UC San Diego

UC San Diego Previously Published Works

Title

Inhibition of venom phospholipases A2 by manoalide and manoalogue. Stoichiometry of incorporation.

Permalink

https://escholarship.org/uc/item/1v72p3gs

Journal

Journal of Biological Chemistry, 266(25)

ISSN

0021-9258

Authors

Reynolds, LJ
Mihelich, ED
Dennis, EA

Publication Date

1991-09-01

DOI

10.1016/s0021-9258(18)55330-8

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 266, No. 25, Issue of September 5, pp. 16512-16517, 1991
Printed in U.S.A.
© 1991 by The American Society for Biochemistry and Molecular Biology, Inc.

Inhibition of Venom Phospholipases A_2 by Manoalide and Manoalogue

STOICHIOMETRY OF INCORPORATION*

(Received for publication, April 17, 1991)

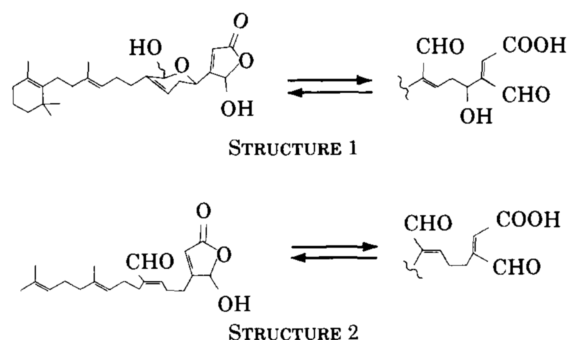
Laure J. Reynolds‡§, Edward D. Mihelich¶, and Edward A. Dennis‡||

From the ‡Department of Chemistry, University of California, San Diego, La Jolla, California 92093-0601 and ¶Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, Indiana 46285

We have previously described the irreversible inhibition of cobra venom phospholipase A_2 (PLA_2) by the marine natural product manoalide (MLD) (Lombardo, D., and Dennis, E. A. (1985) *J. Biol. Chem.* 260, 7234–7240) and by its synthetic analog, manoalogue (MLG) (Reynolds L. J., Morgan, B. P., Hite, G. A., Mihelich, E. D., and Dennis, E. A. (1988) *J. Am. Chem. Soc.* 110, 5172–5177). We have now made a direct comparison of the action of these two inhibitors on PLA_2 from cobra, bee, and rattlesnake venoms and have found that MLG behaves kinetically similarly to MLD in all cases with only minor differences. The time courses of inactivation differ significantly between the three enzymes, however, with the inactivation of bee and rattlesnake $PLAs_2$ occurring much faster than does the inactivation of the cobra venom enzyme. The enzymes also differ in their sensitivity to the presence of Ca^{2+} during the inactivation. Of the three enzymes, the most Ca^{2+}-sensitive is the rattlesnake enzyme, which shows a much faster rate of inactivation in the presence of Ca^{2+} than in the presence of EGTA. However, the same rate of inactivation was also observed when the inhibitor Ba^{2+} was substituted for Ca^{2+}, indicating that catalytic activity is not required for inactivation of the enzyme. To probe the mechanism of inactivation and to determine the stoichiometry of incorporation, we have synthesized ^3H-labeled MLG and have found that inactivation of cobra PLA_2 is accompanied by an incorporation of 3.8 mol of [^3H]MLG/mol of enzyme. The same amount of ^3H incorporation was observed when *p*-bromophenacyl bromide-inactivated PLA_2 was incubated with [^3H]MLG, again indicating that catalytic activity is not required for the reaction of PLA_2 with MLG. All together, these results suggest that MLD and MLG are not suicide inhibitors of PLA_2. A portion of the incorporated radioactivity was acid-labile, and dialysis of the radiolabeled PLA_2 under acidic conditions resulted in a loss of about one-third of the enzyme-associated radioactivity, leaving 2.4 mol of [^3H]MLG/mol of PLA_2. In previous studies, amino acid analysis, which also included acid treatment, indicated that MLG-modified cobra phospholipase A_2 contained 2.8 mol of Lys less than the native enzyme. Thus, 1 mol of [^3H]MLG is incorporated per mol of Lys lost. The implications of this 1:1 stoichiometry of MLG to Lys on the mechanism of reaction of these inhibitors is discussed.

We have previously described the inhibition of cobra venom phospholipase A_2 (PLA_2)[1] by the marine natural product manoalide (MLD) (1) and by its synthetic analog, manoalogue (MLG) (2). Both of these compounds cause a partial irreversible inactivation of the enzyme. This inactivation occurs with a loss of ~3-4 lysine residues as detected by amino acid analysis. The inhibition of PLA_2 by manoalide may play a role in the anti-inflammatory activity of this compound *in vivo* as first recognized by Jacobs *et al.* (3). Thus, understanding the mechanism of action of these compounds on PLA_2 is of considerable interest.

