Autocatalytic Acylation of Phospholipase-like Myotoxins†

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ABSTRACT: Several snake venoms contain a phospholipase A_2 in which position 49 in the active site is occupied by a lysine or a serine instead of the aspartate residue normally found. Although these proteins do not bind Ca^{2+} and are devoid of catalytic activity, they are still highly specific myotoxins and have recently been shown to induce membrane leakage by a new type of cytolytic mechanism. Three of these toxins, myotoxin II from *Bothrops asper*, ammodytin L from *Vipera ammodytes*, and the K49 protein from *Agkistrodon piscivorus piscivorus*, were examined for their interaction with fatty acids and were found to bind long-chain fatty acids covalently by a rapid, spontaneous, autocatalytic process. The fatty acids could be released by treatment with 1 M NH_2OH or NaOH, but not with 1 M NaCl or by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Electron spin resonance studies using spin-labeled fatty acids showed that only the carboxyl headgroup of the fatty acid was linked to protein amino acid, the carbon chain had free mobility and did not bind tightly to the protein surface. Stearic acid methyl esters and short-chain fatty acids did not bind to the toxins. Acylated myotoxins bound to the surface of liposomes and isolated muscle membranes, with the fatty acid moiety inserted into the lipid bilayer and possibly acting as an anchor. The phospholipase-like myotoxins represent the first group of proteins able to undergo acylation by spontaneous reaction with free fatty acids. On the basis of their high homology with active phospholipases, it is proposed that this ability may be the only remaining part of a complete phospholipase reaction mechanism.

Snake venoms contain a large variety of PLA_2's[1] with different toxic effects and specificities. Despite their functional differences, most of them share common biochemical and immunological characteristics with the type II PLA_2. Although these toxins generally are active phospholipases, the role of enzyme activity in cytotoxicity is not clear, and much evidence now indicates that not all of their toxic properties depend on the phospholipase activity (Rosenberg, 1990; Fletcher & Jiang, 1993).

In the last decade, a new class of phospholipase-like proteins completely devoid of enzyme activity has been discovered in the venoms of different snakes (Maraganore et al., 1984; Yoshizumu et al., 1990; Francis et al., 1991). The absence of catalysis notwithstanding, the purified proteins have been shown to act as potent myotoxins (Lomonte & Gutiérrez, 1989; Díaz et al., 1991; Krizaj et al., 1991). A common feature of this new class of toxins, which maintain a high degree of amino acid and structural homology with active phospholipases, is the substitution of the highly conserved aspartate in position 49. Normally the position is occupied by a lysine, as in the *Bothrops asper* and *Agkistrodon piscivorus piscivorus* proteins (Maraganore et al., 1984; Francis et al., 1991), but in the *Vipera ammodytes* protein a serine is found (Krizaj et al., 1991). The aspartate is essential for the binding of Ca^{2+} in the active site; the substitution no longer allows binding to take place and thus prevents the catalytic cleavage of the phospholipid, as documented by van den Bergh et al. (1989). The primary site of action of this group of myotoxins is believed to be the plasma membrane of muscle fibers, as suggested by morphological and biochemical evidence (Gutiérrez et al., 1984; Díaz et al., 1991; Lomonte et al., 1994), but the mechanism of toxicity is still unknown.

Recent studies carried out in our laboratories demonstrated that two such myotoxins, ammodytin L and myotoxin II, interacted with artificial lipid bilayers in a manner different from that of active phospholipases (Díaz et al., 1991; Rufini et al., 1992; Pedersen et al., 1994). Current hypotheses of interfacial catalysis by PLA_2 assume the transfer of phospholipid from the aggregated substrate to the catalytic site through a hydrophobic channel (Scott et al., 1990; Ramirez & Jain, 1991), without any changes in the enzyme structure or substrate aggregate order. Unlike the active PLA_2's, the myotoxins strongly perturb the hydrophobic core of lipid membranes, most likely through insertion into or even penetration of the bilayer. Addition of myotoxins to different types of loaded liposomes causes very rapid leakage of their contents, as in the case of vesicles composed of ether-linked phospholipids, which cannot be hydrolyzed even by active PLA_2's (Rufini et al., 1992; Pedersen et al., 1994). It seems plausible that this lytic activity could form the basis for the

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[1] Abbreviations: ESR, electron spin resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PLA_2, phospholipase A_2; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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physiological effects of these toxins, but the molecular mechanism is still unknown.

