Chemical Modification of the α-Amino Group in Snake Venom Phospholipases A_2. A Comparison of the Interaction of Pancreatic and Venom Phospholipases with Lipid–Water Interfaces†

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ABSTRACT: Phospholipases isolated from the venoms of *Crotalus atrox*, *Vipera berus*, and *Naja melanoleuca* rapidly react with glyoxylic acid in the presence of Cu^{2+} ions, acetate buffer, pH 5.5, and 4 M tetramethylurea to yield proteins with an α-keto acid at the N terminus. The modified proteins have no activity when tested with micellar substrates but partially retained their activity toward substrates in monomeric form or on the substrate organized at the air–water interface. Modification lowers but does not abolish the affinity of the proteins for micelles of the substrate analogue *n*-hexadecylphosphocholine, as judged from gel filtration and ultraviolet difference spectroscopy. A model is proposed to explain how the modified proteins interact with micellar phospholipids but do not hydrolyze them despite their potential catalytic power, as indicated by the hydrolysis of monomeric substrate.

Phospholipase A_2 (EC 3.1.1.4) has been isolated from the venoms of bees and snakes and from mammalian pancreas (Tu, 1977). The enzymes isolated from the vertebrate sources show a high degree of sequence homology (Heinrickson et al., 1977). These enzymes all hydrolyze phospholipids stereospecifically; they show an absolute requirement for calcium ions and are resistant to denaturating conditions. Despite these similarities, diverse results have been reported for studies concerning the mechanism of action of enzymes from different sources (Tinker et al., 1978; Tinker & Wei, 1979). It has been reported that those venom enzymes which are present as dimers even in dilute solutions are exclusively active as dimers during the catalytic event (Wells, 1971; Shen et al., 1975). For cobra venom phospholipase A_2, which is present as a monomer at low concentrations, a substrate-induced dimerization has been proposed (Deems & Dennis, 1975). For the enzyme from porcine pancreas, no data pertaining to a functional dimer could be obtained (De Araujo et al., 1979). In addition, venom phospholipases show a variety of pharmacological activities; one of the most intensively studied properties is the ability of certain phospholipases to interfere with neurotransmitter release at the neuromuscular junction (Karlsson, 1979) whereas other phospholipases are inert in this system.

The activity of all phospholipases is strongly dependent upon the nature of the substrate: although monomers are hydrolyzed, the enzyme only becomes fully active in the presence of aggregated substrate. Monomeric substrates with fatty acids of chain length 4–8 carbon atoms are hydrolyzed with comparable V_{max} values, around 10 μmol min^{-1} mg^{-1}, by both pancreatic and venom phospholipases (Roholt & Schlamowitz, 1961; Pieterson, 1973; Wells, 1974, Viljoen & Botes, 1979). With micelles of these substrates, the values obtained for enzymes from different sources fall in the range 20–20 000 μmol min^{-1} mg^{-1} (Pieterson, 1973; Wells, 1974; Algyer & Wells, 1979).

For the enzymes from mammalian pancreas, it has been shown that the affinity of these proteins for organized substrate is critically dependent on the presence of a (protonated) α-amino group (van Dam-Mieras et al., 1975). For the venom phospholipases, no such relationship has been established. The transamination reaction which is known to occur between glyoxylic acid and the α-amino group of proteins (Dixon & Fields, 1972) gives rise to a protein with an α-keto acid instead of an α-amino acid at the N-terminal part. The reaction was successfully applied to mammalian phospholipases (A. J. Slotboom et al., unpublished results). We report here the transamination of three phospholipases from the venoms of *Vipera berus*, *Crotalus atrox*, and *Naja melanoleuca*, respectively. The modified proteins were characterized by their ability to degrade monomeric or organized substrates. Furthermore, the affinities of the modified proteins for micelles of the product analogue *n*-hexadecylphosphocholine have been determined. The results are compared with available data on pancreatic enzymes.

Materials and Methods

Phospholipases. Phospholipase A_2 from *Vipera berus* was a generous gift of Dr. Boffa, Paris. The enzyme from *Crotalus atrox* (Sigma, St. Louis, MO) was purified as described by Hachimori et al. (1971) except that the crude venom was first precipitated with $NdCl_3$ (Wells, 1975). The venom of *Naja melanoleuca* (Sigma, St. Louis, MO) was fractionated according to the procedure described by Joubert & van der Walt (1975); fraction De-III was used in this study. Porcine pancreatic phospholipase A_2 was isolated as described by Nieuwenhuizen et al. (1974). Protein concentrations were determined spectrophotometrically by using $E^{1\%}_{280}$ values of 10 (*V. berus*), 21 (*C. atrox*), 23 (*N. melanoleuca*), and 13 (porcine pancreas).