Manoalide contains two reactive ring structures, a hemiacetal ring and a γ-lactone ring (4), which open at high pH to generate α,β-unsaturated aldehydes as shown in Structure 1 (1). Manoalogue contains the lactone ring and the α,β-unsaturated aldehyde portion of the hemiacetal ring, but lacks the hydroxyl part of the hemiacetal ring as well as a portion of the terminal cyclohexenyl ring (Structure 2). Previous studies (2) have demonstrated that both the opening of the lactone ring and the presence of the free aldehyde group are required for irreversible inhibition. The structure of the MLD-PLA_2 adduct remains unknown. However, various mechanisms for inactivation have been proposed that are based on a reaction of lysine residues with the two unsaturated aldehyde groups (2, 5–7). One model proposes that 2 lysine residues in the enzyme react with a single inhibitor molecule (6). Other models propose the reaction of a single lysine residue/inhibitor (2, 7). Determining the stoichiometry of inhibitor incorporated per lysine modified could help distinguish between these mechanisms.



STRUCTURE 1

STRUCTURE 2

* This work was supported in part by National Institutes of Health Grant GM 20501 and National Science Foundation Grant PCM 88-17392. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by National Institutes of Health Postdoctoral Fellowship GM 12202.

|| To whom correspondence should be addressed.

[1] The abbreviations used are: PLA_2, phospholipase A_2; MLD, manoalide; MLG, manoalogue; thio-PC, 1,2-bis(decanoylthio)-1,2-dideoxy-*rac*-glycero-3-phosphatidylcholine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

The phospholipases A_2 that have been sequenced to date have been divided into three main classes based on sequence homology (8). In this report, we present the first side-by-side comparison of manoalide and manoalogue using PLA_2 from each of the three classes: cobra (Type I), rattlesnake (Type II), and bee (Type III) venoms. We also describe the synthesis of tritium-labeled manoalogue that we have used to determine the stoichiometry of MLG incorporation into PLA_2 during the inactivation of the enzyme. Together, these studies give us important insights into the mechanism of action of these inhibitors.

EXPERIMENTAL PROCEDURES

Materials—Phospholipase A_2 from cobra venom (*Naja naja naja*) was purchased from Miami Serpentarium (Punta Gorda, FL) and purified as described elsewhere (9, 10). PLA_2 from rattlesnake (*Crotalus adamanteus*) was purchased from Worthington. Bee venom PLA_2 was obtained from Boehringer Mannheim and Sigma. Nonradiolabeled manoalogue ((*E,E*)-2-[3-(2,5-dihydro-2-hydroxy-5-oxo-3-furanyl)propylidine]-6,10-dimethyl-5,9-undecadienal) was synthesized as described previously (2). Manoalide was the generous gift of Dr. Paul Scheuer (University of Hawaii at Manoa, Honolulu, HI). Sodium boro[^{3}H]hydride was purchased from Amersham Corp. 1,2-Bis(decanoylthio)-1,2-dideoxy-*rac*-glycero-3-phosphatidylcholine (thio-PC) was synthesized as previously described (11). Dipalmitoyl-*sn*-glycero-3-phosphorylcholine was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL), and Triton X-100 was from Calbiochem. Hydroxylamine hydrochloride and 4,4′-dithiodipyridine were from Aldrich. Bovine serum albumin and *p*-bromophenacyl bromide were purchased from Sigma. Scintillation counting was performed in Safety-Solve scintillation fluid (Research Products International Corp., Mt. Prospect, IL) with a TM Analytic Beta Trac 6895 counter.

Preparation of [^{3}H]Manoalogue—Radiolabeled manoalogue was synthesized from methylated manoalogue following a procedure previously described for the synthesis of nonradiolabeled manoalogue (2). Tritium was incorporated at the free aldehyde position by reduction with sodium boro[^{3}H]hydride (196 mCi/mmol). The reduction was followed by the hydrolysis of the methoxyl group on the lactone ring and then the reoxidation of the alcohol to the aldehyde with pyridinium dichromate (Fig. 1). Products were purified at each step by preparative thin-layer chromotography on Uniplate-T taper plates with Silica Gel GF from Analtech, Inc. (Newark, DE). The final product had a specific activity of ~25 mCi/mmol with 25,000 cpm/nmol.

Enzyme Assays—PLA_2 activity was measured in a spectrophotometric assay using a racemic thio-PC substrate (12, 13). The substrate was prepared by drying the appropriate amount of thio-PC in chloroform solution under a stream of nitrogen. The lipid was solubilized into mixed micelles by the addition of Trixon X-100 in buffer, followed by heating to 40°C and vortexing. The final assay solution contained 0.5 mM thio-PC, 2 mM Triton X-100, 10 mM $CaCl_2$, 0.1 M KCl, and 25 mM Tris-HCl (pH 8.5). A 0.3-ml volume of this solution was added to a cuvette (2 × 10 mm) along with 5 μl of 4,4′-dithiodipyridine (50 mM in ethanol) and equilibrated to 30 °C. The reaction was initiated by the addition of 5 μl of PLA_2 solution containing ~70 ng of protein. The production of free thiols was monitored by observing their reaction with 4,4′-dithiodipyridine to form a product that absorbs at 324 nm. Rattlesnake and bee $PLAs_2$ behave similarly to the cobra enzyme in this assay, with rates of 45–80 μmol/min/mg and half-maximal velocity occurring at ~50–80 μM.

