Carbodiimide modification enhances activity of pig pancreatic phospholipase A₂

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Pig phospholipase A₂, pig iso-phospholipase A₂ and bovine pancreatic phospholipase A₂ were reacted in solution with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, in the presence of *N*-hydroxysulfosuccinimide, at pH 7. The influence of micellar protectants was analyzed. In the presence of *n*-hexadecylphosphocholine, the losses of activity in micellar diheptanoyl-lecithin were 80, 35, and 10% in bovine phospholipase A₂, pig iso-phospholipase A₂, and pig phospholipase A₂, respectively. With 1-oleoylglycerophosphocholine, the bovine enzyme lost 40% activity, but the pig enzyme was activated sevenfold. The modified pig enzyme showed pre-micellar activation on monomeric diheptanoyl-lecithin, and either reduced or increased activities on mixed micelles of bile salt with egg phosphatidylcholine, depending on the composition of the micelles. This activation is consistent with previous protein-engineering studies of pig pancreatic phospholipase A₂. In this study, we present new information concerning the specificity and interfacial recognition behaviour of this enzyme in relation to this activation.

Sources of extracellular phospholipases A₂ (PLA₂) include mammalian pancreas, cobra and other snake venoms (Waite, 1987). These enzymes act preferentially on phospholipids present at interfaces. This phenomenon, known as interfacial recognition, is not well understood and has instigated detailed structural characterization of these enzymes. In particular, chemical modification studies that assigned the functionality of several different residues have been conducted (Verheij et al., 1981). In one of these studies, the reaction of water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) with bovine pancreatic PLA₂ showed that Asp49, a well-conserved residue in PLA₂ proteins, is involved in the binding of the cofactor calcium (Fleer et al., 1981).

Previously, we have used a modification of the carbodiimide method for covalent immobilization of PLA₂ (Ferreira et al., 1993). Mild reaction conditions were employed, with the pH increased to pH 7.0 and using *N*-hydroxysulfosuccinimide as a reaction enhancer (Staros et al., 1986). In the present study, modification in the soluble form of pancreatic PLA₂ proteins under similar reaction conditions is investi-

gated. Different variables are analyzed, such as pH, micellar protectants, and different nucleophiles. Pig PLA₂, pig iso-PLA₂ and bovine pancreatic PLA₂ are compared. In each case, activities in micellar short-chain lecithin are determined. Under certain conditions, the pig enzyme showed a several-fold activation. The results are compared with data from previous enzyme-engineering studies.

MATERIALS AND METHODS

Enzyme preparation

Pig pancreatic PLA₂ was purchased from Boehringer Mannheim as a suspension in 3.2 M ammonium sulfate. This preparation was extensively dialyzed against cold deionized water. To destroy any trypsin activity, the dialyzed solution was heated at 65°C for 1 h. This treatment did not affect activity, but increased the enzyme stability in solution. Concentrated solutions were aliquoted and stored frozen. Bovine pancreatic PLA₂ was obtained as a lyophilized powder from Sigma.

Other materials

N-Hexadecylphosphocholine (PamPCho) was a product of Calbiochem and all other phospholipids were from Avanti Polar Lipids. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and *N*-hydroxysulfosuccinimide were from Pierce, and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMEC) was from Aldrich. All other chemicals were of the highest purity available.

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Abbreviations. PLA₂, phospholipase A₂; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; CMEC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; PamPCho, *n*-hexadecylphosphocholine; PtdCho, phosphatidylcholine; OleGroPCho, 1-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphocholine; (Hpo)₂GroPCho, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine.

Enzyme. Phospholipase A₂ (EC 3.1.1.4).

Fig. 1. Modification of protein carboxylic groups with carbodiimides. Pathways in the absence (upper) and presence (lower) of *N*-hydroxysulfosuccinimide as an enhancer are shown.

