# Influence of Chemistry in Immobilization of Cobra Venom Phospholipase $A_2$: Implications as to Mechanism†

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*Received April 14, 1993; Revised Manuscript Received May 18, 1993*

ABSTRACT: Phospholipase $A_2$ from *Naja naja kaouthia* venom was covalently coupled onto agarose beads using two different chemistries. The effect of micellar competitive inhibitors in the coupling media was evaluated. Enzyme bound to *N*-hydroxysuccinimide-activated agarose, which is reactive primarily toward $\epsilon$-amino groups, had 20% activity retention against micellar diheptanoylphosphatidylcholine (DiC7-PC). Enzyme bound through carboxylic groups, using a modification of the carbodiimide method, had 50% retention. Similar relative activities were observed, for both conjugates, in monomeric dihexanoyl-PC and in mixed micelles of Triton X-100 with dipalmitoyl-PC or dioleoylphosphatidylethanolamine. The soluble form of the enzyme showed premicellar activation against monomeric DiC7-PC, while the immobilized form showed interfacial recognition at concentrations around the critical micellar concentration. These results suggest that the enzyme activity lost upon immobilization is a result of the inherent chemical modification of the enzyme and that enzyme oligomerization and interfacial recognition are not cause–effect phenomena.

Phospholipases $A_2$ (PLA2)[1] are lipolytic enzymes that hydrolyze the $S_N2$ ester bond of 1,2-diacylphosphatides. Extensive work has been carried out regarding their structure and mechanism, particularly for the extracellular mammalian pancreatic and snake venom PLA2s. A striking feature of these enzymes is their 10–100-fold enhancement in catalytic turnover when the substrates become organized into lipid–water interfaces (Verheij *et al.*, 1981b). Despite the detailed structural information about these PLA2s, this interfacial recognition behavior is still not clearly understood (Scott *et al.*, 1990).

Previously, Lombardo and Dennis used *Naja naja naja* PLA2 covalently attached to tresyl chloride-activated Sepharose as a probe of catalysis (Lombardo & Dennis, 1985). The activity of the insoluble enzyme against aggregated phospholipids was less than 10% of the soluble form. Probably due to these low activity retentions, PLA2 immobilization has not been carried out in further studies. Our initial motivation for immobilizing PLA2 was for the development of a novel potential therapy for hypercholesterolemia, based on an extracorporeal shunt containing a column of Sepharose–PLA2 conjugate (Labeque *et al.*, 1993). This application required stable linkages and improved activity retentions. In the present study, two different chemistries of immobilization were investigated for the cobra venom enzyme *Naja naja kaouthia* PLA2. The influence of micellar protectants during the immobilization step was also analyzed. Kinetic properties of the different conjugates are compared and rationalized along with known structure–function correlations of cobra PLA2s. From the results, inferences for the mechanism of PLA2 catalysis are made.

## MATERIALS AND METHODS

*Materials.* PLA2 from the venom of *Naja naja kaouthia* was purchased from Fluka (Ronkonkoma, NY). Enzyme purity was routinely checked by sodium dodecylsulfate gel electrophoresis, which showed a single band under silver staining. *N*-Hydroxysuccinimide-activated agarose (Affi-Gel 15) was a product of Biorad (Richmond, CA). DAPA, EDC, and NHS were from Pierce (Rockford, IL). C16-PN was from Calbiochem (San Diego, CA), and all other phospholipids were from Avanti Polar Lipids (Alabaster, AL). Iodine-125 (17.4 Ci/mg in 0.1 N NaOH solution) was a product of NEN Research Products (Boston, MA), and IODOBEADS was from Pierce; counting of radioactive samples was done on a LKB-Wallac ClinicGamma 1272.