Some activities were also determined with the pH-stat assay. The activity toward dipalmitoyl-*sn*-glycero-3-phosphorylcholine-mixed micelles was measured using a Radiometer pH-stat in a 2-ml assay volume with 5 mM dipalmitoyl-*sn*-glycero-3-phosphorylcholine, 20 mM Triton X-100, and 10 mM $CaCl_2$ at 40 °C and pH 8.0 as described previously (14). Assays were initiated by the addition of 10 μl (400 ng) of bee venom PLA_2 or 5 μl (70 ng) of cobra PLA_2.

Inhibition of PLA_2—The inactivation of PLA_2 by manoalide and manoalogue was studied by preincubating the enzyme with the inhibitor at 40 °C. At different times, aliquots (usually 5 μl) were removed from the preincubation mixture and diluted into the thio-PC or pH-stat assays to determine remaining enzyme activity. A typical preincubation mixture contained 1 μM PLA_2 in a 200-μl volume of 0.1 M Tris-HCl (pH 8.0). Preincubations were initiated by the addition of inhibitor, dissolved in methanol or dimethyl sulfoxide, to a final concentration of 300 μM or, in the case of the controls, by the addition of solvent. The solvent concentration in the preincubation was usually 1.2%. Incubation mixtures testing for calcium effects contained either 10 mM $CaCl_2$, 10 mM $BaCl_2$, or 1 mM EGTA. Variations to these general conditions are noted in the figure legends.

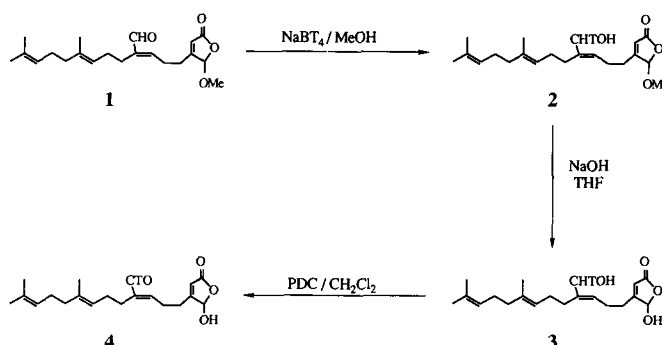


FIG. 1. **Radiolabeled manoalogue 4 was synthesized from the methoxybutenolide 1 by reduction of the aldehyde with sodium boro[^{3}H]hydride, followed by removal of the methyl group and reoxidation to the aldehyde with pyridinium dichromate (*PDC*).** *THF*, tetrahydrofuran.

With cobra venom PLA_2, inactivation was very slow, and the remaining enzyme activity for each sample was calculated relative to its own activity immediately after mixing. With the bee and rattlesnake enzymes, which were inactivated rapidly, a significant amount of activity was lost during the first 0.5 min. Thus, the remaining activity in these samples was calculated relative to the controls. Triton X-100 (50 μM) was added to the preincubation mixtures in some of the experiments to minimize loss of enzyme to the walls of the microcentrifuge tubes. The presence of Triton X-100 in the preincubation mixture at this low concentration did not affect the results of the experiment.

Manoalogue was also tested for its ability to inhibit in the thio-PC assay in the absence of preincubation. MLG in dimethyl sulfoxide was added to thio-PC/Triton X-100-mixed micelles to a final concentration of 300 μM, and the solution was sonicated for 2 min in bath sonicator. PLA_2 activity on these mixed micelles was then determined as described above for the thio-PC assay.

[^{3}H]Manoalogue-labeled PLA_2—Cobra venom PLA_2 (10–30 μM) was incubated with a 20-fold excess of [^{3}H]MLG in 20 mM Tris-HCl (pH 8.0) at 40 °C. After 4 h, maximum inhibition was reached, and excess [^{3}H]MLG was removed by passing the enzyme through a Pharmacia LKB Biotechnology PD-10 Sephadex G-25 column and eluting with H_2O. The enzyme fractions were dialyzed exhaustively against H_2O for 3 days. Identically sized aliquots of the dialysate were then removed for scintillation counting and protein quantitation. Protein concentrations were measured by the method of Lowry *et al.* (15) with bovine serum albumin as a standard using the correction factor for *N. naja naja* PLA_2 determined by Darke *et al.* (16). The stoichiometry of labeling was calculated by comparing the moles of tritium (based on 25,000 cpm/nmol) to the quantity of protein present in the dialysate. The result reported is an average of five experiments ± S.D.

The stability of [^{3}H]MLG-labeled PLA_2 under acidic conditions was questioned following a series of high pressure liquid chromatography experiments, which showed a slightly lower recovery of ^{3}H label following exposure of the protein to solutions containing 0.1% trifluoroacetic acid. After dialysis against H_2O, some of the [^{3}H]MLG samples were dialyzed for 2–4 additional days against either H_2O (control) or 0.1% trifluoroacetic acid (pH 2.0). Samples were then recounted and assayed for protein as described above. The results reported represent the average of four experiments. $NaBH_4$-reduced [^{3}H]PLA_2 was prepared by treating [^{3}H]MLG-labeled PLA_2 with sodium borohydride essentially as described by Glaser *et al.* (17).