Separation of isoforms

The commercial preparation of pig pancreatic PLA₂ was subjected to reverse-phase high-performance liquid chromatography (Hewlett Packard, LC 1090) in a Vydac C₄ column (0.46 mm×25 mm) using a gradient of 0.1% trifluoroacetic acid (solvent A) and 80% acetonitrile/0.085% trifluoroacetic acid (solvent B) as follows: 2–30% solvent B for 0–15 min; 30–75% solvent B for 15–90 min; 75–99% solvent B for 90–100 min; isocratic elution at 99% solvent B for 100–110 min. This was followed by a 20-min equilibration at 2% solvent B. The flow rate was 0.72 ml/min. The peaks corresponding to the two isoforms were collected, dried on a speed-vacuum concentrator (Heto, Denmark), and redissolved in MilliQ water. The identification of isoforms was performed by peptide sequencing of the first 19 residues of both proteins, using an Applied Biosystems model 477 protein sequencer.

Protein concentrations

The protein content in solution was determined by measuring the absorbance at 280 nm, using $A^{1\%} = 13$ for all pancreatic enzymes (Jain et al., 1991), or by the microbicinchoninic acid assay (Pierce); the calibration for this assay was carried out with pig PLA₂ at a known concentration.

Enzyme modification

Unless otherwise specified, modification reactions were carried out in 15 mM Mops, 150 mM CaCl₂, 0.5 M taurine, pH 7.0, containing 5 mM 1-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphocholine (OleGroPCho); the PLA₂ concentration was 60 µg/ml (4.3 µM). Reactions were initiated by adding *N*-hydroxysulfosuccinimide and EDC from freshly prepared stock solutions in water, to give final concentrations of 4 mM and 30 mM, respectively. The pH was immediately adjusted to pH 6.95–7.0, and the reaction was monitored for the following 5–10 min at this value. Mixtures were allowed to stand overnight. These conditions are designated the standard conditions.

Separation of modified enzyme

Reaction mixtures were first concentrated in a centrifugal concentrator (Amicon) and subjected to reverse-phase HPLC. The column and elution conditions were the same as those used in the separation of pig PLA₂ isoforms (see above). The modified enzyme was collected, dried, and redissolved in MilliQ water.

Activity assays

PLA₂ activities were always measured titrimetrically (Waite, 1987). Assays with 1,2-diheptanoyl-*sn*-glycero-3-

Table 1. Effect of pH and micellar protectant during modification of pig PLA₂. Standard modification conditions were used, except that *N*-hydroxysulfosuccinimide was omitted at pH 5.0. Activities were measured in 8 mM (Hpo)₂GroPCho, and represent averages of two or more independent experiments, differing by no more than 10%.

pH	Micellar protectant	Specific activity µmol · min ⁻¹ · mg ⁻¹
Native	—	92
5.0	—	12
5.0	PamPCho	43
5.0	OleGroPCho	56
7.0	—	23
7.0	PamPCho	84
7.0	OleGroPCho	875

phosphocholine [(Hpo)₂GroPCho] were carried out in 1 mM borate (pH 8.0) or 1 mM Mes (pH 6.0), 0.1 M NaCl, 25 mM CaCl₂, at pH 8.0 and 31°C. Mixed micelles of sodium cholate and egg PtdCho were prepared by vortexing lyophilized lecithin in 1 mM borate, 0.1 M NaCl, 10 mM CaCl₂, containing 10 mM or 50 mM sodium cholate; assays were performed at pH 8.0 and 25°C in 2 ml. For each sample, the average values of two or more assays, differing by no more than 10%, were determined. Furthermore, for any set of conditions, the values represent averages of two or more independent experiments.

