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Cobra Venom Phospholipase A_2 Inhibition by Manoalide

A NOVEL TYPE OF PHOSPHOLIPASE INHIBITOR*

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Manoalide, an unusual nonsteroidal sesterterpenoid recently isolated from sponge, antagonizes phorbol-induced inflammation but not that induced by arachidonic acid, suggesting that manoalide acts prior to the cyclooxygenase step in prostaglandin synthesis, possibly by inhibiting phospholipase A_2. We have now studied the inhibitory effect of manoalide on a homogeneous preparation of phospholipase A_2 from cobra venom. For a given concentration of manoalide, the inhibition of phospholipase A_2 activity toward dipalmitoylphosphatidylcholine/Triton X-100 mixed micelles is time-dependent and plateaus at about 85% inhibition of the initial velocity even after extensive preincubation. Metal ions (Ca^{2+}, Ba^{2+}, Mn^{2+}) increase the inhibition, while lysophosphatidylcholine and substrate micelles protect. Increasing manoalide concentration shows increasing inhibition of the initial velocity until a plateau is reached, giving a typical saturation curve with a linear double-reciprocal plot. Under typical conditions (20-min preincubation, 40 °C, pH 7.1), 50% inhibition is achieved at a manoalide concentration of about 2×10^{-6} M. The data indicate that manoalide is a potent inhibitor of the cobra venom phospholipase A_2. Manoalide is now shown to react irreversibly with lysine residues in the enzyme. Surprisingly, the cobra venom phospholipase normally acts poorly on phosphatidylethanolamine as substrate, but after reaction with manoalide, the enzyme is somewhat more active toward this substrate rather than being inhibited. This suggests that a lysine residue may be important in understanding the substrate specificity of phospholipase A_2.

Phospholipase A_2 is a ubiquitous enzyme (1) found in the exocrine secretion of the pancreas (2), insect, and reptile venoms (3) and intracellularly (4). This enzyme catalyzes the hydrolysis of the ester bond in the *sn*-2 position of phospholipids (1). Experiments in our laboratory on various substrates for the cobra venom (*Naja naja naja*) enzyme suggest that the active enzyme has two kinds of functional sites: an activator site with minimum specificity for phosphorylcholine-containing lipid and a catalytic site with little specificity for the polar group of phospholipids (5, 6). The enzyme first binds a phospholipid in one of the sites, thereby binding to the lipid-water interface. This enhances catalysis of the substrate. At the present time, it is not clear if the active form of the enzyme is a monomer or higher order aggregate (1).

Recent interest in marine natural products has led to the isolation of several new terpenoids from sponges (7) which are probably secondary metabolites. Among them, manoalide (8) (Fig. 1) was shown by Jacobs and co-workers (9) to be analgesic and to antagonize phorbol-induced inflammation but not that induced by arachidonic acid. This suggests that manoalide may act before the cyclooxygenase step in prostaglandin synthesis. Furthermore, Jacobs and co-workers (10) showed that manoalide can inhibit the neurotoxic action of β-bungarotoxin and bee venom phospholipase hydrolysis of phosphatidylcholine *in vitro*. Because a phospholipase could be the site of manoalide action *in vivo*, we have undertaken to examine in detail the mechanism of action of this possible new type of anti-inflammatory agent on a well-characterized phospholipase A_2. Also as reported herein, these studies provide new information on the important role of lysines in the action of the cobra venom phospholipase toward various substrates. A preliminary report of these results has been presented (11).

EXPERIMENTAL PROCEDURES

Materials—Phospholipase A_2 from cobra venom (*Naja naja naja*) was purchased from the Miami Serpentarium and purified to homogeneity as previously described by Deems and Dennis (12) and modified by Hazlett and Dennis (13). Palmitoyllysophosphatidylcholine (lyso-PC[1]) and dipalmitoylphosphatidylcholine (dipalmitoyl-PC) were obtained from Calbiochem-Behring. Sphingomyelin and oleic acid were from Sigma. Dodecylphosphorylcholine was a gift of Dr. H. S. Hendrikson, (St. Olaf College, Northfield, MN). Diheptanoyl-PE was synthesized according to a method described elsewhere.[2] The product gave a single spot by one-dimensional thin layer chromatography on silica gel (Analtech) using chloroform/methanol/water (65:25:4 by volume) as developing solvent and iodine vapor and molybdate spray (14) for detection. Diheptanoyl-PC was obtained from Avanti, dipalmitoyl-PE from Mann, Triton X-100 from Rohm and Haas, and 2,4-dinitrophenylhydrazine (DNPH) from Aldrich. All other products and solvents used were of the best available grade.

Manoalide was originally isolated from the sponge *Luffariella variabilis* (8). The pure, characterized product was a generous gift from Dr. D. J. Faulkner (Scripps Institution of Oceanography, La Jolla, California). Manoalide was stored in ethylene glycol (5 mM stock solution).

Enzyme Assays—Phospholipase A_2 activity toward long chain

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This paper is dedicated to Professor Eugene P. Kennedy on the occasion of his 65th birthday.

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[1] The abbreviations used are: lyso-PC, 1-palmitoyl-*sn*-glycero-3-phosphorylcholine; diacyl-PC, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine; diacyl-PE, 1,2-diacyl-*sn*-glycero-3-phosphorylethanolamine; MOPS, 4-morpholinepropanesulfonic acid; DNPH, 2,4-dinitrophenylhydrazine.

