Highly efficient immobilization of phospholipase A_2 and its biomedical applications

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Summary A new method for the immobilization of phospholipase A_2 (PLA_2) has been developed to enhance the activity retention of immobilized PLA_2. When PLA_2 from the venom of *Agkistrodon piscivorus piscivorus* was pretreated with 4-nitro-3-octanoyloxybenzoic acid to acylate ϵ-amino groups of two lysines (Lys-7 and Lys-10) and the resulting acylated enzyme was covalently coupled onto carbonyldiimidazole-activated cross-linked agarose beads, the immobilized acylated enzyme showed high retention of activity toward various aggregated phospholipids. Toward densely packed phospholipid bilayers, such as large unilamellar vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, the immobilized acylated *A. p. piscivorus* PLA_2 was 25-fold more active than the soluble *A. p. piscivorus* PLA_2. The general applicability of our immobilization protocol was demonstrated by the high retention of activity achieved for the immobilized acylated PLA_2 from the venom of *Naja naja naja*. In particular, full activity retention of the immobilized acylated *A. p. piscivorus* PLA_2 toward phospholipids on the surface of human low density lipoproteins suggests its potential usefulness in a newly developed PLA_2-based therapy for hypercholesterolemia.—**Shen, Z., and W. Cho.** Highly efficient immobilization of phospholipase A_2 and its biomedical applications. *J. Lipid Res.* 1995. **36:** 1147–1151.

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Phospholipase A_2 (PLA_2; E.C.3.1.1.4) catalyzes the hydrolysis of the fatty acid ester in the 2-position of 3-*sn*-phospholipids (for a most recent review see ref. 1). PLA_2 can catalyze the hydrolysis of a wide range of phospholipid aggregates, including micelles, monolayers, and bilayers. In particular, PLA_2 was shown to readily hydrolyze phospholipids present in human serum low density lipoproteins (LDL) (2). Subsequently, it was shown that LDL modified by PLA_2 was removed from the circulation to the liver more rapidly than unmodified LDL (3). Based on this finding, a novel therapy for hypercholesterolemia has been recently developed that utilizes immobilized PLA_2 contained in an extracorporeal shunt. Unfortunately, however, the immobilization of PLA_2 generally results in a significant loss of enzymatic activity, limiting biomedical applications of immobilized PLA_2. To date, the immobilization has been carried out primarily with cobra venom enzymes using two different coupling methods; the coupling through ϵ-amino groups of lysines and through carboxyl groups of asparatates and glutamates (4, 5). When the ϵ-amino groups are coupled to a solid support, the coupling yield is high but the activity of immobilized enzyme toward aggregated phospholipid substrates decreases drastically, which has been ascribed to the modification of lysyl residues that play critical roles in the interaction of PLA_2s with anionic interfaces. On the other hand, the coupling of protein carboxylic groups to the solid support improves the retention of enzymatic activity up to 50% of original activity but is hampered by a lower coupling yield and modification of catalytically important residues. We previously reported a chemical method to specifically acylate lysines of PLA_2 that are involved in its interaction with anionic interfaces. In particular, we acylated two lysines (Lys-7 and Lys-10) in the amino-terminal region of PLA_2 from the venom of *Agkistrodon piscivorus piscivorus* (App-D49) (6) and the resulting 7,10-diacylated App-D49 was up to 250 times more active than nonacylated enzyme toward phosphatidylcholine monolayers and bilayers (7). To take advantage of this superior catalytic power of acylated PLA_2, we have designed a new strategy for the immobilization of PLA_2 that involves the acylation of PLA_2 prior to immobilization. The immobilization of acylated PLA_2 could offer great advantages over the conventional immobilization method in that the acylation not only activates PLA_2 but also protects critical lysines from undesired modification during the immobilization. Herein, we report kinetic properties of immobilized acylated PLA_2s that demonstrate their high activity retention toward various aggregated phospholipids, including phospholipids on the surface of LDL particles.

MATERIALS AND METHODS

Materials

App-D49 was purified from lyophilized snake venom (Sigma) as described (8). PLA_2 from the venom of *Naja naja naja* was purchased from Sigma. 1,2-Dibutyryl-*sn*-glycero-3-phosphocholine (DBPC), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar lipids. 1,1'-Carbonyldiimidazole activated 6% cross-linked beaded agarose [Reacti-Gel (6X)] and Triton X-100 were from Pierce. Large unilamellar vesicles (LUV; an average diameter of 100 nm) of POPC were prepared by multiple extrusion through a 0.1-mm polycarbonate filter (Millipore) in a microextruder Liposofast (Avestin, Ottawa, Ontario) (9). Small unilamellar vesicles (SUV, an average diameter of 25 nm) were prepared by sonication (10). Mixed micelles of Triton X-100 and DLPC were prepared as

Abbreviations: PLA_2, phospholipase A_2; LDL, low density lipoprotein; DBPC, 1,2-dibutyryl-*sn*-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphocholine; NOB, 4-nitro-3-octanoyloxybenzoic acid; App-D49, Asp-49 PLA_2 from the venom of *Agkistrodon piscivorus piscivorus;* App-diC8, 7,10-dioctanoyl-App-D49; HPLC, high performance liquid chro matography; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles.

