Site-Specific ϵ-NH_2 Monoacylation of Pancreatic Phospholipase A_2. 1. Preparation and Properties†

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ABSTRACT: The lipid-binding domain of pancreatic phospholipases A_2 contains a number of exposed, hydrophobic amino acid side chains that are involved in the binding of the enzyme to organized lipid–water interfaces. Besides these apolar residues, at least two positively charged lysine groups are present in positions 10 and 116 of the lipid-binding domain. In order to investigate the possible function of these basic side chains in the lipid-binding process, a number of specifically acylated enzyme mutants were prepared, and their kinetic and lipid-binding properties have been compared with those of the native enzymes. It is concluded that the attachment of a long-chain acyl group in an amide linkage to Lys^{10} or Lys^{116} phospholipase A_2 has only a minor influence on the catalytic properties of the enzyme. On the other hand, the lipid-binding properties of the mutant enzymes appear to be considerably reinforced.

Phospholipase A_2 (EC 3.1.1.4) catalyzes the hydrolysis of the 2-acyl ester bond in 1,2-diacyl-*sn*-glycero-3-phospholipids with the formation of 1-acyllysophospholipids and free fatty acids. The enzyme is absolutely dependent on Ca^{2+} ions. The two richest sources are the mammalian pancreas and the venom of snakes. Notwithstanding the high sequence homology between snake venom phospholipases and the pancreatic enzymes (Slotboom et al., 1982) and their similar three-dimensional X-ray structures (Dijkstra et al., 1981, 1983; Renetseder et al., 1985), a few interesting differences have been noted.

First, the pancreatic phospholipases are synthesized in a precursor form, prophospholipase, that has an N-terminal extension of seven amino acids as compared to the sequence of the active enzymes. Tryptic cleavage of the heptapeptide converts the zymogen into active phospholipase A_2 (PLA).[1] For the venom enzymes a zymogen form has never been demonstrated.

Second, most venom phospholipases preferentially hydrolyze neutral lecithin molecules, whereas the pancreatic enzymes clearly prefer negatively charged phospholipids such as cardiolipin, phosphatidylglycerol, or phosphatidic acid, and they hardly attack long-chain zwitterionic lecithin dispersions (Van Deenen & De Haas, 1963; Hendrickson et al., 1981).

It is well-known that differences exist in the molecular packing characteristics of the various phospholipids. Natural lecithins form closely packed bilayer structures, while the anionic phospholipids in the presence of Ca^{2+} often organize themselves in the hexagonal H_{II} phase. Compared to most venom enzymes, the pancreatic phospholipases have been shown to possess very weak penetrating properties in monomolecular surface films. It is possible that they may enter into more loosely packed hexagonal structures but not enter the bilayer organization. The pancreatic phospholipases A_2 have been shown to possess two functionally and topographically distinct binding sites for lipids. The first is the classical active site where monomeric substrate molecules are bound and slowly hydrolyzed. This site is present in the zymogen as well. In addition, the active enzyme contains a so-called lipid-binding domain, a three-dimensional cluster of hydrophobic amino acids located at the surface of the protein. This second binding site binds the phospholipase to the organized lipid–water interface.

The high-resolution X-ray structure of the bovine and porcine pancreatic phospholipases A_2 shows that in the lipid-binding domain at least two positively charged side chains are present: Lys^{10} and Lys^{116}. These residues might also be responsible for the preferential attack of anionic phospholipids by the pancreatic enzymes. How can we improve the lipid-binding properties of the pancreatic enzymes? One possibility is the introduction of a long hydrophobic anchor, covalently attached to a side chain of a specific amino acid in the lipid-binding domain.

This paper describes the preparation and catalytic properties of several monoacylated enzymes carrying a single fatty acyl group in an amide linkage to the ϵ-NH_2 function of either Lys^{10} or Lys^{116}. The accompanying paper (Van der Wiele et al., 1988) describes the behavior of various monoacylated phospholipases A_2 on monomolecular lecithin films, on lecithin

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[1] Abbreviations: PLA, phospholipase A_2; PREC, prophospholipase A_2; AMPREC, fully ϵ-amidinated prophospholipase A_2; AMPREC-8, porcine phospholipase A_2 in which all but one Lys are amidinated; AMPA, fully ϵ-amidinated phospholipase A_2; ^{Am}Lys, ϵ-amidinated lysine; Boc, *tert*-butyloxycarbonyl; For, formyl; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; DFP, diisopropyl fluorophosphate; DEAE, diethylaminoethyl; CM, carboxymethyl; FPLC, fast protein liquid chromatography; LC, liquid chromatography; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; dpm, disintegrations per minute; DMF, *N*,*N*-dimethylformamide; Pal, palmitoyl; Lau, lauroyl; Ol, oleoyl; Cap, caprinoyl; Ac, acetyl; Mal, maleyl; Osu, hydroxysuccinimide ester; DNFB, 2,4-dinitrofluorobenzene; TNBS, 2,4,6-trinitrobenzenesulfonic acid; DNP, 2,4-dinitrophenyl; di-C_8GS, 1,2-dioctanoyl-*sn*-glycerol 3-sulfate; DABITC, 4-(dimethylamino)azobenzene isothiocyanate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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vesicles, and on natural membranes.