Bromophenacyl Bromide-labeled PLA_2—*p*-Bromophenacyl bromide-inactivated cobra venom PLA_2 was prepared by incubating PLA_2 (20 μM) with a 100-fold excess of *p*-bromophenacyl bromide in 50 mM Tris-HCl (pH 8.0). After 2 h, no enzymatic activity was detected by the pH-stat assay. Excess *p*-bromophenacyl bromide was removed by chromatography on a Pharmacia LKB Biotechnology PD-10 column, followed by dialysis against H_2O. Incubation with [^{3}H]MLG was performed as described above. The result reported is

an average of two experiments and is compared to matched controls (no *p*-bromophenacyl bromide), which were run on the same days.

RESULTS

The high concentration of inhibitor used in the preincubation mixtures in this study was chosen to maximize the effect of these compounds on the rate and extent of inactivation to facilitate their comparison. The concentration chosen (300 μM) is also near their limit of solubility (1). MLD and MLG do inhibit venom $PLAs_2$ effectively at much lower concentrations of inhibitor. Previous studies have reported IC_{50} values of 7.5 μM for the inhibition of cobra PLA_2 by MLG (2) and from 0.05 to 2 μM for the MLD inhibition of bee (18), cobra (1), and rattlesnake (*Crotalus durissus*) (7) $PLAs_2$. However, inactivation is slower at these lower concentrations.

In this study, the rate and extent of inactivation varied slightly from day to day. For example, in the presence of 300 μM MLG or MLD, the maximum inhibition of cobra PLA_2 typically reached between 50 and 60% of the original activity over a period of 2–3 h. The inactivation of bee and rattlesnake venoms was generally much more consistent and showed less variability than did the cobra venom. Each experiment was performed on at least two separate occasions, and the same effect was observed each time. Representative experiments are shown in the figures.

Inactivation of Cobra Venom PLA_2—In the absence of added Ca^{2+} or EGTA, the inhibition of cobra venom PLA_2 by 300 μM MLG was almost identical in the rate and extent of inactivation to that by MLD. As described above, with both inhibitors, 50–60% inactivation was observed in 2–3 h. This result was independent of the type of assay used since it was observed using both the thio-PC and pH-stat assays. Slight differences were observed between the two inhibitors when $CaCl_2$ or EGTA was included in the preincubation mixtures (Fig. 2). With MLD, incubation in the presence of $CaCl_2$ resulted in a somewhat faster rate of inactivation than in the presence of EGTA, as was reported earlier (1). With MLG, little difference in rate between $CaCl_2$ and EGTA was observed. Thus, inactivation by MLD appears to be slightly more sensitive to the presence of Ca^{2+} than is that by MLG. Control samples, which contained $CaCl_2$ or EGTA, but no inhibitors, lost $<2\%$ of their activity in 4 h.

Inactivation of Bee Venom PLA_2—The inactivation of bee venom PLA_2 by MLD resulted in the loss of 95% of the enzymatic activity in 30 min (Fig. 3). The rates observed with

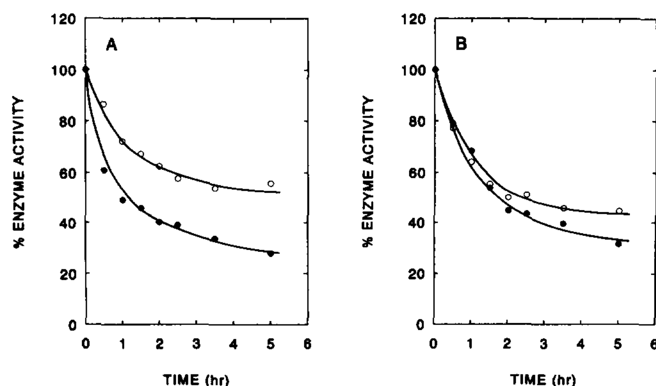


FIG. 2. **Cobra venom PLA_2 (1 μM) was incubated in 20 mM Tris-HCl (pH 8.0) at 40 °C in the presence of 300 μM MLD (*A*) or MLG (*B*), 1.2% methanol, and either 10 mM $CaCl_2$ (●) or 1 mM EGTA (○).** At various times, 5-μl aliquots were removed and assayed for enzymatic activity in the thio-PC assay. Percent enzyme activity is calculated relative to the activity of each sample at time 0.

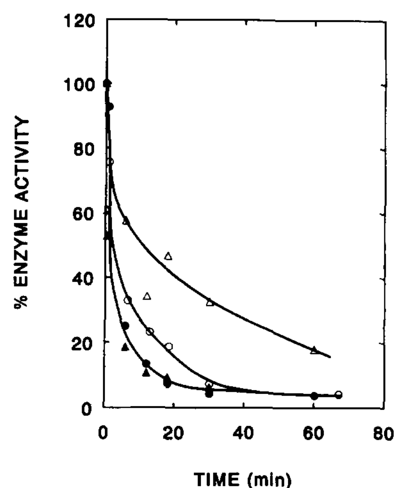


FIG. 3. **Bee venom PLA_2 (2.5 μM) was incubated at 40 °C in 20 mM Tris-HCl (pH 8.0) and 4% methanol in the presence of 50 μM (▲) or 300 μM (●) MLD or 50 μM (△) or 300 μM (○) MLG.** At various times, 10-μl aliquots were removed and assayed in the pH-stat assay. Percent enzyme activity is calculated relative to the control, with no inhibitor, at time 0.