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described (11). Radiolabeled 4-nitro-3-octanoyloxybenzoic acid (NOB) was synthesized from [1-^{14}C]octanoic acid (American Radiolabeled Chemicals Inc.) and 4-nitro-3-hydroxybenzoic acid as described (12, 13). Fresh human plasma LDL was a generous gift from Drs. G. Retzinger (University of Cincinnati) and S. Meredith (University of Chicago). The protein concentration of the LDL preparation was 1.5 mg/ml. Phospholipid concentration of LDL was calculated according to the protein/phospholipid ratio (ca. 1.2) reported by Kleinman et al. (14).

Preparation of acylated PLA_2 and immobilization of acylated PLA_2

Detailed descriptions of the preparation and characterization of 7,10-dioctanoylated App-D49 (App-diC8) were reported elsewhere (6, 7). Radiolabeled App-diC8 was prepared using radiolabeled NOB in the same manner. Also, *N. n. naja* PLA_2 was acylated and purified by the same protocol. Briefly, after the treatment of *N. n. naja* PLA_2 with radiolabeled NOB, a major acylated form was separated from the mixture of acylated enzymes by reverse-phase HPLC using a Vydac C4 column. For the coupling, 1 ml of Reacti-Gel (6X) was extensively washed with cold deionized water and then dispersed in 2 ml of 0.1 M borate buffer, pH 9.0. The coupling of acylated PLA_2 (0.1 mM) to the gels was allowed to proceed for 2 h at room temperature with gentle agitation. After the coupling, gels were incubated in 1.0 M ethanolamine buffer, pH 9.0, for 3 h to block all remaining reactive groups, washed extensively with cold deionized water, and finally stored in 0.16 M KCl solution containing 0.02% sodium azide at 4°C. The amount of acylated PLA_2 coupled to the gels was determined by measuring the radioactivity of the weighed gel. Nonacylated PLA_2 was immobilized in the same manner. The amount of immobilized nonacylated PLA_2 was determined by the micro bicinchoninic acid method (Pierce) (15). Immobilized acylated enzymes were stable over several weeks when stored at 4°C.

Kinetic measurements

All the kinetic experiments were performed at 37°C and at pH 7.4. Detailed descriptions of the kinetic conditions and data analysis were reported elsewhere (7). Kinetics of PLA_2-catalyzed hydrolysis of vesicles were measured in the presence of 0.5 mM POPC, 0.1 mM BSA, 0.16 M NaCl, and 10 mM $CaCl_2$. The hydrolysis of mixed micelles was performed in the presence of 2 mM Triton X-100, 0.5 mM DLPC, and 10 mM $CaCl_2$ and the hydrolysis of DBPC was measured with 1 mM DBPC, 0.16 M NaCl, and 10 mM $CaCl_2$. The hydrolysis of LDL phospholipids was performed in the presence of 50 μM phospholipids, 0.1 mM BSA, 0.16 M NaCl, and 10 mM $CaCl_2$. Time courses of the hydrolysis of phospholipids were monitored with a computer-controlled Metrom pH stat (Brinkmann) in a thermostated vessel. Under the experimental conditions used, all the PLA_2-catalyzed reactions exhibited apparent first-order kinetics. Thus, rate constants were determined from the non-linear least-squares analysis of the progress curve using an integrated first-order rate equation. Apparent specificity constants ($(k_{cat}/K_m)_{app}$) were calculated by dividing the pseudo-first-order constant by enzyme concentration. For all the kinetic experiments, enzyme concentrations were adjusted so as to keep the half-life of reaction below 5 min. Concentration of immobilized PLA_2 was determined by measuring the radioactivity of the gels added to the reaction mixture. Immobilized acylated enzymes were recovered from the reaction mixture and used multiple times for kinetic measurements without any significant loss of catalytic activity.