We used fluorescence polarization measurements of probes inserted in the membrane to study the binding of myotoxins to liposomes and their effects on the organization of the lipid bilayer (Rufini et al., 1992). Experiments were also made to examine this effect using a different technique: electron spin resonance measurements of spin-labeled membranes. Surprisingly, the results suggested a direct interaction between myotoxins and fatty acid probes. In the present work, we report that three phospholipase-like myotoxins, *B. asper* myotoxin II, *A. piscivorus piscivorus* K49, and *V. ammodytes* ammodytin L, are able to bind a fatty acid covalently in an autocatalyzed reaction. This reaction takes place spontaneously without phospholipid hydrolysis and does not require Ca^{2+} as a cofactor.

EXPERIMENTAL PROCEDURES

Materials. Myotoxin II was prepared from crude, dried venom as previously described (Gutiérrez et al., 1984; Lomonte & Gutiérrez, 1989). Ammodytin L was prepared according to Gubenšek et al. (1980). The K49 protein from *A. piscivorus piscivorus* was purified from crude venom (Sigma) by a two-step procedure using FPLC (Pharmacia). Isolated sarcolemma membranes prepared from rat soleus muscle (Incerpi & Luly, 1989) were the kind gift of Prof. S. Incerpi. Bee venom PLA_2, *Naja naja* PLA_2, fatty acid free bovine serum albumin, dipalmitoyl-PA, and dimyristoyl-PC were from Sigma and used without further purification; 5-doxyl stearic acid, 5-doxylstearic acid methyl ester, 12-doxyl stearic acid, and 4-doxylpentanoic acid were from Molecular Probes (Eugene, OR). [^{14}C]Arachidonic acid (55 mCi/mmol) and [^{3}H]myristic acid (55 Ci/mmol) were from Amersham.

Liposomes. Small unilamellar liposomes, composed of either pure PC or PC/PA with a molar ratio of 9:1, were prepared by sonication as previously described (Rufini et al., 1992). The final lipid concentration in the samples was 0.9−1.0 mM.

Electron Spin Resonance Measurements. Three different buffer systems were tested: 50 mM Tris buffer (pH 7.6); 20 mM Hepes (pH 7.2); and 10 mM phosphate containing 3 mM KCl (pH 7.4). Samples were normally prepared with a final volume of 50−100 μL in a small test tube. The spin labels were deposited as a film at the bottom by evaporation of 1−2 μL of stock solution in ethanol, whereupon buffer, toxins, or other components were added and mixed briefly. The samples were drawn into thin-walled glass capillaries, plugged, and placed inside a standard 3 mm quartz tube. Flat glass capillaries were used to gain a factor of 2 in intensity for samples with very weak signals (Pedersen & Cox, 1988). Measurements were made at room temperature with a Bruker ESP300 instrument equipped with a high-sensitivity TM_{110} mode cavity. Normally 100 mW microwave power at 9.83 GHz was applied; since thermal equilibration is slow at this power level (3−4 min), control experiments were done with 10 mW power to detect rapid spectral changes and exclude saturation distortions. Spectra were recorded using 1.0 G modulation, a scan time of 42 s, and a time constant of 20 ms; up to 80 scans were accumulated to improve the signal to noise ratio. Correlation times were calculated according to Marsh (1981). The spectra are drawn at different levels of amplification in order to allow the visual comparison of line shapes.