RESULTS

Effect of pH and micellar protectant

When pig pancreatic PLA₂ was reacted with water-soluble EDC, the activity of the modified protein depended strongly on pH. Table 1 shows activities with micellar (Hpo)₂GroPCho as substrate for the enzyme modified at both pH 5.0 and pH 7.0. In the absence of any micellar protectant, the modified enzyme had 13–25% residual activity. Inclusion of OleGroPCho or PamPCho in the reaction media protected the enzyme against deactivation. At pH 5.0, and in the presence of lipids, approximately 45–60% residual activity was observed. When the enzyme was modified at pH 7, and protected by PamPCho, this value increased to 90%. Surprisingly, in the presence of OleGroPCho, the modified enzyme had average activity 875 ± 62 ($n = 8$), compared with 92 ± 6 U/mg for the native enzyme.

Effect of protein concentration

When all other variables were kept constant, the specific activity of the bulk reaction mixture varied with protein concentration (Fig. 2). Decreasing protein concentrations increased the specific activity against micellar (Hpo)₂GroPCho up until approximately 100 µg/ml; no further increase was observed at lower concentrations. Reaction mixtures above 100 µg/ml were subjected to reverse-phase HPLC for separation of the modified enzyme; after recovery, the peaks of the modified enzyme had similar activities to enzyme modified at less than 100 µg/ml.

Effect of carbodiimide and *N*-hydroxysulfosuccinimide

A second carbodiimide, CMEC, was used for modification of pig PLA₂, both in the presence and absence of *N*-

Fig. 2. Effect of pig PLA₂ on activity of bulk reaction mixture. All other conditions were standard (see Materials and Methods section). Activities were measured in 8 mM (Hpo)₂GroPCho. The estimated errors are less than 10%.

Table 2. Effect of carbodiimide and *N*-hydroxysulfosuccinimide on the modification of pig PLA₂. Activities were measured in 8 mM (Hpo)₂GroPCho, and represent averages of two or more experiments differing by no more than 10%. The carbodiimide and *N*-hydroxysulfosuccinimide concentrations were 30 mM and 4 mM, respectively.

Carbo- diimide	<i>N</i> -Hydroxy-sulfo- succinimide	Specific activity μmol · min ⁻¹ · mg ⁻¹
CMEC	—	128
CMEC	+	506
EDC	—	522
EDC	+	875

Table 3. Effect of nucleophile in modification of pig pancreatic PLA₂. Standard modification conditions were used, except that the nucleophile was varied. Activities were measured in 8 mM (Hpo)₂GroPCho. Estimated errors are less than 10%.

Nucleophile	Specific activity μmol · min ⁻¹ · mg ⁻¹
Taurine	875
Ethanolamine	851
Diaminepropane	561
Glycine ethyl ester	113
Heptylamine	32

hydroxysulfosuccinimide (Table 2). However, the greatest activation was observed with EDC and *N*-hydroxysulfosuccinimide. CMEC with *N*-hydroxysulfosuccinimide led to lower activation. EDC alone was able to activate the enzyme to a great extent, but CMEC alone did not significantly increase enzyme activity. These results suggest different yields of the modified, activated form of the enzyme.

Effect of different nucleophiles

The activities of pig PLA₂ reacted in the presence of different nucleophiles are shown (Table 3). Highest activation was observed with taurine, followed by ethanolamine, di-

Fig. 3. Separation of modified enzyme by reverse-phase HPLC. Chromatogram of a mixture of native and modified pig PLA₂. The native and modified peaks are centered at 34 min and 46 min, respectively.

Table 4. Modification of different pancreatic PLA₂ enzymes. Activities were measured in 8 mM (Hpo)₂GroPCho, and represent averages of two or more independent experiments differing by no more than 10%.

Micellar protectant	Specific activity of	
	bovine PLA ₂	pig iso-PLA ₂
	μmol · min ⁻¹ · mg ⁻¹	
Native	50	535
PamPCho	10	352
OleGroPCho	29	695

aminepropane and glycine ethyl ester. In the presence of heptylamine, deactivation was observed.

