Structural Importance of the Amino-Terminal Residue of Pancreatic Phospholipase A_2†

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ABSTRACT: To study the structural importance of the NH_2-terminal Ala^1 residue of pancreatic phospholipase A_2, several mutants were prepared by a stepwise semisynthetic approach. ^{13}C NMR spectroscopy of 90%-enriched [[3-^{13}C]Ala^1]phospholipases A_2 shows the pK values of the α-NH_3^+ groups of porcine enzyme, porcine isoenzyme, bovine enzyme, and equine enzyme to be 8.4, 8.8, 8.9, and 8.8, respectively. A group titrating with a pK of ~6.3, present only in the porcine and equine phospholipases A_2, presumably originating from Glu^{71}, disappears in the presence of 0.2 M Ca^{2+}, while the pK values of their α-NH_3^+ groups shift to 9.3 and 9.0, respectively. No such effects were observed for the porcine isoenzyme and bovine enzyme, which lack an acidic side chain in position 71. It can thus be concluded that the equine phospholipase A_2, like the porcine enzyme, possesses in addition to the catalytic Ca^{2+} binding site also a second, low-affinity Ca^{2+} binding site, which is not present in the porcine isophospholipase A_2 and bovine phospholipase A_2. From the titration behavior of the α-NH_3^+ group of equine and porcine [[3-^{13}C]-Ala^1]phospholipases A_2 in the presence of micelles of *n*-hexadecylphosphocholine, it seems very likely that at alkaline pH the equine phospholipase A_2, like the porcine enzyme, requires the second Ca^{2+} ion for optimal binding to neutral lipid–water interfaces. Semisynthetic porcine phospholipase A_2 analogues in which the position of the α-NH_3^+ group is varied have lost their affinity toward neutral lipid–water interfaces and consequently their catalytic activity on *micellar* substrates. Most of these phospholipase A_2 analogues retain, however, some of their enzymatic activity on *monomeric* substrate. Substitution of the side chain of Ala^1 in porcine phospholipase A_2 by hydrophobic side chains abolishes almost all activity due to the loss of affinity for neutral lipid–water interfaces. In contrast, NH_2-terminal residues having more polar side chains affect only slightly phospholipase A_2 activity. Compared to the native enzyme, these latter phospholipase A_2 analogues show an increased penetration capacity for monolayers of 1,2-didecanoyl-*sn*-glycero-3-phosphocholine. Probably, the interaction of hydrophobic amino acid residues at the 1-position with other hydrophobic side chains present in their vicinity prevents the correct positioning of the α-NH_3^+ group, thereby leading to loss of catalytic activity. Finally, [Gly^1]phospholipase A_2 possesses an intermediate position between native [L-Ala^1]-phospholipase A_2 and [D-Ala^1]phospholipase A_2 as concluded from kinetic, direct binding, ^1H NMR photo-CIDNP, fluorescence, and ^{13}C NMR studies.

Phospholipase A_2 (EC 3.1.1.4) catalyzes in the presence of Ca^{2+} ions the hydrolysis of the ester bond at the C_2 position of 3-*sn*-phosphoglycerides (van Deenen & de Haas, 1964). In the mammalian pancreas the enzyme is synthesized as a precursor, called prophospholipase A_2. Just as the precursors of proteolytic enzymes (Neurath & Walsh, 1976), the zymogens of pancreatic phospholipases A_2 differ from the active enzymes by the presence of short activation peptides covalently linked to the N-terminal amino acids of the enzymes. In the duodenum, the prophospholipase A_2 is converted into the active enzyme by liberation of the N-terminal heptapeptide upon tryptic cleavage of the Arg^{-1}–Ala^1 bond (de Haas et al., 1968, 1969). During this tryptic activation, there is a conformational change in the protein, which can easily be monitored by fluorescence spectroscopy of the unique Trp at position 3 (Abita et al., 1972). According to proton titration studies, the newly generated α-NH_3^+ group of the N-terminal Ala^1 of the porcine enzyme has a pK of 8.3 (Janssen et al., 1972). Both the proenzyme and the active enzyme have low, comparable activities toward monomeric substrates, indicating the presence of a functionally active site in the zymogen as well (Pieterson et al., 1974; Volwerk et al., 1979). Only the active enzyme, however, is able to display its full enzymatic activity when present in substrate–protein complexes (Pieterson et al., 1974; Hille et al., 1983). For the porcine pancreatic enzyme, it has been shown that the affinity for *neutral* lipid–water interfaces is critically dependent on the presence of an α-ammonium group (Slotboom & de Haas, 1975; van Dam-Mieras et al., 1975). Semisynthetic studies clearly showed that the amino acid residues Leu^2, Trp^3, and Arg^6 located in the N-terminal part of the pancreatic enzymes are important for optimal lipid binding properties (van Scharrenburg et al., 1981, 1983). Modification studies revealed that also Tyr^{69}, Leu^{19} (=Tyr^{19} in equine phospholipase A_2), and Met^{20} are involved in the interaction of the porcine enzyme with lipid–water interfaces (Meijer et al., 1979; Meijer, 1979). Verheij et al. (1981) used the transamination procedure (Dixon & Fields, 1972) to selectively modify the α-amino group of three snake venom phospholipases A_2. These modified snake venom phospholipases A_2, having an α-keto acid instead of an α-amino group, still bind, though with decreased affinity, to neutral lipid–water interfaces but do not hydrolyze these micellar substrates. Transamination of the snake venom phospholipase A_2 from *Naja naja oxiana* leads to the loss of enzymatic activity toward organized substrates (Aianyan et al., 1982). The X-ray structure of both the bovine (1.7 Å) and the porcine (2.6 Å) enzymes showed that the α-amino group is buried in the protein and fixed to other protein atoms via H bridges (Dijkstra et al., 1981, 1983). In both cases, hydrogen bonds

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exist between the α-NH_3^+ function, the $O^{\epsilon 1}$ of the nearly invariant Gln,[4] and a fully enclosed H_2O molecule, which is also in contact with the active site residues Asp^{99}, His^{48}, and Asp^{49}·Ca^{2+} (Verheij et al., 1980). A recent semisynthetic study revealed, however, that in contrast to the bovine enzyme the porcine phospholipase A_2 does not require the $O^{\epsilon 1}$ of a Glx at position 4 for enzymatic activity or affinity toward neutral aggregated substrates (van Scharrenburg et al., 1984). This result demonstrates that even between these two very homologous pancreatic enzymes striking differences exist in the microenvironment of the essential α-amino group. The present paper describes a comparative study of the titration behavior of the N-terminal Ala of four different [3-^{13}C]Ala^1-labeled pancreatic enzymes, prepared by a stepwise semisynthetic approach (Slotboom & de Haas, 1975; DiMarchi et al., 1979). The titration behavior of the α-NH_3^+ group of labeled equine phospholipase A_2 in the presence of micellar *n*-hexadecylphosphocholine is also investigated. Until now, all studies concerning the N-terminus of phospholipase were focused on the α-amino group. In this paper, also the influence of the side chain of the N-terminal amino acid residue on the enzymatic properties of phospholipase A_2 has been investigated. For this purpose, the N-terminal Ala^1 of the best characterized pancreatic enzyme, viz., porcine phospholipase A_2, has been replaced by Nle, Leu, Phe, AibU, Gly, Asn, Asp, and Asp(OBu^t).[1,2] Special attention has been paid to the porcine [[1-^{13}C]Gly^1]enzyme, a fully active mutant displaying a clearly different titration behavior compared to that of the native enzyme.

