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Conformational Changes Associated with Activation of Bee Venom Phospholipase A_2[1]

Tanweer Ahmed,* Sharon M. Kelly,† Anthony J. Lawrence,‡,2 Mokdad Mezna,§ and Nicholas C. Price†

**Protein Crystallography, Joseph Black Building, University of Glasgow, Glasgow, G12 8QQ, UK;* †*Department of Biological and Molecular Sciences, University of Stirling, Stirling, FK9 4LA, UK;* ‡*Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, G12 8QQ, UK; and* §*School of Biochemistry, University of Birmingham, Birmingham, UK*

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Bee venom PLA_2 possesses a binding site for long-chain fatty acids that can be acylated by long-chain fatty acid imidazolides [Drainas, D. and Lawrence, A.J. (1978) *Eur. J. Biochem.* 91, 131-138]. Occupation of the site either by oleic acid or the oleoyl residue enhances the catalytic activity by 45.7-fold in the presence of 20% 1-propanol and occupation of the site by the oleoyl residue increases the lytic activity against rabbit erythrocytes by 60-fold. Treatment of the enzyme with oleic acid and glutaraldehyde is known to produce irreversible activation [Lawrence, A.J. and Moores, G.R. (1975) *FEBS Lett.* 49, 287-291]. Here we show that reduction of the glutaraldehyde-treated enzyme with borohydride stabilizes the activated state and enables the fatty acid to be removed, revealing that a large proportion of the induced activation does not require the presence of oleic acid and indicating that activation is due to a change in the conformation rather than the hydrophobicity of the protein. A kinetic study of enzyme activated by oleoyl imidazolide showed that this modification stabilizes the protein against reversible inactivation by 1-propanol. Comparison of the CD spectra of native and oleoyl imidazolide-activated enzyme shows a change in secondary structure with apparent increase in both α-helix and β-sheet content. During reaction of the enzyme with oleoyl imidazolide, the protein fluorescence shows a biphasic response with an initial fall attributed to occupation of the binding site followed by a progressive decrease with a shift of the emission maximum from 341 to 348 nm. The rate of the second phase closely matched the rate of increase in catalytic activity of the enzyme. Free oleic acid caused a rapid fall in fluorescence emission without the subsequent slow change. These results support the proposal that oleic acid or the oleoyl residue occupy a very similar site on the protein and that occupation of this site increases the exposure of one or both of the Trp residues to the aqueous environment. Binding studies show that activation by oleoyl imidazolide does not increase the affinity of the enzyme for the erythrocyte membrane. It is proposed that occupation of a long-chain fatty acid binding site on the enzyme enhances catalytic activity by changing the conformation of the protein rather than acting as a hydrophobic anchor to the substrate surface.

Key words: activation, acylation, circular dichroism, conformation, fluorescence, phospholipase A_2.

Venom PLA_2 enzymes are small compact proteins with a high disulfide bond content (*1, 2*), which act at a lipid-water interface. Their catalytic properties are modulated in a variety of interesting ways by amphiphilic molecules that intercalate into the lipid phase and modify the properties of the interface. Neutral detergents, which can promote the transition from bilammelar to micellar morphology in the substrate, may increase hydrolysis rates by 1-2 orders of magnitude (*3*), but within either surface type subtle variations are possible that can have significant effects on reaction rates (*4, 5*). The effects of detergents on the substrate may obscure the possibility that some lipophilic modulators of PLA_2 activity could act directly on the enzyme.

Long-chain fatty acids, for example, are thought to activate phospholipase enzymes by increasing the charge density of the lipid surface (*6*), but an observation that the activation of bee venom PLA_2 by oleic acid could be stabilized by glutaraldehyde treatment indicated not only that the fatty acid bound to the enzyme, but also that it activated by changing the protein conformation (*7*). Subsequent experiments which showed that a sub-class of PLA_2 enzymes could be irreversibly activated by moderately reactive acylating derivatives of long-chain fatty acids

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[2] To whom correspondence should be addressed. Tel: +44-141-330 5196, Fax: +44-141-330 4501, E-mail: gbaa09@udcf.gla.ac.uk

Abbreviations: DOPC, dioctanoylphosphatidylcholine; GPC, glycerophosphorylcholine; PC, phosphatidylcholine; PLA_2, phospholipase A_2; CD, circular dichroism; TLC, thin layer chromatography; SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

strengthened the possibility that the free acids were allosteric modifiers of enzymic activity (*8, 9*).

Although the activated enzymes were extremely stable under non-denaturing conditions (*10*), all attempts to characterize the acylation sites have failed, principally because the linkage is labile upon partial denaturation and the acyl residue is rapidly lost during acidic or basic urea PAGE. These results suggested that a novel, highly labile, acylation could be involved: this might form the basis of a reversible regulation mechanism that would escape detection by conventional labeling experiments. One of the attractive models for activation of phospholipase enzymes by long-chain fatty acylation is that the modified side chain acts as a surface anchor at the lipid interface (*11, 12*). However, a number of pieces of evidence, including the conformational stability of the activated enzyme, the lability of the linkage on denaturation and the fact that a bound acyl group would have very different anchoring properties from free fatty acids support the earlier model that activation results from a conformation change in the protein.

In this paper we present evidence that both free oleic acid and oleoyl imidazolide interact with bee venom PLA_2 to produce a conformation change and that the conformation change is, to a large extent, responsible for the large enhancement of catalytic activity.