Reaction with different PLA₂ enzymes

The activities of other pancreatic PLA₂ enzymes, reacted under similar conditions, are shown in Table 4. When OleGroPCho was used as a protectant, pig iso-PLA₂ was activated by only 30%, and the bovine enzyme was deactivated by 40%. Like pig PLA₂, these two enzymes were deactivated with PamPCho as a micellar protectant.

Isolation of modified enzyme

Reverse-phase HPLC was used to isolate the activated, modified form of the enzyme from the non-activated form, and from OleGroPCho. The modified enzyme has higher hydrophobicity, eluting as a relatively broad peak, at higher acetonitrile concentrations than the native enzyme (Fig. 3).

Kinetic studies

Fig. 4 shows the activity/concentration profiles for native and modified (reverse-phase HPLC purified) pig PLA₂ against (Hpo)₂GroPCho. As expected, the native enzyme had close to background activities at monomeric concentrations of (Hpo)₂GroPCho (<1.5 mM), and a pseudo Michaelis-Menten behavior in the micellar region, with $V_{\max}$ 126 U/mg

Table 5. Effect of pH on activities of native and modified pig PLA₂ and pig iso-PLA₂. Activities were measured in 8 mM (Hpo)₂-GroPCho. Estimated errors are less than 10%.

PLA ₂	Activity at	
	pH 6.0	pH 8.0
	$\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$	
Pig, native	128	92
Pig, modified	586	875
Pig iso-PLA ₂ , native	404	535
Pig iso-PLA ₂ , modified	484	695

Fig. 4. Activity/concentration profiles in (Hpo)₂GroPCho. Initial hydrolysis rates for native (○) and modified (●) pig PLA₂ at varying concentrations of substrate. The error bar represents $\pm$ SD of activities for eight independent modification experiments.

and an apparent Michaelis constant, K_{app} 3.85 mM (in micellar concentration). These values are in agreement with other studies (Kuipers et al., 1989). The modified enzyme had a striking profile (Fig. 4). The activity increased rapidly with concentration in the monomeric region, attained a maximum at the approximate critical micellar concentration (1.5 mM), and showed no further increase in the micellar region. Thus, the k_{cat} of the modified enzyme was sevenfold higher than the value of the native enzyme.

The activities at pH 6.0 and pH 8.0 in micellar (Hpo)₂-GroPCho were compared, for native and modified forms of pig PLA₂ and pig iso-PLA₂ (Table 5). The pig enzyme was more active at pH 6.0, but modified pig PLA₂, and native and modified pig iso-PLA₂ were more active at pH > 7.

Other interesting observations occurred in the kinetics for mixed micelles of bile salts and egg PtdCho (Table 6). Modified pig PLA₂ showed both decreased and increased activities with these micelles, depending upon the ratio of bile salt/egg PtdCho. At low ratios (2:1 molar), modification lowered the k_{cat} value; at high bile salt concentration (10:1), the modified enzyme was 4–5 times as active as the native enzyme. Pig iso-PLA₂ had a similar behavior, but activity of the modified form in the high bile salt concentration micelles was less than two times the native value.

Table 6. Activities of native and modified pancreatic PLA₂ in mixed micelles of sodium cholate with egg PtdCho. Values are initial rates of reaction, and represent averages $\pm$ SD of two or more independent experiments.

PLA ₂	Cholate/egg PtdCho	
	10 mM/5 mM	50 mM/5 mM
	$\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$	
Pig, native	1143 $\pm$ 75	42 $\pm$ 2
Pig, modified	496 $\pm$ 18	199 $\pm$ 2
Pig iso-PLA ₂ , native	947 $\pm$ 81	95 $\pm$ 3
Pig iso-PLA ₂ , modified	555 $\pm$ 42	177 $\pm$ 25

Fig. 5. Calcium dependence of modified pig PLA₂. Hanes' plot of the activity of modified pig PLA₂ as a function of calcium concentration. Assays were carried out in 1 mM borate, 0.1 M NaCl, 8 mM (Hpo)₂GroPCho, pH 8.5, and 31°C. Estimated errors of activities are less than 10%.