Experimental Procedures

Materials

Porcine prophospholipase A_2, porcine isoprophospholipase A_2, bovine prophospholipase A_2, and equine pancreatic prophospholipase A_2 were purified and converted into their corresponding phospholipases A_2 as described previously [see Nieuwenhuizen et al. (1974), van Wezel & de Haas (1975), Dutilh et al. (1975), and Evenberg et al. (1977), respectively]. The pancreatic prophospholipases A_2 were ϵ-amidinated (AMPRECs), purified, and converted into the corresponding ϵ-amidinated phospholipases A_2 (AMPAs) as described (Slotboom & de Haas, 1975; van Scharrenburg et al., 1981). *N-t*-Boc-Asn-ONp, *N-t*-Boc-Asp(OBu^t), and *N-t*-Boc-Nle-diCHA salt were purchased from Sigma. α-Aminoisobutyric acid (AibU) was from Aldrich (Belgium). *N-tert*-Butyloxycarbonyl-*N*-hydroxysuccinimide esters of Leu and Phe were obtained from Fluka A.G. (Switzerland). [3-^{13}C]Ala and [1-^{13}C]Gly (90% enriched) were purchased from Merck Sharp & Dohme (Canada). *N-tert*-Butyloxycarbonyl-*N*-methylalanine was purchased from Bachem (Switzerland). *n*-Alkylphosphocholines were prepared as described before (van Dam-Mieras et al., 1975). 1,2-Didecanoyl- and 1,2-dioctanoyl-*sn*-glycero-3-phosphocholines were prepared as described by Cubero Robles & van den Berg (1969). [2,3-Bis(hexanoylthio)propyl]phosphocholine was synthesized as described by Volwerk et al. (1979). Porcine des-Ala^1-AMPA, des-Ala^1,Leu^2-AMPA, des-Ala^1,Leu^2,Trp^3-AMPA, des-Ala^1–Arg^6-AMPA, [[3-^{13}C]Ala^1]AMPA, [[1-^{13}C]Gly^1]AMPA, [[1-^{13}C]Gly^{-1}]AMPA, [[3-^{13}C]-D-Ala^1]AMPA, [Asp^1]AMPA, [Asp^1(OBu^t)]AMPA, [Phe^1]AMPA, [Nle^1]AMPA, [*N*-Me-Ala^1]AMPA, [Leu^1]AMPA, and [AibU^1]AMPA were prepared and purified according to the procedures described by Slotboom & de Haas (1975). Porcine [Asn^1]AMPA was prepared by coupling of *N-t*-Boc-Asn *o*-nitrophenyl ester to des-Ala^1-AMPA. Bovine and equine [[3-^{13}C]Ala^1]AMPAs and porcine [[3-^{13}C]Ala^1]-iso-AMPA were prepared as described for the porcine AMPA analogues (Slotboom et al., 1977; Jansen et al., 1979). These enzymes were purified on CM- and DEAE-cellulose columns as described for the native phospholipases A_2 (Dutilh et al., 1975; Evenberg et al., 1977; van Wezel & de Haas, 1975). All the AMPA analogues were homogeneous on slab gel electrophoresis at pH 9.6, and dansylation showed in all cases exclusively the expected N-terminal amino acid. The kinetic parameters of the different [[3-^{13}C]Ala^1]AMPAs were found to be identical within the experimental error with those of the corresponding native AMPAs. All other chemicals used were of the highest purity available.

Methods

Amino acid analyses were performed by the method of Spackman et al. (1958), on a Kontron liquid chromatograph Liquimat III. Samples were hydrolyzed for 24 h at 110 °C in evacuated, sealed tubes with 5.8 N HCl. Tryptophan was determined similarly by addition of 4% of thioglycollic acid as described by Matsubara & Sasaki (1969). Protein concentrations were determined from the absorbances at 280 nm by using an $E_{1cm}(1\%)$ of 13.0 for the ϵ-amidinated porcine and bovine phospholipase A_2 analogues and porcine ϵ-amidinated isophospholipase analogues, except for porcine des-Ala^1–Trp^3- and des-Ala^1–Arg^6-AMPA, for which a value of 8.8 was used. Calculations of the protein concentration of ϵ-amidinated equine phospholipase A_2 and porcine prophospholipase A_2 were done with an $E_{1cm}(1\%) = 12.3$, whereas the concentration of equine ϵ-amidinated prophospholipase A_2 was calculated with an $E_{1cm}(1\%) = 11.8$. Slab gel electrophoresis was performed for 1 h at 50 mA on 7.5% polyacrylamide gels at pH 9.6 with the apparatus as described by Studier (1973). The N-terminal amino acids were determined by dansylation according to Gray (1972). The amino acids [3-^{13}C]Ala, [1-^{13}C]Gly, [3-^{13}C]-D-Ala, and α-aminoisobutyric acid were reacted with di-*tert*-butyl dicarbonate according to the procedure of Moroder et al. (1976), to yield their N-*t*-Boc-protected derivatives. N-Hydroxysuccinimide esters of the N-*t*-Boc-protected [3-^{13}C]Ala, [3-^{13}C]-D-Ala, [1-^{13}C]Gly, AibU, Nle, and Asp(OBu^t) were prepared by using the procedure of Anderson et al. (1964). The purity of all N-*t*-Boc-protected amino acid *N*-hydroxysuccinimide esters was checked by thin-layer chromatography on silicic acid with $CHCl_3$–MeOH–concentrated NH_3 (85:20:2 v/v). The undesired presence of [(succinimidooxy)carbonyl]-β-alanine *N*-hydroxysuccinimide ester in the N-*t*-Boc-protected amino acid *N*-hydroxysuccinimide ester preparations (Gross & Bilk, 1968a,b) was checked. The usual 6 N HCl hydrolysates of all the N-*t*-Boc-protected amino acid N-hydroxysuccinimide esters used were subjected to amino acid analyses, and the β-alanine content in each sample was determined. When β-alanine was found to be present, the N-*t*-Boc-protected amino acid *N*-hydroxysuccinimide esters were further purified by crystallization and/or SiO_2 chromatography with $CHCl_3$–MeOH mixtures until no β-alanine

[1] Abbreviations: PLA, phospholipase A_2; AMPREC, ϵ-amidinated prophospholipase A_2; AMPA, ϵ-amidinated phospholipase A_2; *t*-Boc, *tert*-butyloxycarbonyl; CIDNP, chemically induced dynamic nuclear polarization; [^{2}H]TSP, sodium trimethylsilyl[2,2,3,3-$^2H_4$]propionate; NMR, nuclear magnetic resonance; ppm, parts per million; CHA, cyclohexylammonium; ONp, *o*-nitrophenyl; Bu^t, *tert*-butyl; C_{16}PN, *n*-hexadecylphosphocholine; C_{18}PN, *n*-octadecylphosphocholine; CPK model, Corey–Pauling–Koltun space-filling molecular model; Me, methyl; AibU, α-aminoisobutyric acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; pH*, uncorrected pH meter readings in ^2H_2O.