Materials and Methods

Most of the materials and methods used in this study have been described recently (De Haas et al., 1987; Van Scharrenburg et al., 1984). Bovine des(Ala[1]–Am-Lys[10])AMPA was prepared from bovine AMPREC by CNBr cleavage at Met[8] (Van Scharrenburg et al., 1981) followed by two successive Edman degradations to remove Ile[9] and amidino-Lys[10] (Slotboom & De Haas, 1975). 1,2-Dioctanoyl-*sn*-glycerol 3-sulfate was prepared as described before (Van Oort et al., 1985). N^{α}-*t*-Boc-N^{ϵ}-palmitoyl-Lys and *N*-hydroxysuccinimide esters of caprinic, lauric, palmitic, and oleic acid were prepared as outlined by Lapidot et al. (1967) and were purified by crystallization and/or chromatography on silica. [1-^{14}C]-Palmitic acid, [1-^{14}C]lauric acid, and [1-^{14}C]acetic anhydride were purchased from Amersham (England).

Preparation of Bovine Pal-ε-NH_2-Lys[10]-AMPA. N^{α}-*t*-Box-N^{ϵ}-palmitoyl-Lys was coupled to bovine des(Ala[1]–Am-Lys[10])AMPA using the mixed-anhydride procedure (Meienhofer, 1979; Van Scharrenburg et al., 1981). Excess reactant was removed by elution of the reaction mixture on a Sephadex LH-20 column using 90% DMF. After dialysis, the protein-containing fraction was treated with an equal volume of a 2 M H_2NOH solution (pH 7.5). The N^{α}-*t*-Boc group was then removed by treatment with TFA (Slotboom & De Haas, 1975) and the polypeptide chain elongated with Boc-Ile and Boc-Met by using their respective *N*-hydroxysuccinimide esters; the Boc groups were removed by treatment with TFA. The intermediate, des(Ala[1]–Met[8])[Pal-Lys[10]]AMPA, was purified on CM-cellulose. Finally, N^{α}-*t*-Boc-Ala-Leu-Trp(For)-Gln-Phe-Arg-Gly was coupled to des(Ala[1]–Gly[7])[Pal-Lys[10]]AMPA by the mixed-anhydride procedure (Van Scharrenburg et al., 1981). After removal of the formyl and Boc groups (Van Scharrenburg et al., 1981), the protein was purified on DEAE-cellulose in the presence of 25% acetonitrile.

Preparation of Porcine ε-NH_2-Lys-AMPREC. To a solution of porcine pancreatic prophospholipase A_2 (0.5 g) and 1,2-dioctanoyl-*sn*-glycerol 3-sulfate (0.17 g) in 90 mL of buffer (20 mM sodium tetraborate containing 5 mM EDTA, pH 9.5) was added 20 mL of a freshly prepared solution of methyl acetimidate hydrochloride (0.75 g) in the same buffer (20 mL), readjusted to pH 9.5 with NaOH solution. The reaction mixture was stirred at room temperature, and after 40 min, the reaction was stopped by addition of acetic acid to pH 3.8 and the mixture stirred for another 30 min at 4 °C. The precipitate of the high molecular weight complex of partially amidinated prophospholipase A_2 and substrate, after centrifugation, was dissolved in 25 mL of 20 mM sodium tetraborate buffer (pH 8.5) containing 5 mM Ca^{2+} and 0.4 mM DFP and incubated overnight with 5 mg of native porcine PLA to degrade the substrate. In order to obtain pure species with one or two nonamidinated ε-NH_2-lysyl residues, these residues were reacted with maleic anhydride, enabling their separation by ion-exchange chromatography. Maleination was done by addition of 10 aliquots of 0.1 mL of a maleic anhydride solution (100 mg of maleic anhydride/mL of absolute dioxane) at intervals of 2 min to a magnetically stirred solution of crude amidinated pro-PLA (0.25 g) in 25 mL of buffer (0.1 M sodium tetraborate, pH 8.5). The pH was maintained at 8.5 by addition of NaOH solution, and the mixture was stirred for 45 min at room temperature. To split maleyl esters of tyrosyl residues, an equal volume of 2 M H_2NOH solution (pH 7.5) was added and stirring continued for another 20 min at room temperature. The crude maleinated-amidinated pro-PLA was purified by chromatography on DEAE-cellulose. Three fractions, A, B, and C, were obtained in yields of 40, 50, and 10%, respectively. Fraction A was determined to be fully ε-amidinated pro-PLA. It elutes at the same position as authentic fully ε-amidinated pro-PLA, and upon tryptic activation it gives the same specific activity in the egg-yolk assay as found for fully ε-amidinated PLA. Fractions B and C elute later from the DEAE column due to the presence, respectively, of one or two negatively charged maleyl groups (vide infra). Both fractions were demaleinated at pH 2.5 (HCl) for 24 h at 50 °C (Butler et al., 1969) and were purified by chromatography on DEAE-cellulose. By use of the DNFB and TNBS procedures, the pure demaleinated fractions B and C were found to contain 0.97 and 1.99 free ε-NH_2-lysyl groups per protein molecule, respectively.