MATERIALS AND METHODS

Organic solvents were analytical grade from BDH. Chemical reagents and honey bee (*Apis meliffera*) venom were purchased from Sigma chemical. Bee venom phospholipase A_2, (PLA_2) was purified from bee venom as described (*10, 13*). The purity was established by electrophoresis on acid/urea and alkaline/urea polyacrylamide gel electrophoresis (*14, 15*) and the concentration was determined by amino acid analysis. Egg lecithin, prepared by the acetone extraction procedure (*16*) was further purified by chromatography on alumina followed by exhaustive deionization on a mixed bed resin and used to prepare glycerophosphorylcholine (GPC) as described (*16*). Diacyl phosphatidyl choline derivatives were synthesized by a modification of the method of Patel *et al.* (*17*), using a pyrrolidinopyridine catalyst, but acylating the GPC free base directly. The derivatives were purified by chromatography on alumina, dried and redissolved in methanol and then exhaustively deionized with Dowex MR-3 mixed bed resin. The products gave single phosphomolybdate-positive, ninhydrin-negative bands on TLC. Lyso-phosphatidyl choline was prepared by bee venom catalyzed hydrolysis of egg lecithin in solution in 20% 1-propanol with 1 mM $CaCl_2$ maintained at pH 8.0 by addition of NaOH. After reaction the product was extracted with chloroform, washed with ethyl acetate, dried, redissolved in chloroform and purified by alumina chromatography and by treatment with a mixed-bed ion exchange resin. Lyso palmitoyl phosphatidyl choline was prepared from dipalmitoyl phosphatidyl choline by the same procedure. Monoacyl phosphatidyl choline derivatives were reacylated as described above and the products purified by standard methods. [9,10(*n*)-^{3}H]oleic acid was purchased from Amersham International plc. as a solution in toluene, dried and diluted 1:15 with unlabeled oleic acid (to give a specific activity of 74 Gbq/mmol) and used as a 1 mg per ml solution in dry acetone for measurements of oleic acid binding. [^{3}H]Oleoyl imidazolide was prepared from 0.5 ml of this solution by addition of 0.7 mg of carbonyl diimidazole (*15*).

Fluorescence measurements were made at 25°C using a Perkin-Elmer LS 50 spectrofluorimeter. CD measurements were made at 25°C using a JASCO-J-600 spectropolarimeter and secondary structure analysis was undertaken using the CONTIN procedure (*18*).

Assays of phospholipase A_2 activity were made by conductimetric methods using a modified apparatus with eight 2 ml capacity reaction cells (*19*). Hydrolysis of phosphatidyl choline derivatives was carried out in 10 mM triethanolamine HCl buffer at pH 8.0 and where stated mixed with 1-propanol and the solvent concentration defined on a volume percent basis (*i.e.* 20% 1-propanolic buffer contains 200 ml of 1-propanol in a total volume of 1 liter). Assays were calibrated as described (*19*). Erythrocyte leakage assays were by conductimetric measurement as described using a 0.3% v/v suspension of rabbit erythrocytes in isotonic sucrose medium buffered with 10 mM morpholinoproprionate-Na^+, pH 7.4 (*20*).

RESULTS

Methods for Activating Bee Venom PLA_2—The original demonstration of activation by long-chain fatty acids was obtained using a conductimetric modification of the standard titrimetric assay in which egg phosphatidyl choline and its hydrolysis products were solubilized by the inclusion of 20% 1-propanol. The conductimetric assay had several advantages over the titration method, but most importantly for this study it monitored precise events from the inception of reaction and it indicated the level of contamination of the substrate by ionic species. This method revealed that reaction progress curves were biphasic with sigmoidal character that was greatly enhanced by deionizing the substrate using a mixed-bed resin (*20*). Kinetic studies then showed that the activating product was indeed the long-chain fatty acid. It was then found that the requirement for free fatty acid could be by-passed by two methods. In the first case the concentration dependence of fatty acid activation was abolished by adding glutaraldehyde to a concentrated solution of enzyme in the presence of an activating level of oleic acid. The "fixed" enzyme retained activation after dilution whilst the control did not. The second method involved treatment of the enzyme with a stoichiometric equivalent amount of a moderately reactive long-chain fatty acyl derivative leading to the introduction of an acyl moiety into a 1-propanol-resistant location in the protein (*21*).

The Effect of 1-Propanol on Activation—An intriguing feature of the activated enzyme was its apparent substrate specificity. Thus, treatment with oleoyl imidazolide, that activated 50-fold using egg lecithin as substrate in 20% 1-propanolic medium or against rabbit erythrocytes in the presence of albumin, produced no activation against dioctanoyl phosphatidyl choline. Thus the enzyme seemed to possess a specific binding site for long-chain fatty acids which could be irreversibly occupied by a covalently linked acyl group, but only increased the catalytic activity under a limited range of reaction conditions. However no further studies of the role of 1-propanol in the activation had been

carried out. In part this was because the lower limit of 1-propanol concentrations in which the conductimetric assay could be applied was limited to > 15% for the natural substrate. Below this level the long-chain fatty acid reaction products ($>C_{10}$) formed non-conducting calcium adducts. To circumvent this we tested the effect of 1-propanol on the catalytic activity of the enzyme against short chain diacyl phosphatidyl choline derivatives. As expected the hydrolysis rate in a pure aqueous medium peaked with the DOPC and fell smoothly with acyl chain length (Fig. 1). However very significant differences were observed between the didecanoyl and dilauroyl derivatives where triton X-100 changed from being a weak inhibitor to a powerful activator. In addition the effect of 20% 1-propanol changed from being strongly inhibitory to weakly activating. This is consistent with the model that dilauroyl phosphatidyl choline is the smallest member of the series to adopt liposomal rather than micellar morphology in pure aqueous medium.