Phospholipase Assays. The activities were routinely determined in a titrimetric assay (Nieuwenhuizen et al., 1974) consisting of 25 mL of a mixture containing one egg yolk in 300 mL of H_2O, 6 mM $CaCl_2$, and Triton X-100 at a final concentration of 5 mM (*V. berus* and *C. atrox*) or 25 mM (*N.* melanoleuca) at pH 8.0. The enzymes were also tested at pH 8 for their activity toward micellar dioctanoyllecithin (de Haas et al., 1971) and for their activity on monomolecular films of didodecanoyllecithin (Verger et al., 1973). The activity on monomeric solutions of DL-[2,3-bis(hexanoylthio)propyl]-phosphocholine was measured at pH 7.5 as described by Volwerk et al. (1979).

Ion-Exchange Chromatography. DEAE-cellulose (DE-52) and CM-cellulose (CM-32) were Whatman products and were

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0006-2960/81/0420-0094$01.00/0

used in accordance with the manufacturers' instructions. SP-Sephadex C-50 was obtained from Pharmacia. Chromatography on DE-52 was performed in 10 mM tris(hydroxymethyl)aminomethane hydrochloride in 10 mM succinate, pH 7.0, and on SP-Sephadex in 10 mM sodium succinate at pH 4.2. The protein was loaded on the column at amounts of 1 mg/mL ion exchanger, and elution was performed with linear gradients of NaCL with 10 column volumes of buffer in the mixing beaker.

Estimation of Reactive Carbonyl Groups. In the modified protein, the content of keto groups was determined by reaction with 2,4-dinitrophenylhydrazine essentially by the third method as described by Fields & Dixon (1971). The reaction was continuously followed at 370 nm with a Shimadzu UV 200 double-beam spectrophotometer with a reagent solution as a blank, until no further change in absorbance was observed. Standard curves were prepared by using α-ketoglutaric acid.

Amino Acid Analysis. Protein samples were hydrolyzed for 24 h with 6 N HCl in vacuo at 110 °C. The samples were analyzed with a Technicon TSM. The values of Thr and Ser were corrected for losses by 5% and 10%, respectively.

Modification with Glyoxylate. The reaction was carried out at room temperature with the protein (2 mg/mL) dissolved in a solution containing 10 mM Cu^{2+}, 2 M sodium acetate, and 0.4 M acetic acid. When tetramethylurea was included, the buffer (2 times concentrated) was mixed with an equal volume of tetramethylurea to give a final concentration of about 4 M with respect to the latter component.

The reaction was started by the introduction of a 1 M solution of glyoxylic acid in the same buffer to obtain a final concentration of 0.1 M. Aliquots were tested for enzymatic activity in the egg yolk assay. After the modification, the solution was rapidly desalted by using a Sephadex G-25 coarse column which was run in 1% acetic acid. After being desalted, the proteins were dialyzed against 50 mM sodium bicarbonate at pH 8 for 72 h at 4 °C, followed by dialysis against distilled water for 24 h. Final purification of the modified proteins was achieved on DEAE-cellulose at pH 8 (proteins obtained from *C. atrox* and *N. melanoleuca*) or on CM-cellulose at pH 7.0 (*V. berus*). The elution patterns showed one major peak well separated from the traces of native phospholipase. The main peak was pooled, desalted, and lyophilized; typical yield was 70%.

Binding of the Proteins to Phospholipids. The binding of phospholipase to micelles of C_{16}-PN[1] was qualitatively determined by gel filtration of a mixture of 0.5 mg of protein and 5 mg of lipid on a column (0.9 × 80 cm) of Sephadex G-100 equilibrated with a buffer containing 10 mM $CaCl_2$, 100 mM NaCl, and 50 mM Tris-HCl, pH 8.0, essentially by the procedure as described by Slotboom et al. (1977). Quantitative measurements were obtained by measuring the ultraviolet difference spectra as a function of C_{16}-PN concentration (van Dam-Mieras et al., 1975) and analyzing the data by fitting them to a nonlinear iterative program similar to the one described by de Araujo et al. (1979) and Fletcher & Powell (1963). Further details about its application to spectroscopic measurement will be published elsewhere (M. R. Egmond et al., unpublished results).