Dependence of activity on calcium concentration

The Hanes' plot ($[Ca]/V$ versus $[Ca]$) of the dependence of enzymic activity on calcium concentration for the modified enzyme is shown in Fig. 5. All data in the 0–10 mM calcium range are fitted with one straight line, indicating the existence of a single calcium-binding site. An apparent dissociation constant value of K_{Ca} 0.24 mM was estimated.

DISCUSSION

Chemical modification of carboxylic groups in proteins is frequently carried out using water-soluble carbodiimides as activators, forming an *O*-acylisourea intermediate that reacts with a nucleophile (Hoare and Koshland, 1967). In the presence of *N*-hydroxysulfosuccinimide, the reaction leads to the formation of a *N*-hydroxysulfosuccinimide ester of the carboxylic group (Fig. 1), that is displaced by strong nucleophiles (Staros et al., 1986; Staros, 1988). This ester is more resistant to hydrolysis than the *O*-acylisourea adduct, thus permitting higher yields in the final aminolysis step (Staros, 1988). Both ester formation and aminolysis reactions proceed at pH values of approximately pH 7.

Using this chemistry, and in the presence of OleGroPCho as protectant, a modified form of pig pancreatic enzyme that

was the sevenfold more active toward small micelles than native enzyme was obtained. Under analogous reaction conditions, bovine PLA₂ was not activated, and pig iso-PLA₂ was activated by 30% (this slight activation results, most likely, from the presence of some pig isoform in the preparation). Comparing the sequences of the three enzymes, pig PLA₂ has a glutamate residue at position 71, where the bovine enzyme and pig iso-enzyme have an asparagine residue. There is no additional carboxylic group in pig PLA₂ without an equivalent (group) in either the bovine enzyme or pig iso-enzyme. The fact that activation occurred in the presence of micellar phospholipid suggests that the underlying modification involves residue(s) of the interfacial recognition site. The X-ray crystal structure of the pig enzyme indicates that several residues of the loop at positions 62–72 are involved in interfacial binding (Dijkstra et al., 1983), thus reinforcing the hypothesis of Glu71 modification. Glu71 is involved in the binding of a second calcium ion, which is responsible for the effective enzyme interaction with organized lipid interfaces at alkaline pH > 7 (Donnée-Op den Kelder et al., 1983). A mutant pig PLA₂ with Glu71 substituted by an asparagine residue (van den Bergh et al., 1989) lacked the second calcium-binding site. Analogously, this carbodiimide-modified enzyme has only one calcium-binding site, as shown by the dependence of enzymic activity upon calcium concentration (Fig. 5). For native pig pancreatic PLA₂, the Hanes' plot shows a transition between two different slopes at 1–2 mM calcium (van den Bergh et al., 1989). Furthermore, the $V_{\max}$ value in micellar dioctanoyl-lecithin of the modified enzyme (4821 U/mg, at 25°C) was nearly identical to the value reported for the above mutant (5043 U/mg; van den Bergh et al., 1989).

Micellar OleGroPCho can promote modification of a residue in the vicinity of the interfacial binding site in two ways. Either the binding to the interface changes its environment and/or conformation, making it more reactive, or EDC can partition to OleGroPCho micelles, and thus facilitate contact with that residue. Meyer et al., while performing tyrosine modification in pancreatic PLA₂ enzyme, found that tetranitromethane was incorporated into lysophosphatidylcholine micelles, resulting in a higher rate of modification (Meyer et al., 1979). The fact that activation is observed when the enzyme is modified at pH 7.0, and not at pH 5.0, can also result from this partitioning. It is likely that the surface charge of the micelle is different at these two pH values, favoring partitioning of the positively charged carbodiimides at pH 7. It is interesting, however, that activation was not observed with PamPCho as a micellar protectant. The same partitioning effect could still be the reason for the lower reactivity of the carbodiimide CMEC compared to EDC (Table 2). Alternatively, the presence of two bulky rings in the structure of CMEC may hinder the access to the residue responsible for the activation.