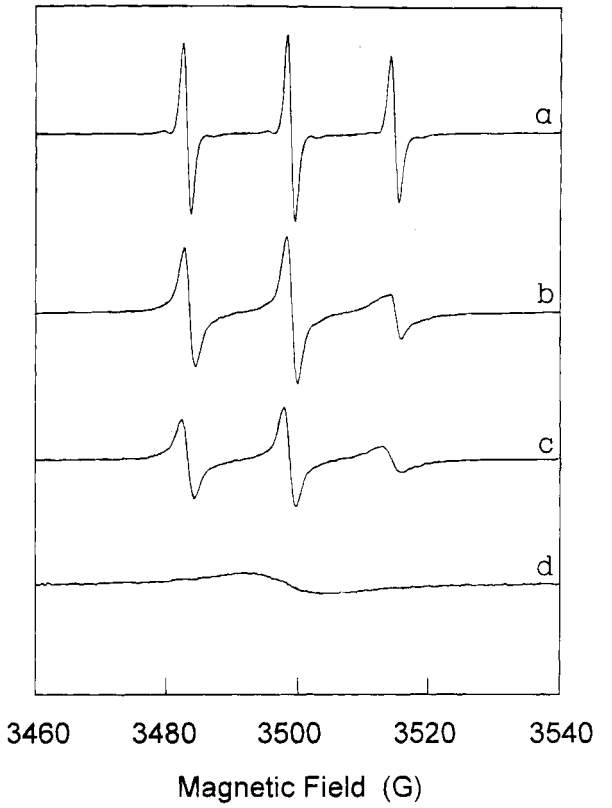

FIGURE 1: ESR spectra showing the interaction of spin labels with myotoxin II. The samples contained 50 μM 5-doxylstearic acid (a−c) or 50 μM 5-doxylstearic acid methyl ester (d) in Tris buffer, without toxin (a) or with the addition of 0.35 mg/mL (b) or 0.82 mg/mL (c, d) myotoxin II. The line width in (a) is so narrow that even the ^{13}C satellite lines are seen.

Fatty Acid Binding Assay. Proteins (3 nmol) were dissolved in 40 μL Tris-HCl (pH 7.3) and labeled by the addition of 1 μL of ethanol containing 10 nmol of [^{14}C]-arachidonic acid or [^{3}H]myristic acid; control measurements were made with the same volume of ethanol. After a 30 min incubation at room temperature, the reaction was stopped by the addition of 80 μL of Laemmli solution (Laemmli, 1970), and the samples were boiled for 5 min and analyzed by SDS gel electrophoresis according to Schagger and von Jagow (1987) using 10% polyacrylamide and 1 mM mercaptoethanol. The gel was fixed and the proteins were blotted on nitrocellulose using a Trans-Blot transfer cell (Bio-Rad), as described by Towbin et al. (1979). The autoradiographic film (X-Omat S, Kodak, New Haven, CT) was exposed to the nitrocellulose sheets for 4 and 30 days for ^{14}C-labeled and ^{3}H-labeled samples, respectively. Gels were stained with Coomassie Blue.

RESULTS

ESR spectra of dilute solutions of stearic acid spin labels in buffer showed the characteristic sharp three-line signal of a rapidly moving nitroxide compound (Figure 1a); in contrast to their unlabeled fatty acid counterparts, the fatty acid spin labels in fact have relatively high solubility in aqueous media (Gaffney et al., 1983). The spectra were almost completely isotropic with narrow line widths, and the correlation times were calculated to approximately 6×10^{-11} s irrespective of the position of the oxazolidine moiety on

the carbon chain. When increasing amounts of purified myotoxin II were added to the spin label samples, a second component appeared in the spectrum with a simultaneous decrease in the sharp three-line spectrum (Figure 1b,c). The freely mobile spin label signal had largely disappeared at a protein:spin label ratio of 1:1. The resulting spectrum was typical for a partly immobilized spin label, with an apparent correlation time τ_B of approximately 9×10^{-10} s. All changes in the ESR signal were completed within the time needed to prepare the sample and initiate measurements, ca. 30 s. No subsequent changes could be detected when the samples were incubated at room temperature or 37 °C over a period of several hours.

The amount of stearic acid spin label bound to the myotoxins was consistently found to be substoichiometric. Typically, a spin label:protein ratio of 0.7−0.8 was measured, but the ratio varied very much for different toxin preparations, being as low as 0.2 in some cases. The most likely explanation was thought to be that part of the protein already had unlabeled fatty acid bound at the end of the purification procedure. Considering the source of these proteins, it is obvious that working under fatty acid-free conditions was not the primary concern during the collection of the crude material for purification.