[2] All amino acid residues except Gly and AibU have the L configuration unless stated otherwise.

was present in the hydrolysates.

NMR Spectroscopy. All ^{13}C NMR and ^1H photo-CIDNP NMR spectra were obtained with a Bruker HX-360 spectrometer at the S.O.N.-Facility in Groningen, The Netherlands. Photo-CIDNP spectra (Kaptein, 1978) were recorded at 360 MHz as previously described (Jansen et al., 1978; Egmond et al., 1980), with 3-(carboxymethyl)lumiflavin as the dye. Chemical shifts were calculated relative to TSP, with dioxane as internal standard. All ^{13}C spectra were recorded at 90.5 MHz with quadrupole detection. Two-thousand transients were accumulated with a repetition time of 0.82 s and a spectral width of 10000 Hz. An exponential multiplication of the free-induction decay corresponding to a line broadening of 6 Hz was applied to improve the signal to noise ratio. Chemical shifts were measured at 25 °C from benzene for 1.0–2.0 mM protein solutions, dissolved in 50 mM Hepes or Tris buffer, containing 10% D_2O. Adjustment of the pH was performed with 2 M NaOH or HCl in 10% D_2O. In the plots of the ^{13}C resonance as function of the pH (Figures 1 and 2), the drawn curves have been calculated by using an iterative least-squares fitting procedure, which was based on the program of Fletcher & Powell (1963), with as adjustable parameters the upper and lower chemical shift levels, the pK, and the Hill coefficient of the titration curve.

The enzymatic activities of the pancreatic (ϵ-amidinated) phospholipases A_2 and their semisynthetic analogues were routinely determined by using the titrimetric assay procedure with egg yolk lecithin as substrate (Nieuwenhuizen et al., 1974). In contrast to this procedure, 2-fold higher Ca^{2+} and 1.5-fold higher sodium deoxycholate concentrations were used. Kinetic measurements with micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine and monomeric [2,3-bis(hexanoylthio)propyl]phosphocholine were performed as described before [see de Haas et al. (1971) and Volwerk et al. (1979), respectively]. Direct binding studies were carried out by using ultraviolet difference spectroscopy as previously described by Hille et al. (1981). The ultraviolet difference spectra were recorded at 25 °C on an Aminco DW-2a spectrophotometer equipped with a MIDAN data analyzer, coupled to an Apple II desk-top computer. Fluorescence spectroscopy was done with a Perkin-Elmer MPF3 spectrofluorometer as described by van Dam-Mieras et al. (1975).

Monolayer Experiments. The zero-order trough with two compartments and the surface barostat technique used to measure substrate hydrolysis were identical with those described by Verger & de Haas (1973). Calculation of the induction time (τ) was performed as previously described by Verger et al. (1973). Surface pressure π was measured by the Wilhelmy plate method, with a thin platinum plate (perimeter 3.94 cm) attached to a Beckman R II C Model LM 600 microbalance.

Results

The lysine residues in porcine isoprophospholipase A_2 and porcine, bovine, and equine prophospholipases A_2 can be converted into ϵ-acetimidolysines by treatment of these precursors with methyl acetimidate. The ϵ-amidinated phospholipases A_2 (AMPAs) possessing only one free amino group, viz. the α-NH_2 of Ala^1, are obtained by limited tryptic treatment of the ϵ-amidinated precursors (AMPRECs). The bovine, equine, and porcine AMPAs as well as porcine iso-AMPA possess enzymatic activities of about 70–80% of the native PLAs on micellar short-chain lecithins: V_{max} values of the various AMPAs on L-dioctanoyllecithin are 2300, 4500, 2000, and 2000 μequiv min^{-1} (mg of protein)$^{-1}$, respectively. The Ca^{2+} and substrate binding properties of the AMPAs as well as their kinetic behavior on monolayers are almost the same as those for the native enzymes (van Scharrenburg et al., 1981; Pattus et al., 1979a). This selective blocking of the ϵ-NH_2 groups permits specific substitution or labeling of amino acid residues at the N-terminus by a preparative Edman procedure and subsequent replacement with activated N-protected amino acid residues. The feasibility of the procedures applied during this semisynthetic approach was already shown by Slotboom & de Haas (1975).

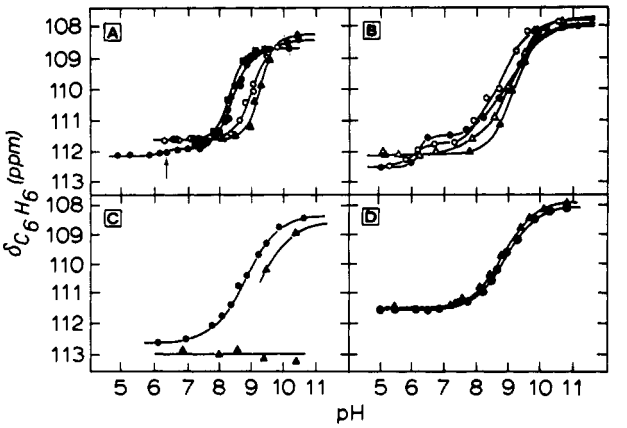

FIGURE 1: Plots of the chemical shifts (ppm from C_6H_6) of the 90%-enriched ^{13}C nuclei in the different pancreatic [[3-^{13}C]Ala^1]-AMPAs as a function of the pH at various Ca^{2+} concentrations: (A) (porcine AMPA) no Ca^{2+} (●), 5 mM Ca^{2+} (■), 50 mM Ca^{2+} (○), and 200 mM Ca^{2+} (▲); (B) (equine AMPA) no Ca^{2+} (●), 5 mM Ca^{2+} (○), 50 mM Ca^{2+} (△), and 200 mM Ca^{2+} (▲); (C) (porcine iso-AMPA) no Ca^{2+} (●) and 200 mM Ca^{2+} (▲); (D) (bovine AMPA) no Ca^{2+} (●) and 200 mM Ca^{2+} (▲).