Molecular Weight Estimations. Gel filtration for molecular weight estimation was performed in a column (0.9 × 80 cm) of Sephadex G-100 in buffer containing 10 mM $CaCl_2$, 100 mM NaCl, and 50 mM Tris-HCl, pH 8.0. Volumes of the fractions were determined by weight. The same column was calibrated by using a Boehringer protein calibrating kit (Size II) and with equine pancreatic phospholipase and its covalently linked dimer which is a by-product of nitration of this enzyme (Meyer et al., 1979a). The elution profile was followed by the measurement of phospholipase A_2 activity in the egg yolk assay and by ultraviolet absorption (active enzymes) or by ultraviolet absorption alone (modified enzymes).

Disc Gel Electrophoresis. Disc gel electrophoresis was carried out on cylindrical gels containing 7.5% acrylamide as described by Jovin et al. (1964). Gels were run at room temperature (2 mA/tube) with glycine–Tris (pH 9.5) or β-alanine–acetic acid (pH 4.5) as electrode buffers. Proteins were stained with a 1% (w/v) solution of Amido Black in 7% (v/v) acetic acid. Destaining was done with the same solvent.

Dansylation. Dansylation was carried out as described by Gray (1972). In order to avoid aspecific losses, the hydrolysates were dissolved in 50% pyridine and spotted directly on the polyamide sheets (Schleicher & Schüll, Dassel, GFR). The sheets were run in solvent systems 1 and 2 as described by Woods & Wang (1967) except that benzene was replaced by toluene. Finally, the sheets were rerun in the second dimension in *n*-butyl acetate–acetic acid–methanol (270:30:30 v/v).

Results

Modification with Glyoxylic Acid. Under standard conditions (10 mM Cu^{2+}, 2 M sodium acetate, and 0.4 M acetic acid, pH 5.5), proteins usually react rapidly in the presence of 100 mM glyoxylic acid, the reaction being complete within 1 h (Dixon & Fields, 1972). However, the phospholipases were quite inert under these conditions. The inactivation of *V. berus* phospholipase was under these conditions a pseudo-first-order process with a half-life time of 117 min. When the reaction was carried out in the presence of *N,N,N',N'*-tetramethylurea, the rate of inactivation increased about 60-fold. The enzymes from *C. atrox* and *N. melanoleuca* showed similar behavior. In the absence of glyoxylic acid, the proteins showed no loss of enzymatic activity during 24 h.

As glyoxylic acid contains two vicinal carbonyls, it is a potential arginine reagent and could react with ϵ-amino groups forming a Schiff base. Therefore, the modification reaction was performed in the presence of [^{14}C]glyoxylic acid. When the activity in the egg yolk assay had dropped to a value of about 1%, the protein was rapidly desalted on a Sephadex G-25 coarse column (120 × 1 cm). It was found that all three proteins after complete inactivation (≤1% residual activity) showed incorporation levels of 0.4–0.6 mol of glyoxylic acid/mol of protein. The radioactive label was not removed upon repeated gel filtration. However, extensive dialysis at 4 °C against 50 mM sodium bicarbonate at pH for 72 h removed more than 90% of the incorporated label. The slow release of label is consistent with the reversible labelling of arginine or lysine residues.

Properties of the Modified Proteins. The purified proteins which were homogeneous as judged from disc gel electrophoresis at pH 9.2 or 4.5 lacked enzymatic activity (as observed immediately after the modification reaction) when assayed with egg yolk or 1,2-dioctanoyllecithin as substrates. When the modified proteins were assayed for the presence of keto groups, a positive reaction was obtained with an incorporation level of 0.9 ± 0.2. Amino acid analyses showed no differences except for the loss of one residue of serine each in the protein from *V. berus* and *C. atrox*. The analyses of modified *N. melanoleuca* phospholipase did not significantly differ from those of the native protein, which can be explained by the presence of 19 Asx residues in addition to the N-ter-

[1] Abbreviations used: C_{16}-PN, *n*-hexadecylphosphocholine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Table I: Activity of Native and Modified Phospholipases toward Monomeric Substrate[a]

enzyme	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
native *V. berus*[b]			2148
α-keto *V. berus*[b]			269
native *C. atrox*	0.18 ± 0.03	3.5 ± 0.4	4550
α-keto *C. atrox*[b]			466
α-keto *N. melanoleuca*[b]			964
porcine pancreatic	1.2 ± 0.2	4.8 ± 0.5	966