The appearance of the new spectral component clearly indicated binding of the fatty acid spin label to myotoxin II. However, the ESR spectrum still showed a high degree of mobility and was completely different from the strongly anisotropic spectra of 5-doxylstearic acid bound on a protein surface or moving within a membrane lipid bilayer. This suggested a type of binding directly to the carboxyl group, rather than hydrophobic interactions involving the long carbon chain. In agreement, it was found that the corresponding fatty acid methyl ester spin label did not bind to the myotoxin (Figure 1d). Due to the low solubility of the methyl ester derivative in aqueous samples, the sharp three-line signal from dissolved spin labels could not be detected; this lipid formed micelles leading to exchange broadening of the nitroxide ESR signal. As a control, the samples were incubated for 24 h, followed by dilution of the spin label in the micelles through the addition of unlabeled stearic acid methyl ester; this procedure did not reveal any binding of the methyl ester spin label to the protein.

In a series of parallel experiments, the two other myotoxins tested were also found to be able to bind fatty acids with similar characteristics (Figure 2a,b). Spin-labeled stearic acid bound to ammodytin L had a higher mobility, as reflected in the τ_B of approximately 5×10^{-10} s. In contrast, a much more immobilized spin label was seen with the K49 protein; the correlation time could not be estimated in this case, both because the spectra were insufficiently resolved and because the high degree of anisotropy did not allow the calculation of τ_B based on few spectral parameters.

Control experiments with the addition of active PLA_2's from *N. naja* or bee venom did not result in any change in the fatty acid spin label spectrum, in the absence or presence of 1 mM Ca^{2+} (Figure 2c). Similarly, the neurotoxic phospholipase β-bungarotoxin did not show any sign of fatty acid binding (results not shown). Addition of albumin gave the expected strongly anisotropic signal of a spin label located in the fatty acid binding cleft of the protein (Ge et al., 1990), and in this case the 5-doxylstearic acid methyl ester gave an almost identical spectrum (Figure 2d).

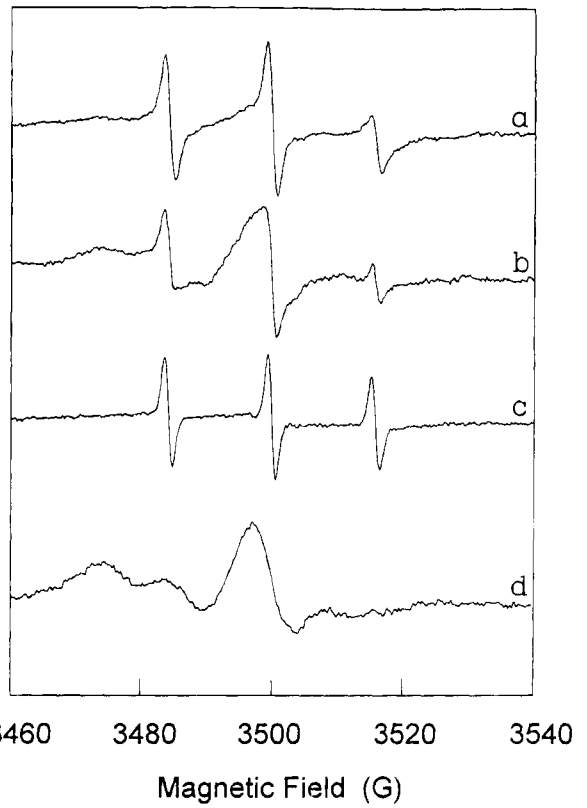

FIGURE 2: ESR spectra of spin labels interacting with proteins. Samples contained 20−50 μM 5-doxylstearic acid and the following proteins: (a) 0.7 mg/mL ammodytin L; (b) 1.2 mg/mL *Agkistrodon* K49 protein; (c) 1.1 mg/mL bee venom PLA_2 (identical spectra were obtained in the presence of 1 mM Ca^{2+} or with 1.1 mg/mL *N. naja* PLA_2 and 0.8 mg/mL β-bungarotoxin); (d) 1.0 mg/mL bovine serum albumin (a similar spectrum was obtained using the 5-doxylstearate methyl ester).

