A study on the functional subunits of phospholipases A₂ by enzyme immobilization

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Pancreatic and venom phospholipases A₂ have complex and distinct oligomerization behaviour. Pancreatic enzymes are monomeric in solution, but their quaternary structure at interfaces is unknown. On the other hand, certain crotalid venom phospholipases A₂ are dimeric in solution, and different reports have proposed either the monomer or the dimer as the catalytically functional subunit. In this study, enzyme immobilization was used as a tool for determining the functional subunits of these enzymes. The dimeric *Crotalus atrox* phospholipase A₂ was covalently attached to agarose beads, via either the amine or the carboxylic groups of the protein. In the first case immobilization led to an 80% loss of activity as compared with the soluble form, and measured by using micellar

diheptanoylphosphocholine. Inclusion of micellar protectants in the coupling media did not improve the activity. Enzyme immobilized via carboxylic groups was 2–3-fold more active than the amine-coupled form. In a second approach, *Crotalus atrox* enzyme was immobilized with single-subunit attachment. The removal, with denaturing washes, of the non-covalently bound units involved in monomer–monomer interactions, caused a large decrease in specific activity of the support-bound enzyme. This suggests the dimeric form as the fully active one. Similar procedures were also carried out with pig pancreatic and *Naja naja* phospholipases A₂. The results indicated that these enzymes are active as monomers.

INTRODUCTION

The most well-characterized phospholipases A₂ (PLA₂s) include the extracellular mammalian pancreatic and cobra and snake venom enzymes. Although these enzymes share high degrees of similarity in their primary, secondary and tertiary structures (White et al., 1990), they vary in their tendency to oligomerize. Pancreatic PLA₂s are monomeric in solution and seem to act as such on monomolecularly dispersed substrates (Hazlett et al., 1990); however, with micellar n-alkylphosphocholines, two enzyme molecules per complex were observed (Donnée-Op den Kelder et al., 1981; Hille et al., 1981; Soares de Araujo et al., 1979), raising the possibility of the dimer being the active form at interfaces. Cobra venom enzymes aggregate in water at moderate concentrations, and in the presence of monomeric substrates (Hazlett and Dennis, 1988). In a third type of behaviour, some PLA₂s from crotalid venoms, e.g. from the rattlesnakes *Crotalus adamanteus* and *C. atrox*, form very strong homodimers, even in dilute solutions (Hachimori et al., 1971; Keith et al., 1981). These PLA₂s have the highest turnover numbers at many phospholipid-containing interfaces; as a result, the possible role of dimerization on PLA₂ catalysis has been questioned for both the cobra venom (Roberts et al., 1977) and pancreatic enzymes (Cho et al., 1988; Tomasselli et al., 1989). On the other hand, crystal structures (Brunie et al., 1985; Renetseder et al., 1985), and kinetic data for the *C. atrox* PLA₂ (Jain et al., 1991), suggest that the dimers may dissociate upon contact with interfaces.

Enzyme immobilization can elucidate the role of oligomeriz-

ation in catalysis: if an oligomeric enzyme is immobilized through one of its subunits, the activity of the enzyme-bound oligomer and the enzyme-bound subunit can be compared, after dissociating the former with a denaturing agent (Chan, 1976). We report here the covalent immobilization of *C. atrox* PLA₂ on Sepharose beads, through protein amine or protein carboxylic groups; the kinetic properties of these conjugates are compared. The denaturation technique is then used to prepare bound monomers of the enzyme. To investigate differences among PLA₂s, similar experiments were carried out with the monomeric pig pancreatic and *Naja naja* venom enzymes.

Apart from these mechanistic studies, immobilization of PLA₂s may have several important applications, such as in the production of fatty acids or lysophospholipids, the development of radioimmunoassays, or in a novel extracorporeal treatment of hypercholesterolaemia (Labeque et al., 1993).