Localization of the Nonamidinated Lys Residue(s) in ε-NH_2-Lys-AMPREC. ε-NH_2-Lys-AMPREC was incubated with 0.1% (w/w) trypsin at pH 8.0 (10 mM Tris, 5 mM Ca^{2+}) to obtain ε-NH_2-Lys-AMPA. The latter protein was purified on DEAE-cellulose. The enzyme, dissolved in 10 mM Tris-HCl and 5 mM Ca^{2+} (pH 8.0) was incubated with TPCK–trypsin (15% w/w) at room temperature. The progress of the reaction was followed by FPLC (Pharmacia, Sweden) as shown in Figure 2. After 3 h, when less than 5% of enzymatic activity was left, the incubation was stopped and the resulting mixture separated on a DEAE-cellulose column. An elution pattern similar to that shown in Figure 2 was obtained. The first eluting peak (IV, Figure 2) (90% yield) was found to be pure on a reversed-phase column and analyzed correctly for the N-terminal hexapeptide Ala-Leu-Trp-Gln-Phe-Arg. Fractions corresponding to peaks II and III (Figure 2) analyzed correctly for des(Ala[1]–Arg[6])ε-NH_2-Lys-AMPA and were obtained in 30 and 70% yield, respectively. Upon renewed incubation of the protein present in peak II (Figure 2) with TPCK–trypsin, this material was almost quantitatively converted into peak III. Peak III was subjected to Edman degradation using the DABITC procedure (Chang, 1983). As a result Ser + Asn, Met + Leu, and Ile + Asp were identified in the first, second, and third step, respectively. In order to unambiguously assign the nonamidinated Lys residue, peak III was oxidized with performic acid (Hirs, 1967) and the mixture, after lyophilization, separated on a Sephadex G-25 fine column (0.05 M NH_4HCO_3, pH 8). Two peaks were obtained: one eluting in the void volume (III_A) and one eluting later (III_B). Upon amino acid analysis, fraction III_A analyzed correctly for the peptide 7–116. Fraction III_B, which was found to be pure on LC, gave the following amino acid analysis: $Asx_{2.0}Thr_{0.96}$cysteic $acid_{0.9}Leu_{1.0}$(Lys + ε-amidinated $Lys)_{1.7}Tyr_{+}$, in good agreement with the C-terminal sequence Asn[117]-Leu-Asp-Thr-^{Am}Lys-^{Am}Lys-Tyr-Cys[124]. Both the peptide 7–116 (III_A) and 117–124 (III_B) were obtained in 85% yield.

Preparation of Porcine Acyl-Lys[116]-AMPAs. To a magnetically stirred solution of ε-NH_2-Lys[116]-AMPREC in a mixture of DMF and 0.02 M Hepes buffer (pH 8.35) (9:1 v/v) was added [1-^{14}C]palmitoyl-*N*-hydroxysuccinimide ester (20-fold molar excess) dissolved in DMF. The reaction mixture was kept overnight at room temperature. Excess reactant was removed by elution of the reaction mixture on a Sephadex LH-20 column using 50% DMF. The protein-containing fraction was dialyzed and treated for 20 min with an equal volume of 2 M H_2NOH at pH 7.5 at room temperature. After exhaustive dialysis the desired [[1-^{14}C]Pal-Lys[116]]AMPREC (0.94 palmitoyl moiety/protein molecule) was obtained. The acylated protein was found to be pure by

FPLC (Mono Q and S columns) using mixtures (1:1 v/v) of appropriate buffer and acetonitrile. [[1-^{14}C]-Pal-Lys^{116}]AMPREC, dissolved in 5 mM Tris-HCl buffer (pH 8.0) containing 5 mM $CaCl_2$, was activated by 0.1% (w/w) trypsin to obtain [[1-^{14}C]Pal-Lys^{116}]AMPA.