^{13}C NMR Spectroscopy. (A) Titration Behavior of Porcine, Equine, and Bovine [[3-^{13}C]Ala^1]AMPAs and Porcine Iso-[[3-^{13}C]Ala^1]AMPA. Replacement of the N-terminal amino acid Ala^1 of pancreatic AMPA by a 90%-enriched [3-^{13}C]Ala^1 allows a direct determination of the pK value of the α-NH_3^+ function under dynamic conditions. The pH titration curves of porcine, equine, and bovine [[3-^{13}C]Ala^1]AMPAs and porcine iso-[[3-^{13}C]AMPA are shown in Figure 1. The α-amino group of porcine AMPA appears to have a pK of 8.4 in the absence of Ca^{2+} ions, which is rather high as compared to the pK value of 7.8, measured for the α-amino group of the N-terminal hexapeptide Ala^1–Arg^6 of porcine AMPA. Furthermore, the curve in Figure 1A shows the titration of a group with a pK of 6.3 ± 0.2 and a chemical shift difference of 15 Hz (0.17 ppm). Addition of 5 mM Ca^{2+}, enough to saturate the catalytic Ca^{2+} binding site of porcine AMPA at basic pH values, has hardly any effect on the pK of the α-NH_3^+ function. In the presence of 200 mM Ca^{2+} ions, the pK of the N-terminus of porcine AMPA shifts to 9.3, and the group titrating with a pK value of 6.3 is no longer observed. Addition of 250 mM NaCl instead of 200 mM Ca^{2+} has no influence on the pK of the N-terminus, and the group titrating with a pK of 6.3 does not disappear. These effects of Ca^{2+} ions on the titration behavior of the N-terminus, together with other evidence (Slotboom et al., 1978; Pattus et al., 1979b), indicate the presence of a second calcium binding site in this porcine enzyme, which influences the N-terminal region. The equine [[3-^{13}C]Ala^1]AMPA shows a titration behavior (Figure 1B) comparable to that of the porcine enzyme. As compared to the porcine enzyme, the equine AMPA possesses a more basic α-amino group with a pK of 8.8 in the absence of Ca^{2+} ions. Upon saturation of the first catalytic Ca^{2+} binding site with 5 mM Ca^{2+}, there is a small decrease in pK of the α-NH_3^+ group to 8.6. The presence of 200 mM Ca^{2+} increases the pK to 9.0. In the absence of Ca^{2+}, there is again a group titrating

Table I: k_{cat}/K_M (s^{-1} M^{-1}) of Hydrolysis of Monomeric Bis(thiohexanoyl)lecithin by Native and N-Terminally Modified Porcine Pancreatic ϵ-Amidinated Phospholipases A_2

AMPA analogues	monomeric bis(thiohexanoyl)lecithin k_{cat}/K_M (s^{-1} M^{-1})[a]	AMPA analogues	monomeric bis(thiohexanoyl)lecithin k_{cat}/K_M (s^{-1} M^{-1})[a]
"native" AMPA	555	des-Ala^1	150
AMPREC	274	des-Ala^{1}Leu^2	93
D-Ala^1	122	des-Ala^1–Trp^3	nd[b]
N-Me-Ala^1	373	des-Ala^1–Arg^6	nd

[a] Determined at pH 8.5 and 25 °C as described by Volwerk et al. (1979). [b] Not detectable.

with a p*K* of 6.3, causing a larger chemical shift of the [3-^{13}C]Ala^1 resonance in the equine enzyme compared to the porcine enzyme. At high Ca^{2+} concentrations this group titrating between pH 5.5 and 7.0 disappears completely. This result points strongly to a relation between a group with p*K* = 6.3 and the binding of a second Ca^{2+} ion with low affinity. As shown in Figure 1C,D, both porcine iso-AMPA and bovine AMPA have rather basic α-NH_3^+ functions, comparable to that of equine AMPA, with p*K* values of 8.8 and 8.9, respectively. In contrast to the equine AMPA, the p*K* values of the α-NH_3^+ functions of bovine AMPA and porcine iso-AMPA are not influenced by the addition of 5 mM Ca^{2+} (data not shown). Furthermore, the group titrating with a p*K* of 6.3 is absent in the pH titration curves of porcine iso-[[3-^{13}C]Ala^1]AMPA and bovine [[3-^{13}C]Ala^1]AMPA. This result is already an indication that both the porcine iso-AMPA and bovine AMPA do not possess the group with a p*K* of approximately 6.3 involved in the binding of a second Ca^{2+} ion, as found for the porcine and equine AMPAs. The p*K* value of the α-NH_3^+ group of bovine AMPA is hardly influenced by the addition of 200 mM Ca^{2+}. In contrast, the titration behavior of the α-NH_3 group of Ala^1 of porcine iso-AMPA changes quite drastically in the presence of high Ca^{2+} concentrations. In the presence of 200 mM Ca^{2+}, two ^{13}C resonances of the Ala^1 of porcine iso-AMPA are visible at basic pH (Figure 1C). One ^{13}C resonance is present at a fixed chemical shift of 113 ppm. The other resonance, which titrates, appears above pH 9 and grows in intensity with increasing pH at the expense of the ^{13}C resonance at 113 ppm. Such a titration behavior is due to the presence of two populations of α-NH_3 groups in slow exchange on the NMR time scale (vide infra).

(B) Titration Behavior of Equine [[3-^{13}C]Ala^1]AMPA in the Presence of the Substrate Analogue n-Hexadecylphosphocholine (C_{16}PN). Upon addition of the micellar substrate analogue C_{16}PN to the equine ^{13}C-labeled AMPA, the ^{13}C-signal of the methyl group of Ala^1 disappears completely at acidic and neutral pH (Figure 2). At these pH values, the enzyme binds to the lipid–water interfaces, which most likely causes a severe broadening of the ^{13}C resonances, due to motional restrictions of the methyl group of Ala^1 in the enzyme–micelle complex. Figure 2 shows that the [3-^{13}C]Ala^1 resonance of the equine AMPA appears again above pH 7.0 before the deprotonation of the α-NH_3^+ function starts. The 3-^{13}C resonance of the labeled porcine AMPA shows the same behavior in the presence of C_{16}PN micelles as the equine AMPA (data not shown). Recent binding and kinetic studies on the porcine and equine PLAs (Donné-Op den Kelder et al., 1981, 1983) revealed that above pH 6.25 there is a large decrease in the affinity toward neutral lipid–water interfaces and, as a consequence, a drop in the enzymatic activity of these enzymes on aggregated substrates at low Ca^{2+} concentrations. Addition of 200 mM Ca^{2+} at basic pH values, which means saturation of the second, low-affinity Ca^{2+} binding site, restores the affinity of both porcine and equine AMPA for C_{16}PN micelles.

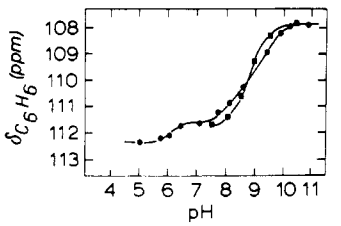

FIGURE 2: Plots of the chemical shifts (ppm from C_6H_6) of the 90%-enriched nucleus of equine [[3-^{13}C]Ala^1]AMPA as a function of pH: (●) no Ca^{2+}; (■) no Ca^{2+} + 100 mM C_{16}PN.