Binding of fatty acids to the three myotoxins could be observed with three different buffer systems (Tris, Hepes, and phosphate buffers), with different ionic strengths and slightly different pH values (not shown). More interestingly, it was found that once the fatty acid was attached to the protein it was not possible to remove it with high concentrations of various mono- or divalent cations; even 1 M NaCl had no effect on the ESR spectrum. This indicated a stronger binding of the acyl carboxy group than mere electrostatic interactions. To verify the existence of a covalent bond, the myotoxins were labeled with [^{14}C]arachidonic acid (Figure 3) or [^{3}H]myristic acid (data not shown) and subjected to denaturing SDS gel electrophoresis. As can be seen, the fatty acid marker remained bound to the protein, confirming the permanent nature of the acyl−protein linkage.

Additional experiments provided further information on the protein−fatty acid interaction. Stearic acid labeled with the oxazolidine group close to the end of the carbon chain (16-doxylstearate) bound to myotoxin II in the same way as 5-doxylstearate, although the spectrum was slightly more isotropic, $\tau_B = 8 \times 10^{-10}$ s, confirming that the long carbon chain is not bound to the protein surface (Figure 4a). In contrast, a short-chain fatty acid analog, 4-doxylpentanoic acid, did not bind at all to the myotoxins (Figure 4b).

Preincubation of myotoxin II with 100 μM butyrate did not affect the subsequent binding of 50 μM stearic acid spin label (not shown). Competition studies with unlabeled stearic acid turned out to be difficult to interpret: because of its

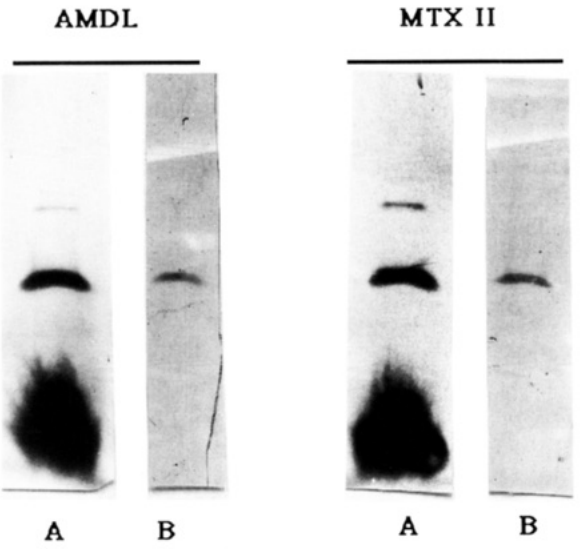

FIGURE 3: SDS−polyacrylamide gel analysis of ammodytin L (left) and myotoxin II (right) labeled with [^{14}C]arachidonic acid and visualized by autoradiography (A) and Coomassie staining (B). The large blurred spot at the bottom of the gels is due to free arachidonic acid, which forms mixed micelles with SDS.

very low solubility the stearate formed micelles, and the ESR spectrum was completely dominated by the signal from spin-labeled micelles. Incubation of myotoxin II with spin-labeled fatty acid at pH's below 4 did not result in binding (Figure 4c), but if the bond was formed by preincubation at neutral pH, it would also resist at low pH (Figure 4d). Release of the bound spin label could be achieved through incubation with 1 M NaOH for 60 min at 37 °C (Figure 4e). Another standard method for releasing fatty acid residues from acylated proteins, incubation with NH_2OH, was not applicable in the ESR experiments due to the fast reduction of the nitroxide reporter group. However, SDS gel electrophoresis of [^{3}H]myristoylated ammodytin L treated with 1 M NH_2OH for 60 min at 37 °C confirmed the complete separation of the fatty acid from the toxin (results not shown).

The relevance of covalent fatty acid binding to the membrane-perturbing ability of these toxins was tested in liposome experiments. Due to the relatively low phospholipid concentrations used, approximately 1 mM, liposomes labeled with 5-doxylstearate gave typical two-component spectra (Figure 5a). The three sharp lines belong to the very small fraction of stearic acid labels moving freely and rapidly in the aqueous phase; these lines are superimposed on the broad anisotropic signal from the bulk of the spin labels located within the lipid bilayer. Upon the addition of increasing concentrations of myotoxin II, the sharp water phase signal gradually disappeared; this would normally reflect favored partitioning of the label into the lipid phase due to a general decrease in membrane viscosity (Figure 5b,c). But in this case a much more anisotropic membrane component appeared, as indicated by the drastic increase in the outer splitting parameter, $A_{||}$, corresponding to much more restricted motion of part of the spin labels in the membrane. In the light of the results presented earlier, these effects were interpreted as specific binding of myotoxin II to the labeled fatty acids and insertion of the fatty acid moiety into the membrane. To verify this hypothesis, myotoxin II was acylated with 5-doxylstearate by preincubation and subsequently added to the liposomes; the spectrum of the protein-bound spin label became identical to the spectrum in Figure