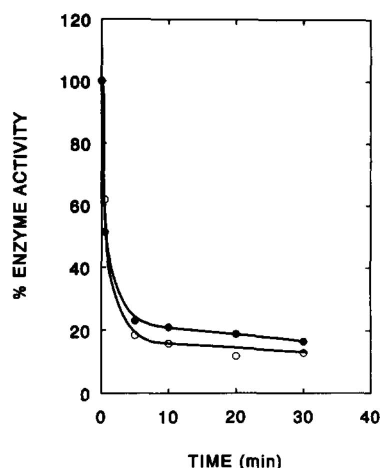


FIG. 4. ***C. adamanteus* PLA_2 (1 μM) was preincubated at 40 °C in the presence of 300 μM MLD (●) or MLG (○) with 0.1 M Tris-HCl (pH 8.0) and 1.2% methanol.** At various times, 5-μl aliquots were removed and assayed for enzymatic activity in the thio-PC assay. Percent enzyme activity is calculated relative to the activity of the control, with no inhibitor, at time 0. Each point represents the average of duplicate samples.

50 and 300 μM MLD were the same. The rate of inactivation by MLG was slower than that observed by MLD, but reached the same extent of inhibition. This slower inactivation is most pronounced at the lower inhibitor concentration. The control samples, in this experiment, lost 6% of their activity over 60 min. Similar to a previous report on MLD (18), no difference in the rate of reaction was observed for either MLG or MLD (300 μM) between samples incubated in the presence of $CaCl_2$ or EGTA.

Inactivation of Rattlesnake Venom PLA_2—Rattlesnake venom PLA_2 was inactivated extremely rapidly in the presence of MLD and MLG. With both inhibitors, at 40 °C, ~80% of the enzymatic activity was lost within 5 min (Fig. 4), with little further loss of activity up to 2 h. When the preincubation was performed at room temperature, a gradual loss of activity was observed over the first 5 min with 300 μM MLG. This inhibition is concentration-dependent, and the inactivation is slower with lower concentrations of inhibitor. At 50 μM, MLG

caused a loss of 55% activity in the first 5 min, whereas a sample with 5 μM MLG lost 15% activity in 5 min (data not shown). By 30 min, the activity of these samples was down to 75 and 50% of the initial activity, respectively.

Unlike the bee and cobra venom enzymes, rattlesnake venom PLA_2 was inactivated by MLG at a considerably faster rate when incubated in the presence of $CaCl_2$ than in the presence of EGTA (Fig. 5). Preincubations were also performed in the presence of $BaCl_2$ to test whether this faster inactivation was due to catalytic activity of the enzyme in the presence of Ca^{2+} or to some other effect. Rattlesnake venom PLA_2 showed no activity in the thio-PC assay when $CaCl_2$ was replaced by $BaCl_2$. However, inactivation of rattlesnake PLA_2 by MLG in the presence of $BaCl_2$ was almost identical to that observed with $CaCl_2$. Inactivation of the enzyme by MLD was also faster in the presence of $CaCl_2$ than in the presence of EGTA (data not shown). The remaining enzyme activity in these experiments was compared to the activity of the controls, which contained $CaCl_2$, $BaCl_2$, or EGTA, but no inhibitor. Control samples with EGTA showed slightly higher activity than the other controls.

As described above, MLD and MLG caused a partial inactivation of all three enzymes. The extent of inactivation was not affected significantly by further incubation (up to 24 h) or by the addition of a second dose of inhibitor. The addition of hydroxylamine hydrochloride (600 mM) in either Tris-HCl (pH 7.0) or glycine (pH 9.0) buffer to each of the three inactivated enzymes resulted in a recovery of 0–20% of the enzymatic activity; however, the data for these experiments were not very reproducible. No differences were observed between incubations performed with either methanol or dimethyl sulfoxide as solvents.

The loss of PLA_2 activity observed upon incubation of the enzyme with MLG was not due to an effect of the inhibitor of the thio-PC assay itself or to a competitive inhibition during the assay. Enzyme solutions were diluted 60-fold from the preincubation mixture into the assay solution, bringing the inhibitor concentration from 300 μM in the preincubation mixture down to 5 μM in the cuvette. When enzyme activity was tested on substrate-mixed micelles composed of 0.5 mM racemic thio-PC (*i.e.* 0.25 mM L-thio-PC), 2 mM Triton X-100, and 300 μM MLG, all three enzymes retained >75% of the activity observed in the absence of inhibitor. This relatively small decrease in activity at such a high inhibitor concentration can be explained by substrate dilution in the interface (19) and by inactivation of the enzymes during the time course of the assay.