EXPERIMENTAL

PLA₂s

Freeze-dried venom of the rattlesnake *C. atrox* was purchased from Miami Serpentarium (Salt Lake City, UT, U.S.A.), and PLA₂ was purified from it by the procedure of Hachimori et al. (1971). *Naja naja kaouthia* PLA₂ was a product of Fluka (Ronkonkoma, NY, U.S.A.). Pig pancreatic PLA₂ was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.) as a suspension in 3.2 M (NH₄)₂ SO₄. Enzyme purities were verified by SDS/PAGE, followed by silver staining.

Abbreviations used: PLA₂, phospholipase A₂; DAPA, diaminodipropylamine-derivatized agarose; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; NHS, N-hydroxysulphosuccinimide; C₁₆-PN, n-hexadecylphosphocholine; C_{18:1}-LPC, 1-(*cis*-9-octadecenoyl)-sn-glycero-3-phosphocholine; DiC₇-PC, 1,2-diheptanoyl-sn-glycero-3-phosphocholine; GdHCl, guanidinium hydrochloride.

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Other materials

N-Hydroxysuccinimide-activated agarose (Affi-Gel 15) was from Bio-Rad (Richmond, CA, U.S.A.). DAPA, EDC and NHS were from Pierce (Rockford, IL, U.S.A.). C_{16} -PN was from Calbiochem (San Diego, CA, U.S.A.) and all other phospholipids were from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Materials and methods for radiolabelling PLA_2 s have been reported previously (Ferreira et al., 1993). Other reagents were of the highest purity available.

Immobilization of *C. atrox* PLA_2

^{125}I -labelled enzyme was immobilized on to Affi-Gel 15 and DAPA by using the procedures described for cobra venom PLA_2 (Ferreira et al., 1993). Initial protein concentrations were in the range 40–90 μ g/ml. EDC and NHS concentrations in DAPA couplings were 25 mM and 4 mM respectively.

Preparation of matrix-bound monomers of *C. atrox* PLA_2

When the final objective was to prepare DAPA-bound monomers of *C. atrox* PLA_2 , the protein concentration in the coupling solution was increased to 0.6–1.6 mg/ml, and that of EDC was decreased to 5 mM. After coupling, beads were extensively washed for removal of non-specifically bound enzyme (Ferreira et al., 1993). Samples of these DAPA- PLA_2 conjugates (0.5–1.0 g of wet gel) were then transferred to the upper chamber of a 5 μ m-pore membrane spin filter (USA/Scientific Plastics, Ocala, FL, U.S.A.), and suspended in 0.1 M Mops/7 M guanidinium hydrochloride (GdHCl), pH 7.5. The suspension was orbital-shaken for 1 h, then centrifuged (300 g/min), and the radioactivity of the denaturing buffer was directly measured on a γ -radiation counter (LKB-Wallac ClinicaGamma 1272). This procedure was repeated twice, the intention being to release non-covalently attached enzyme involved in protein-protein interactions, leaving only covalently bound monomers. Then the beads were washed several times with 0.1 M Mops buffer, and suspended in 1 mM borate/0.1 M NaCl/25 mM $CaCl_2$, pH 8.0. Activities of GdHCl-treated and untreated conjugates against micellar DiC_7 -PC were evaluated.

Analogous coupling and denaturing procedures were carried out with the ^{125}I -labelled pig pancreatic and *Naja naja kaouthia* PLA_2 s.

Activity assays

PLA_2 activities were measured titrimetrically, in 2 ml volumes of 1 mM borate/0.1 M NaCl/25 mM $CaCl_2$, containing 5 mM (for cobra venom PLA_2 s) or 8 mM (pig PLA_2) DiC_7 -PC, at 25 °C.

RESULTS

Binding and activity retention of immobilized *C. atrox* PLA_2

Table 1 contains information on protein binding and normalized activity (soluble = 1.0) for enzyme immobilized on *N*-hydroxysuccinimide-activated agarose, which is reactive toward ligand amine groups. Bound enzyme lost more than 80 % activity against micellar short-chain phosphatidylcholine. Inclusion of micellar substrate analogues, C_{16} -PN or $C_{18:1}$ -LPC, did not improve activity retention.