*Radioiodination of PLA2.* $Na^{125}I$ (1 mCi) in solution was incubated for 5 min with 4–6 IODOBEADS. To this was added 2–3 mg of enzyme in 0.5–1 mL of 0.1 M phosphate buffer, pH 7.4. After a 15-min reaction, the mixture was gel-filtered (Econo-Pac 10 DG, Biorad), and the protein fractions with highest radioactivity were pooled. For a more complete separation of free iodine, the labeled protein solution was repeatedly ultrafiltered in a Centricon-10 concentrator (Amicon, Beverly, MA). Final enzyme preparations had an average incorporation of radioactivity of 120 $\mu$Ci/mg protein.

*Protein Concentration.* Protein concentrations were determined by MICRO-BCA assay (Pierce). The calibration curve was prepared from a solution of *Naja naja* PLA2 in water, whose concentration was determined by absorption at 280 nm, using $A_{1\%} = 25.9$ (Karlsson & Pongsawasdi, 1980).

† This research was supported by W. R. Grace & Co.-Conn. J. Ferreira also acknowledges Invotan (Portugal) for contribution for his support.

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[1] Abbreviations: PLA2, phospholipase $A_2$; CMC, critical micellar concentration; DAPA, diaminodipropylamine-derivatized agarose; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; NHS, *N*-hydroxysulfosuccinimide; C16-PN, *n*-hexadecylphosphocholine; C18:1-LPC, 1-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphocholine; DiC7-PC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine; DiC6-PC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; DiC16-PC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DiC18:1-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; SM, sphingomyelin.

0006-2960/93/0432-8098$04.00/0 

*Covalent Coupling to Affi-Gel 15 Beads.* Mixtures of unlabeled and $^{125}I$-labeled PLA2 were prepared, generally using less than 20% of the labeled form. The specific decay of the mixture (cpm/$\mu$g PLA2) was evaluated by measuring the radioactivity and amount of protein (MICRO-BCA assay) in small aliquots. The remaining solution was used for activity assays and immobilization studies.

Coupling solution was prepared by diluting the protein solution in 0.1 M MOPS, pH 7.5, containing 100 mM $CaCl_2$ and, when present, 5 mM C16-PN or C18:1-LPC. The Affi-Gel was extensively washed with cold water, suction-dried, and weighed prior to being mixed with the solution. A ratio of 1 mL of solution to 1 g of wet gel was used. Coupling was allowed to proceed overnight at 4 °C with gentle agitation.

*Coupling to DAPA.* Coupling solutions were prepared as in Affi-Gel immobilization, using as coupling buffer 15 mM MOPS, pH 7.0. After addition of wet gel, NHS and EDC were added from freshly prepared solutions in water, to final concentrations of 4 and 25 mM, respectively. The pH of the reaction was monitored over the next 10-min period and adjusted to 6.95–7.0 with 0.1 M NaOH and 0.1 M HCl. Coupling reactions were allowed to proceed overnight at room temperature.

*Washing of the Beads.* After coupling, suspensions were transferred to a sintered glass funnel and washed successively with 2 × 5 gel volumes of 0.1 M sodium carbonate buffer, pH 8.0, containing 1 M NaCl, and then with 0.1 M sodium acetate, pH 5.5, and then with 1 mM borate, 0.1 M NaCl, and 25 mM $CaCl_2$, pH 8.0 (activity buffer). The procedure was repeated twice. Radioactivity in the third cycle of washes was reduced to background, indicating that this procedure is efficient in eliminating uncovalently bound protein from the gel. Finally, the beads were equilibrated and suspended in activity buffer prior to kinetic analysis.

*Kinetic Assays.* All kinetic assays were followed titrimetrically (Waite, 1987). Short-chain lecithins, obtained as lyophilized powders, were quickly weighed and dissolved in activity buffer; assays were conducted in 2-mL reaction volumes at pH 8.0 and at 25 or 31 °C. Mixed micelles of triton X-100 and phospholipids in 1 mM borate and 10 mM $CaCl_2$ buffer were prepared as previously reported (Reynolds & Dennis, 1991), and reactions were monitored as above at pH 8 and 25 °C. Accurate determination of the amount of soluble or agarose-conjugated PLA2 in each assay was possible by measurement of the radioactivity of the reaction mixture. The data for each sample represent the average of at least two assays differing by no more than 10%. Protein from the same preparation was used to compare the activity of immobilized enzyme to that of the soluble form. Furthermore, for any set of immobilization conditions, the results reflect the averages of two or more independent experiments.