The effect of substrate hydrophobicity was further investigated using phosphatidyl choline derivatives which possessed a short acyl chain in the 2-position. Briefly the 2-acyl derivatives of egg lysophosphatidyl choline were good substrates for the bee venom enzyme, but the 2-nonanoyl and higher derivatives gave biphasic progress curves where the transition could be abolished by neutral detergents, including the product monoacyl phosphatidyl choline species, but not by the product fatty acid (Fig. 2A). Very similar responses were obtained using the more precisely defined compounds based on 1-palmitoyl lysophosphatidyl choline, eliminating the possibility that rate transitions could reflect substrate heterogeniety. These results indicated that the biphasic character was determined by a transition between low susceptibility liposomal and high-susceptibility micellar morphology, which is the accepted basis for detergent mediated activation for symmetrical diacyl derivatives. When the 2-nonanoyl derivative of 1-palmitoyl phosphatidyl choline was used as a substrate to compare activated and native bee venom PLA_2 it became clear that the effects of activation were greater on the early than on

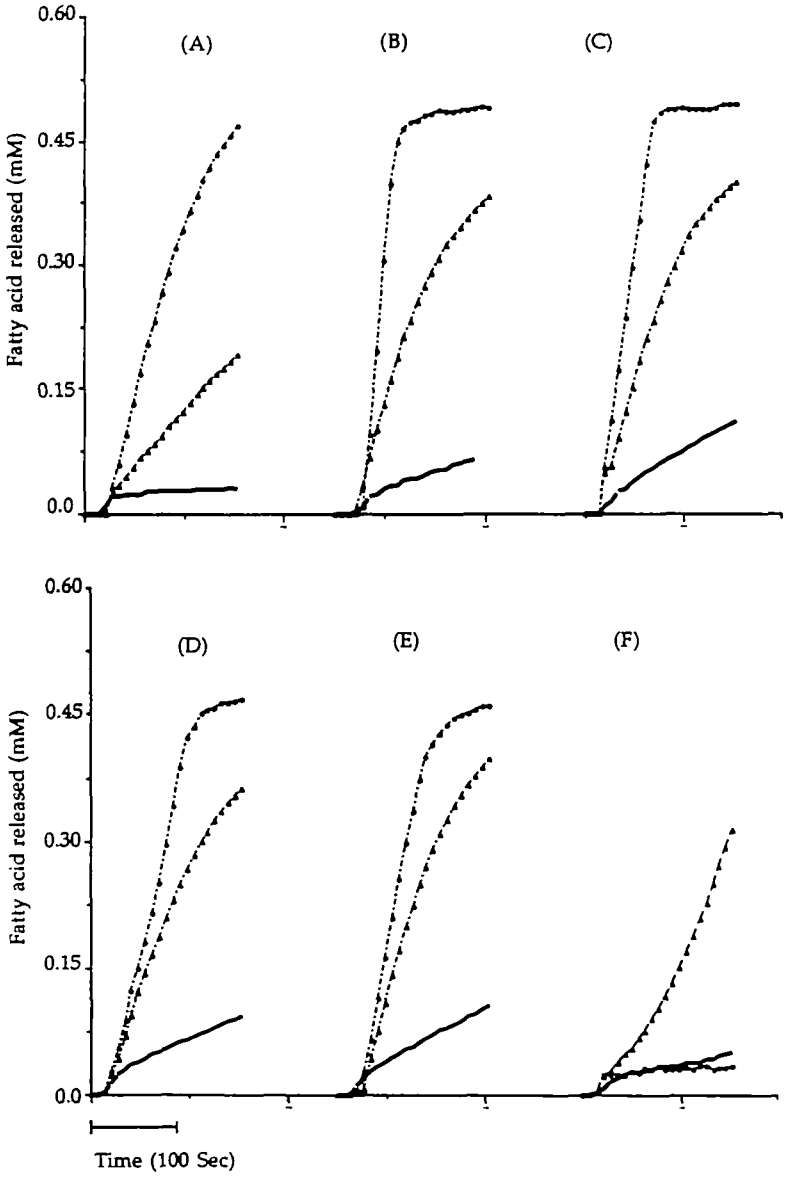

Fig. 1. **Modulation of the catalytic activity of bee venom PLA_2 by triton X-100 and 1-propanol.** The hydrolysis of different symmetrical diacyl derivatives of phosphatidyl choline by bee venom PLA_2 was measured by conductimetric analysis using reaction cells containing 2 ml of 10 mM triethanolamine/HCl, pH 8.0 at 37°C, containing 0.5 mM phospholipid in each case. Reaction was started by addition of 2 μl of 1 mg/ml PLA_2 either under the conditions described (—·—), or in the presence of 0.025% triton X-100 (—··—), or 20% v/v of 1-propanol (——). The results are shown for: (A) diheptyl, (B) dioctanoyl, (C) dinonanoyl, (D) didecanoyl, (E) diundecenyl, and (F) the dilauroyl derivatives of phosphatidyl choline, respectively.

the late phase of the reaction.