[[1-^{14}C]Lau-Lys^{116}]-, (0.91 lauroyl moiety/protein molecule) [Cap-Lys^{116}]-, and [Ol-Lys^{116}]AMPRECs and -AMPAs were prepared similarly as described for the palmitoyl analogue.

Preparation of Porcine [[1-^{14}C]Acetyl-Lys^{116}]AMPA. To a magnetically stirred solution of 25 mg of ϵ-NH_2-Lys^{116}-AMPREC in 5 mL of 0.5 M sodium tetraborate (pH 10) was added 0.5 mL of [1-^{14}C]acetic anhydride in 2 mL of dry dioxane in five aliquots over a period of 30 min at room temperature. The pH is maintained at 10 by addition of 1.5 M NaOH solution. After the last addition, the mixture was stirred for another 20 min at room temperature. Subsequently, an equal volume of a 2 M H_2NOH solution (pH 7.5) was added and the mixture was stirred at room temperature for 20 min. After desalting, the acetylated AMPREC was purified by chromatography on a DEAE-cellulose column. Pure [[1-^{14}C]acetyl-Lys^{116}]AMPREC (1.18 acetyl groups/protein molecule) was obtained that upon tryptic activation yielded [[1-^{14}C]acetyl-Lys^{116}]AMPA.

Free ϵ-NH_2-lysyl groups were determined by the DNFB procedure (Wofsy & Singer, 1963) and by the TNBS procedure (Habeeb, 1966; Okuyama & Satake, 1960). ^{14}C radioactivity was determined in a Packard Tricarb scintillation counter, using Packard instagel as scintillation liquid.

Results

For the specific introduction of a palmitoyl chain in position 10 of phospholipase A_2, the following semisynthetic approach was used. As starting material fully ϵ-amidinated bovine prophospholipase A_2 (AMPREC) was preferred over the porcine enzyme because the former protein contains only a single Met residue located at position 8 of the polypeptide chain. Cyanogen bromide cleavage, followed by two preparative Edman degradations of the large C-terminal fragment, yielded the peptide H_2N-Cys^{11}...Cys^{123} with all seven disulfide bridges intact. This large polypeptide was N-terminally elongated by three successive chemical couplings using, in order, appropriately protected N^{ϵ}-palmitoyl-Lys, Ile, and Met. The resulting polypeptide H_2N-Met^8-Ile^9-Pal-Lys^{10}-Cys^{11}... Cys^{123} was finally transformed into an active phospholipase mutant by coupling with the protected heptapeptide Boc-Ala^1-Leu-Trp(For)-Gln-Phe-Arg-Gly, yielding [Arg^6,Pal-Lys^{10}]AMPA.

In order to allow specific monoacylation of Lys^{116} in phospholipase A_2, the technique of "differential labeling" was used (Figure 1). Amidation of porcine prophospholipase A_2 with methyl acetimidate yields the fully ϵ-amidinated zymogen AMPREC, which contains nine amidinated Lys residues. When the same reaction was carried out in the presence of the substrate 1,2-dioctanoyl-*sn*-glycerol 3-sulfate, a crude AMPREC preparation was obtained that contained 0.7–0.9 free ϵ-NH_2 group/mol of protein. This suggested that the anionic substrate provided partial protection of one or more lysine side chains against amidination. Purification of the crude AMPREC by ion-exchange chromatography was unsuccessful. This is not surprising if one takes into account the similar positive charges of the ϵ-NH_3^+ in unmodified Lys and the NHC(CH_3)=NH^+ group in ϵ-amidinated lysine side chains. Blocking of the free ϵ-NH_3^+ groups in the crude AMPREC with maleic anhydride transformed these positively

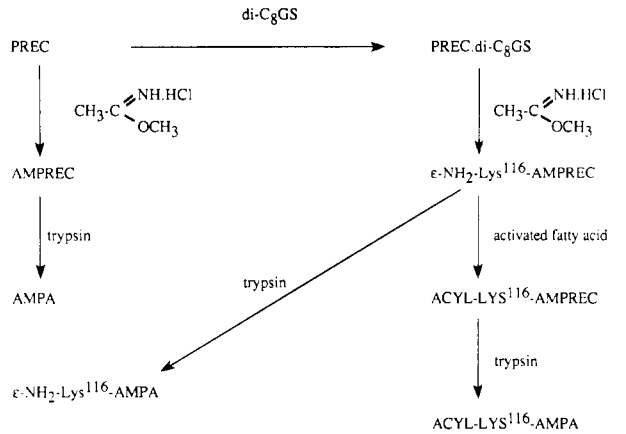

FIGURE 1: Scheme depicting conversion of porcine pancreatic prophospholipase A_2 (PREC) into various acyl-Lys^{116}-ϵ-amidinated phospholipases A_2 (acyl-Lys^{116}-AMPA). Acyl = $C_{16}H_{31}O$: [Pal-Lys^{116}]AMPA. Acyl = $C_{18}H_{33}O$: [Ol-Lys^{116}]AMPA. Acyl = $C_{12}H_{23}O$: [Lau-Lys^{116}]AMPA. Acyl = $C_{10}H_{19}O$: [Cap-Lys^{116}]-AMPA. Acyl = C_2H_3O: [Ac-Lys^{116}]AMPA. Acyl = COCH=CHCOOH: [Mal-Lys^{116}]AMPA.