## RESULTS

*Protein Binding.* The *N*-hydroxysuccinimide activation of Affi-Gel 15 is reactive primarily toward $\epsilon$-amino groups of lysine residues (Dean *et al.*, 1985). In the absence of any phospholipid, the support bound practically the entire 100 $\mu$g of enzyme/mL of original solution (Table I). When C18:1-LPC or C16-PN was included in the media, the fractions of protein coupled were reduced to 20% or 10%, respectively.

Carbodiimides attack carboxyl groups, forming an *O*-acylurea intermediate that can be subsequently displaced by a nucleophile to form an amide linkage (Hoare & Koshland, 1967). Reaction generally takes place at pH 4.5–5.5. Staros *et al.* developed a modification of this procedure, using *N*-hydroxysulfosuccinimide as a reaction enhancer (Staros *et al.*, 1986). In this method, the pH is raised to neutral or slightly alkaline values. In addition, a low carbodiimide concentration (25 mM) was used, making reaction conditions very mild. This explains the lower percent of protein bound, compared with Affi-Gel, when lipids were absent (Table II). However, in this case, inclusion of micellar substrate analogues inhibited the coupling reaction by a maximum of 3-fold.

Table I: Binding and Normalized Activities (Soluble = 1.0) in Micellar DiC7-PC, for Immobilizations of *Naja naja kaouthia* PLA2 onto Affi-Gel 15[a]

| protectant (5 mM) | fraction bound (average %) | activity retention (4 mM DiC7-PC) |
|---|---|---|
| | 97 | 0.15 ($N$=2) |
| C18:1-LPC | 19 | 0.19 ($N$=2) |
| C16-PN | 11 | 0.23 ($N$=2) |

[a] Initial protein loadings were in the range 90–190 $\mu$g/mL.

Table II: Binding and Normalized Activities (Soluble = 1.0) in Micellar DiC7-PC, for Immobilizations of *Naja naja kaouthia* PLA2 onto DAPA[a]

| protectant (5 mM) | fraction bound (average %) | activity retention (4 mM DiC7-PC) |
|---|---|---|
| | 45 | 0.25 ($N$=2) |
| C18:1-LPC | 35 | 0.38 ± 0.07 ($N$=3)[b] |
| C16-PN | 21 | 0.50 ± 0.07 ($N$=7)[b] |

[a] Initial protein loadings were in the range 145–200 $\mu$g/mL. [b] Confidence intervals based on Student's $t$ distribution, with $P$ = 0.05.

*Activity against Short-Chain Lecithins.* In each experiment, activity of immobilized enzyme against saturating conditions of micellar diheptanoylphosphatidylcholine (DiC7-PC) was compared with the activity of the soluble form of the same preparation. Table I lists normalized activities (soluble = 1.0) for PLA2 immobilized onto Affi-Gel. In agreement with the results for *Naja naja naja* PLA2 (Lombardo & Dennis, 1985), binding of this cobra venom enzyme through amine groups leads to a drastic loss of enzymatic activity. The addition of micellar C18:1-LPC and C16-PN brought negligible improvements in activity retention, albeit the strong affinity of cobra PLA2 for those micelles [e.g., the binding constant to C16-PN micelles is (1–2) × $10^5$ $M^{-1}$ (Teshima *et al.*, 1983)]. With respect to reproducibility, the results of two independent experiments differed by no more than 3%.