RESULTS AND DISCUSSION

Immobilization of App-D49 and activity of immobilized App-D49

Immobilization of protein to the Reacti-Gel (6X) support occurs primarily through the reaction of ϵ-amino groups of lysyl residues of protein. As expected from the large number of lysines (13 lysines) present in App-D49 (8), the coupling yield was high (1.9 mg protein/mg gel). Under the same conditions, however, the coupling yield for the App-diC8 was relatively low (0.2 mg protein/mg gel). We previously showed (7, 16) the critical involvement of the two lysines, Lys-7 and Lys-10, in the interaction with anionic interfaces. The observed tenfold decrease in coupling yield for App-diC8 again indicates that these two lysines are highly reactive surface groups. Also, this result is consistent with previous findings (4, 5) that phospholipid aggregates decreased the coupling yield of PLA_2 by blocking its interfacial binding site. Despite slower coupling reaction, an elongated coupling of App-diC8 with the gels increased the coupling yield up to 1.0 mg protein/mg gel without impairing the activity of immobilized enzyme (data not shown).

To investigate the effects of acylation on the activity retention of immobilized App-D49, we measured kinetic properties of App-D49 and App-diC8, in soluble and immobilized forms, toward a wide variety of phospholipid aggregates, including LDL. Results are summarized in **Table 1.** We recently reported (7) that App-diC8 was slightly more active than App-D49 toward monodispersed substrate, Triton X-100/DLPC mixed micelles and POPC SUV, but ca. 250-fold more active than App-D49 toward POPC LUV. When LDL was used as a substrate, App-diC8 was only modestly more active than App-D49, indicating that the physical state of phospholipids on the LDL surface is similar to that of POPC SUV rather than to that of POPC LUV. This finding is

TABLE 1. Activities of soluble and immobilized App-D49 and App-diC8

Enzyme	$(k_{cat}/K_m)_{app}$				
	DBPC	Triton X-100/DLPC	POPC SUV	POPC LUV	LDL
	$10^2 \times M^{-1} s^{-1}$		$10^5 \times M^{-1} s^{-1}$		
Soluble App-D49	4.1 ± 0.4	51 ± 10	24 ± 5.0	0.04 ± 0.01	8.0 ± 0.9
Immobilized App-D49	2.5 ± 0.3	0.6 ± 0.1	0.3 ± 0.1	N.D.	0.1 ± 0.02
Soluble App-diC8	7.9 ± 0.5	56 ± 10	27 ± 5.0	10 ± 1.5	10 ± 1.0
Immobilized App-diC8	5.5 ± 0.3	45 ± 8.0	9.0 ± 1.0	1.0 ± 0.1	7.0 ± 0.9

Experimental conditions for kinetic experiments and methods for the calculation of parameters are described under Material and Methods. Values represent mean values ± standard errors determined from a minimum of three measurements; N.D., not detectable.

consistent with the previous finding that the size of LDL particle is similar to SUV (17). After immobilization, both App-D49 and App-diC8 retained 60–70% of their original activity toward mono-dispersed DBPC substrate, indicating that the coupling reaction did not significantly interfere with the catalytic apparatus of these enzymes. By contrast, the acylation of App-D49 exerted dramatic effects on the activity retention of immobilized enzyme when the activity was assayed with aggregated substrates. For instance, the immobilization reduced the activity of App-D49 to ca. 1% toward Triton X-100/DLPC mixed micelles and POPC SUV. Also, the immobilized App-D49 up to 10 μM showed no detectable activity toward POPC LUV. For App-diC8, however, the immobilization showed much less drastic effects on the enzymatic activity; the activity retention varied from 10% toward POPC LUV up to 33% toward POPC SUV and 80% toward Triton X-100/DLPC mixed micelles. Lombardo and Dennis (4) pointed out that for immobilized PLA_2, diffusion of large aggregated phospholipids inside the agarose gel matrix might become a rate-limiting process. They also estimated that 4:1 Triton X-100/phospholipids mixed micelles with an average mass of ca. 1×10^5 daltons should be able to freely diffuse into 6% agarose gel which has an exclusion limit of 4×10^6 daltons. Also, Huang and Mason (18) estimated that hydrodynamic mass of egg phosphatidylcholine (mainly POPC) SUV with a diameter of 25 nm was ca. 2×10^6 daltons. Accordingly, one would expect that Triton X-100/DLPC mixed micelles, and presumably POPC SUV, will have a relatively free assess to App-D49 and App-diC8 immobilized inside the pore of 6% agarose gel used in these studies. Thus, the 100-fold decrease in activity for immobilized App-D49 toward Triton X-100/DLPC (or toward POPC SUV) should be mainly due to the modification of lysines in the interfacial binding site, most notably Lys-7 and Lys-10. This notion is supported by the fully retained interfacial activity of immobilized App-diC8 toward Triton X-100/DLPC. By contrast, the hydrolysis of POPC LUV appeared to be limited by the diffusion of vesicular particles inside the gel as evidenced by the tenfold decrease in activity caused by the immobilization of App-diC8. Yet, the immobilized App-diC8 was 25 times more active than soluble App-D49 toward POPC LUV due to the high intrinsic activity of App-diC8 toward POPC LUV. Finally, we measured the activity of the immobilized PLA_2s toward phospholipids on LDL particles. Aggerbeck, Kézdy, and Scanu (2) reported that all the phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine on the surface of the LDL were conducive to the PLA_2 hydrolysis. LDL particles have a molecular mass of 3×10^6 daltons and a diameter of 22 nm and are, therefore, expected to be similar to POPC SUV in size (17). Toward LDL phospholipids, however, the immobilized App-diC8 showed higher activity retention (70%) than observed with POPC SUV (33%). As a result, the immobilized App-diC8 was as active as App-D49 toward LDL phospholipids. Presumably, the unique molecular shape of LDL particles enables them to diffuse more freely into the gel matrix than SUV particles with comparable size. Most importantly, these results demonstrate that the acylation of PLA_2 prior to its immobilization allows the full retention of enzymatic activity toward aggregated phospholipid substrates, most notably LDL phospholipids.