[a] The activity was determined at pH 7.5 in a buffer containing 100 mM $CaCl_2$, 100 mM NaCl, and 100 mM Hepes at 25 °C. The amount of enzyme used varied between 0.5 and 2.5 μg according to the enzymatic activity; the substrate was varied between 0 and 0.7 mM which is well below the critical micellar concentration (1.2 mM) of DL-[2,3-bis(hexanoylthio)propyl]phosphocholine. For experimental details, see Volwerk et al. (1979). [b] No individual data for K_m and V_{max} could be obtained because no saturation kinetics were observed. Instead, the values for k_{cat}/K_m are given. The standard deviations therein are 10–20%.

minal asparagine present in this enzyme (Joubert, 1975).

When 10 nmol of the modified proteins were dansylated in the presence of 2 nmol of phospholipase A_2 from porcine pancreas as an internal standard (N-terminal Ala), only Ala was found as an N-terminal residue and no Ser (*V. berus*, *C. atrox*) or Asx (*N. melanoleuca*) was detectable. Under these conditions, a 5% contamination of a protein with an intact α-amino group would have been readily identified.

Activity of Modified Proteins toward Monomeric Substrates. As the modified proteins have lost their activity toward micellar substrates, it was of interest to test their activity using monomeric substrate solutions. Table I shows that the modified proteins hydrolyzed monomers of DL-[2,3-bis(hexanoylthio)propyl]phosphocholine. For comparison, data for unmodified venom and pancreatic phospholipases are included. In some cases, separation of the kinetic parameters K_m and V_{max} was not possible because their values were correlated. Therefore the specificity constants (k_{cat}/K_m) are also included in Table I. Modification of the α-amino group lowers the specificity constants about 10-fold. The observation that after modification no saturation kinetics could be obtained suggests that the reduction in catalytic activity is mainly due to a decreased binding.

Micellar Binding Experiments. Gel filtration offers a rapid procedure to test a number of proteins for their ability to bind to phospholipid micelles. In the absence of binding, a column of Sephadex G-100 will readily separate micelles of the product analogue *n*-hexadecylphosphocholine (M_r 95 000; de Araujo et al., 1979) from uncomplexed protein (M_r 13 500–28 000). On the other hand, if the affinity is high, the protein will elute together with the micelles even without "tailing" due to protein leaving the complex during the run of the column. In fact, this was observed for the three different native and modified venom phospholipases: fractions containing the micelles contained virtually all (≥90%) of the protein applied to the column, indicating a high affinity of the enzyme for micelles.

Quantitative data have been obtained by measuring the binding of *Vipera berus* and *Naja melanoleuca* enzymes to alkylphosphocholine micelles by using UV difference spectroscopy. Both at pH 6 (data not shown) and at pH 7.5 binding was observed. Figure 1 shows the spectra obtained

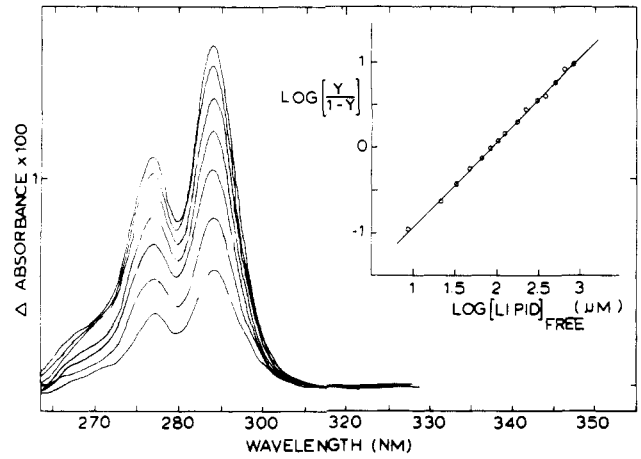


FIGURE 1: Ultraviolet difference spectra of transaminated *Vipera berus* phospholipase (33 μM) in the presence of 71.5–246 μM C_{16}-PN. The buffer contained 100 mM NaCl, 25 mM $CaCl_2$, and 50 mM Hepes, pH 7.5. (Inset) Hill plot of the titration curve obtained from data collected from the spectral change at 288 nm. Note that $[lipid]_{free}$ rather than $[lipid]_{added}$ is plotted.

