be eliminated as a likely participant in the enzyme inactivation mechanism due to the absence of free cysteine residues on bee venom PLA_2. (3) In contrast to Cys, the MLD–Lys reaction requires Lys in a polymeric form to reduce MLD activity, an optimal form of the Lys polymer appears to be the L_4 peptide, and some selectivity was observed when the Lys residues were in a 1,4-Lys (Lys-Trp-Gly-Lys) arrangement. A 1,4-Lys does exist on bee venom PLA_2 [11] and is adjacent to the hypothesized active site His [15]. (4) The chromophore development and rate of inactivation of bee venom PLA_2 appear to be independent processes. The chromophore development may be associated in the irreversible binding of MLD.

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