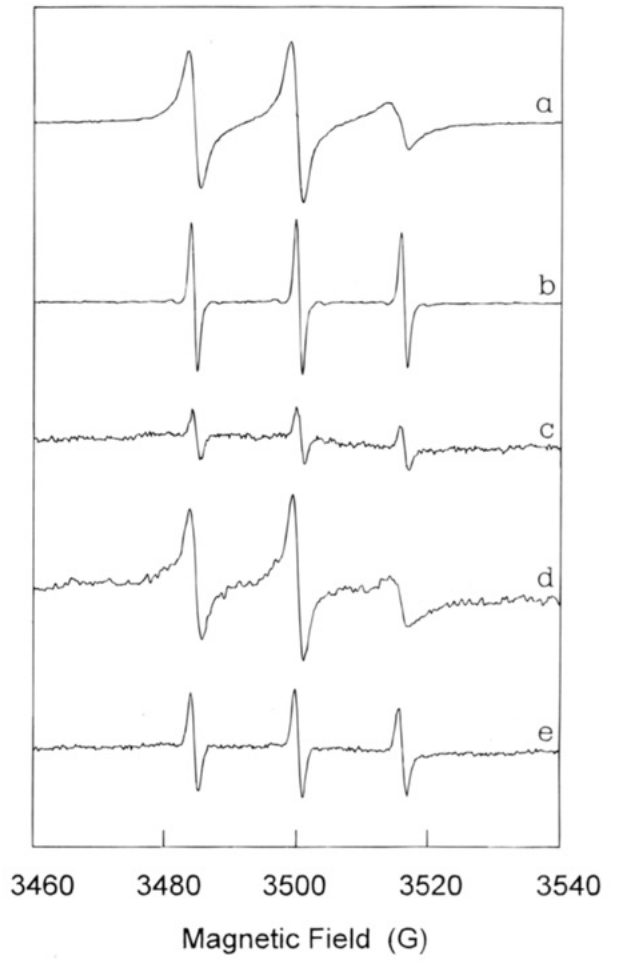

FIGURE 4: Characterization of the toxin−stearate binding. All samples contained 0.8 mg/mL myotoxin II and 50 μM spin label (similar spectra were obtained using ammodytin L): (a) 16-doxylstearic acid; (b) 4-doxylpentanoic acid; (c) 5-doxylstearate, toxin added to Tris-HCl buffer at pH 3.5; (d) 5-doxylstearate, toxin preincubated with spin label in Tris buffer at pH 7.6, followed by adjustment of pH to 3.5; (e) 5-doxylstearate, toxin preincubated with spin label in Tris buffer at pH 7.6, followed by incubation with 1 M NaOH for 60 min at 37 °C. The signal in (c) is relatively small because most of the stearic acid spin label is protonated and forms micelles.

5c, demonstrating that the acylated myotoxin was indeed bound to the membrane (not shown). In contrast, the spectrum of liposomes containing the fatty acid methyl ester spin label was not affected by the addition of high concentrations of myotoxin II (Figure 5d,e); this result confirmed the complete lack of interactions between the myotoxins and the fatty acid ester.

The effects of myotoxin II could be seen with both pure PC liposomes and mixed PC/PA vesicles, indicating that surface charge was not an essential parameter. The findings described earlier are not restricted to liposomes; similar results were obtained when ammodytin L was tested on a preparation of isolated muscle membranes, the physiological target membrane for these toxins. Also in this case, addition of the toxin (0.7 mg/mL) strongly reduced the mobility of stearic acid spin labels inserted in the membrane, whereas no significant changes were detected for membranes labeled with the methyl ester spin label (data not shown).