When the enzyme was coupled through carboxylic groups, considerably higher activities were observed (Table II). In this case, the micellar protectants had a significant effect on the final activity, which reached 50–60% of the soluble form, against short-chain lecithin micelles. Figure 1A shows the activity–concentration profiles for soluble and immobilized enzyme (C16-PN protected) against DiC6-PC, which has a CMC of 10–13 mM. The immobilized enzyme has a profile similar to that of the soluble form, with lower specific activity overall. Immobilized enzyme exhibited interfacial recognition (Figure 1A). In the micellar region, a pseudo-Michaelis–Menten behavior was followed; graphs of $1/V$ vs $1/C_{mic}$, with $C_{mic} = C_{total} - CMC$ (Figure 1B), permitted calculation of $V_{max}$ values of 4063 and 2414 units/mg PLA2 for soluble and immobilized forms, respectively. The apparent $K_m$, in micellar concentrations, were 0.69 and 0.51 mM, respectively.

At monomeric concentrations of DiC6-PC, conjugated enzyme also had lower activities than the soluble form. Kinetic constants could not be calculated, as this enzyme does not follow regular behavior against monomeric lecithins (see also

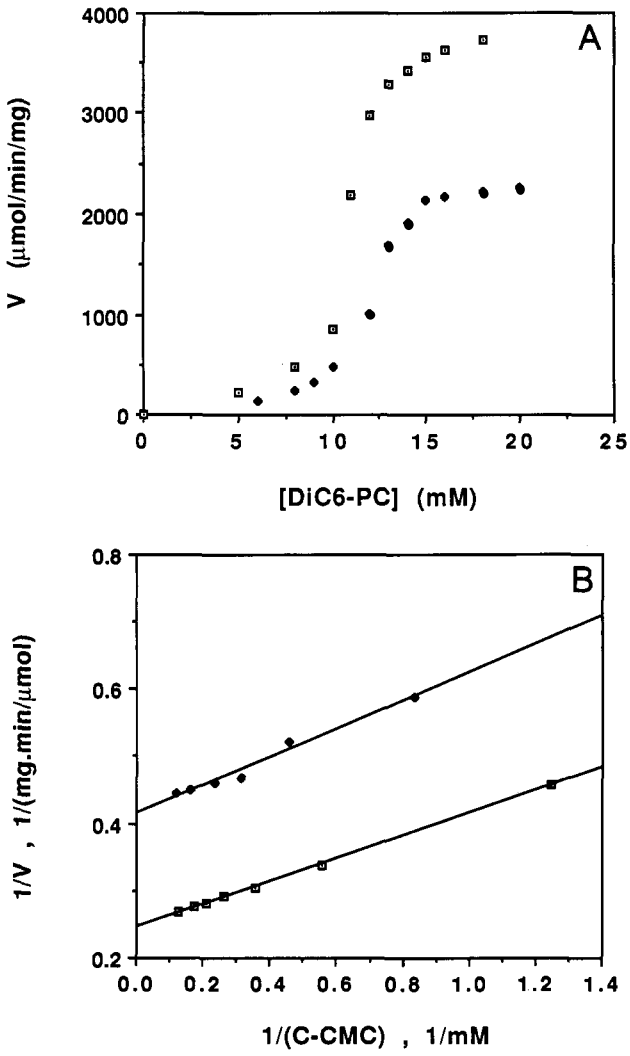


FIGURE 1: (A) Activity–concentration profiles for soluble (□) and DAPA-immobilized (◆) *Naja naja kaouthia* PLA2 against DiC6-PC. Assays were carried out at pH 8.0, 31 °C, using 0.07 μg of enzyme per assay. (B) Lineweaver–Burk plot for the micellar region.