In an alternative chemistry, protein was linked through its carboxylic groups to an amine-derivatized support (DAPA), by using a modification of the carbodi-imide method (Ferreira et al., 1993; Staros et al., 1986). Couplings were carried out at neutral

pH, low carbodi-imide concentration (25 mM), and with NHS as an enhancer. Immobilizations carried out in the absence of any lipid or in the presence of 5 mM C_{16} -PN yielded bound enzymes with 40–50 % of the specific activity of the soluble form (Table 2). As shown by the activity-concentration profile in DiC_7 -PC (Figure 1), immobilized enzyme exhibited interfacial recognition at concentrations around the critical micellar concentration (1.5 mM). Curiously enough, enzyme immobilized in the presence of LPC lost more than 90 % of activity (Table 2).

Table 1 Protein binding and activity retentions of *C. atrox* PLA_2 immobilized on Affi-Gel 15

Coupling media contained 40–90 μ g/ml enzyme, with or without micellar phospholipid as a protectant. Activities were measured in micellar DiC_7 -PC, as described in the Experimental section, and values are normalized for the soluble enzyme activity (soluble = 1.0).

Protectant (5 mM)	Fraction bound (average %)	Activity retention (4 mM DiC_7 -PC)
—	73	0.18 ($n = 2$)
$C_{18:1}$ -LPC	58	0.06 ($n = 2$)
C_{16} -PN	41	0.17 ($n = 1$)

Table 2 Protein binding and activity retentions of *C. atrox* PLA_2 immobilized on DAPA

Coupling media contained 40–55 μ g/ml enzyme, with or without micellar phospholipid as a protectant. Activities were measured in micellar DiC_7 -PC, as described in the Experimental section, and values are normalized for the soluble enzyme activity (soluble = 1.0).

Protectant (5 mM)	Fraction bound (average %)	Activity retention (4 mM DiC_7 -PC)
—	63	0.45 ($n = 2$)
$C_{18:1}$ -LPC	71	0.09 $\pm$ 0.07 ($n = 3$)*
C_{16} -PN	60	0.39 $\pm$ 0.05 ($n = 5$)*

* Confidence intervals based on Student's *t*-distribution, with $P = 0.05$.

Figure 1 Activity against short-chain phosphatidylcholine

Activity-concentration profiles for soluble (●) and DAPA-immobilized (○) *C. atrox* PLA_2 in DiC_7 -PC. Assays were carried out as described in the Experimental section, at several DiC_7 -PC concentrations.

Table 3 Preparation of different DAPA-PLA₂ conjugates

Coupling conditions and final percentage of protein bound in the preparation of DAPA-PLA₂ conjugates used in GdHCl treatments (Table 4).

Expt.	PLA ₂	[EDC] (mM)	[NHS] (mM)	Protectant (5 mM)	Protein loading (μg/g of gel)	Bound (%)
A	Pig	5	—	—	1600	32
B	<i>Naja naja</i>	5	4.0	—	1420	8.5
C	<i>Naja naja</i>	5	4.0	C ₁₆ -PN	2050	3.2
D	<i>Naja naja</i>	25	4.0	—	183	30
E	<i>C. atrox</i>	5	—	—	600	18
F	<i>C. atrox</i>	5	4.0	—	1590	24
G	<i>C. atrox</i>	25	4.0	C ₁₆ -PN	42	61

Activity of supernatant enzyme

In order to determine the possible reasons for activity loss on immobilization, the activity of uncoupled enzyme, remaining in solution after the immobilization reaction, was analysed. Supernatant enzyme of C_{18:1}-LPC- and C₁₆-PN-protected immobilizations reported in Table 2 had an average activity of 9 % (*n* = 2) and 40 % (*n* = 3) of native enzyme value respectively.

Preparation of DAPA-PLA₂ conjugates for GdHCl treatments

Table 3 presents the coupling conditions for DAPA-PLA₂ conjugates used in determining the functional role of subunits. In most cases, 5 mM EDC concentrations and 1.4–2.0 mg/ml protein loadings were used. The binding efficiency was highest with pig pancreatic PLA₂, followed by *C. atrox* and *Naja naja kaouthia* PLA₂ respectively.