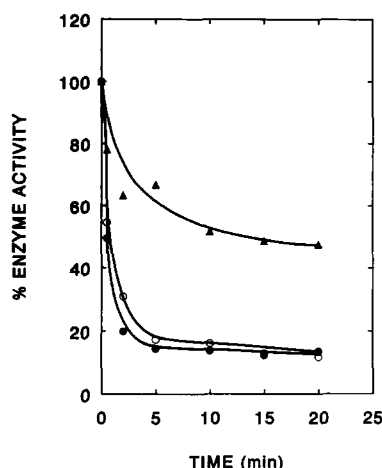


FIG. 5. ***C. adamanteus* PLA_2 (1 μM) was preincubated at room temperature in 0.1 M Tris-HCl (pH 8.0) with 300 μM MLG, 50 μM Triton X-100, 1.2% dimethyl sulfoxide, and 1 mM EGTA (▲), 10 mM $CaCl_2$ (●), or 10 mM $BaCl_2$ (○).** At various times, 5-μl aliquots were removed and assayed in the thio-PC assay. Controls, with no inhibitor, contained EGTA, $CaCl_2$, or $BaCl_2$. Percent enzyme activity is calculated relative to the activity of the appropriate control at each time point. Each point represents the average of duplicate samples.

Incorporation of [^{3}H]MLG—When incubated with [^{3}H] MLG, cobra venom PLA_2 showed a loss of activity over 3 h. In this same time period, a gradual increase in the amount of tritium associated with the enzyme was observed. At 4 h, 3.8 ± 0.6 mol of tritium were observed per mol of PLA_2. Incubation for up to 19 h resulted in only an 8% increase in the amount of protein-associated radioactivity. Further dialysis of the MLG-labeled protein in 0.1% trifluoroacetic acid resulted in a loss of about one-third of the protein-associated radioactivity, leaving 2.4 ± 0.4 mol of [^{3}H]MLG/mol of PLA_2. The samples dialyzed against H_2O showed no change in radioactivity. The loss of radioactivity in acid appeared to occur in the first 24 h of dialysis and did not increase when dialysis was extended for up to 5 days. The loss of tritium was not prevented by reducing the [^{3}H]MLG-labeled PLA_2 with sodium borohydride prior to acid dialysis. PLA_2 inactivated by *p*-bromophenacyl bromide incorporated roughly the same amount (106%) of radioactivity/mole of enzyme compared to the native enzyme. Thus, incorporation of radiolabel occurs in the absence of catalytic activity. The presence of trifluoroacetic acid or MLG in the samples did not interfere with the values obtained in the assay of Lowry *et al.* (15).

DISCUSSION

Inactivation of PLA_2—In this study, with all three venom enzymes tested, the rate and extent of inactivation by MLG were very close to those observed with MLD. This similarity in MLD and MLG reactivity suggests that these inhibitors have the same mechanism of reaction. The two inhibitors are not identical, however; and there are some slight differences between the two, such as the slower reactivity of MLG with bee venom PLA_2. The faster inactivation by MLD in this case could reflect a more facile ring opening or a more favorable binding to the enzyme.

Whereas the two inhibitors behaved similarly to each other on a particular enzyme, the resulting time courses of inactivation differed significantly from enzyme to enzyme. The rattlesnake enzyme is inactivated extremely rapidly and loses ~80% of its activity in 5 min; the bee enzyme reacts slightly slower, but is almost completely inactivated; and the cobra enzyme reacts much slower than the other two, losing only half of its activity in 3 h. Differences in the sensitivity of phospholipases to MLD have also been observed by Bennett *et al.* (7). This difference between enzymes could reflect different mechanisms of reaction for each enzyme. However, since inactivation presumably involves the formation of inhibitor-lysine adducts and since lysine residues are not conserved between enzymes (8), it more likely reflects a different distribution of reactive lysine residues near the phospholipid-binding sites of these enzymes (17). Differences between the binding of the inhibitors to the three enzymes or some other unidentified protein structural differences (7) could also play a part.

In addition to different time courses of reaction, the three enzymes also differ in their sensitivity to the presence of Ca^{2+} in the preincubation mixture (1, 18). Rattlesnake PLA_2 is inactivated much faster in the presence of $CaCl_2$ than in the presence of EGTA; bee venom PLA_2 shows no difference in the rate of inactivation with or without $CaCl_2$; and cobra

venom PLA_2 is inactivated slightly faster with $CaCl_2$ when MLD is the inhibitor, but shows little or no preference with MLG. The venom PLA_2 enzymes require the presence of Ca^{2+} for catalytic activity (20). The fact that the inactivation of rattlesnake PLA_2 is faster in the presence of Ca^{2+} may suggest that inactivation requires catalysis. However, this inactivation occurs just as fast in the presence of $BaCl_2$ as in the presence of $CaCl_2$, as was reported for the cobra enzyme (1). Ba^{2+} competes with Ca^{2+} and has been shown, with the cobra (21) and rattlesnake (22) enzymes, to be an inhibitor of PLA_2 activity. Thus, the results with $BaCl_2$ suggest that enzymatic catalysis does not play a role in the inactivation of PLA_2 by MLG and MLD. The fact that inactivation does occur with EGTA, albeit slower in the case of the rattlesnake, also supports this conclusion. Rattlesnake PLA_2, unlike the cobra and bee enzymes, requires an ordered addition of Ca^{2+} and phospholipid substrate (22). The faster inactivation seen for the rattlesnake enzyme in the presence of $CaCl_2$ and $BaCl_2$ could be due to a conformational change in the enzyme on cation binding that makes some reactive residue more accessible or to an enhanced binding of the inhibitors to the enzyme in the presence of Ca^{2+} and Ba^{2+}.