The effect of 1-propanol with this substrate differed significantly from that seen using DOPC. Here the two phases of reaction responded differently with the early phase being activated whilst the late phase was initially activated and then inhibited (Fig. 2B). In consequence the curves became monophasic at higher propanol concentration. These results showed that the major difference between native and activated enzyme was the ability of the latter to resist 1-propanol induced inactivation.

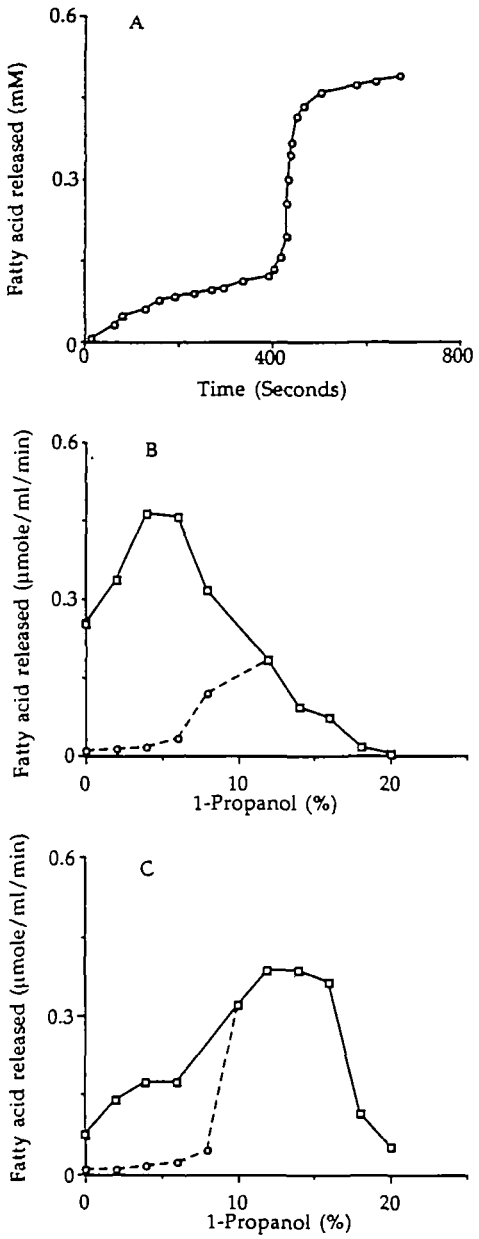

Fig. 2. **Differential stabilization of normal and activated bee venom PLA_2 against 1-propanol by a hydrophobic substrate.** Native bee venom PLA_2 and PLA_2 prepared by treatment of a 1 mg per ml solution in 10 mM triethanolamine/HCl, pH 8.0, with 10 μl per ml of 4 mM solution of oleoyl imidazolide in acetone were used to hydrolyze an 0.5 mg per ml solution of 1-palmitoyl, 2-nonanoyl phosphatidyl choline in 10 mM triethanolamine/HCl buffer, pH 8.0 at 37°C. (A) The time course of hydrolysis of 1-palmitoyl, 2-nonanoyl phosphatidyl choline showing the measurement of initial and of maximum rates. (B) The dependence of the rates of the initial and late phases of hydrolysis of 1-palmitoyl, 2-nonanoyl phosphatidyl choline on 1-propanol concentration. Reactions were carried out as is (a) using 1 μg per ml of native PLA_2 at a range of difference concentrations of 1-propanol. Results are displayed as the initial (–○–) and the maximum (–◇–) reaction rates. The progress curves become monophasic at 12% 1-propanol concentration. (C) As in B but using 1 μg per ml of oleoyl imidazolide activated enzyme. Here the curves become monophasic at 10% 1-propanol concentration.

Activation by Glutaraldehyde—An earlier study showed that bee venom PLA_2 could be activated for attack against long chain phosphatidyl choline substrates in 20% 1-propanol by mild treatment with glutaraldehyde in the presence (but not in the absence) of oleic acid followed by subsequent dilution for assay under conditions where the fatty acid would normally dissociate from the enzyme (Fig. 3) (*6*). In the absence of oleic acid, glutaraldehyde treatment slightly reduces the basal activity of the enzyme, but strongly inhibits the development of sensitivity to activation by fatty acid reaction products, or to exogenous free oleic acid. Enzyme activated by this method was not stable and gradually lost its activity. Here we attempted to stabilize the modified enzyme by borohydride reduction and then to determine whether or not the long-chain fatty acid could be removed whilst maintaining activation. Figure 4 shows that borohydride reduction during activation halted the progress of reaction and did indeed stabilize