[2] Pluckthun, A., Rohlfs, R., Davidson, F., and Dennis, E. A. (1985) *Biochemistry*, in press.

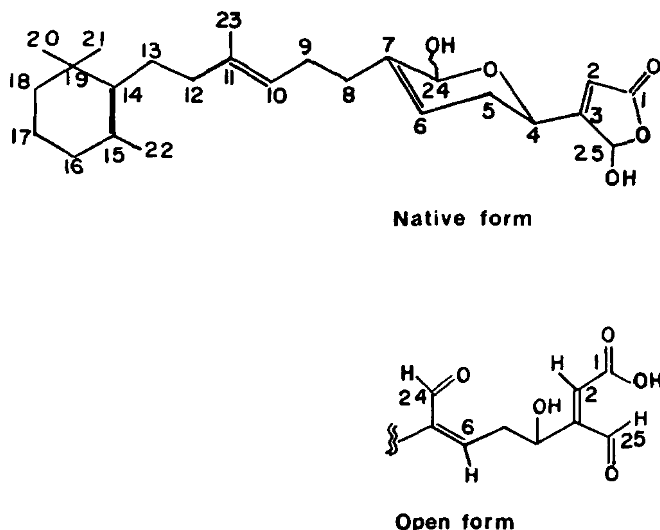

FIG. 1. **Structure of manoalide, a sesterterpenoid antibiotic isolated from *L. variabilis*, a marine sponge.** The *Open form* shows the numbering for the two rings which could possibly be reactive; see "Discussion."

phospholipids (5 mM) in mixed micelles with Trixon X-100 (20 mM) in the presence of $CaCl_2$ (10 mM) was determined by pH-stat (12) at 40°C and pH 8.0. Short chain phospholipids were assayed by the same method except that Triton X-100 was omitted.

Protein—Protein was determined according to Lowry *et al.* (15).

Incubation of Phospholipase A_2 with Manoalide—Incubations of phospholipase A_2 with manoalide were performed in 0.1 M buffer (Tris-HCl or borate as specified) at pH 8.0 and 40 °C for 20 min. The ratio of the manoalide concentration to the protein concentration was maintained at about 500:1 (unless otherwise specified). The per cent of inhibition was obtained by enzyme assays after incubation with and without manoalide under the same conditions. When a larger quantity of inhibited enzyme was prepared, the excess manoalide was removed by gel filtration on a Sephadex G-25 column (1 × 25 cm) and eluted with distilled water.

Amino Acid Analysis—Amino acid analysis was performed on a Beckman 117 or 121 amino acid analyzer. Suitable amounts of the native or modified protein were dissolved in 6 M HCl. The samples were then hydrolyzed in vacuum-sealed tubes at 110 °C for 20–22 h.

Determination of Carbonyl Groups—About 290 μmol of manoalide were incubated in 0.5 ml of 0.1 M buffer for 20 min at 40 °C. Buffers used were sodium phosphate, pH 5.6–7.8, and Tris-HCl, pH 7.6–9.5. 0.4 mM 2,4-dinitrophenylhydrazine in 2 N HCl/methanol (1:1 by volume) was prepared, and 1.0 ml was added to 100 μl of the manoalide solution and incubated again for 20 min at 40 °C, then centrifuged for 10 min (Eppendorf 5412 model bench centrifuge). The bright yellow precipitate was then dried and dissolved in 1 ml of *n*-butyl alcohol, and the optical density was read at 370 nm (λ_{max}) relative to a control without added manoalide (16). Assay of carbonyl groups on the modified protein was performed according to Fields and Dixon (16).

^1H NMR—^1H NMR spectra were obtained at 360 MHz on a modified Varian instrument equipped with quadrature phase detection, a 1180E FT Nicolet data processor, and a Nicolet 293B programable pulser. Experimental conditions included a sweep width of ± 3000 Hz, and a 16K memory was employed to accumulate 100 scans for nondecoupled spectra. The water peak was suppressed using a WEFT sequence with a selective DANTE inversion (17). The DANTE pulse consisted of 20 (π/20) pulses with a repetition rate of 400 μs. For these experiments, manoalide (3.0 mM) was dissolved in D_2O/CD_3OD (50:50 by volume). The apparent pH, not corrected for D_2O or CD_3OD, was adjusted to the desired value with NaOD or DCl. Chemical shifts were measured relative to methanol.

RESULTS

Phospholipase A_2 Inhibition by Manoalide—Under the standard experimental conditions employed, about 50% inhibition is obtained after about 20 min (Fig. 2). Longer times

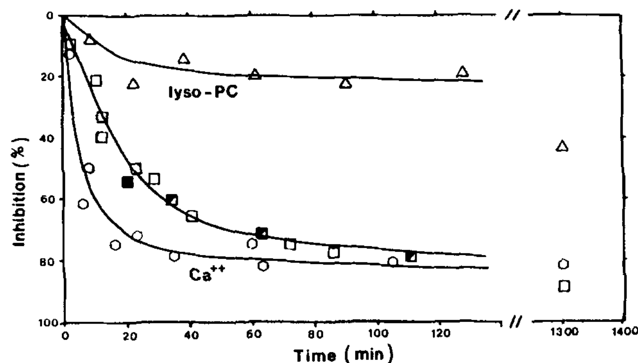

FIG. 2. **Inhibition of cobra venom phospholipase A_2.** Assays were performed after incubation in a 500-fold molar excess of manoalide to phospholipase A_2. Incubations were performed in 0.1 M borate buffer, pH 8.0, in the presence of 0.5 mM lyso-PC (△), 10 mM $CaCl_2$ (○), or in the absence of added ligand (□). Data was also obtained in Tris-HCl buffer, pH 8.0 in the absence of added ligand (■).