Table III: Normalized Activities (Soluble = 1.0) of *Naja naja kaouthia* PLA2 Immobilized through Amine and Carboxylic Groups (C16-PN Protected) in Monomeric DiC6-PC (at 31 °C) and Mixed Micelles of Triton X-100 (at 25 °C)[a]

| | | Triton X-100 mixed micelles (20 mM) | | |
|---|---|---|---|---|
| chemistry | DiC6-PC (5 mM) | DiC16-PC (5 mM) | DiC18:1-PE (5 mM) | DiC18:1-PE/SM (5/1 mM) |
| (soluble) | (136) | (505) | (69) | (1095) |
| $NH_2$ | 0.20 | 0.13 | 0.13 | 0.12 |
| COOH | 0.49 | 0.52 | 0.41 | 0.24 |

[a] Values in parentheses are specific activities (μmol/min/mg) for soluble enzyme.

below). However, relative activities at 5 mM concentration were close to 50% (Table III), a value similar to retention against micellar lecithins.

Regarding the profile against DiC7-PC (Figure 2), the soluble enzyme showed an uncommon, but known, pattern: enzyme activity increased sharply in the monomeric region, followed by a leveling off in the micellar region ($C > 1.5$ mM). Similar behavior was reported (van Eijk *et al.*, 1983b) for the snake enzyme *Naja melaneuca* PLA2 (fraction DE III). Curiously enough, the immobilized enzyme did not exhibit premicellar activation and showed interfacial recognition at concentrations about the CMC.

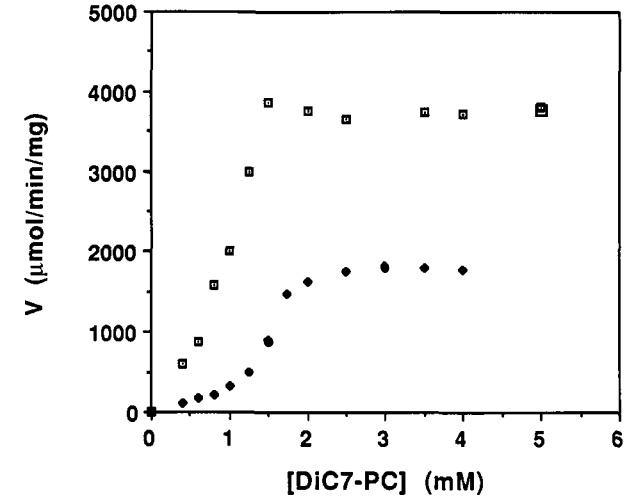


FIGURE 2: Activity–concentration profiles for soluble (□) and DAPA-immobilized (◆) *Naja naja kaouthia* PLA2 against DiC7-PC. Assays were done at pH 8.0, 25 °C, using 0.07 μg of enzyme per assay.

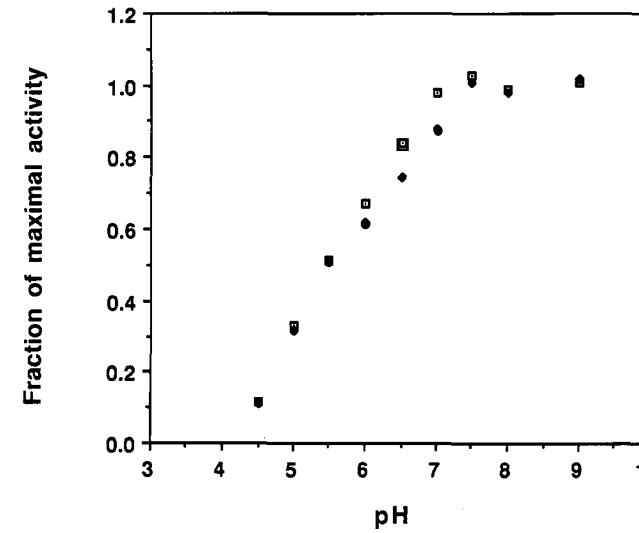


FIGURE 3: Activity–pH profiles for soluble (□) and DAPA-immobilized (◆) *Naja naja kaouthia* PLA2. Assays were carried out in 1 mM MES (pH 4.5–6.0), 1 mM HEPES (pH 6.5–7.5), or 1 mM borate (pH 8.0–9.5) with 0.1 M NaCl, 25 mM $CaCl_2$, and 4 mM DiC7-PC, 25 °C.