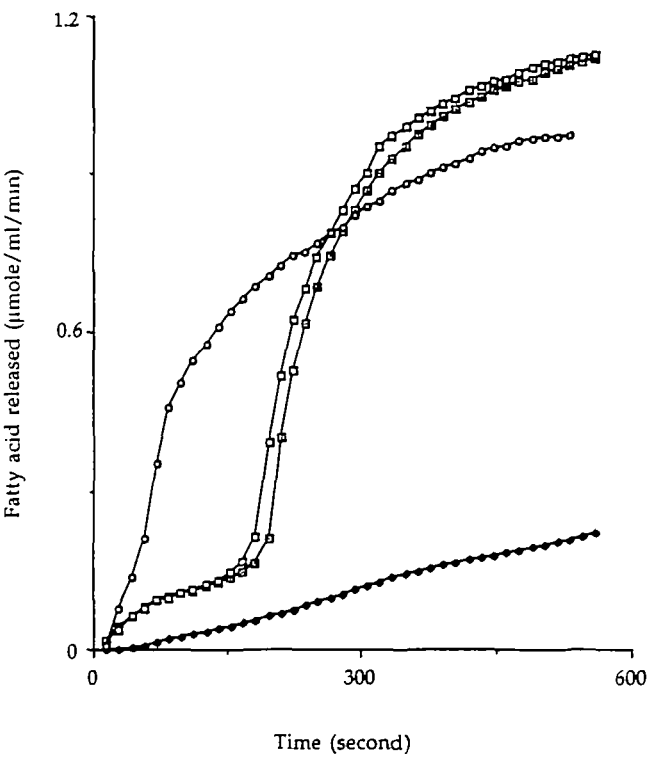

Fig. 3. **Stabilization of fatty acid activation of bee venom PLA_2 by glutaraldlehyde.** A solution of bee venom PLA_2 at 1 mg/ml in 10 mM triethanolamine/HCl, pH 8.0, containing 20% v/v 1-propanol, was incubated at 25°C either alone (–⊡–), with 0.1 mM oleic acid (–□–), with 0.25% glutaraldehyde (–●–), or with 0.1 mM oleic acid followed by 0.25% glutaraldehyde (–○–). After 5 min, 2 μl samples were withdrawn for conductimetric measurement of the time course of hydrolysis of purified egg phosphatidyl choline dissolved at 1 mg/ml in the same buffer at 37°C.

the activated state. Enzyme was now activated by glutaraldehyde in the presence of ^{3}H-labeled oleic acid, reduced by borohydride and subjected to gel filtration on Biogel P30 in 20% 1-propanol (Fig. 5). Table I shows the activation status of the enzyme at all stages in these procedures in comparison with activation produced by oleoyl imidazolide treatment. The results demonstrated very clearly that the fatty acid free enzyme retained a significant degree of activation. Many PLA_2 enzymes are known either to dimerize at high concentration, or else to be active as dimers (*22, 23*). One possible mode of action of fatty acids or bound acyl residues would be to stabilize a dimeric state and glutaraldehyde could further stabilize such a dimer. Furthermore the reduction of glutaraldehyde activated enzyme by borohydride would render the linkage irreversible. However, no form of the activated enzyme shows a change in M_r as detected by gel filtration using Biogel P30 in 20% 1-propanol (data not shown), or on acidic urea PAGE (*14*) or basic urea PAGE (*15*). More cogently dimer was not detected on SDS-PAGE (data not shown).

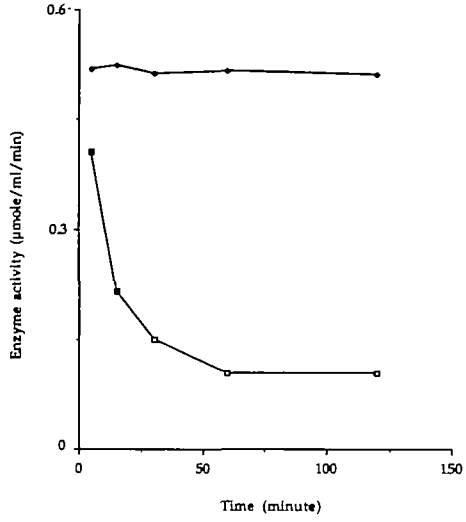

Fig. 4. **Borohydride reduction of glutaraldehyde treated bee venom PLA_2.** Two samples of bee venom PLA_2 were activated by treatment with 0.25% glutaraldehyde in the presence of 0.1 mM oleic acid as in Fig. 3. One sample (−◆−) was treated with sodium borohydride and 2 μl aliquots withdrawn for assay as in Fig. 3 at measured times. The other sample (−⊡−) was not treated with sodium borohydride.

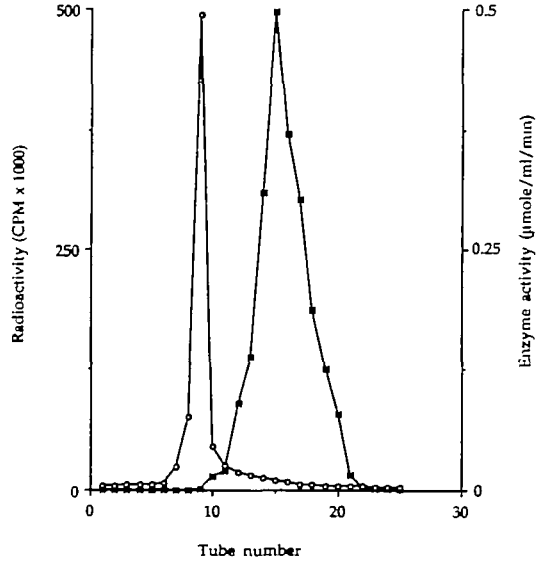

Fig. 5. **Activation of PLA_2 by glutaraldehyde does not require the continued presence of oleic acid.** Bee venom PLA_2 was activated by treatment with 0.25% glutaraldehyde in the presence of 0.1 mM ^{3}H-labeled oleic acid, reduced with borohydride, and then passed through a column (10×1 cm) of Biogel P30 prepared in 10 mM triethanolamine/HCl containing 20% v/v of 1-propanol and the enzyme tested for the retention of activation. Enzyme activity (−○−) and radioactivity (−■−).