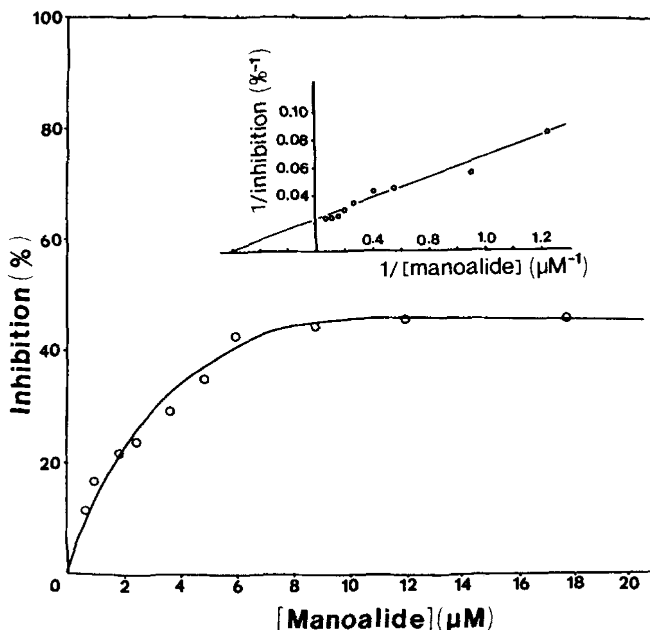

FIG. 3. **Dependence of the inhibition of phospholipase A_2 on the manoalide concentration.** Phospholipase A_2 (0.48 μM) was incubated for 20 min at 40 °C in 0.1 M Tris-HCl buffer, pH 7.1, in the presence of various concentrations of manoalide. The double-reciprocal plot is shown in the *inset*.

lead to a maximum of 80–85% inhibition no matter what buffer is used, borate or Tris-HCl. Addition of excess manoalide does not modify this plateau value. A semilogarithmic plot of per cent inhibition *versus* time (not shown) suggested that the overall rate of inhibition does not appear to be consistent with any simple scheme. $CaCl_2$ at a final concentration of 10 mM increases the rate of inhibition; 5–7 min of incubation are then enough to induce 50% inhibition. Again, only 80–85% inhibition is reached with long incubation times. Lyso-PC in micellar concentration (0.5 mM) protects phospholipase A_2 against the inhibition induced by manoalide. Fig. 3 shows the effect of increasing concentration of manoalide on the phospholipase A_2 (5 μM) inhibition. This inhibition, observed after 20 min of incubation, increases with increasing concentration of manoalide, until a plateau value is reached. The inhibition at this plateau is not increased by increasing

the manoalide concentration up to 600 μM. This is a typical saturation curve as shown by the linearity of the double-reciprocal plot. Half-inhibition is obtained at a manoalide concentration of about 1.7 μM.

The inhibition of phospholipase A_2 by manoalide appears to be irreversible. As shown in Table I, an 80% inhibited phospholipase A_2 preparation was passed through a Sephadex G-25 column to remove excess manoalide. The per cent inhibition was also not significantly changed after a 16-h dialysis against water at 4 °C.

Effects of Ligands and Metal Ions—The effect of various ligands on inhibition of the phospholipase A_2 by manoalide is shown in Table II. The per cent of inhibition was obtained after 20 min of incubation of the phospholipase A_2 (0.5 μM) with manoalide (0.2 mM) in the presence of ligand or metal ion at the final concentration indicated. Micellar substrates such as dipalmitoyl-PC and dipalmitoyl-PE solubilized by Triton X-100 protect the enzyme against the inhibition, while the detergent by itself has less effect. Sphingomyelin, an excellent activator for cobra venom phospholipase A_2 acting on PE, shows no protective effect toward the inhibition. Products of the phospholipid hydrolysis, such as lyso-PC and free fatty acid, also protect, even if the effect observed with the former is lower. From these data, one must conclude that manoalide acts in or close to the active site of phospholipase A_2. Ca^{2+}, a divalent metal ion essential for phospholipase A_2 activity, increases the rate of the inhibition by manoalide (Table III). Other metal ions, such as Ba^{2+} or Mn^{2+} which are known to antagonize the Ca^{2+} effect, have qualitatively the same effect as Ca^{2+}; thus, the effect of metal ions on the inhibition by manoalide appears to be nonspecific.

Activity of the Modified Phospholipase A_2 on Various Substrates—The phospholipase A_2 was assayed toward various substrates (Table IV). The loss of enzyme activity was similar on long chain dipalmitoyl-PC solubilized by Triton and on micellar short chain PC. Surprisingly, using PE, whatever the chain length and in the presence or absence of Triton X-100, we observed an increased activity compared to the native enzyme. But when substrates such as diheptanoyl-PE were tested in the presence of an activator (phosphorylcholine containing compounds such as dodecylphosphorylcholine), the activity decreased relative to native enzyme and the per cent of inhibition reached that of diacyl-PC. These data are consistent with a modification of the catalytic properties of the enzyme due to the action of manoalide.