Table II: Binding of Native and Modified Phospholipases to Micelles Measured by Ultraviolet Difference Spectroscopy[a]

enzyme	K_D (μM)	N	C (M^{-1} cm^{-1})	NK_D (μM)
native *V. berus*	1.6	5.1	1305[b]	8.2
α-keto *V. berus*	11.4	7.9	789[b]	90.1
native *N. melanoleuca*	5.3	5.1	3958[c]	27.0
α-keto *N. melanoleuca*	14.4	9.0	1677[c]	130.0
porcine pancreatic	2.5	38	1950[c]	110.0

[a] Conditions used: protein concentrations 20–30 μM in a buffer containing 25 mM $CaCl_2$, 100 mM NaCl, and 50 mM Hepes, pH 7.5. Aliquots of a solution of *n*-hexadecylphosphocholine (4–10 mM) in the same buffer were added. For experimental details, see van Dam-Mieras et al. (1975). K_D is the dissociation constant of the complex, N represents the ratio of lipid to protein in the complex, and NK_D can be considered as a measure for the effective affinity of the protein for the micelle. C is the molar difference signal when the protein is fully saturated with lipid. [b] Measured at 288 nm. [c] Measured at 292 nm.

with transaminated *V. berus* phospholipase A_2 in the presence of micellar concentrations of C_{16}-PN. The collected data were fitted to a nonlinear iterative program (see Materials and Methods) which permits calculation of the affinity constant, K_D, the stoichiometry of the complex (N), and the molar-difference signal. When the observed signal and the calculated $lipid_{free}$ concentration were plotted in a Hill plot, a straight line with unit slope was obtained. This is indicative of the presence of only one singly binding site or more sites with identical affinities (Tze-Fei Wong, 1975). Similar pictures were obtained for the other native and modified phospholipases in a concentration range 5–36 μM with respect to protein. The results are summarized in Table II.

From Table II it is evident that modification of phospholipase lowers its affinity for micelles whereas the stoichiometry is hardly changed. The molar-difference absorption is lowered. Not only is the signal lowered but also the shapes of the spectra of native and modified proteins are different. The spectrum of the phospholipase A_2 from *V. berus*, which does not contain tryptophan, is characterized by broader peaks than the spectrum of the modified protein (Figure 2). The latter spectrum is similar to a solvent perturbation spectrum of *N*-acetyl-

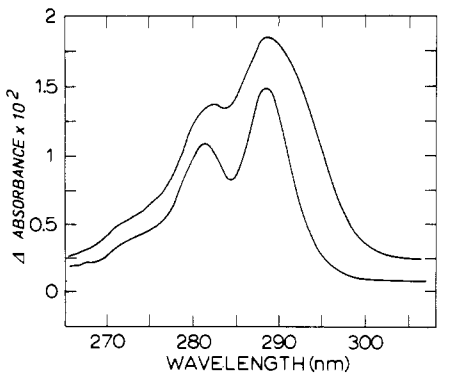


FIGURE 2: Ultraviolet difference spectra of 4.9 μM native *Vipera berus* phospholipase in the presence of 432 μM *n*-hexadecylphosphocholine (upper curve) and of 7.8 μM modified enzyme in the presence of 1070 μM *n*-hexadecylphosphocholine (lower curve). In both cases, the observed spectrum represents more than 90% of the maximal signal. The buffer contained 100 mM NaCl, 25 mM $CaCl_2$, and 50 mM Hepes, pH 7.5.

tyrosine ethyl ester (Donovan, 1969). The spectrum of the native enzyme could be simulated by taking two *N*-acetyltyrosine ethyl ester spectra with relative magnitudes of 2:1 and shifting the smaller spectrum slightly (1.5–2 nm) to longer wavelengths. This suggests that in native *V. berus* phospholipase there are at least two different populations of tyrosine residues that are perturbed by lipid. In contrast, the transaminated protein only contains one population of tyrosine, resulting in the sharp peaks shown in Figure 2 (see also Discussion).