Circular Dichroism—In order to obtain direct evidence for conformation change the circular dichroism (CD) spectra of normal and activated bee venom phospholipase A_2 were compared. The results, Fig. 6, show clearly that there is a significant change in secondary structure on activation. Using the CONTIN procedure (Provencher and Glöckner, 1981) the α-helix and β-sheet contents of normal and activated PLA_2 were 30±1 to 39±2 and 38±1 to 45±2, respectively (*18*).

TABLE I. **Measurement of bee venom PLA_2 activation.** Reaction time courses for the hydrolysis of pure egg phosphatidyl choline were obtained by conductimetric assay in 10 mM triethanolamine/HCl at pH 8.0 containing 20% v/v of 1-propanol at 37°C. The initial and maximum rates of the biphasic progress curves were obtained graphically. The activation factor was determined by dividing the initial rates in each case by the initial rate of the control reaction and product activation was determined from the ratio of the maximum to the initial rate in each case. Results are the means of two independent determinations.

Treatment	Reaction rate (fatty acid released nmol/ml/min)		Activation factor	Product activation
	Initial	Maximum		
Control	19	875	–	46
Oleoyl imidazolide	901	901	47	1.0
Oleic acid+glutaraldehyde	130	529	6.8	4.0
Oleic acid+glutaraldehyde+$NaBH_4$				
a) Before gel filtration	155	845	8.1	5.3
b) After gel filtration	151	654	7.9	4.3

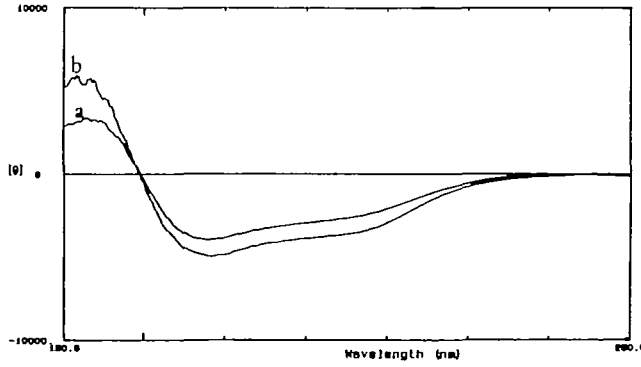

Fig. 6. **Comparison of the CD of native and oleoyl imidazolide activated bee venom PLA_2.** Bee venom PLA_2 (0.2 mg per ml) was activated with molar equivalent of oleoyl imidazolide (solution in acetone) as described. CD spectra of the native (a) and the modified (b) enzymes were made at 25°C using a JASCO J-600 spectropolarimeter. A control spectrum of native PLA_2 in the presence of same concentration of acetone was indistinguishable from that of the enzyme in purely aqueous medium.