charged side chains into the anionic NHCOCH=CHCOO$^-$ derivatives, which allowed effective separation in three fractions, A, B, and C, eluting in that order from DEAE-cellulose. After deblocking of the maleyl-protected lysine residues at low pH and performing quantitative analysis for free ϵ-NH_2 groups in the three fractions, it appeared that fraction A consisted of pure fully ϵ-amidinated zymogen, so-called AMPREC-9. Fractions B and C were shown to contain one and two free ϵ-NH_2 groups/mol of protein, respectively, which suggests the possibility that 1,2-dioctanoyl-*sn*-glycerol 3-sulfate has protected one or two lysyl side chains against amidination.

Specificity of the Protein and Identification of the Free Lys Residue(s). Fraction B after demaleination was purified by ion-exchange chromatography. The resulting pure ϵ-NH_2-Lys^{116}-AMPREC was then converted by limited proteolysis into ϵ-NH_2-Lys^{116}-AMPA (Nieuwenhuizen et al., 1974). By use of FPLC a single symmetric peak (Figure 2, I) is observed. In order to find out which Lys residue(s) is (are) not amidinated, AMPREC-8 was incubated with TPCK-treated trypsin and the composition of the incubation mixture was followed over time by FPLC. From previous work we know that fully ϵ-amidinated porcine phospholipase A_2 is very stable against trypsin attack. High protease concentrations and prolonged incubation periods are required to obtain one single cleavage reaction, i.e., Arg^6-Ser^7. The resulting peptides Ala^1–Arg^6 and Ser^7–Cys^{124} elute on FPLC (Mono Q column) in positions IV and II, respectively. From Figure 2 it is clear that even after 2 h of incubation with trypsin, the Arg^6–Ser^7 bond is still incompletely split. Three hours of incubation are required for complete cleavage of the Arg^6–Ser^7 linkage (disappearance of peak I). The C-terminal fragment Ser^7–Cys^{124} (peak II) is even more slowly degraded with the production of peak III, most probably by an additional tryptic cleavage after unknown free lysine(s). Amino acid analyses of peaks II and III were indistinguishable and differed from that of the starting material (peak I) only by the loss of the N-terminal fragment Ala^1-Leu-Trp-Gln-Phe-Arg^6. This indicates that the tryptic conversion of peak II into peak III involves a cleavage reaction but that the fragments remain together through disulfide bonds. Three consecutive Edman degradations on the highly purified fraction III yielded in step 1 Ser and Asn in step 2 Met and Leu, and in step 3 Ile and Asp. Inspection of the complete amino acid sequence of the porcine phospholipase A_2 shows the origin of Ser, Met, and Ile as Ser^7-Met^8-Ile.[9] The

Table I: Comparison of Various Properties of Native, Fully ε-Amidinated and Acyl-N^{ϵ}-amidinated Porcine and Bovine Phospholipases A_2

enzyme	sp act.[a] (egg-yolk assay) (μequiv min^{-1} mg^{-1})	V_{max}[b] (micellar L-dioctanoyl-lecithin assay) (μequiv min^{-1} mg^{-1})	parameters for binding of phospholipases to micellar *cis*-9-octadecenylphosphocholine[c]: N (no. of lipid monomers per enzyme molecule)	K_D (micellar dissociation constant) (μM)	NK_D (μM)	$T_{1/2}$ (half-time values for inactivation by *p*-bromophenacyl bromide)[d] (min)
porcine						
PLA	1300	2200	32	1.8	58	30
AMPA	300	1650	42	1.8	76	60
ε-NH_2-Lys^{116}-AMPA	650	2000	40	1.1	44	40
[Ac-Lys^{116}]AMPA	45	755	38	1.0	38	95
[Mal-Lys^{116}]AMPA	30	405	25	1.7	43	125
[Cap-Lys^{116}]AMPA	470	2100	13	2.5	33	75
[Lau-Lys^{116}]AMPA	250	2500	15	1.9	29	260
[Pal-Lys^{116}]AMPA	330	2200	8	1.5	12	360
[Ol-Lys^{116}]AMPA	500	2600	10	1.8	18	325
bovine						
PLA	145	3000	50	13.2	660	2
AMPA	50	2300	50	64	3200	2
[Arg^6]AMPA	120	5900	48	2.5	120	3
[Arg^6,Pal-Lys^{10}]AMPA	50	5600	12	1.7	20	9