N-Terminally Modified Porcine ϵ-Amidinated Phospholipase A_2 Analogues. (A) Modifications and Substitutions That Affect the α-Amino Group of Porcine AMPA. The various N-terminally modified porcine AMPA derivatives, mentioned in Table I, possess only residual enzymatic activities toward *micellar* L-dioctanoyllecithin of about 5% compared to native AMPA. The α-NH_3^+ function of pancreatic phospholipase A_2 is essential for the optimal enzymatic activity of the enzyme on micellar substrates. These N-terminally modified or shortened AMPA analogues, including AMPREC, possess furthermore no detectable binding to neutral micelles of the substrate analogue *n*-octadecylphosphocholine (C_{18}PN), indicating the absence of a functional lipid binding domain.

As can be seen from Table I, most of these porcine AMPA analogues, of which the α-amino group is affected, are still able to hydrolyze *monomeric* substrate. The active sites of most of these porcine analogues seem to be rather intact. The [*N*-Me-Ala^1]AMPA, for example, possesses over 60% of the k_{cat}/K_M value of the native enzyme on the monomeric substrate. Upon shortening of the N-terminus of porcine AMPA by more than two amino acid residues, no enzymatic activity on monomeric substrate could be detected. The effect of the removal of amino acid residues from the N-terminus was also studied by laser photo-CIDNP ^{1}H NMR (Egmond et al., 1980). The photo-CIDNP ^{1}H NMR technique monitors only the resonances originating from aromatic residues, like Tyr, Trp, and His, which are exposed to the solvent. Figure 3 shows the CIDNP spectra obtained with the porcine AMPA and the N-terminally shortened analogues. The CIDNP spectrum of native porcine AMPA (Figure 3A) shows absorptive signals at approximately 7.3–7.7 ppm arising from Trp^3 aromatic resonances. The strong signal at 6.9 ppm is assigned to the H-3 and -5 protons of Tyr^{69} and Tyr^{123}, which overlap. Removal of the N-terminal Ala^1 induces a shift of H-3 and -5 proton resonances of Tyr^{69} downfield of approximately 0.2 ppm (Figure 3B). This downfield shift indicates a change in the conformation of the des-Ala^1-AMPA in the region of Tyr^{69}, which is part of the lipid binding domain (Dijkstra et al., 1984). The Trp^3 resonances of des-Ala^1-AMPA are rather weak compared to those of the native enzyme. The des-Ala^1–Trp^3-AMPA spectrum (Figure 3C) of course does not show Trp^3 resonances. Furthermore, in the CIDNP spectrum of this latter protein, a broad emissive signal of tyrosines is present at 6.8 ppm. This signal cannot only arise from Tyr^{69}, but other tyrosine residues, which become exposed upon removal of the

Table II: Kinetic Parameters and Micellar Binding Properties of Native and N-Terminally Modified Porcine ε-Amidinated Pancreatic Phospholipases A_2

AMPA analogues	egg yolk assay sp act.[a]	micellar dioctanoyllecithin V_{max}[b]	monomeric bis(thiohexanoyl)lecithin k_{cat}/K_M[c]	binding of micellar *n*-octadecylphosphocholine, NK_D (mM)[d]
native AMPA	300	2000	555	0.40
Asn^1	146	930	408	0.63
Asp^1	30	590	306	0.20
Asp^1OBu^t	25	770	360	0.07
$AibU^1$	nd[e]	90	506	nd
Phe^1	nd	110	71	nd
Nle^1	10	240	129	nd
Leu^1	nd	40	265	nd
Gly^1	100	1600	141	2.0

[a] Determined at pH 8.0 and 40 °C as described under Methods and expressed in microequiv min^{-1} (mg of protein)$^{-1}$. [b] Determined by titration of fatty acids liberated with 10.05 mM NaOH at pH 6.0 and 45 °C (50 mM $CaCl_2$, 10 mM acetate, and 0.1 M NaCl) as described previously (de Haas et al., 1971). Appropriate aliquots of enzyme solutions were added from stock solutions (0.2–0.4 mg of enzyme/ml) and when necessary diluted 50–100-fold in distilled H_2O containing bovine serum albumin (10 mg/mL) to avoid irreversible adsorption to the glass. Expressed in microequiv min^{-1} (mg of protein)$^{-1}$. [c] Determined at pH 8.5 and 25 °C as described by Volwerk et al. (1979) and expressed in s^{-1} M^{-1}. [d] Determined by ultraviolet difference spectroscopy (van Dam-Mieras et al., 1975). Conditions used were as follows: 50 mM acetate, 0.1 M NaCl, 0.1 M $CaCl_2$, pH 6.0, 25 °C. *N* represents the ratio of lipid to protein in the complex and NK_D can be considered as a measure for the effective affinity of the protein for the micelle (Hille et al., 1981; Donné-Op den Kelder et al., 1981). [e] Not detectable.

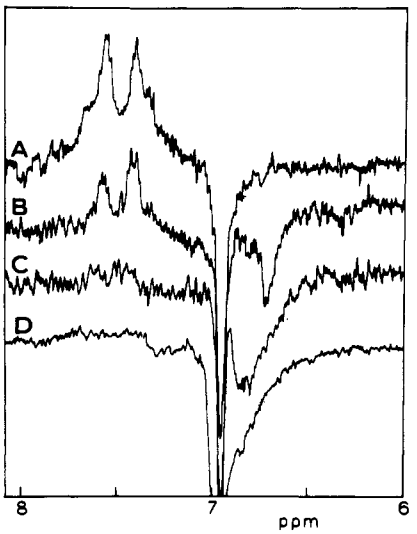

FIGURE 3: The 360-MHz photo-CIDNP ^1H NMR spectra of porcine AMPA (A), des-Ala^1-AMPA (B), des-Ala^1–Trp^3-AMPA (C), and des-Ala^1–Arg^6-AMPA (D).

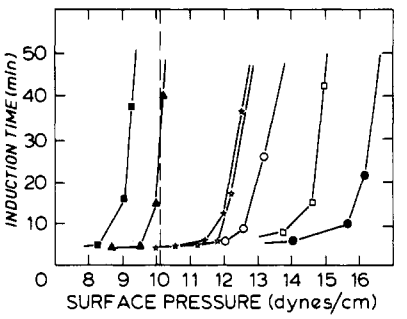

FIGURE 4: Influence of surface pressure of a 1,2-didecanoyl-*sn*-glycero-3-phosphocholine monolayer on the induction time of hydrolysis, by porcine AMPA (☆), [Gly^1]AMPA (solid star), [Asn^1]AMPA (□), [Asp^1]AMPA (○), [$Asp^1(OBu^t)$]AMPA (●), [Nle^1]AMPA (▲), and [Leu^1]AMPA (■). The dashed line (– –) marks the upper limit of the penetration capacity of porcine AMPA analogues lacking detectable affinity for micelles of $C_{18}PN$.