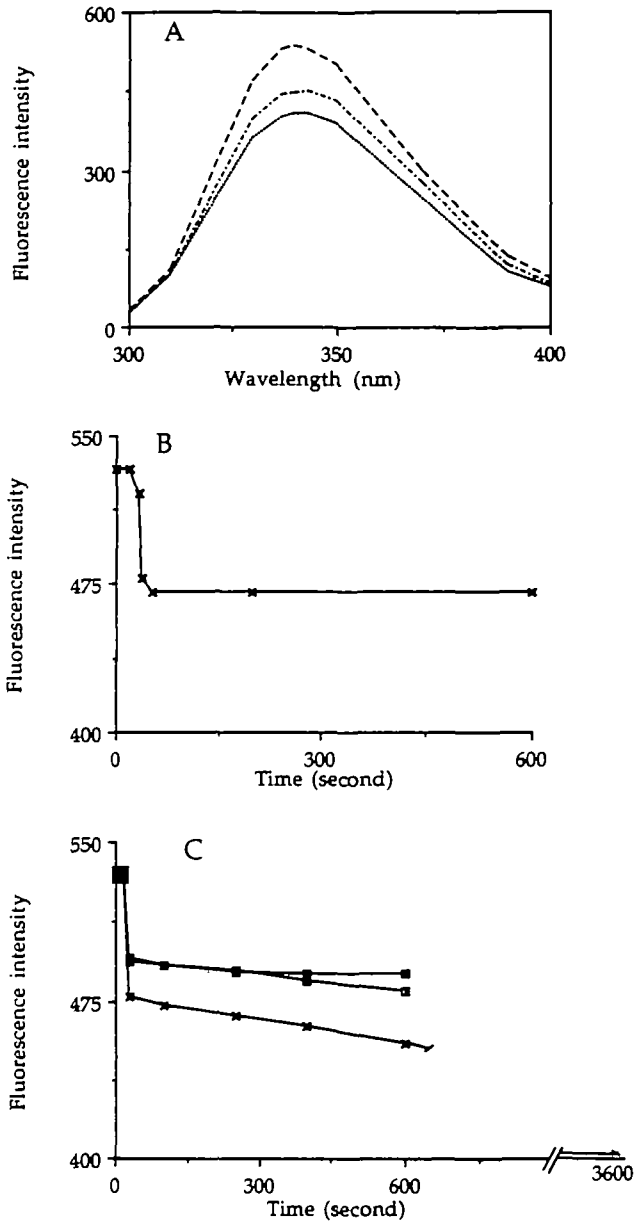

Fig. 7. **Perturbation the tryptophan fluorescence of bee venom PLA_2 by oleic acid and oleoyl imidazolide.** Fluorescence measurements were made at 25°C using a Perkin-Elmer LS 50 spectrofluorimeter. Enzyme solutions were prepared at 0.1 mg per ml (*ca.* 7 μM) and the emission spectrum for excitation at 290 nm obtained. (A) Emission spectra over the range 300-400 nm were recorded for native enzyme (– –), enzyme after 10 min of treatment with 8 μM oleoyl imidazolide (····), and enzyme after 60 min of treatment with 8 μM oleoyl imidazolide (·· ··). (B) Time course of fluorescence emission at 340 nm for enzyme treated as above but with 8 μM of free oleic acid. (C) Time course of fluorescence emission at 340 nm for enzyme treated as above but with 8 μM oleoyl imidazolide at pH 7 (–◆–), 8 (–×–), and 9 (–◇–).

Tryptophan Fluorescence—Bee venom PLA_2 contains two tryptophan residues (*24*) one near the N-terminus, Trp-8 and one near the C-terminus, Trp-128. In addition it has 8 tyrosine residues widely distributed through the sequence. The fluorescence emission spectrum of the native protein ($\lambda_{max} = 341$ nm) showed that the Trp residues were in a moderately hydrophobic environment (*25*). When the enzyme was treated with a molar equivalent of oleoyl imidazolide at pH 8.0 the fluorescence emission decreased sharply and then declined progressively, undergoing a small, but detectable red shift, Fig. 7A. The time course of the slow phase corresponded to a half-life of *ca.* 5 min which is in reasonable agreement with the kinetics of activation (*20*). The simplest interpretation of these results is that the highly hydrophobic reagent binds to the enzyme very rapidly, perturbing the environment of one of the tryptophan residues, and then undergoes a relatively slow reaction in which the oleoyl group is transferred to an acceptor residue; this leads to further perturbation of the tryptophan environment. It was therefore anticipated that free fatty acid might mimic the first part of this process, but not the second and the results (Fig. 7B) are consistent with this interpretation.

A preliminary investigation of the effect of pH on fluorescence emission changes showed that the rate of the slow phase was 2-3 fold lower at pH 7 than at pH 8, and very slightly lower at pH 9 than pH 8, (Fig. 7C) in accord with the known effect of pH on activation kinetics (*26*).

Erythrocyte Binding—Activation of the bee venom PLA_2 with oleoyl imidazolide has a very dramatic effect on the ability of the enzyme to lyse erythrocytes in the presence of albumin (*27*). Because this effect occurs in an aqueous medium it indicates that activation by free fatty acids or acylation might have a physiological role. Although neither the native nor the activated enzyme are lytic in the absence of albumin, the enhanced activity of the activated enzyme in the absence of albumin has been clearly demonstrated. Enhancement of the lytic action of the enzyme could be attributed either to increased binding of the enzyme to the erythrocyte membrane or else to increased lytic properties of the enzyme itself. To investigate the former possibility erythrocytes were incubated with low quantities of normal or activated enzyme and after a brief incubation the cells pelletted and the residual PLA_2 present in the supernatant was assayed against DOPC substrate in the presence of 1 mM $CaCl_2$ to give maximum sensitivity. When this experiment was carried out in the presence of albumin, the recovery of activity from solution was 97.6±2% for native enzyme and 96.1±3% for activated enzyme whilst in the absence of albumin recovery fell to 63±4% for native and 61±3% for the activated enzyme. These results indicate that both forms of the enzyme bind more strongly to albumin than to the erythrocyte membrane, but the affinity for the membrane is not altered significantly by acylation.

DISCUSSION

Before the present study only two assays have been available to study phospholipase A_2 activation by acyl imidazolides, the erythrocyte leakage assay which is done under physiological conditions and the hydrolysis of long-chain substrates in the highly non-physiological 1-propanol-containing medium. These methods have now been complemented by the use of tryptophan fluorescence which, together with CD measurements enables the structural studies only possible in the 1-propanolic medium to be extended to the aqueous medium.

It is quite clear from our results that 1-propanol induces a low activity conformation of the enzyme and that this change can be counteracted by the combination of a long-

chain substrate and a free fatty or bound acyl residue. The fact that the bound acyl residue also activates the enzyme in a purely aqueous medium indicates that the same mechanism is involved. The major problem is to determine whether or not the apparent activation site is a site for free fatty acids or for long-chain acyl groups. Investigation of fatty acid activation in aqueous media tends to give ambiguous results because it is virtually impossible to distinguish between ligand binding to the enzyme or to the substrate, but no ambiguity is present when the enzyme is modified by long-chain fatty acylation. At present neither the nature of the bond between the acyl group and the enzyme, nor the relationship between the fatty acid binding site and the acylation site is known although the two sites appear to be closely related.