[a] Determined at pH 8.0 as described previously (Van Scharrenburg et al., 1981). [b] Determined by titration of fatty acid liberated with 10.3 mM NaOH at pH 6.0 and 45 °C (50 mM $CaCl_2$, 10 mM acetate, and 0.1 M NaCl) as described previously (Van Scharrenburg et al., 1981). [c] Determined by ultraviolet difference spectroscopy (Hille et al., 1981). Conditions: 50 mM acetate, 0.1 M $CaCl_2$, and 0.1 M NaCl, pH 6.0, 25 °C. [d] Determined at pH 6.0 and 30 °C as described by Volwerk et al. (1974). Conditions: 0.1 M sodium cacodylate, 0.1 M NaCl; [enzyme], 20 μM.

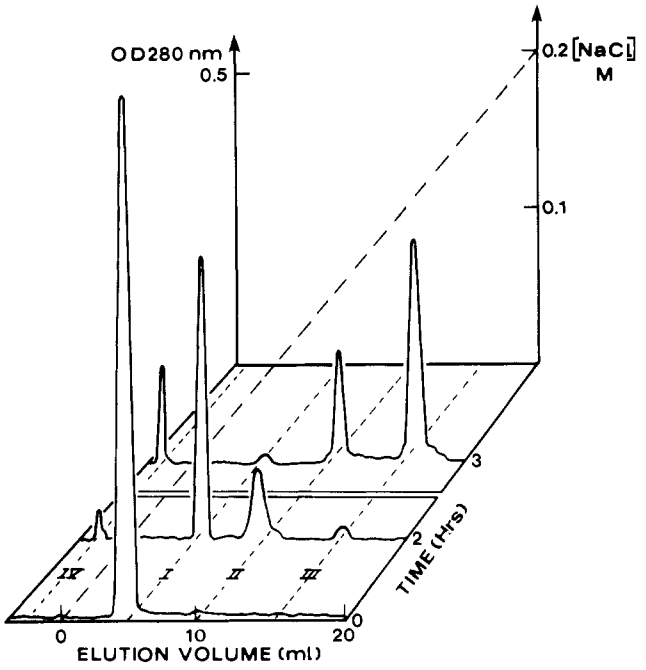

FIGURE 2: Elution patterns of porcine ε-NH_2-Lys-AMPA upon incubation with TPCK–trypsin obtained by FPLC (Mono Q column HR-5/5, equilibrated with 5 mM Tris, pH 7.5, and developed with 20 mL of a linear salt gradient reaching 0.2 M NaCl). The column was run at room temperature. (For further details see Materials and Methods.) Peak I: ε-NH_2-Lys-AMPA. Peak II: des(Ala^1–Arg^6)-ε-NH_2-Lys-AMPA. Peak III: des(Ala^1–Arg^6)$\Delta^{116,117}$ε-NH_2-Lys-AMPA (see Results and Discussion). Peak IV: Ala^1–Arg^6.

sequence Asn-Leu-Asp, however, occurs twice in the porcine enzyme, namely, Asn^{57}-Leu^{58}-Asp^{59} and Asn^{117}-Leu^{118}-Asp^{119}. In both cases these sequences are preceded by a Lys residue. In order to differentiate between tryptic cleavage after Lys^{56} or Lys^{116}, fraction III was oxidized with performic acid and the resulting peptide mixture eluted on Sephadex G-25. A low molecular weight peptide could be isolated in high yield analyzing correctly for the C-terminal octapeptide of porcine phospholipase A_2: Asn-Leu-Asp-Thr-Lys-Lys-Tyr-Cys. This latter result proves that the single free ε-NH_2 group in AMPREC-8 (fraction I) belongs to Lys^{116}.

Preparation of [Monoacyl-Lys^{116}]-ε-Amidinated Phospholipases A_2. The N-terminal amino acid in AMPREC-8 is pyroglutamic acid, so the only free NH_2 group in the protein is the ε-NH_2 function of Lys^{116}. Reaction of AMPREC-8 with various activated fatty acids yields the monoacylated zymogens, which after tryptic activation give the active phospholipases. The above-described reactions are schematically given in Figure 1, and the properties of the various mutant enzymes are compiled in Table I.