Effect of pH—The pH profile of the phospholipase A_2 inhibition by manoalide was determined. Fig. 4 shows that increasing the pH increased the inhibition under the conditions used. The pH dependence of the inhibition of phospholipase A_2 appears to have a pK_a of about 7. It is possible that it is actually a biphasic process with the first pK_a at about 6.5

TABLE I
Irreversibility of the inhibition

Step	Conditions	Inhibition
		%
1.	Inhibited enzyme[a]	78.1
2.	Dialysis[b]	76.4

[a] Enzyme was passed through a Sephadex G-25 column to remove excess manoalide.
[b] Enzyme from step 1 was dialyzed 16 h against water at 4 °C.

TABLE II
Effect of ligands on the inhibition

Ligand	Concentration	Triton X-100	Inhibition
	mM	*6 mM*	%
None		−	50.0
None		+	36.0
Dipalmitoyl-PC	2	+	19.3
Dipalmitoyl-PE[a]	1.5	+	18.5
Sphingomyelin	1	−	48.5
Lyso-PC	0.5	−	22.5
Oleic acid	1.7	−	35.6

[a] 3 mM Ca^{2+} included.

TABLE III
Effect of divalent metal ions on the inhibition

Metal or ligand	Concentration	Inhibition
	mM	%
None		50.0
EDTA	1	41.9
Ca^{2+}	20	73.0
Ba^{2+}	20	63.3
Mn^{2+}	20	62.3

TABLE IV
Activity of the inhibited Phospholipase A_2 toward various substrates

Substrate (5 mM)	Triton (20 mM)	Dodecyl-phosphoryl-choline (1 mM)	Specific activity: Native	Specific activity: Inhibited
			μmol min⁻¹ mg⁻¹	
Dipalmitoyl-PC	+	−	1330	640
Dipalmitoyl-PE	+	−	7.5	18
Diheptanoyl-PC	−	−	4500	2240
Diheptanoyl-PE	−	−	200	380
Diheptanoyl-PE	−	+	1970	980

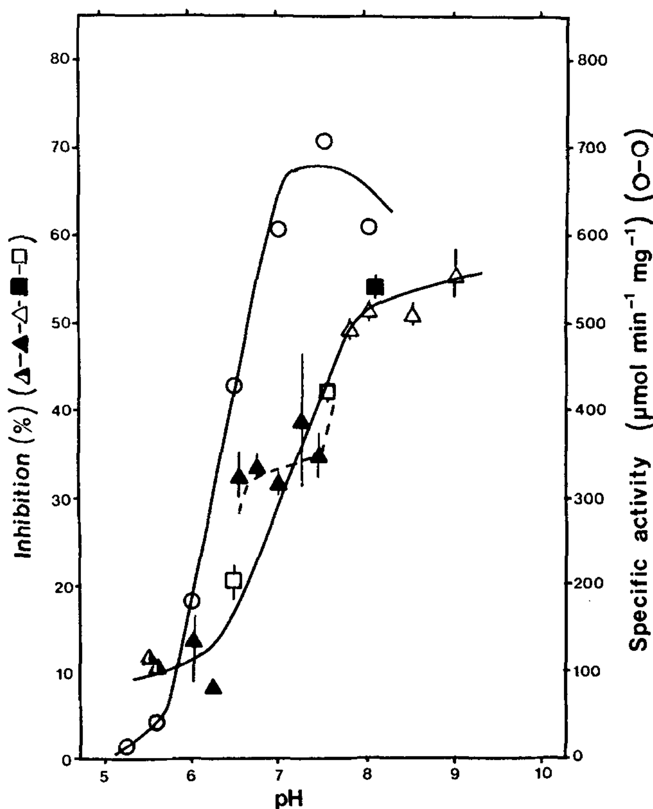

FIG. 4. pH dependence of the rate of hydrolysis of dipalmitoyl-PC in Triton X-100 micelles by phospholipase A_2 (O) and of the inhibition of phospholipase A_2 by manoalide in sodium acetate (Δ) at pH 5.5–5.7, MOPS (□) or sodium phosphate (▲) at pH 6.0–7.6, and Tris-HCl (■) or sodium borate (△) at pH 7.7–9.5 buffers.

and a second one at about 7.7 with a plateau value between 6.5 and 7.5. The maximum inhibition is reached around pH 8. Interestingly, the apparent pK_a is close to the apparent pK_a for the enzymatic reaction as shown by the dependence of enzyme activity on pH, as shown in Fig. 4. The apparent pK_a for the enzyme activity is about 6.9.

Carbonyl Formation Dependence on pH—As shown in Fig. 5, the relative optical density of the DNPH derivative of manoalide doubles in going from pH 5.6 to 9.5, suggesting that the quantity of free carbonyl groups doubles. Presumably, the hemiacetal is converted to the DNPH derivative under the experimental conditions employed. As the starting pH is increased, more of the lactone is apparently in the open form, and when quenched to the acidic pH conditions of the DPNH reaction, it still reacts to give the DNPH derivative. Thus, it appears that between pH 5 and 9, the inhibitory process (Fig. 4) can be correlated with the production of carbonyl groups from the lactone ring.