Monolayer Experiments. From Tables I and II it is clear that the modified enzymes have retained enzymatic activity on monomers and that they still bind to micelles with an affinity high enough to obtain saturation of the enzyme with micelles. However, no hydrolysis takes place with mixed micelles composed of Triton X-100 and long-chain phospholipids or with micelles of dioctanoyllecithin. In the micelles the packing of the phospholipid molecules is high and cannot be varied. The monolayer technique offers an opportunity to study the hydrolysis over a wide range of phospholipid packing at the air–water interface (Verger & de Haas, 1976). Therefore we have studied the hydrolysis of monomolecular films of didodecanoyllecithin by native and modified phospholipases from *V. berus* and *N. melanoleuca*. The results obtained for the *N. melanoleuca* enzymes are shown in Figure 3. The native enzyme hydrolyzes the film up to about 16 dyn/cm. At this pressure, a loss in activity is accompanied by a rapid increase in the induction time, τ. This has been interpreted as a measure of the capability of the enzyme to penetrate the film (Verger & de Haas, 1976). The modified enzyme showed a considerable activity at low pressures but looses its activity at pressures above 13 dyn/cm. A similar picture was obtained for the *V. berus* phospholipases: the native enzyme could penetrate the film up to pressures of about 21 dyn/cm, the modified enzyme only to a pressure of about 17 dyn/cm.

It should be kept in mind that the relative activities in Figure 3 are minimum values since they are not based on the amount of protein associated with the monolayers but rather on the total amount added to the trough. In the case of the modified protein with a lower affinity to lipid–water interfaces (Table II), the amount bound to the monolayer might very well be smaller than that of the native enzyme.

Estimation of Molecular Weights. The phospholipases from *V. berus* and *N. melanoleuca* eluted from Sephadex G-100 columns as monomeric proteins (M_r 13 500–15 000; V_e/V_0 = 2.16 ± 0.08). As expected (Hachimori et al., 1971), the enzyme from *C. atrox* eluted with an apparent M_r of 28 200 (V_e/V_0 = 1.81 ± 0.05). Transamination of this enzyme slightly shifted the elution volume according to an apparent M_r of 26 000. The modified *V. berus* and *N. melanoleuca* phospholipases coeluted with the native enzymes. The molecular weights of the protein–lipid complexes were estimated on the same column. When either of the venom phospholipases was applied to the column together with a 10 or 100 molar excess of C_{16}-PN, the enzymatic activity was recovered together with the phospholipid peak. The observed molecular weights based on the elution volumes varied from 80 000 to 94 000 (see also Discussion).

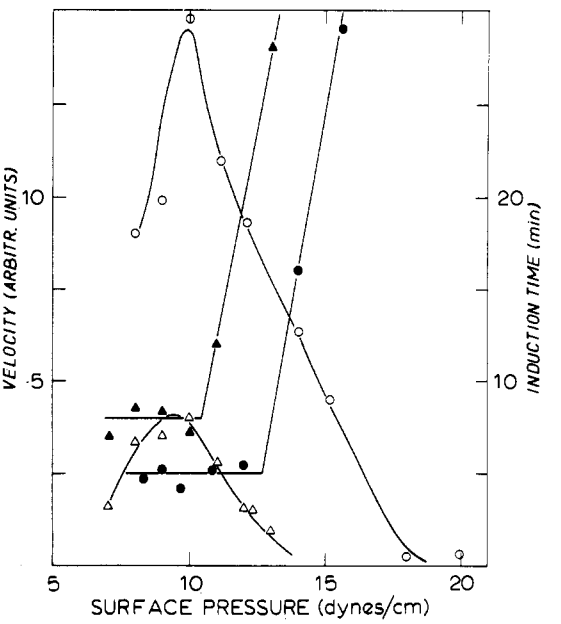


FIGURE 3: Activity of native (○) and modified (△) *Naja melanoleuca* phospholipase on monomolecular films of didodecanoyllecithin. The induction time τ is also indicated: (●) native and (▲) modified phospholipase. The buffer contained Tris-HCl, pH 8, and 10 mM $CaCl_2$. Enzyme injected: 40 (native) or 110 ng (modified). For experimental details, see Verger et al. (1973).

Discussion

Modification of the α-amino group destroys the activity not only of pancreatic phospholipase (Slotboom et al., 1977) but also of venom phospholipases when the enzyme is assayed using *micellar* substrates. Despite their loss of activity toward micellar substrates, the modified enzymes turned out to be quite active on monomolecular films of didodecanoyllecithin although at lower surface densities than the corresponding native proteins. A loss of enzymatic activity after a small change of the N-terminal residue is not unique: replacement of the natural L-Ala[1] residue by D-Ala[1] at the N terminus of porcine phospholipase abolishes the activity on micelles but leaves the activity on monomolecular films largely intact at low (8 dyn/cm) surface pressures (Slotboom et al., 1977). Until now this has been explained by the fact that at very low surface densities aspecific adsorption to the air–water interface may play an important role. However, this explanation cannot be used in the present study since the modified venom phospholipases can penetrate the film to about the same (*N. melanoleuca*) or even much higher (*V. berus*) pressures than any native pancreatic phospholipase. The latter enzymes show a high activity in the egg yolk assay or the dioctanoyllecithin assay.