Discussion

Recently, Van Oort et al. (1985) showed that porcine pancreatic PLA in the presence of submicellar concentrations of anionic substrates aggregates into high molecular weight complexes containing several enzyme molecules and 30–40 substrate monomers. In these premicellar aggregates the enzyme is highly active. The zymogen forms similar aggregates; however, the proenzyme does not show enhanced catalytic activity. Upon amidination of the zymogen, present in a high molecular weight aggregate with 1,2-dioctanoyl-*sn*-glycerol 3-sulfate, about eight of the nine lysine residues were amidinated. This observation suggested the possibility that in the zymogen–detergent complex one lysine side chain was protected against modification. Purification of the crude amidinated zymogen mixture first required removal of the anionic substrate that was produced by a short incubation with a trace of native phospholipase A_2 in the presence of Ca^{2+}. PLA rapidly transforms the diacylglycerol sulfate into the lyso derivative and octanoic acid, products easily removable by dialysis. From the detergent-free protein mixture a pure zymogen could be isolated in high yield containing one free ε-NH_2 group per protein molecule. Trypsin treatment, followed by oxidative cleavage of the disulfide bonds and analysis of the resulting peptides, clearly demonstrated that the single free ε-NH_2 group in the zymogen belonged to Lys^{116}. This so-called AMPREC-8 was subsequently converted into a series of monoacylated zymogen mutants containing fatty acids of different chain lengths. Table I compares the kinetic properties, direct binding characteristics with micelles of a substrate

analogue, and inactivation kinetics of the various monoacylated PLA mutants.

The kinetic properties were measured by using dioctanoyllecithin as substrate and the more physiological mixed micellar system of egg lecithin and sodium deoxycholate. It is clear that full amidination of all nine ϵ-NH_2-lysine functions in porcine PLA (=AMPA) considerably lowers the specific activity in the egg-yolk assay, whereas k_{cat} on the short-chain dioctanoyllecithin drops only 25%. Much more remarkable is the high catalytic activity in both assays of ϵ-NH_2-Lys^{116}-AMPA. This mutant, having eight of nine lysine side chains amidinated, retains 50 and 91% of the activity of native PLA in the egg-yolk and short-chain lecithin assays, respectively. This result points to an important effect of the free ϵ-NH_2 group of Lys^{116} on catalytic efficiency. Blocking of this ϵ-NH_3^+ group by a short acetyl or maleyl chain reduces k_{cat} in the egg-yolk assay to a few percent of the value of that of the native enzyme. This effect cannot be attributed, however, to the loss of the positive charge at pH 8.0 on Lys^{116} because acylation with caprinic, lauric, palmitic, or oleic acid yields mutants with high specific activity.

With regard to the binding properties to the single-chain substrate analogue *cis*-9-octadecenylphosphocholine, it has been shown previously (De Araujo et al., 1979; Donné-Op den Kelder et al., 1981) that the pancreatic enzymes bind to the detergent micelles, forming well-defined lipid–protein complexes. The dissociation constants are given in Table I as NK_D. It seems evident that the presence of a covalently attached long-chain fatty acid on Lys^{116} considerably improves the binding of the mutants to the zwitterionic detergent. It has to be taken into account that the NK_D values are apparent dissociation constants, based on experimental saturation curves in which complexation is plotted as function of free detergent concentration. We know, however, that the interaction of pancreatic PLA with these neutral detergent micelles is not just an additive process in which one enzyme molecule remains bound to the micellar surface of the lipid micelle. After this initial interaction, the enzyme–micelle complex reorganizes itself, and the thermodynamically stable complex consists of two enzyme molecules attached to a lipid aggregate containting only about half the number of detergent monomers as compared to the original detergent micelle. In other words, upon binding to zwitterionic micelles, the pancreatic PLAs convert the detergent micelles into well-defined lipid–protein complexes containing N lipid monomers/enzyme molecule. From Table I it is clear that the differences in the apparent dissociation constant NK_D between the various PLA mutants are mainly caused by the number N of lipid monomers per enzyme molecule in the final aggregates. While the micellar dissociation constants K_D of the various mutants vary little, the "splitting power" of the acylated PLA mutants increases with the apolar character of the acyl chain and the resulting enzyme–detergent complexes contain progressively less lipid molecules. The same phenomenon has been reported for several highly penetrating snake venom phospholipases A_2 (Van Eijk et al., 1983).

Because of the above-discussed effects of an apolar acyl chain in the lipid-binding domain on the catalytic properties of PLA, it seemed of interest to investigate whether the kinetics of active-site inhibition by apolar halo ketones are changed by Lys^{116} modification. From Table I it is evident that the presence of a long fatty acyl chain on Lys^{116} does, indeed, provide an important protection against active-site-directed modification of His^{48}. This suggests that in the absence of substrate the acyl chain in the mutant PLAs blocks, at least partially, the entrance of the active site. The observation that the long acyl-chain mutants display high enzymatic activity indicates, however, that in the presence of substrates the localization of the acyl chain will be more directed to the lipid–water interface.