The activity–pH profiles of soluble and carbodiimide-bound enzyme against micellar DiC7-PC are practically superimposable (Figure 3). This suggests that the pH of the microenvironment of this support is not shifted significantly from the value in bulk solution. Assays were performed at pH 8.0, well within the maximum activity plateau.

Another important consideration when dealing with immobilized enzymes is the possibility of diffusional limitations. Lombardo and Dennis showed, using a similar enzyme and support, that this did not occur with short-chain lecithin micelles as substrate (Lombardo & Dennis, 1985). We corroborated this conclusion by using decreasing protein loadings and observed no further improvement in specific activity (data not shown).

*Activity against Mixed Micelles of Triton X-100 and Phospholipids.* Mixed micelles of nonionic surfactants with long-chain phospholipids have been frequently used as model substrates for cobra venom PLA2s (Roberts *et al.*, 1979). Activity retentions of immobilized *Naja naja kaouthia* PLA2 against Triton X-100/DiC16-PC mixed micelles (Table III) were similar to retentions observed in pure lecithin micelles.

Phosphatidylethanolamines are poor substrates for cobra PLA2, but the addition of a choline-containing compound strongly activates their hydrolysis (Lombardo & Dennis, 1985; Roberts *et al.*, 1979). In both immobilization chemistries, the relative activities against Triton X-100/DiC18:1-PE mixed micelles (Table III) are analogous to those of DiC7-PC or triton X-100/DiC16-PC micelles. Upon addition of 1 mM sphingomyelin, the DAPA–PLA2 conjugate was not activated as much as the soluble enzyme.

## DISCUSSION

Cobra venom PLA2 was covalently immobilized onto cross-linked agarose, using two different chemistries; the effect of micellar competitive inhibitors was evaluated. Precise determination of the amount of protein coupled to the beads and the amount of immobilized or soluble catalyst present in every activity assay was possible by using a radioactive tracer. The method used for labeling is mild, and $^{125}$I-PLA2 solution had an activity against micellar DiC7-PC that was 70–80% of the activity of native enzyme.

*Naja naja* PLA2 immobilized through amine groups showed a severe loss of enzyme activity. Addition of micellar protectants strongly inhibited the coupling reaction and had little effect on activity retention. This suggests that after interfacial binding there might be few, if any, exposed amine groups. In addition, bound enzyme lost 80% activity, indicating that important Lys groups were affected. Previous chemical modification studies have revealed the importance of certain amino groups in snake and pancreatic PLA2s. van Eijk *et al.* found that 4-chloro-3,5-dinitrobenzoate attacks almost specifically the Lys-6 of *Naja melanoleuca* PLA2 (van Eijk *et al.*, 1983a), which is a conserved residue in all cobra venom enzymes. The modified form had practically no activity against aggregated substrates. Curiously enough, the deactivation rate was retarded 40-fold in the presence of *n*-tetradecylphosphocholine. The crystal structure of *Naja naja atra* PLA2 confirmed that Lys-6 is part of the interfacial binding surface (White *et al.*, 1990). Other studies (Ghomashchi *et al.*, 1991; Reynolds *et al.*, 1991) have shown that manoalogue also modifies Lys in cobra venom enzyme, with loss of 70–80% of enzyme activity. Moreover, modification by glyoxylic acid of the $\alpha$-amino group in the *Naja melanoleuca* enzyme led to similar observations (Verheij *et al.*, 1981a). These modifications did not affect the affinity for interfaces but lowered the catalytic efficiency. This might explain the loss in activity of amine-immobilized PLA2, toward both micellar and monomeric substrates. We note, however, that in some previous studies (Ghomashchi *et al.*, 1991; Lombardo & Dennis, 1985) deactivation against monomeric substrates was not observed.