N-terminal tripeptide, must also contribute. The des-Ala^1–Arg^6-AMPA CIDNP difference spectrum (Figure 3D) indicates an even greater loss of native tertiary structure. A very strong emissive signal, arising from several tyrosine residues, is observed at 6.9 ppm, the random-coil position for tyrosine aromatic resonances.

(B) Substitution of Ala^1 of Porcine AMPA by Other Amino Acid Residues. Substitutions in the side chain of Ala^1 in porcine AMPA have different consequences for the kinetic and binding properties of the porcine analogues. The [Asn^1]-, [Asp^1]-, and [$Asp^1(OBu^t)$]AMPAs bind to micelles of the substrate analogue *n*-octadecylphosphocholine ($C_{18}PN$) with a dissociation constant in the same order as that found for "native" AMPA. The esterification of the side chain of Asp^1 with 2-methyl-2-propanol clearly improves the affinity toward lipid–water interfaces. The N-terminal substitutions of Ala^1 by Asn, Asp, or $Asp(OBu^t)$ cause, however, a decrease in catalytic activity in all three assay systems used, compared to native AMPA. This decrease in enzymatic activity is most pronounced in the egg yolk assay.

Compared to native AMPA, [Gly^1]AMPA has also a low specific activity in the egg yolk assay and a low second-order rate constant (k_{cat}/K_M) in the assay with monomeric substrate. On micelles of L-dioctanoyllecithin, however, [Gly^1]AMPA possesses 80% of the V_{max} value of native AMPA (Table II). The affinity of [Gly^1]AMPA for micelles of $C_{18}PN$ is decreased by a factor of 5 compared to native AMPA.

Substitution of the N-terminal Ala^1 by amino acid residues having hydrophobic side chains like Phe, Leu, or Nle or an additional methyl group, like AibU, completely eliminates the affinity toward neutral lipid–water interfaces. No binding of the [Phe^1]-, [Leu^1]-, [Nle^1]-, and [$AibU^1$]AMPA analogues to micelles of $C_{18}PN$ can be detected, and this result explains the very low enzymatic activities of these analogues on micellar substrates. All these analogues possess catalytic activity on the monomeric substrate [2,3-bis(hexanoylthio)propyl]-phosphocholine. The $AibU^1$ analogue shows even over 95% of the catalytic activity of native porcine AMPA. In contrast, [Phe^1]- and [Nle^1]AMPas have low k_{cat}/K_M values for the hydrolysis of the monomeric substrate as compared to the k_{cat}/K_M value found for the native enzyme.

As can be seen in Figure 4, the N-terminally substituted AMPA analogues, which have no detectable affinity for micelles of $C_{18}PN$, are only able to penetrate monolayers of 1,2-didecanoyl-*sn*-glycero-3-phosphocholine up to 10 dyn/cm, about 2 dyn/cm lower than the native porcine AMPA. The [Asn^1]-, [Asp^1]-, and [$Asp^1(OBu^t)$]AMPA analogues have significantly higher penetrating capacities of the lecithin monolayer than the native enzyme (Figure 4). Probably due to the presence of the *tert*-butyl moiety, [$Asp^1(OBu^t)$]AMPA is able to penetrate the lecithin monolayer, till a surface pressure of 16 dyn/cm, which is a striking improvement

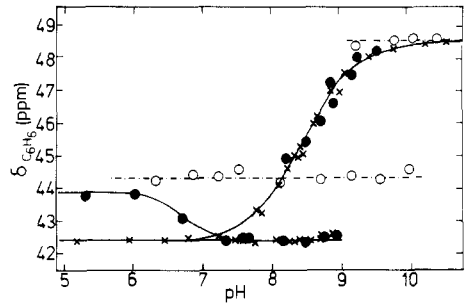

FIGURE 5: Plot of chemical shift of 90%-enriched ^{13}C nucleus in [[1-^{13}C]Gly^1]AMPA as a function of the pH in the absence (×) and presence (●) of 100 mM $C_{16}PN$ or with 100 mM $C_{16}PN$ + 60 mM Ca^{2+} (○).

compared with porcine AMPA.

^{13}C NMR and Photo-CIDNP ^1H NMR Studies of Porcine [[1-^{13}C]Gly^1]AMPA. pH titration of porcine [[1-^{13}C]Gly^1]-AMPA (Figure 5) shows a behavior quite different from that of the native enzyme (Figure 1A). The Gly at position 1 induces at pH values above 7.0 two ^{13}C resonances. Apparently, two different conformations are present.[3] In one conformation of [Gly^1]AMPA, the N-terminus shows a normal titration curve with a pK value of 8.4 of the α-NH_3^+ function, while in the other conformation the α-NH_3^+ function remains protonated to high pH values. The intensity of the ^{13}C resonance titrating with a pK of 8.4 increases at the expense of the ^{13}C resonance at the chemical shift of 42.5 ppm. Addition of small and high amounts of Ca^{2+} ions has hardly any effect on the pK values of the two states (data not shown). No group titrating with a pK of 6.3 affects the chemical shift of the ^{13}C resonance observed. These results could suggest the absence of a second Ca^{2+} binding site in this [Gly^1]AMPA in contrast to the native enzyme. Titrations of [[1-^{13}C]Gly^1]AMPA in the presence of micelles show, however, that the presence of Ca^{2+} ions is essential for binding to lipid–water interfaces at basic pH, just as found for the native enzyme. Addition of micelles of $C_{16}PN$ to [[1-^{13}C]Gly^1]AMPA in the absence of Ca^{2+} ions appears to have no effect on the titration curve above pH 7.0 (Figure 5). Below pH 6.0, the chemical shift of the ^{13}C resonance of [[1-^{13}C]Gly^1]AMPA becomes 44 ppm. It is known from direct binding studies that [Gly^1]AMPA binds to micelles in the absence of Ca^{2+} ions at acidic pH values. Apparently, the affinity toward micelles in the absence of Ca^{2+} ions is lost between pH 6.0 and 7.2, as found for the native porcine enzyme. Addition of 50 mM Ca^{2+} restores the affinity of [Gly^1]AMPA toward lipid–water interfaces at basic pH values, as can be concluded from the chemical shift of the [1-^{13}C]Gly^1 resonance (Figure 5). The pK of the amino group in the lipid–protein complex is increased to a value above 9.0 and, in addition, a slow exchange is observed between the α-NH_2 and the α-NH_3^+ forms. No gradual shift of the resonance positions is observed but a change in the intensity ratio of the two ^{13}C resonances. These results strongly suggest the presence of a second Ca^{2+} binding site regulating the lipid binding properties of [Gly^1]AMPA at basic pH as found for native porcine AMPA. In the absence of Ca^{2+} ions, a group with a pK value of about 6.3 is directly involved in the loss of affinity of [Gly^1]AMPA toward micelles. This group is presumably the same as that observed in the titration curves

[3] The inactive porcine [[1-^{13}C]Gly^{-1}]AMPA shows a normal titration behavior with a *single* pK of 8.4, which is decreased upon addition of Ca^{2+} ions or NaCl (data not shown). This result proves that the two conformations of [[1-^{13}C]Gly^1]AMPA above pH 7.0 are a consequence of the Gly at position 1 of the enzyme.