Transamination of the α-amino group in the venom phospholipases lowers the specificity constant for the hydrolysis

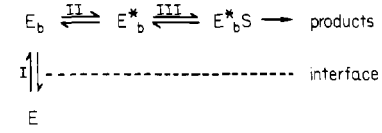
of monomeric substrates but does not abolish it. In fact, the modified venom enzymes are about equally effective as native porcine pancreatic phospholipase A_2 (Table I). Recently it has been shown by Dijkstra (1980) that the α-amino group of bovine pancreatic phospholipse is hydrogen bonded to the C_α-carbonyl oxygens of Gln-4 and Asn-71 and also via a water molecular to Tyr-52 and Asp-99. A structural role was assigned to the α-amino group. After modification of the snake venom enzymes kinetic studies on monomeric substrate did not yield individual data for K_m and V_{max}. The observed 10-fold decrease in k_{cat}/K_m (Table I) is most probably due to increased K_m values rather than decreased k_{cat} values, as the observed kinetics were far from saturation in the monomeric substrate range.

As a result of the modification, the affinity for micelles is lowered 5- to 10-fold. However, this reduced affinity cannot explain the loss of activity toward micellar substrates since the enzyme can easily be saturated with substrate.[2] In fact, the affinity for micelles of the modified venom phospholipases is close to that of the native porcine enzyme (Table II). With the latter enzyme, transamination abolishes the affinity for micelles or shifts it to values which cannot be accurately measured (A. J. Slotboom et al., unpublished results).

For equine pancreatic phospholipase it has been shown (Meyer et al., 1979b) that besides the N-terminal region also Tyr-19 and Tyr-69 are involved in binding to micelles, and we may assume that also in venom phospholipases this binding takes place over an extended surface area with a number of hydrophobic sites. As mentioned above, the micellar binding properties of pancreatic phospholipases are more sensitive to chemical modification than those of the venom phospholipases. Although this might be a fundamental difference between both classes of enzymes, it could also be that the difference is only gradual. If one assumes that the (weak) binding of pancreatic phospholipases is mainly induced by the N-terminal region with little support from other sites, one can expect a large effect of the modification. On the other hand, if in venom phospholipases other sites also strongly support interfacial binding, one can expect a relatively small effect of transamination.

The precise molecular differences between protein–lipid complexes of native and transaminated venom phospholipases remain obscure. The observation that transamination lowers the ultraviolet difference signal and alters the shape of the spectrum (Table I, Figure 2) might suggest that fewer groups are perturbed and that they are perturbed in different ways. Despite the binding to interfaces, no hydrolysis of the micelles takes place. This can be explained in two ways: (1) the modified enzyme is bound, but its active site is not properly oriented toward the interface; or (2) for binding and activity, a specific conformation is required which is stabilized by the N-terminal amino group. Micelles of the substrate (a weak detergent) denature the modified protein but not the native protein.

Scheme I



The low stoichiometry (1 mol of enzyme/5–10 mol of lipid, Table II) raises the question about the actual size of this complex. This question is also of interest since venom phospholipases have been reported to be active as dimers only. A complex of 5–10 C_{16}-PN molecules/protein monomer has a molecular weight of 17 000–18 000. Sephadex gel filtration of venom phospholipases applied together with a 10–100 molar excess of C_{16}-PN indicated a molecular weight of the complex of more than 80 000. Since the column was not equilibrated with lipid, it cannot be excluded that part of the complex dissociates on the column, and consequently the observed molecular weight must be considered as a minimum value. If we assume that the value of 80 000 daltons is correct, it means that the actual complex contains about 5 protein molecules together with a relatively small (~40) number of lipid molecules. On the basis of spectroscopic measurements using a large range of protein to lipid ratios (10:1 up to 1:1000), only one type of complex was observed. This means that the low-stoichiometry complex is the preferred and stable one and must be suspected to be the catalytically active species. It is of interest to note that low stoichiometries (5–7 lipid molecules/protein molecule) have been observed in studies using vesicles of long-chain lecithin and lecithin-phosphatidylserine and venom phospholipases from several snake venoms (Prigent-Dachary et al., 1980). Apparently the venom phospholipases reduced the vesicle structure to much smaller aggregates. These observations do not exclude a possible role of dimeric phospholipases, but, in the low-stoichiometry complexes, contact between *all* proteins present seems to be unavoidable.