Carbodiimides can also attack tyrosine residues, forming an *O*-arylisourea adduct that is quite stable at pH 7 and pH < 7 (Carraway and Koshland, 1968). As phenolic groups are more reactive at higher pH values, the possibility of the carbodiimide attacking the neighboring Tyr69 or Tyr63 cannot be completely excluded. However, the data suggests that tyrosine modification, if it occurs, is not the primary reason for activation. For instance, *N*-hydroxysulfosuccinimide does enhance the yield of modified enzyme, as observed with CMEC (Table 2). Based on the proposed reaction mechanism (Staros et al., 1986), *N*-hydroxysulfosuccinimide is unlikely to interfere with the *O*-arylisourea derivative.

A plausible reason for the increased catalytic activity of modified pig PLA₂ is the change in the conformation of the loop at positions 62–72, involved in interfacial binding. Cobra venom enzymes have higher turnover numbers than the pancreatic enzymes, and they lack some residues of that loop (Kuipers et al., 1989). A mutant pig PLA₂ lacking residues 62–66 had a k_{cat} against micellar (Hpo)₂GroPCho 16 times higher than the native enzyme (Kuipers et al., 1989). Another site-directed-mutagenesis study of bovine pancreatic PLA₂ (Noel et al., 1991) showed that replacement of Lys56 by a neutral or hydrophobic residue imparted different conformations in that same loop, with a 3–4-fold improvement in the k_{cat} in short-chain lecithin micelles.

The modified pig PLA₂ had approximately 50% native activity in 10 mM cholate/5 mM egg PtdCho micelles, but that value increased with cholate concentration, surpassing the value for the native enzyme by more than fourfold in 50 mM cholate/5 mM egg PtdCho micelles. Analogous observations were obtained with 10 mM sodium cholate/3 mM dilauroylphosphoglycerol and 50 mM sodium cholate/3 mM dilauroylphosphoglycerol micelles where dilauroylphosphoglycerol is negatively charged (data not shown). The size and shape of mixed micelles of bile salts with lecithins vary with composition (Mazer et al., 1980; Nichols and Ozarowski, 1990; Shankland, 1970). Molecular masses of 100 kDa and 6.8 kDa were reported for the 10 mM:5 mM and 50 mM:5 mM cholate/egg PtdCho mixed micelles, respectively (Shankland, 1970). Taken together, these results suggest that (a) activation against zwitterionic (Hpo)₂GroPCho is not due to a charge effect and (b) micellar packing and/or phospholipid conformation are the probable reasons for the lowered activities in large mixed micelles. The results presented for the native pig enzyme are in agreement with previously reported kinetic studies (Hoffman et al., 1983).

One other study proposed that activation of PLA₂ occurred upon acylation of Lys56 (Tomasselli et al., 1989); the acylated enzyme had a high tendency to dimerize and this would be the fully active form of the pancreatic PLA₂ enzyme at interfaces. Several observations support the hypothesis that dimerization is not an issue in this modification: (a) the pH dependency of the activation (Table 1), (b) increasing protein concentration in the medium lowered the overall activity (Fig. 2), (c) SDS/PAGE of the modified enzyme showed that covalent cross-linking was minimal, (d) PLA₂ that was first immobilized onto agarose beads (Ferreira et al., 1993) was subjected in a second step to the standard chemical modification; the immobilized enzyme, prevented from oligomerization, was activated.

In this study, and in studies with recombinant enzymes (Kuipers et al., 1989; Noel et al., 1991), we find examples of the effects of changes in enzyme conformation in PLA₂ catalysis. However, the reversal in relative activities between modified and native forms in different cholate/egg PtdCho micelles points to the influence of interface quality in the process.

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