Stoichiometry of Incorporation—The inactivation of phospholipases A_2 by manoalide and manoalogue is believed to involve a reaction between the inhibitors and lysine residues on the enzyme (1). The involvement of lysine residues has been implicated by the disappearance of lysine residues upon amino acid analysis of the inactivated PLA_2 (1, 2, 17), by the reaction observed between MLD and lysine-containing peptides (23), and by the identification of possible MLD-Lys adducts by amino acid sequencing (2, 17). The ring open forms of manoalide and manoalogue contain several functional groups that could potentially react with lysine residues. Schiff base formation could occur at either of the two free aldehyde groups; Michael addition could occur at the β-unsaturated bonds to the two aldehydes; or amide formation could occur at the carboxylic acid. Michael addition could also occur on the closed form of the lactone ring. Previous studies (2, 5, 6) with manoalide analogs have indicated that irreversible inactivation of PLA_2 requires the presence of both the lactone ring and the unsaturated aldehyde portion of the hemiacetal ring. Thus, the mechanism of action of these inhibitors seems to entail reactions with two of the available functional groups. Glaser *et al.* (6) have proposed that the mechanism of action of MLD involves Schiff base formation between 2 lysine residues on the enzyme and the two free aldehyde groups of the inhibitor. Thus, one inhibitor molecule would cross-link 2 lysine residues. This mechanism was inferred by studies with lysine-containing peptides that suggested that MLD reacts with short peptides containing 2 lysine residues in a 1,4-sequence (Lys-*X*-*X*-Lys) (23).

Based on our experience with cobra venom PLA_2, however, the two-Schiff base mechanism seemed unlikely for two reasons. First, Schiff bases are known to be unstable under acidic conditions, and the inhibitor adducts have apparently survived the acid treatment prior to amino acid analysis. Second, the cobra enzyme contains 6 lysines, only 2 of which (Lys^6 and Lys^{10}) are close to each other in the primary sequence (24) or crystal structure (25). Amino acid sequencing of the MLG-modified protein indicated that Lys^6 is apparently modified, whereas Lys^{10} is still intact (2). Thus, we proposed that MLG and MLD react with Lys residues on the enzyme in a 1:1 ratio.

One of the goals of this study was to distinguish between these 1- and 2-lysine mechanisms for covalent modification of PLA_2. Manoalogue was used in this study since we can synthesize radiolabeled MLG relatively easily. The kinetic experiments described earlier suggested that MLG and MLD are reacting by similar mechanisms. Thus, any reaction mechanism inferred from the incorporation of radiolabeled MLG most likely reflects the mechanism of MLD as well.

In this study, an average of 3.8 mol of [^{3}H]MLG were retained per mol of cobra phospholipase A_2 after dialysis in H_2O. Enzyme that had been inactivated by *p*-bromophenacyl bromide, an inhibitor that reacts with the active-site histidine (26), incorporated the same amount of [^{3}H]MLG as did the native enzyme. This result indicates that catalytic activity is not required for incorporation of the radiolabel and suggests that the inhibitor is not incorporated into the catalytic site itself. Dialysis of the labeled protein under acidic conditions caused a partial loss of radiolabel. This loss of label is probably not due to the reversal of a Schiff base since it was not prevented by reduction with sodium borohydride. The retention of 2.4 mol of [^{3}H]MLG/mol of enzyme following extensive dialysis under acidic conditions suggests that the retained label is also not a simple Schiff base adduct. The partial loss of ^{3}H label could reflect a rearrangement of the MLG adduct in acid or an exchange of the ^{3}H label with ^{1}H from solvent. However, the acid-labile ^{3}H as well as the partial recovery of activity following hydroxylamine treatment suggest that there may be some other, less stable, secondary acyl-enzyme adducts formed in addition to the proposed lysine adducts.

In our previous study (2), amino acid analysis of the MLG-labeled PLA_2 showed 2.8 lysine residues less than that of the native enzyme. In this study, after acid dialysis, 2.4 mol of [^{3}H]MLG were found per mol of PLA_2. Thus, 1 mol of [^{3}H] MLG is incorporated per mol of lysine lost. This 1:1 stoichiometry is inconsistent with the two-Schiff base mechanism. Since inactivation by MLG and MLD is believed to involve a reaction with two functional groups on the inhibitor, a 1:1 stoichiometry means that a multistep reaction with a single enzyme residue is likely to occur. We have previously proposed two mechanisms for the inactivation of cobra PLA_2 by MLG (2). These mechanisms involve an initial attack by lysine on the butenolide ring by either conjugate addition or Schiff base formation. This first step would be followed by an intramolecular conjugate addition by the same lysine to the second unsaturated aldehyde, leading to the formation of an irreversible tertiary amine. Currently, we have no evidence to favor one of these mechanisms over the other.