Immobilization of PLA2 through carboxylic groups, as well, could lead to extensive enzyme inactivation. PLA2s have two conserved Asp residues involved in catalysis: Asp 49 interacts with the cofactor $Ca^{2+}$ and Asp 99 is a residue of the catalytic domain (Fleer *et al.*, 1981; Scott *et al.*, 1990). Few studies were done on chemical modification of carboxylic groups in PLA2s, except for the ones that determined the role of those two residues. Fleer *et al.* (1981) have shown that, after extensive reaction of bovine pancreatic PLA2 with EDC at pH 5.5, all but Asp 39 and Asp 99 carboxylic groups were modified; there was no residual activity. In the presence of $Ca^{2+}$, Asp 49 remained unmodified and the enzyme had 15% residual activity. In order to avoid such inactivation, immobilizations were carried out with considerably milder conditions: low carbodiimide concentrations, pH of reaction raised to 7.0, calcium and micellar substrate analogues always present in saturating concentrations. Significantly higher activity retentions resulted, attaining 50–60% in micellar short chain lecithins.

Several observations suggest that the activity lost upon immobilization was due not to the immobilization per se but rather to reaction of EDC with important carboxylic groups: activity retentions against other substrates, either monomeric DiC6-PC or micellar PE, are similar to the values observed in micellar short-chain lecithins. In most cases, activity of unbound protein, remaining in solution after reaction, was similar, or even lower, than activity of agarose-bound enzyme (the only exceptions were the C16-PN protected immobilizations that we attribute to a lower extent of modification).

The fact that phosphatidylethanolamines alone are poor substrates for cobra venom PLA2 but become excellent ones in the presence of choline-containing compounds has been explained by the existance of an activator site, distinct from the catalytic site (Adamich *et al.*, 1979). Ortiz *et al.* constructed a molecular model of *Naja naja kaouthia* PLA2 that also suggested the presence of a specific recognition site for choline head groups, comprised of residues Glu 55, Trp 61, Tyr 63, Phe 64, and Lys 65 (Ortiz *et al.*, 1992). Involvement of either Glu 55 or Lys 65 in immobilizations is a possible factor of deactivation. In particular, it might explain the reduced activation of DAPA–PLA2 upon addition of sphingomyelin to TX-10/DiC18:1-PE micelles (Table III).

An important but controversial point in PLA2 catalysis, particularly for cobra venom PLA2s, is the role of enzyme aggregation—dimerization or higher order aggregation—with respect to enzyme activation. These venom enzymes undergo a concentration-dependent aggregation in solution (Hazlett & Dennis, 1988); also, ultracentrifugation, gel filtration, and cross-linking experiments (Hazlett & Dennis, 1985; Hazlett & Dennis, 1988; Roberts *et al.*, 1977) have indicated that substrate analogues induce aggregation. These observations have led to the hypothesis that interfacial activation is related to enzyme dimerization (Roberts *et al.*, 1977). The fact that the immobilized *Naja naja naja* enzyme had minimal interfacial recognition (Lombardo & Dennis, 1985) was in support of this theory. On the other hand, kinetic studies on negatively charged vesicles (Jain *et al.*, 1991) suggest that all PLA2s are catalytically active at the interface as monomers. The present work supports the observation that immobilization of cobra venom enzyme through amine groups severely affects enzyme activity, but this is probably due to the chemical modification inherent to the coupling process. In an alternative chemistry and under analogous conditions, immobilized enzyme did show interfacial recognition (Figure 2). This would mean that enzyme aggregation is not significant for catalysis in cobra venom PLA2.

Another interesting observation is the fact that the soluble enzyme showed premicellar activation in monomeric DiC7-PC, while agarose-coupled enzyme did not. A similar activation in *Naja melaneuca* has been explained by the formation of premicellar aggregates, containing 30–90 lipid and 2–4 enzyme molecules (van Eijk *et al.*, 1983b). The fact that premicellar activation did not occur with the insoluble enzyme may be rationalized by the impossibility of formation of these lipid–enzyme aggregates. However, the same enzyme still showed interfacial activation at concentrations around CMC, suggesting, once again, that enzyme aggregation and interfacial recognition may not be related.

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