DISCUSSION

The results presented here unequivocally demonstrate that all three phospholipase-like myotoxins examined undergo

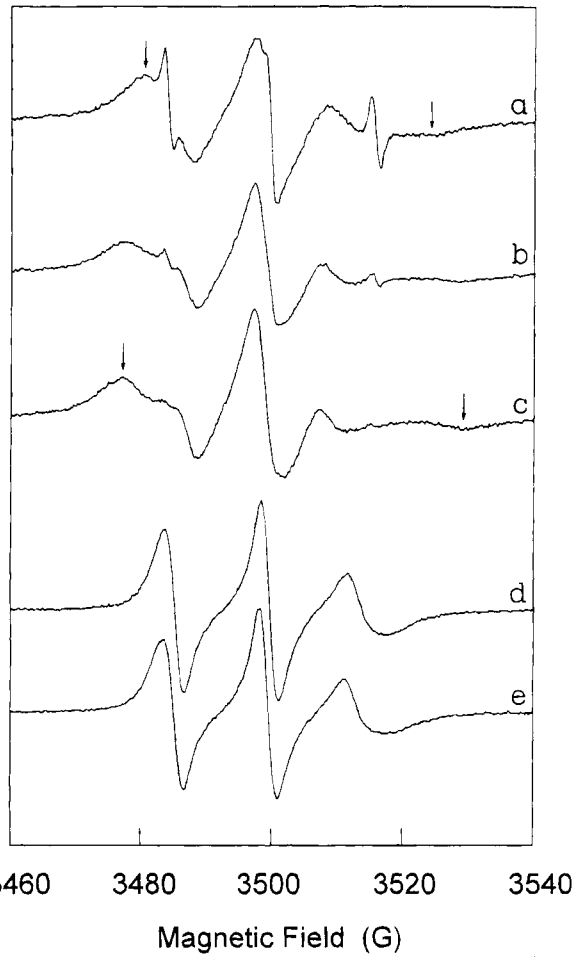

FIGURE 5: Effect of myotoxins on spin-labeled liposomes. Samples contained 50 μM 5-doxylstearic acid (a–c) or 50 μM 5-doxylstearic acid methyl ester (d, e). The liposomes were composed of PC/PA in Tris buffer, without toxin (a, d) or with the addition of 0.18 mg/mL (b) or 0.82 mg/mL (c, e) myotoxin II. The outer splitting parameter $2A_{||}$ was measured as indicated by the vertical markers.

acylation in the presence of stoichiometric amounts of fatty acids. The lack of an effect of treatment with high ionic strength and the SDS electrophoresis experiments permit us to affirm that the binding between fatty acid and protein must be covalent. Since amino acids are not known to react with fatty acids spontaneously, it is reasonable to consider the acylation a product of autocatalysis. Numerous other types of proteins are now known to be acylated, but the acylation reaction is always catalyzed by an apposite enzyme, such as *N*-myristoyl transferase (Gordon et al., 1991) or *O*-palmitoyl transferase (Kasinathan et al., 1990). In enzymes involved in lipid metabolism an acyl–protein intermediate may be formed transiently (Muderhwa et al., 1992), but permanent acylation does not take place. The unique property of the myotoxins appears to be the *spontaneous and autocatalyzed acylation.*

Myotoxin acylation took place within seconds at room temperature, was calcium independent, and did not require phospholipid cleavage. The active venom PLA_2's tested were unable to bind fatty acids under the same conditions, and the same was true for β-bungarotoxin, which is known to be a fusogenic phospholipase (Rufini et al., 1990). But even though the activities of myotoxins and active PLA_2's are clearly different, it is likely that the autoacylation reaction has its origin in the phospholipase character of these toxins.

When the four known amino acid sequences of phospholipase-like myotoxins are compared with the sequences of active nontoxic phospholipases, highly conserved residues are found in all regions of the sequences. In one extreme case, 90% nucleotide homology was found between the myotoxin ammodytin L from *V. ammodytes* and a nonmyotoxic PLA_2 from the same species (Pungercar et al., 1990). Crystallographic studies of the inactive K49 and the active D49 PLA_2's from *A. piscivorus piscivorus* showed that the catalytic network and hydrophobic channel were largely identical in the two proteins (Scott et al., 1992). It is therefore reasonable to suggest that acylation of these toxins is not an independently acquired property, but simply represents the only remaining part of the phospholipase catalytic cycle. PLA_2-like myotoxins lack the ability to coordinate Ca^{2+} ions in the catalytic network, and consequently a hydrolysis transition state cannot be stabilized, but the protein might maintain the capacity to activate and transfer an acyl chain to an amino acid residue. Heinrikson and co-workers reported the autocatalytic acylation of lysine residues in both porcine pancreatic PLA_2 and the D49 active PLA_2 of *A. piscivorus piscivorus*, but the process required substrate cleavage and was Ca^{2+} dependent (Cho et al., 1988; Tomasselli et al., 1989; Noel et al., 1991).