Known modifications of proteins by long chain fatty acids include stable modification of the N-terminal glycine amino group by myristate (*28-30*) and the thio-esterification of cysteine by palmitoyl residues (*31, 32*). Whereas the former derivative is extremely stable, the latter is labile both to alkaline conditions and also to cellular deacylating enzymes. Nevertheless both types of derivatives are sufficiently stable to be characterized by SDS-PAGE (*33*). Many features of the present modification indicate that a novel bond type is involved which is extremely labile once the protein has been denatured. Thus the activated enzyme shows no detectable modification after acid urea PAGE (*14*) and although [^{3}H]oleoyl imidazolide introduces a labeled group that is resistant to extraction by organic solvents (*9*) or by albumin, the label is rapidly lost on SDS-PAGE (*26*) or under the mild conditions required for reduction and carboxymethylation of the protein.

The function of long-chain acyl residues in proteins is a matter for debate. They may stabilize protein oligomers (*34*), or facilitate both stable or transient protein-membrane interactions (*28, 35*) where the acyl chain may extrude from the protein and penetrates the lipid layer acting as a hydrophobic anchor. Such a model would appear to be a logical feature for a phospholipase and enable the protein to "scoot" (*36*) on the substrate surface. Nevertheless the results obtained here provide good reason to believe that it does not apply in the present case.

Studies of activation in 1-propanolic medium indicate that the major effects is to stabilize the enzyme against denaturation by the solvent. Thus there appears to be a site on the protein that can bind long-chain fatty acids or fatty acyl residues and reverse the inactivating effect of 1-propanol. However activation is substrate-dependent and not observed for a short-chain compound such as DOPC, but is present with the 1-palmitoyl, 2-nonanoyl PC derivative, hence the presence of both activator and long-chain substrate is required to reverse the denaturing effect of 1-propanol. Proof that a conformation change occurs has been obtained by glutaraldehyde fixation in the presence of oleic acid where the present results show that the major component of activation by this modification persists even when the fatty acid has been removed.

To extrapolate from stabilization against organic solvent to a role for the activating modification in aqueous medium it is necessary to show that the enzyme is capable of undergoing a conformation change under these conditions and that it can be correlated with the activating modification. Although the three dimensional structure of the enzyme is stabilized by five disulfide bridges (*1*), the protein nevertheless undergoes a significant conformational change on activation as shown by the changes in far U.V. CD.

This conclusion was strengthened by the changes in tryptophan fluorescence which indicate that occupation of the fatty acid binding site causes a conformation change. On the basis of the red-shift in the emission maximum, the conformational change increases the exposure of at least one tryptophan residue to the solvent. As anticipated, the effect is instantaneous when induced by the free fatty acid, but when produced by oleoyl imidazolide has an appropriate rate and pH dependence to correlate with activation. Of the two Trp residues present in this protein, Trp-8 is in a relatively internal hydrophobic environment near the active site, whilst Trp-128 is in a less compact region of the protein with a lower density of hydrophobic residues, hence Trp-8 is the more likely source of the observed changes. It is of interest that the acidic PLA_2 isoform from the spitting cobra *Naja mossambica mossambica* has three Trp residues (*vis.* Trp-18, Trp-19, and Trp-60), and on reaction with oleoyl imidazolide it gives significantly larger fluorescence changes than the bee venom enzyme (our unpublished work) suggesting that the two adjacent Trp residues could be involved.

The bulk of the evidence presented here shows that the enzyme is capable of conformation change and that such change does accompany activation associated with occupation of a hydrophobic site on the enzyme. Nevertheless the observations do not entirely preclude the alternative model that the acyl group occupies a surface location and acts as a hydrophobic anchor linking the enzyme to the substrate. Based on the apparent equivalence of free fatty acid activation and acyl group activation, strong linkage of the fatty acid to the enzyme must involve the carboxylate group and a proximal part of the acyl chain with the distal part of the acyl chain penetrating the substrate. We have shown elsewhere (*20, 37*) that a chain length >6 carbon atoms is required for specific reaction at the activating site. It would now be of interest to use the fluorescence technique to study the relationship between structural changes in the enzyme and activation as a function of acyl chain length to see if the requirements for activation and for conformation change differ, as would be expected for the hydrophobic tail model, but not for the conformation change model of activation.

The most cogent argument against the hydrophobic tail model is that direct determination of the binding of the enzyme to erythrocyte membranes is not significantly altered by activation. Thus on balance the most plausible model is that acylation involves a buried acyl residue that forces a conformation change which makes the enzyme more effective against long-chain substrates whilst having no effect against short-chain substrates. This includes the possibility that contact with the substrate surface itself actually forces the enzyme into a low activity conformation and the effect of the activator is to overcome this induced change.

The present study has been confined to the bee venom enzyme for the principal reason that it is the only such enzyme that can be activated by the combination of glutaraldehyde and free fatty acid and therefore provides unique evidence for conformation change. Cobra venom enzymes

that can be activated by acyl imidazolides clearly do not possess the fortuitous spacing of primary amino groups that allows the activated conformation to be stabilized by cross-linking. A study of the snake venom enzymes, which provide a series of natural mutants with varied susceptibility to acyl imidazolides, should allow the nature of the fatty acid site and the acylation site to be determined.

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