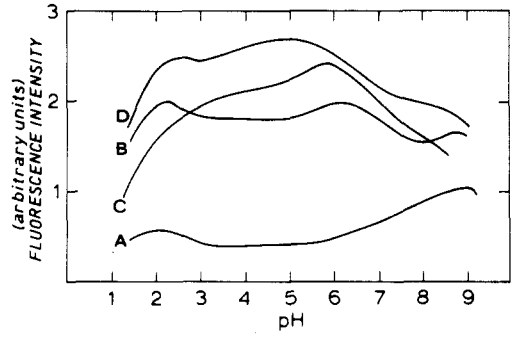

FIGURE 6: Fluorescence intensities (arbitrary units) of porcine AMPA (A), [Gly^1]AMPA (B), [D-Ala^1]AMPA (C), and AMPREC (D) as a function of the pH. Protein concentrations were 0.01 mM.

of both porcine and equine [[3-^{13}C]Ala^1]AMPAs (Figure 1A,B) in the absence of Ca^{2+} ions. It is surprising that the increase in the pK of the α-NH_3^+ group as well as the disappearance of the group titrating with a pK of 6.3 by Ca^{2+} ions can be observed only in the presence of lipids. The rather low pK value of the α-NH_3^+ function of [Gly^1]AMPA of 8.4 in the absence of lipids and the high pK value (9.0) in the presence of lipids and Ca^{2+} ions indicate the induction of a functional lipid binding domain upon addition of micelles, as was already suggested by Pattus et al. (1979c).

The Trp^3 present in porcine AMPREC, [D-Ala^1]AMPA, and [Gly^1]AMPA shows a high fluorescence intensity as compared to the Trp^3 of native porcine AMPA (Figure 6). These data suggest that the Trp^3 of [Gly^1]AMPA is in a less quenching environment like the Trp^3 of AMPREC and [D-Ala^1]AMPA as compared to the Trp^3 present in the native enzyme. On the basis of the pK value of the α-NH_3^+ function of 8.4 in the absence and presence of Ca^{2+} ions and the high fluorescence intensity of Trp^3, the N-terminal part of [Gly^1]AMPA has a conformation resembling that of the N-terminus of [D-Ala^1]AMPA and AMPREC. These latter two proteins have no affinity for neutral lipid–water interfaces in contrast to [Gly^1]AMPA.

The photo-CIDNP ^1H NMR spectra of Figure 7 indicate that the H-3 and -5 resonances of Tyr^{69} of both [Gly^1]AMPA and native AMPA overlap with the resonances of Tyr^{123} at approximately 6.9 ppm. In contrast, the H-3 and -5 resonances of Tyr^{69} of [D-Ala^1]AMPA and AMPREC are shifted 0.2 ppm downfield. This NMR result indicates, that under the conditions used (1 mM enzyme, pH* 5.5), porcine [Gly^1]AMPA possesses in the environment of Tyr^{69} a conformation comparable to that of the native enzyme, suggesting the presence of a functional lipid binding domain (Dijkstra et al., 1984).

Discussion

The pK values of the N-terminal α-NH_3^+ function of porcine and especially of porcine isophospholipase A_2 and bovine and equine phospholipases A_2 are rather high as compared with those of the N-terminal alanine in small peptides that range from 7.8 to 8.0 (Wilbur & Allerhand, 1977). In general, such a rise in pK is induced by interaction with a negative charge in a hydrophobic environment. The X-ray structure of both the porcine phospholipase A_2 (2.6 Å) (Dijkstra et al., 1983) and the bovine phospholipase A_2 (1.7 Å) (Dijkstra et al., 1981) showed that the α-NH_3^+ groups of Ala^1 are buried in the interior of the enzymes but they do not directly interact with carboxylates. Direct interaction exists, e.g., in the case of porcine phospholipase A_2, between the α-NH_3^+ group and the $O^{\epsilon 1}$ of Gln^4, the carbonyl oxygen of Glu^{71}, and a fully enclosed water molecule, which is also linked with Tyr^{52} and the active site residue Asp^{99} (Figure 8). Such

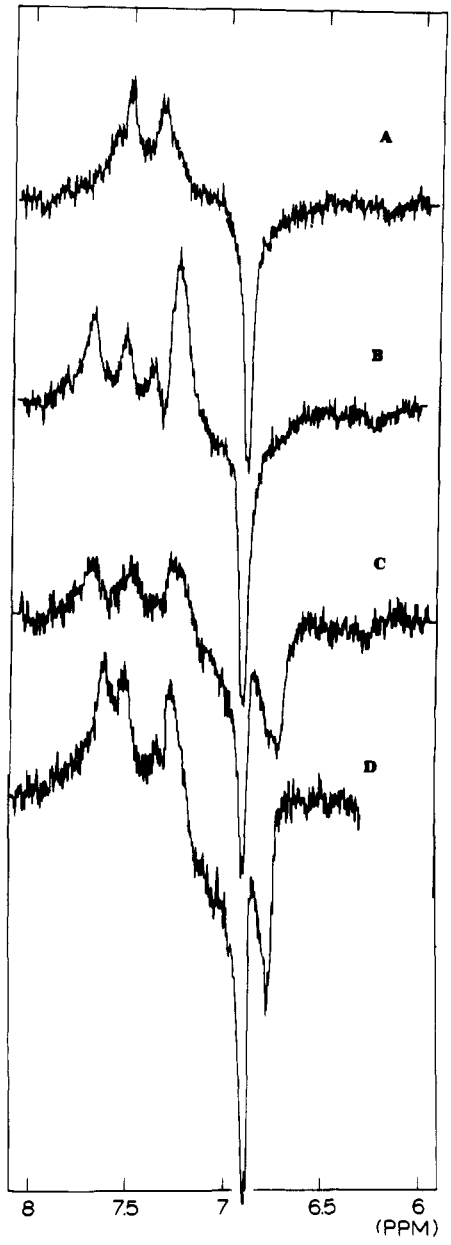

FIGURE 7: The 360-MHz ^1H NMR photo-CIDNP spectra of porcine AMPA (A), [Gly^1]AMPA (B), [D-Ala^1]AMPA (C), and AMPREC (D).

a hydrogen-bonded system around the α-NH_3^+ group will, however, mimic an interaction with a carboxylate.