In the model presented by Verger et al. (1973), which is not fundamentally different from the model of Deems et al. (1975) for the enzyme from *Naja naja*, the catalytic event is preceded by a reversible binding of the enzyme to the interface. Reversible binding and hydrolysis of a single substrate molecule *in* the interface is the next step. In the Verger model, the only bound species considered of interest is the activated form (E*), and other binding steps which might precede it are neglected.

The data presented here would be more readily explained, however, by assuming that the early binding steps do play an important role. For example, enzyme bound to the interface can be present in two conformations, E_b and E^*_b with low and high enzymatic activities. In Scheme I the possible equilibria that might be involved in the association of enzyme E with lipid–water interfaces are indicated. By the enzyme's being brought into contact with increasing concentrations of micelles, equilibrium I will be shifted toward E_b. Once the enzyme is bound, an equilibrium is established between E_b and E^*_b, and we assume that the equilibrium concentrations are governed by the properties of the interface (packing density and orientation of the hydrocarbon chain, hydration of the phospholipid molecules, etc.) as well as by the properties of the protein.[3] In the case of porcine pancreatic enzyme where micelles of dihexanoyllecithin are hydrolyzed only 4- to 5-fold

[2] One might argue that in enzyme–micelle complexes consisting of enzyme bound to micelles of either single chain or double chain phospholipids the individual lipid molecules are bound to the active site in different orientations. Direct binding studies using double-chain phospholipids could resolve this problem, but so far we have not found suitable double-chain compounds. These lipids are either too soluble (high critical micellar concentration) or their solutions are turbid due to formation of coacervates of bilayer structures. Since micelles of single-chain lecithin analogues (glycol lecithin and 2-acyllysolecithin) are good substrates for phospholipase, we routinely measure protein–micelle binding constants with *n*-alkylphosphocholine. It has been shown before (cf. van Dam-Mieras, et al., 1975) that micelles of lysolecithins and alkylphosphocholines have very similar properties.

[3] The emphasis in the scheme shown above is on binding rather than on catalysis. Whether or not enzyme is slowly released from the interface during catalysis (Tinker et al., 1978; Tinker & Wei, 1979) or slowly bound to the interface (Verger et al., 1973) is not considered here.

faster than its monomers, the enzyme will be present in the form E_b rather than E^*_b. When NaCl is included in the assay system, the activity on the aggregated substrates is enhanced about 100-fold (de Haas el al., 1971). This observation would be consistent with a shift of equilibrium II to the right due to subtle changes in the packing and conformation of the lipid molecules in the interface. The importance of the conformation of the lipid molecules has been stressed by several investigators (Wells, 1974; Roberts & Dennis, 1977; Roberts et al., 1978). The observation that phospholipase A_2 from *Crotalus adamanteus* effectively hydrolyzes micelles of dihexanoyllecithin in the absence of salt (Wells, 1974; Algyer & Wells, 1979) once more stresses that for enzymes from different sources equilibrium II can be quite different.

Modification of the α-amino group in pancreatic phospholipase clearly shifts equilibrium I toward the free enzyme, blocking hydrolysis of micellar substrates but not of monomeric substrates. For the modified venom phospholipases, which also have retained their capability to hydrolyze monomeric substrate, addition of increasing concentrations of micelles shifts equilibrium I toward E_b but no appreciable amounts of E^*_b are formed. It should be noted that the actual conformation E_b of the native and the corresponding modified phospholipase could be quite different.

Diverse results obtained with phospholipases from different sources can be explained by assuming that they do not follow a common mechanism. However, in view of the high degree of homology (Heinrickson et al., 1977) between phospholipases isolated from different sources, one might expect their mechanism of action to be similar. For the hydrolysis of monomeric substrates, this is probably true (Dijkstra, 1980; Verheij et al., 1980). The interaction with lipid–water interfaces makes a comparison of the hydrolysis of aggregated substrates more difficult since it includes more equilibria and also is dependent upon more specific properties of the protein. Comparative studies using pancreatic as well as toxic and nontoxic venom phospholipases might yield more information on this problem.

Acknowledgments

We gratefully acknowledge R. Dijkman for the synthesis of *n*-hexadecylphosphocholine and DL-[2,3-bis(hexanoylthio)propyl]phosphocholine. We are indebted to W. C. Puijk for assistance during some of the monolayer experiments and to Dr. M. K. Jain for critically reading the manuscript.

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