The ^1H NMR spectra of manoalide dissolved in D_2O/CD_3OD at pH 6.5 and 9.0 are shown in Fig. 6. The upfield portion of the spectrum displays some differences between pH 6.5 and 9.0, showing that the structure of manoalide is modified by increasing pH. This portion of the spectrum at pH 6.5 does not differ from that of native manoalide in $CDCl_3$ (not shown). Most interesting is the downfield portion where peaks appear at 9.32 ± 0.005 and 10.03 ± 0.03 ppm. These resonances can be attributed to the formation of aldehyde protons from the hemiacetal and/or the lactone rings on carbon 24 and/or 25 (see Fig. 1 for numbering). Assuming that the resonance at 0.96 ± 0.01 ppm arises from six methyl protons on carbons 20 and 21, then the peaks at 10.03 and 9.32 ppm represent, respectively, 0.6 and 0.7 protons. The singlet at 7.17 ± 0.01 ppm (1 proton) and the triplet (J = 7.2 Hz) at 6.77 ± 0.05 ppm (0.7 proton) can be tentatively attributed to protons on carbon 2 and/or carbon 6 which move downfield when the ring(s) are opened from the previous position upfield where they are obscured by the water peak. As the pH is increased, the peaks at 7.17 and 10.03 ppm simultaneously first appear together, followed by the simultaneous appearance of the peaks at 6.77 and 9.32 ppm as the pH is increased further. The intensity of the latter peaks depends on the pH and is constant with time at a given pH. It should be noted that the relative intensities of the lines may be somewhat distorted due to their different relaxation rates and together with integration errors makes it uncertain if the 9.32 and 10.03 ppm peaks together represent 1 or 2 protons (and the same for the 6.77 and 7.17 ppm peaks). When the sample at pH 9.0 was taken back to pH 6.5, the peaks at 10.03, 9.32, 7.17, and 6.77 ppm disappear. The simplest interpretation of these results is that the formation of the aldehyde(s) represents a pH-dependent equilibrium. At neutral pH, both of the rings are closed, while at basic pH one or both appear to be open. There are thus two possibilities. Either the 7.17–10.03 ppm pair and the 6.77–9.32 ppm pair reflect the open form of the hemiacetal and the lactone, or once open the lactone may undergo an isomerization to form *cis/trans* isomers at higher pH and the two pairs reflect the two isomers at slow exchange. Further work is needed to resolve this question.

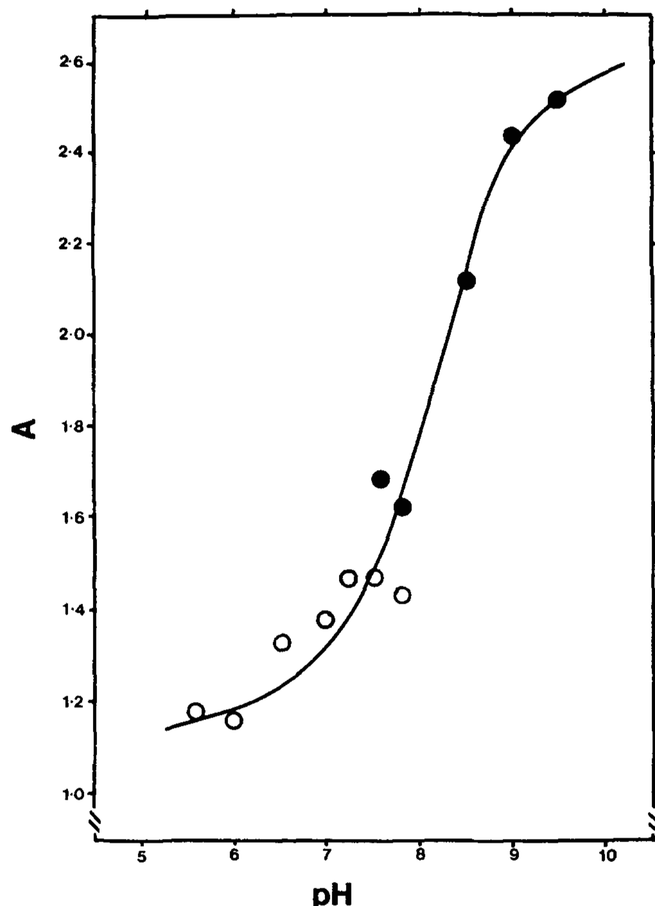

FIG. 5. **Formation of free carbonyls as a function of pH.** Carbonyl formation was followed by reaction with 2,4-dinitrophenylhydrazine and the absorbance (A) of the product was measured at 370 nm. Sodium phosphate (○) and Tris-HCl (●) were employed as buffers.

Amino Acid Analysis of the Manoalide-modified Phospholipase—The phospholipase A_2 was modified using a 50-fold molar excess of manoalide. Incubation was run at pH 8.0 for 20 h at 40 °C. Excess manoalide was removed by a Sephadex G-25 column, followed by an exhaustive dialysis against water for 16 h. The amino acid analysis given in Table V shows that within experimental error only lysine residues are substantially decreased in the modified enzyme as compare with the native enzyme. The values presented here for the native enzyme are in good agreement with that previously reported (18). The histidine residue implicated in the catalytic site is not irreversibly modified by manoalide (19). From these data, we must conclude that the final product of the manoalide reaction with phospholipase A_2 involves the modification of about 4 of the 6 lysine residues present in the enzyme. Note that reaction with additional residues cannot be ruled out within the accuracy of the amino acid analysis and especially since free amino acids could have been regenerated upon acid hydrolysis.