So far all chemical modifications involved the side chain of Lys^{116} that, according to the X-ray structure, is located in the lipid-binding domain of pancreatic PLA. It seemed of interest, however, to include similar specific chemical modifications of other lysine side chains. An attractive possibility seemed to be Lys^{10} located in the N-terminal α-helix of all pancreatic PLAs. From the amino acid sequences of the various pancreatic PLAs, the bovine enzyme was the protein of choice because this enzyme contains only one Met residue in position 8, allowing easy access to position 10. It cannot be denied that, in comparison with porcine PLA, the bovine enzyme has rather different kinetic and lipid-binding properties. As can be seen from Table I, in the egg-yolk assay the native bovine PLA is 8 times less active than the porcine enzyme, but according to the dioctanoyllecithin test, the catalytic machinery of the former enzyme is even better than that of the porcine PLA. Also the lipid-binding properties with the substrate analogue *cis*-9-octadecenylphosphocholine clearly show that the bovine PLA possesses a much lower lipid affinity than the porcine enzyme. As demonstrated before (Van Scharrenburg et al., 1981) these differences between the bovine and porcine PLA are largely caused by nature's variation in position six: Arg in the porcine, Asn in the bovine enzyme. Selective substitution of Asn^6 in the bovine PLA by Arg produces a bovine mutant PLA, which strongly resembles the porcine enzyme (bovine [Arg^6]AMPA vs porcine AMPA). Therefore, bovine [Arg^6]AMPA was transformed into a mutant containing N^{ϵ}-palmitoyl-Lys instead of Lys^{10}. As can be seen from Table I, the presence of a long-chain, apolar acyl group, now in position 10 of the lipid-binding domain, again marginally influences the kinetic properties of the enzyme but considerably reduces the number of detergent monomers in the lipid–protein complexes (12 vs 50). It is also evident that in the bovine enzyme the introduction of a covalently linked palmitic acid chain in position 10 considerably improves the lipid-binding affinity.

Finally, though we have no explanation for the extremely rapid inactivation kinetics of the bovine PLA by halo ketones as compared to the porcine enzyme, it is clear that a palmitic acid chain in position 10 of the bovine PLA provides efficient protection against His^{48} modification.

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Site-Specific ϵ-NH_2 Monoacylation of Pancreatic Phospholipase A_2. 2. Transformation of Soluble Phospholipase A_2 into a Highly Penetrating "Membrane-Bound" Form†

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ABSTRACT: Long-chain lecithins present in bilayer structures like vesicles or membranes are only very poor substrates for pancreatic phospholipases A_2. This is probably due to the fact that pancreatic phospholipases A_2 cannot penetrate into the densely packed bilayer structures. To improve the weak penetrating properties of pancreatic phospholipases A_2, we prepared and characterized a number of pancreatic phospholipase A_2 mutants that have various long acyl chains linked covalently to Lys^{116} in porcine and to Lys^{10} in bovine phospholipase A_2 [Van der Wiele, F. C., Atsma, W., Dijkman, R., Schreurs, A. M. M., Slotboom, A. J., & De Haas, G. H. (1988) *Biochemistry* (preceding paper in this issue)]. When monomolecular surface layers of L- and D-didecanoyllecithin were used, it was found that the introduction of caprinic, lauric, palmitic, and oleic acid at Lys^{116} in the porcine enzyme increases its penetrating power from 13 to about 17, 20, 32, and 22 dyn/cm, respectively, before long lag periods were obtained. Incorporation of a palmitoyl moiety at Lys^{10} in the bovine enzyme shifted the penetrating power from 11 to about 25 dyn/cm. Only the best penetrating mutant, viz., porcine phospholipase A_2 having a palmitoyl moiety at Lys^{116}, was able to cause complete leakage of 6-carboxyfluorescein entrapped in small unilamellar vesicles of egg lecithin under nonhydrolytic conditions. Similarly, only this latter palmitoylphospholipase A_2 completely hydrolyzed all lecithin in the outer monolayer of the human erythrocyte at a rate much faster than *Naja naja* phospholipase A_2, the most powerful penetrating snake venom enzyme presently known.

Long-chain zwitterionic diacylphospholipids such as lecithin that form densely packed bilayer structures in water have been known for a long time to be very poor substrates for pancreatic phospholipase A_2 (Van Deenen & De Haas, 1963; De Haas et al., 1968). Only after transformation of the bilayer packing into mixed-micellar systems with the aid of detergents like sodium deoxycholate do long-chain phosphatidylcholines become good substrates for pancreatic phospholipase A_2. After the first reports of Op den Kamp et al. (1974, 1975) it is now well accepted, however, that small structural irregularities in the bilayer packing, induced by lipid-phase transition, also render the lecithin substrate liable to phospholipase A_2 hy-

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