Another type of acylation has been described for a nonsecreted phospholipase from platelets, which is selective for arachidonic acid-containing phospholipids (Zupan et al., 1992). The catalytic strategy appeared to be different in this case, since the acyl intermediate was bound as a thioester and Ca^{2+} was not an obligatory cofactor. A direct comparison with the myotoxins cannot be made, since substrate hydrolysis appears to be necessary for acylation in the above-mentioned examples; still, these findings confirm that fatty acid binding may occur. The site of acylation in the myotoxins is not known. Binding to a cysteine is not likely since the fatty acid was not released by treatment with mercaptoethanol. The lack of spin label binding to myotoxin II at low pH suggests the involvement of a group with a pK_a between 4 and 6. Interestingly, pK_a for His-48 in the active site has been calculated to be in the range 5.0–5.7 for several active PLA_2's (Nishimura et al., 1992); this histidine is highly conserved in all known myotoxins and PLA_2's.

Various long-chain fatty acids can bind, whereas short-chain analogs do not bind at all; this pattern is commonly found for the acylation of proteins (Gordon et al., 1991; Berthiaume et al., 1994). The spin label experiments provide additional information on the nature of the acylated state. Isotropic rotation correlation times for PLA_2's have been measured to be approximately 1×10^{-8} s (Allegrini et al., 1984; Ludescher et al., 1988). A crude comparison shows that the bound spin label in myotoxin II apparently moves 10 times slower than free labels, but 10 times faster than the protein itself. This confirms that the fatty acid is attached to the protein only through the covalent bond to the carboxyl group; the long carbon chain is moving freely. A more immobilized fatty acid is seen for the *Agkistrodon* K49 protein, and an immobilized component is also found in the spectrum of ammodytin L; this could possibly reflect the formation of dimers or larger aggregates, in analogy with the behavior of active PLA_2's. Studies are in progress to examine this and other aspects of fatty acid binding.

What is the functional role of acylation? An increasing number of viral and cell proteins are known to acquire covalently bound fatty acids, which may influence both the interaction between the acylated proteins and adjacent molecules such as membrane lipids or nucleic acids and also intramolecular processes such as oligomerization or the cooperativity between subunits of a multimeric protein. In particular, it has been suggested that protein acylation may be required for the fusion process between viral and cellular membranes. Chemical modification or site-directed mutagenesis of acylation sites of viral proteins completely abolish, or severely inhibit, the fusogenic activity of several lipid-perturbing viral proteins (Lambrecht & Schmidt, 1986; Naeve & Williams, 1990). In the same way, the involvement of fatty acid binding in the fusion-driven transport of glycoproteins along the secretory pathway has been suggested (Glick & Rothman, 1986). The acylated myotoxins clearly bind to liposomes and cell membranes, with the fatty acid inserted into the bilayer and possibly acting as a lipid anchor (Figure 5). This may explain why these toxins apparently irreversibly attach to liposomes; they do not relocate to other vesicles like the active phospholipases (Rufini et al., 1992). It is known that acylation of PLA_2 can lead to a strongly increased affinity for membranes and influence the capacity of penetration of the protein (Lugtigheid et al., 1993). But the fatty acid might also play a more active role in the reaction mechanism of the toxins; although the acylation of PLA_2 apparently is not essential for the enzyme activity (Noel et al., 1991), acylation of other enzymes has been shown to regulate their activity (Berthiaume et al., 1994). Recently, two other examples of acylated toxins have been discovered, the mollusk toxin ω-conotoxin (Branton et al., 1993) and adenylate cyclase toxin from *Bordetella pertussis* (Hackett et al., 1994), but the function of the lipid moiety is not yet established.

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