Whereas we still do not have an accurate picture of the structure of the MLD or MLG adducts, we do have important insights into the mechanism of action of these compounds. Catalytic activity is not required for either inactivation of the enzyme or incorporation of radiolabeled inhibitor. Thus, MLD is not likely to be a "suicide" inhibitor of PLA_2, as was considered earlier to explain Ca^{2+} effects (5). Since MLG and MLD do not compete well with substrate in micelle assays, they apparently do not have a particularly strong affinity for the active site. They may, however, have a general affinity for hydrophobic sites on the enzyme (6, 7). These observations as well as the multiple labels found per enzyme suggest that the inactivation of PLA_2 by MLD and MLG is not very specific. In fact, there are several other proteins besides phospholipase A_2 that have also been shown to be inactivated by manoalide, including phospholipase C (27) and 5-lipoxygenase (28). Also, since most PLA_2 enzymes tested retain some residual activity, the inhibitors must not be modifying an essential catalytic site residue. The differences in reactivity among the phospholipases A_2 most likely reflect different distributions of lysine residues throughout the proteins. Fi-

nally, a 1:1 stoichiometry of MLG incorporated to lysine lost is inconsistent with a two-Schiff base mechanism.

REFERENCES

1. Lombardo, D., and Dennis, E. A. (1985) *J. Biol. Chem.* **260,** 7234–7240
2. Reynolds, L. J., Morgan, B. P., Hite, G. A., Mihelich, E. D., and Dennis, E. A. (1988) *J. Am. Chem. Soc.* **110,** 5172–5177
3. Jacobs, R. S., Culver, P., Langdon, R., O'Brien, T., and White, S. (1985) *Tetrahedron* **41,** 981–984
4. deSilva, E. D., and Scheuer, P. J. (1980) *Tetrahedron Lett.* **21,** 1611–1614
5. Deems, R. A., Lombardo, D., Morgan, B. P., Mihelich, E. D., and Dennis, E. A. (1987) *Biochim. Biophys. Acta* **917,** 258–268
6. Glaser, K. B., DeCarvalho, M. S., Jacobs, R. S., Kernan, M. R., and Faulkner, D. J. (1989) *Mol. Pharmacol.* **36,** 782–788
7. Bennett, C. F., Mong, S., Clarke, M. A., Krause, L. I., and Crooke, S. T. (1987) *Biochem. Pharmacol.* **36,** 733–740
8. Davidson, F. F., and Dennis, E. A. (1990) *J. Mol. Evol.* **31,** 228–238
9. Hazlett, T. L., and Dennis, E. A. (1985) *Toxicon* **23,** 457–466
10. Reynolds, L. J., and Dennis, E. A. (1991) *Methods Enzymol.* **197,** 359–365
11. Hendrickson, H. S., Hendrickson, E. K., and Dybrig, R. H. (1983) *J. Lipid Res.* **24,** 1532–1537
12. Hendrickson, H. S., and Dennis, E. A. (1984) *J. Biol. Chem.* **259,** 5734–5739
13. Yu, L., and Dennis, E. A. (1991) *Methods Enzymol.* **197,** 65–75
14. Deems, R. A., and Dennis, E. A. (1981) *Methods Enzymol.* **71,** 703–710
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
16. Darke, P. L., Jarvis, A. A., Deems, R. A., and Dennis, E. A. (1980) *Biochim. Biophys. Acta* **626,** 154–161
17. Glaser, K. B., Vedvick, T. S., and Jacobs, R. S. (1988) *Biochem. Pharmacol.* **37,** 3639–3646
18. Glaser, K. B., and Jacobs, R. S. (1986) *Biochem. Pharmacol.* **35,** 449–453
19. Dennis, E. A. (1973) *Arch. Biochem. Biophys.* **158,** 485–493
20. Dennis, E. A. (1983) in *The Enzymes* (Boyer, P., ed) 3rd Ed., Vol. 16, pp. 307–353, Academic Press, New York
21. Roberts, M. F., Deems, R. A., and Dennis, E. A. (1977) *J. Biol. Chem.* **252,** 6011–6017
22. Wells, M. A. (1972) *Biochemistry* **11,** 1030–1041
23. Glaser, K. B., and Jacobs, R. S. (1987) *Biochem. Pharmacol.* **36,** 2079–2086
24. Davidson, F. F., and Dennis, E. A. (1990) *Biochim. Biophys. Acta* **1037,** 7–15
25. Fremont, D. H., Anderson, D., Wilson, I. A., Xuong, N.-H., and Dennis, E. A. (1990) *Am. Crystallogr. Assoc. Ser. 2* **18,** 55
26. Roberts, M. F., Deems, R. A., Mincey, T. C., and Dennis, E. A. (1977) *J. Biol. Chem.* **252,** 2405–2411
27. Bennett, C. F., Mong, S., Wu, H.-L. W., Clark, M. A., Wheeler, L., and Crooke, S. T. (1987) *Mol. Pharmacol.* **32,** 587–593
28. De Vries, G. W., Amdahl, L., Mobasser, A., Wenzel, M., and Wheeler, L. A. (1988) *Biochem. Pharmacol.* **37,** 2899–2905