Leaching and deactivation of DAPA-PLA₂ conjugates with GdHCl treatments

The above conjugates were incubated three consecutive times with the denaturing solution, in order to remove the enzyme units involved in protein–protein interactions that were not covalently bound to the support. The cumulative releases of radioactivity were recorded (Table 4). Specific activities of immobilized enzyme before and after denaturation against micellar DiC₇-PC are also reported. The support containing pig pancreatic PLA₂ (Expt. A) had minimum release of radioactivity;

immobilized enzyme did not lose activity with the 3 h incubation in 7 M GdHCl. We note that some of the radioactivity released might result from free ¹²⁵I strongly adsorbed to the protein before denaturation; that is, the true amount of protein leached is perhaps lower than the radioactivity counts indicate. Slightly higher percentages of the initial radioactivity were released by *Naja naja* PLA₂ conjugates (Expts. B and C), but immobilized enzyme did not change its specific activity.

Different results were obtained with *C. atrox* PLA₂. Approximately one-quarter of the radioactivity was released in the GdHCl solutions (Expts. E and F). When small samples of these washes were assayed in micellar DiC₇-PC, activity was observed after a lag time of approx. 2 min, indicating that protein had been released. No activity was detected in a similar test in *Naja naja* PLA₂ conjugates. Moreover, the specific activities of immobilized *C. atrox* PLA₂ after treatments were less than half of the initial values.

As controls, these leaching experiments were performed in conjugates obtained at higher concentrations of EDC and lower protein loadings (Expts. D and G). Under these conditions, the leaching and deactivation of *C. atrox* conjugates were considerably decreased whereas no significant change from lower EDC concentration was observed for the *Naja naja* enzyme.

DISCUSSION

There is no clear understanding of the quaternary and active structures of extracellular PLA₂ at interfaces. In particular, for the dimeric crotalid PLA₂, Wells (1971) carried out kinetic studies with increasing urea concentrations, and observed that loss of activity and dimer breakdown occurred at similar urea concentrations. The limitation of this approach is that urea can disrupt tertiary structure as well. On the basis of kinetics on phosphatidylcholine monolayers, Shen et al. (1975) also proposed the dimer as the active form. On the other hand, X-ray crystallography of *C. atrox* PLA₂ shows that the catalytic centres of both subunits reside well within the core of the dimer, making access of substrate difficult to understand without some kind of structural re-arrangement (Brunie et al., 1985). Finally, Jain et al. (1991) argued that the enzyme acts as a monomer, on the basis of kinetic studies in vesicles of negatively charged phospholipids.

In continuation of our previous work with cobra venom PLA₂ (Ferreira et al., 1993), we used enzyme immobilization as a probe to determine the catalytically active subunits of different PLA₂s. First, we selected conditions that favoured covalent attachment of both subunits, and then compared binding through protein amine and carboxylic groups. Analogous to the results obtained

Table 4 Protein leaching and deactivation of DAPA-PLA₂ conjugates with GdHCl treatments

The DAPA-PLA₂ conjugates reported in Table 3 were subjected to GdHCl solution washes and then renatured. The fraction of protein leached in the washes was measured by radioactive counting. Activities before and after treatment were measured in micellar DiC₇-PC, as described in the Experimental section.

Expt.	PLA ₂	Protein in beads (μg/g of gel)	Radioactivity leached (%)	Activity (units/mg)		
				Initial	Final	Change (%)
A	Pig	505	2.3	54	53	0
B	<i>Naja naja</i>	121	8.1	635	741	+17%
C	<i>Naja naja</i>	66	6.6	582	608	+4%
D	<i>Naja naja</i>	54	2.4	659	640	–3%
E	<i>C. atrox</i>	106	26	1680	783	–53%
F	<i>C. atrox</i>	382	20	1100	513	–53%
G	<i>C. atrox</i>	26	2.6	2632	2293	–14%

with *Naja naja* enzyme (Ferreira et al., 1993), *C. atrox* PLA₂ immobilized through amine groups had 10–20% activity against aggregated phosphatidylcholine. There are six lysine residues in the *C. atrox* PLA₂ primary sequence, and some of them have been implicated in dimer-stabilization interactions (Brunie et al., 1985). Furthermore, the α -amino terminal has been shown to be essential in maintaining dimeric structure and activity (Randolph and Henrikson, 1982). Therefore, it is likely that covalent binding to its support involves amine groups that are important for catalysis or maintaining protein structure.