The relative molar absorption coefficient of the dinitrophenylhydrazone derivative of manoalide was found to be $E = 11{,}400\ M^{-1}\ cm^{-1}$ based on a sample of manoalide which was incubated in Tris-HCl buffer, pH 9.5, for 1 h and then converted to the DNPH derivative. A similar value was determined for pyruvoylhydrazone using similar conditions (16). When the modified protein was examined for the presence of carbonyl groups (16), it was found that there were 7.7 mol of carbonyl groups/mol of modified phospholipase A_2 compared with a native enzyme control. This would be consistent with the incorporation of four manoalide molecules by reaction with lysine.

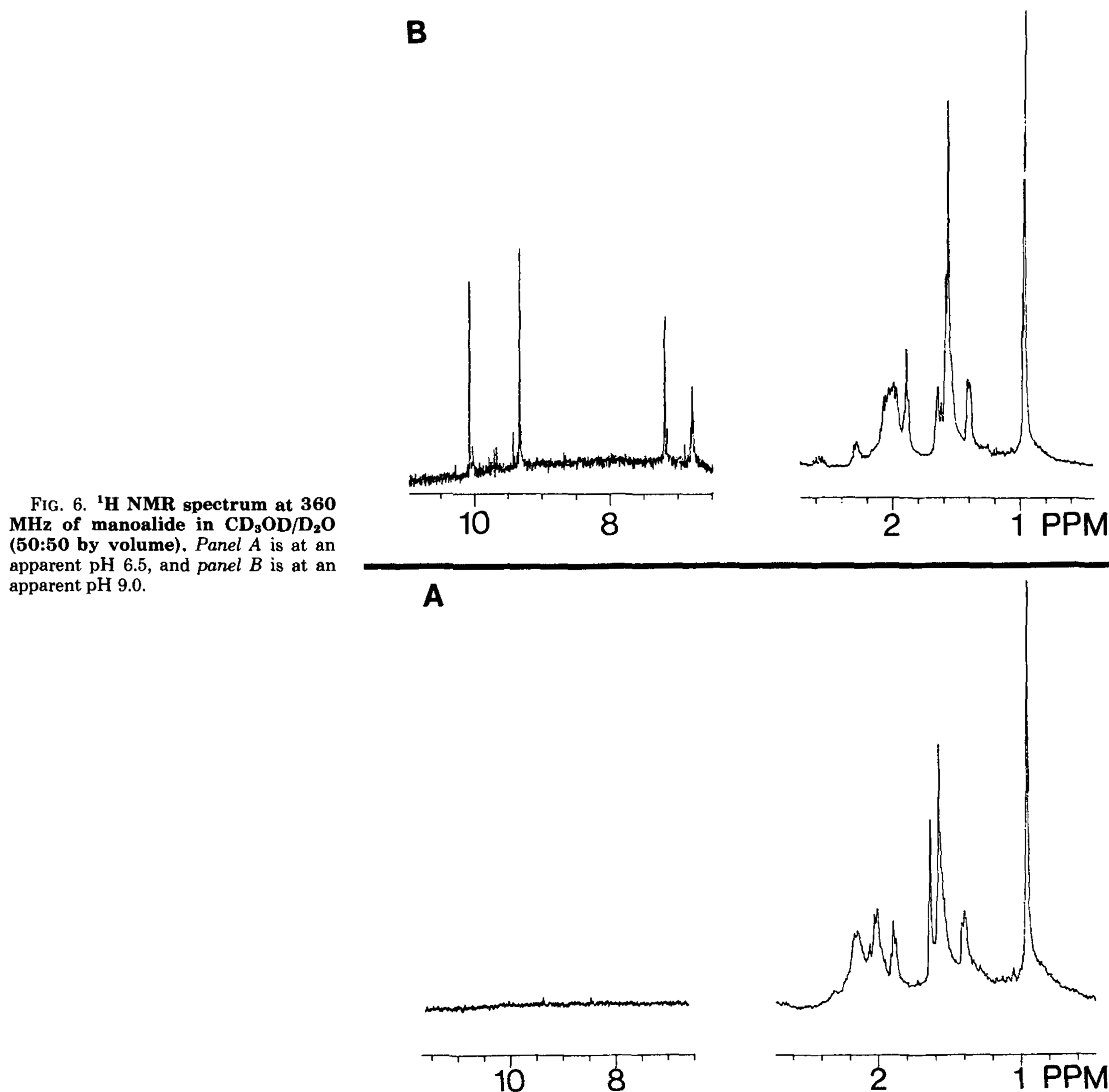

FIG. 6. **^{1}H NMR spectrum at 360 MHz of manoalide in CD_3OD/D_2O (50:50 by volume).** *Panel A* is at an apparent pH 6.5, and *panel B* is at an apparent pH 9.0.

DISCUSSION

Anti-inflammatory Activity of Manoalide—The anti-inflammatory effect of a drug may originate from (i) a direct and irreversible chemical modification of the phospholipase A_2 molecule. One example is the alkylation of the catalytic site histidine by *p*-bromophenacyl bromide (19, 20). (ii) It may be due to an inhibition of intracellular phospholipase A_2 by corticosteroids such as dexamethasone (21). The inhibition has been suggested to be due to the induction of the synthesis of a polypeptide inhibitor of phospholipase A_2 (22). (iii) Inhibiting may occur by preventing phospholipase A_2 activation by the essential Ca^{2+} ion. Some alkaloids (papaverine) or nonsteroidal drugs (mepacrine) have been suggested to have this effect (23).