Immobilization of *N. n. naja* PLA_2 and activity of immobilized *N. n. naja* PLA_2

To evaluate the general applicability of our immobilization strategy, we acylated and immobilized *N. n. naja* PLA_2 as described for App-D49. Determination of the protein concentration and the incorporated radioactivity for a major acylated form of *N. n. naja* PLA_2 showed that it contained ca. one molecule of octanoic acid per enzyme molecule. We measured the activity of the acylated *N. n. naja* PLA_2 toward various substrates. As summarized in **Table 2,** the acylated *N. n. naja* PLA_2 was slightly less active than the nonacylated enzyme toward most substrates including LDL phospholipids. As is the case for the acylated App-D49, however, the acylated *N. n. naja* PLA_2 was ca. 10-fold more active than the nonacylated enzyme toward POPC LUV. When the acylated and nonacylated

TABLE 2. Activities of soluble and immobilized forms of *Naja naja naja* PLA_2 and acylated *Naja naja naja* PLA_2

Enzyme	$(k_{cat}/K_m)_{app}$				
	DBPC	Triton X-100/DLPC	POPC SUV	POPC LUV	LDL
	$M^{-1}s^{-1}$	$10^5 \times M^{-1}s^{-1}$			
Soluble *N. n. naja* PLA_2	38 ± 5	7.5 ± 0.6	21 ± 2	1.3 ± 0.09	14 ± 3.0
Immobilized *N. n. naja* PLA_2	16 ± 4	0.15 ± 0.04	0.6 ± 0.06	0.004 ± 0.001	0.3 ± 0.02
Soluble acylated *N. n. naja* PLA_2	30 ± 5.0	5.1 ± 0.2	13 ± 4.0	12 ± 3.8	12 ± 3.5
Immobilized acylated *N. n. naja* PLA_2	15 ± 3.0	4.5 ± 1.1	5.0 ± 1.5	1.0 ± 0.5	3.0 ± 0.5

Experimental conditions for kinetic experiments and methods for the calculation of parameters are described under Material and Methods. Values represent mean values ± standard errors determined from a minimum of three measurements.

N. n. naja PLA_2s were immobilized to the agarose gels under the same conditions, we found that the coupling yield for the acylated *N. n. naja* PLA_2 (0.05 mg protein/mg gel) was significantly lower than that observed for *N. n. naja* PLA_2 (1.0 mg protein/mg gel). Although we did not determine the location of acylation in this study, this result indicates that the acylation masks the lysyl residue (s) of *N. n. naja* PLA_2 that would otherwise be involved in coupling. In agreement with the previous report, the immobilization of *N. n. naja* PLA_2 drastically decreased the activity toward aggregated substrates while reducing the enzymatic activity toward monodispersed DBPC substrates by ca. 50%. In particular, the activity of immobilized *N. n. naja* PLA_2 toward LDL phospholipids was only 2% of original activity. By contrast, the immobilized acylated *N. n. naja* PLA_2 retained its activity toward most substrates; when compared to soluble *N. n. naja* PLA_2, the activity retention was 60% toward mixed micelles, 24% toward POPC SUV, 77% toward POPC LUV, and 21% toward LDL phospholipids. In particular, the activity retention of immobilized acylated *N. n. naja* PLA_2 toward LDL phospholipids was ten times higher than immobilized nonacylated *N. n. naja* PLA_2, demonstrating the general applicability of our method to the preparation of immobilized PLA_2s with high activity toward LDL phospholipids.

In summary, the lysyl acylation of PLA_2 by NOB prior to the immobilization greatly improves the retention of enzymatic activity of immobilized PLA_2. This simple immobilization method appears to be generally applicable to extracellular PLA_2s that have lysines in the interfacial binding site. High activity of immobilized acylated PLA_2s toward phospholipids on the LDL surfaces will enhance their potential usefulness in the therapy for hypercholesterolemia.

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