Carbodi-imide binding yielded considerably higher activity retentions (Table 2), although some of the 18 aspartate and glutamate residues are catalytic-centre residues, or are also involved in inter-subunit interactions. Expt. G in Tables 3 and 4 shows that enzyme immobilized under those conditions has both subunits covalently bound to the support. Immobilized dimers showed interfacial recognition (Figure 1), indicating that the same catalytic subunit holds for monomolecularly dispersed and aggregated substrates. Furthermore, C₁₆-PN micelles had practically no effect on the activity of immobilized enzyme. This suggests that the structure of micelle-bound enzyme (in particular, aggregation) must be similar to that of enzyme free in solution.

Uncoupled enzyme had the same extent of inactivation as support-bound enzyme. Thus, activity loss was due to modification by EDC of catalytically important carboxylic groups, and not to the immobilization process itself. In this regard, the presence of micellar C_{18:1}-LPC led to almost complete inactivation. Chemical modification of *C. atrox* PLA₂ in solution, under the same reaction conditions as for DAPA immobilization, but with the solid support replaced by an amine nucleophile (0.5 M taurine), led also to the same observation. Since *C. atrox* PLA₂ binds strongly to LPC micelles (dissociation constant $< 10^{-7}$ M; Kupferberg et al., 1981), efficient protection would be expected. One possible explanation might be EDC partitioning to LPC micelles, which permits facilitated contact with an important carboxylic group on the binding site, such as that of Glu-6. This is a non-conserved residue in other PLA₂s; it is included in the protein N-terminus and forms a strong interaction with His-34 and Trp-31 of the other dyad-subunit (Brunie et al., 1985).

In a different approach, we tried to obtain immobilized subunits of *C. atrox* PLA₂. The carbodi-imide coupling seemed more appropriate, as it permitted higher activities. In order to enhance single-subunit attachment, high protein loadings and low EDC concentrations were used in the coupling step (Expts. E and F, Table 3). The amounts of enzyme leached with GdHCl solution were around 25%, that is, only half of the theoretical value if all the enzyme had single-subunit attachment. Although some dissociation during previous washes cannot be excluded, it appears that single-subunit attachment with this support, enzyme and chemistry is difficult to achieve. Nevertheless, release of 20–26% of the protein affected the specific activity of the remaining enzyme by more than 50%. This strongly suggests a role of the dimer unit in *C. atrox* PLA₂ catalysis. Expt. G corroborates this conclusion, as the lower enzyme and higher EDC concentrations favour covalent binding of both subunits, thus minimizing dissociation and deactivation upon GdHCl treatment. The lower specific activities in Expts. E and F, compared with Expt. G or experiments in Table 2, are a result of the higher protein loadings; hindrance effects and diffusional

limitations of the micellar DiC₇-PC associated with the high enzyme turnover number are likely explanations for the decreased specific activities of immobilized *C. atrox* PLA₂, observed at initial protein loadings above 60 μ g/ml. However, this fact does not alter the above conclusions about oligomerization state and functional subunits.

The experiments with the monomeric pig pancreatic (Expt. A) and cobra venom (Expts. B, C and D) PLA₂ served as controls, while testing differences in mechanism among PLA₂s. Release of these enzymes upon treatment with GdHCl was minimal (if any), and there were no variations in their specific activities after renaturation. In one experiment with cobra venom enzyme, a 17% activity increase was observed, which could result from attenuation of hindrance effects after removal of strongly adsorbed enzyme. These results corroborate our previous studies (Ferreira et al., 1993) on kinetics of immobilized *Naja naja* PLA₂, which indicated the monomer as its likely functional subunit. The analogous conclusion holds for the pancreatic PLA₂. The significance of dimerization in certain crotalid enzymes remains to be understood.

This study is an example of using enzyme-immobilization methods as a potential tool for analysing PLA₂'s structure–function relationships.

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