Local anesthetics also decrease phospholipase A_2 activity, and this inhibition could be ascribed to a subtle modification of the membrane structure rendering the phospholipid less accessible to the enzyme (24) or to a prevention of the formation of the enzyme Ca^{2+} substrate complex, resulting in inhibition (25). Thus, we have to distinguish between anti-inflammatory drugs which act directly on the phospholipase A_2 from those less specific, acting on other systems but still leading to a less active phospholipase A_2. The experimental data reported here suggest that manoalide may belong to the first class since its anti-inflammatory effect can be related to the chemical modification of phospholipase A_2. Under the standard condition used, half-inhibition is obtained at a very low concentration of manoalide which makes it a potent inhibitor of phospholipase A_2.

Site of Manoalide Action on Phospholipase A_2—The inhibition of phospholipase A_2 appears to be irreversible and seems most probably due to modification of lysine residues as shown by the amino acid analysis. The rate of this inhibition is increased by metal ions while a lower reactivity of phospholipase A_2 toward *p*-bromophenacyl bromide is observed in their presence (19, 20). Moreover, there is no correlation

TABLE V
Amino acid analysis of native and inhibited Phospholipase A_2

Residue	Native		Inhibited experimental
	Ref. 18	Experimental	
Asx	20.0	21.8	22.5
Thr	4.8	5.3	5.4
Ser	7.9	7.4	7.4
Glx	9.0	7.6	8.0
Pro	4.3	4.0	5.1
Gly	10.3	9.9	10.1
Ala	10.3	10.9	11.4
Cys	13.4	11.6	12.7
Val	3.7	4.3	4.5
Met	1.0	0.8	1.0
Ile	3.8	4.2	4.5
Leu	5.0	5.4	5.6
Tyr	8.0	7.2	7.4
Phe	3.9	4.3	4.9
His	1.0	1.1	1.2
Lys	6.3	6.3	1.8
Arg	4.6	5.2	5.8
Trp	3.0	ND[a]	ND[a]

[a] ND, not determined.

between the inhibitor solubility and the inhibition since the inhibition plateaus for manoalide concentrations higher than 8 μM while the manoalide solubility was estimated to be about 400 μM in 0.1 M Tris-HCl, pH 7.1. The most effective protection is achieved by phospholipid substrates; less protection is observed in the presence of hydrolysis products. Sphingomyelin (even in the presence of 6 mM Triton X-100) does not protect the enzyme against inhibition. This would argue against the involvement of the modifiable lysine residues in the postulated activator site (5, 26).

Substrate and products were micellar, but pure micelles of Triton X-100 do not protect. It is well known that phospholipase A_2 from cobra venom is not able to bind pure micelles of detergent significantly (27). Thus, the protection by substrates could be explained in two ways: 1 (or more) lysine residue is implicated in the catalytic site and occupation of this site prevents modification, or at least one of the lysines can affect substrate binding. Since a complete loss of enzyme activity was not found, even after a long incubation time and extra addition of manoalide, we can conclude that among the 4 modified lysine residues, none of them is directly implicated in catalysis. This assumption agrees with the fact that reagents such as phthalic anhydride and trinitrobenzenesulfonate were shown to modify as many as four lysines on the cobra venom phospholipase A_2, while the enzyme still retained 30–64% of its activity (28).

Recent work shows that the initial binding constant of the protein to an interfacial phospholipid is similar for a thiol analog of PE and PC as was the apparent Michaelis constant for each substrate (29, 30). A variation of these constants could explain the higher activity of the modified enzyme on PE than the native form. Thus, the modified residue(s) could be involved in some way in the binding to the aggregate substrates as found with *Bitis gabonica* phospholipase A_2 (31). Nevertheless, a distortion of the active site of the enzyme due to the introduction of a negatively charged carboxylate group or of a very hydrophobic core coming from manoalide cannot be ruled out. Such an explanation was offered for the results obtained with the Lys-6 modification of the *Naja melanoleuca* enzyme (32).

Active Form of Manoalide—Regarding the mechanism by which manoalide inhibits the phospholipase A_2 activity, several possibilities have to be taken into account. First, to inhibit, manoalide has to form a noncovalent complex with the phospholipase as shown by the saturation kinetics. Half-inhibition was obtained for concentrations as low as 1.7 μM, far below the solubility limit of manoalide (0.4 mM). No evidence for saturation kinetics was obtained with *p*-bromophenacyl bromide, but if so, the K_D of the postulated complex would be considerably larger than the solubility of the haloketone, that means larger than 100 μM (33). Second, the inhibition of the phospholipase A_2 follows the appearance of carbonyl groups on manoalide. This structural modification of manoalide was confirmed by ^1H NMR. These carbonyl groups most probably come from the opening of the hemiacetal and/or the lactone ring leading to the formation of aldehyde functions on carbons 24 and/or 25 (Fig. 1, *Open form*). The opening of the lactone leads to a short-lived *gem*-diol or an aldehyde. The possible apparent biphasic form of the per cent inhibition *versus* pH profile may be due to a sequential ring opening, to a different reactivity between these aldehyde groups, or to the formation of *cis/trans* isomers on the lactone. It appears that an equilibrium between closed rings and opened rings exists, and the ratio depends upon the pH. The exchange between the two states must be slow on the NMR time scale, allowing observation of related peaks.

Third, only lysine residues are apparently covalently modified. This modification is stable to acid hydrolysis and irreversible in neutral conditions. About eight free carbonyl groups are still present on the enzyme after filtration and dialysis, which agree with the modification of four lysines. Taken together, all these results rule out simple nucleophilic addition between a lysine amino group and a carbonyl, leading to the formation of an imine or a Schiff base as they would be expected to be labile. Of course, rearrangement of such an intermediate to a stable adduct would be possible. Both of the possible carbonyl groups formed at basic pH are α,β-unsaturated with a free β-position. Either could undergo a Michael condensation with a good nucleophile such as a free amino group. These two α,β-unsaturated carbonyl groups may react independently since some polymers were detected by polyacrylamide gel electrophoresis-sodium dodecyl sulfate gels (not shown). Unprotonated amino groups are needed for this reaction; increasing pH would also increase the deprotonation and thus favor the conjugate addition. Thus, the inhibition of phospholipase A_2 observed with manoalide appears to be due to reaction of the amino side chain of several exposed lysines with manoalide via Michael addition to the double bond adjacent to a free aldehyde in an open form of the compound. They appear to exist above neutral pH values where both inhibition and enzyme activity is optimal.

Modification of the lysine residues inhibits hydrolysis of PC, while it activates hydrolysis of the poor substrate PE in the absence of activator lipids. This suggests that at least one of the lysine residues is located near the site where substrate binds and differentially affects either binding or catalysis of PC and PE. Thus, manoalide provides a probe of the role of an amino acid residue not previously implicated in the action of this enzyme.

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REFERENCES

1. Dennis, E. A. (1983) in *The Enzymes* (Boyer, P. D., ed) Vol. 16, pp. 307–353, Academic Press, New York

2. Volwerk, J. J., and de Haas, G. H. (1982) in *Lipid-Protein Interactions* (Jost, P. C., and Griffith, O. H., eds) Vol. 1, pp. 69–141, Wiley, New York
3. Tu, A. (1977) *Venoms*, pp. 23–63, Wiley, New York
4. Van den Bosch, H. (1980) *Biochim. Biophys. Acta* **604,** 191–246
5. Adamich, M., Roberts, M. F., and Dennis, E. A. (1979) *Biochemistry* **15,** 3308–3313
6. Roberts, M. F., Adamich, M., Robson, R. J., and Dennis, E. A. (1979) *Biochemistry* **15,** 3301–3308
7. Walker, R. P., and Faulkner, D. J. (1981) *J. Org. Chem.* **46,** 1098–1102
8. de Silva, E. D., and Scheuer, P. J. (1980) *Tetrahedron Lett.* **21,** 1611–1614
9. Blankemeier, L. A., and Jacobs, R. S. (1983) *Fed. Proc.* **42,** 374
10. De Freitas, J. C., Blankemeier, L. A., and Jacobs, R. S. (1984) *Experientia (Basel)* **40,** 864–865
11. Lombardo, D., and Dennis, E. A. (1984) *Fed. Proc.* **43,** 1457
12. Deems, R. A., and Dennis, E. A. (1981) *Methods Enzymol.* **71,** 703–710
13. Hazlett, T., and Dennis, E. A. (1984) *Toxicon*, in press
14. Dittmer, J. C., and Lester, R. L. (1964) *J. Lipid. Res.* **5,** 126–127
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
16. Fields, R., and Dixon, H. B. F. (1971) *Biochem. J.* **121,** 587–589
17. Haasnoot, C. A. G. (1983) *J. Magn. Reson.* **52,** 153–158
18. Darke, P. L., Jarvis, A. A., Deems, R. A., and Dennis, E. A. (1980) *Biochim. Biophys. Acta* **626,** 154–161
19. Roberts, M. F., Deems, R. A., Mincey, T. C., and Dennis, E. A. (1977) *J. Biol. Chem.* **252,** 2405–2411
20. Volwerk, J. J., Pieterson, W. A., and de Haas, G. H. (1974) *Biochemistry* **13,** 1446–1454
21. Vadas, P. (1982) *Life Sci.* **30,** 155–162
22. Flower, R. J., and Blackwell, G. J. (1979) *Nature* **278,** 456–459
23. Vallee, E., Gougat, J., Navarro, J., and Delahayes, J. F. (1979) *J. Pharm. Pharmacol.* **31,** 588–592
24. Seppala, A. J., Saris, N. E-L., and Gauffin, M. L. (1971) *Biochem. Pharmacol.* **20,** 305–313
25. Scherphof, G. L., Scarpa, A., and van Toorenenbergen, A. (1972) *Biochim. Biophys. Acta* **270,** 226–240
26. Pluckthun, A., and Dennis, E. A. (1982) *Biochemistry* **21,** 1750–1756
27. Roberts, M. F., Deems, R. A., and Dennis, E. A. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 1950–1954
28. Darke, P. L. (1982) Ph.D. dissertation, University of California, San Diego
29. Hendrickson, H. S., and Dennis, E. A. (1984) *J. Biol. Chem.* **259,** 5734–5739
30. Hendrickson, H. S., and Dennis, E. A. (1984) *J. Biol. Chem.* **259,** 5740–5744
31. Viljoen, C. C., Visser, L., and Botes, D. P. (1977) *Biochim. Biophys. Acta* **483,** 107–120
32. Van Eijk, J. H., Verheij, H. M., and de Haas, G. H. (1983) *Eur. J. Biochem.* **132,** 177–182
33. Verheij, H. M., Slotboom, A. J., and de Haas, G. H. (1981) *Rev. Physiol. Biochem. Pharmacol.* **91,** 91–203