In addition to the catalytic Ca^{2+} binding site, porcine and equine pancreatic phospholipases A_2 possess another, low-affinity, Ca^{2+} binding site (Slotboom et al., 1978; Donné-Op den Kelder et al., 1983). Without binding of this second Ca^{2+} ion, both the equine and the porcine enzyme lose their affinity toward neutral lipid–water interfaces above pH 6.3 (Figure 2). Taking into account the influence on the pK of the α-amino group, this second Ca^{2+} ion must be located in the direct vicinity of the α-NH_3^+ group. Indeed, the ^{13}C titrations of porcine and equine AMPAs show the relation of a group with a pK of 6.3 and the binding of a second Ca^{2+} ion. Donné-Op den Kelder et al. (1983) recently showed that the acidic side chain of Glu^{71} present in equine and porcine phospholipases A_2 is one of the ligands of the second Ca^{2+} ion. Deprotonation of the Glu^{71} (pK = ±6.3) will affect the hydrogen-bonded system around the α-NH_3^+ and subsequently the structure of the N-terminal part of porcine and equine phospholipases A_2, important for the lipid binding properties of these enzymes.

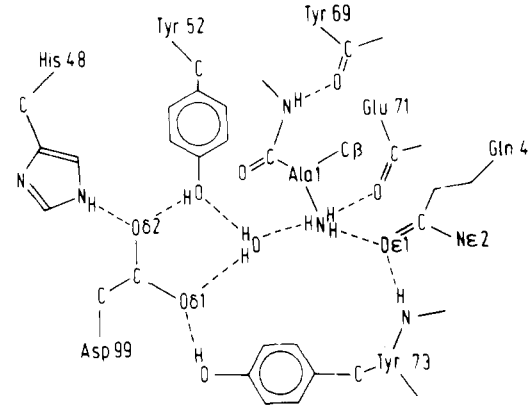

FIGURE 8: Schematic drawing of interaction between the N-terminus and active site of porcine phospholipase A_2 (Dijkstra et al., 1983).

This Glu^{71} controls directly the binding of porcine and equine phospholipases to neutral lipid–water interfaces. Neutralization of the negative charge of the carboxylate moiety of Glu^{71} by a proton or Ca^{2+} ion is essential for optimal binding.

In contrast to the porcine and equine phospholipases A_2, porcine isoenzyme does not require Ca^{2+} ions for optimal binding of neutral lipid–water interfaces (Donné-Op den Kelder et al., 1983). Furthermore, the ^{13}C titration curve of porcine [[3-^{13}C]Ala^1]iso-AMPA and the protein titration experiments with porcine isophospholipase A_2 indicate the absence of a carboxylate having a pK of ±6.3. These findings are thus in good agreement with the fact that porcine isophospholipase A_2 like the bovine phospholipase A_2 possesses at position 71 an Asn instead of a Glu residue (Puijk et al., 1979). The unexpected titration behavior of porcine iso-AMPA in the presence of 200 mM Ca^{2+} is quite different from the effect of high Ca^{2+} concentrations in the ^{13}C titration of porcine and equine AMPAs. Two populations of porcine iso-AMPA seem to be present at high protein (1 mM) and high Ca^{2+} concentrations (200 mM). One population has an α-NH_3^+ function in fast exchange with the deprotonated state, and another population with an α-NH_3^+ function is in slow exchange with the deprotonated form. This slow exchange in the presence of Ca^{2+} ions of a part of the porcine iso-AMPA is most likely due to motional restrictions of the N-terminus. Partial aggregation of the porcine isophospholipase A_2 facilitated by the high protein concentration (1 mM) and aspecific binding of Ca^{2+} ions to the iso-enzyme at these huge concentrations of Ca^{2+} ions (200 mM) might offer an explanation for the restricted mobility of the N-terminus of part of the porcine isoenzyme.

The titration curves of bovine [[3-^{13}C]Ala^1]AMPA indicate the absence of a second, low-affinity Ca^{2+} binding site in this enzyme. Neither the group having a pK of about 6.3 is observed nor is the pK of the α-NH_3^+ function of the bovine enzyme affected by the addition of Ca^{2+} ions (Figure 1). Proton titration experiments with bovine phospholipase A_2 also showed that the abnormal carboxylate (pK = ±6.3) is lacking (Donné-Op den Kelder et al., 1983).

Modification, blocking, or removal of the α-NH_3^+ function of Ala^1 of pancreatic phospholipase A_2 results in an enzyme that has lost its ability to hydrolyze or to bind to micellar neutral substrates.

Even the substitution of Ala^1 by D-Ala distorts the conformation of the enzyme, necessary for effective binding to lipid–water interfaces. The pK of the α-amino group of [D-[3-^{13}C]Ala^1]AMPA has previously been reported to be 7.8 in the presence of 50 mM $CaCl_2$ or 200 mM NaCl (Slotboom et al., 1978). Under the same conditions the α-amino group

of the N-terminal peptide of porcine AMPA, Ala[1]–Arg[6], also has a pK of 7.8. The pK of the α-NH_3^+ function of porcine AMPA, however, ranges from 8.3 to 9.3, depending on the Ca^{2+} concentration (Figure 1A). These findings suggest that the α-amino group of [D-Ala[1]]AMPA and probably that of most of the analogues in Table I are in a position different from that of native AMPA. Consequently, the extended H-bonded network (Figure 8) around the α-NH_2 group is distorted, leading to the loss of affinity for lipid–water interfaces. Most likely, the loss of lipid binding properties is due to an enhanced flexibility of the lipid binding domain as found for the X-ray structure of α-NH_2-transaminated bovine phospholipase A_2 (Dijkstra et al., 1984).

In contrast, the monomeric activity of pancreatic phospholipase A_2 is in most cases largely retained upon modification or blocking of the α-NH_3^+ function. The different values obtained for k_{cat}/K_M on monomeric substrates with the various AMPA analogues, of which the α-amino group is affected, point to a possible involvement of the N-terminus in monomer binding. Removal of more than two amino acid residues from the N-terminus of porcine AMPA leads, according to the NMR results, to a severe loss of native tertiary structure. The absence of any detectable catalytic activity is therefore not surprising.

In contrast to the essential α-amino group of Ala[1] of porcine phospholipase A_2, the side chain of this amino acid residue is less important for enzymatic activity on neutral aggregated substrates. This is not surprising because the snake venom phospholipases A_2 possess N-terminal amino acid residues like Ser, Asn, Asp, and His [e.g., Heinrikson et al. (1977), Joubert (1975), Botes & Viljoen (1974), and Fraenkel-Conrat et al. (1980), respectively). The porcine AMPA analogues, in which Ala[1] is substituted by polar amino acid residues, like Asn or Asp, are enzymes with an affinity toward neutral lipid–water interfaces and a penetrating capacity of lecithin monolayers comparable or better than those of native porcine AMPA. The esterification of the side chain of Asp[1] with a *tert*-butyl group considerably improves the affinity for lipid–water interfaces and the penetrating capacity of lecithin monolayers. The hydrophobic *tert*-butyl moiety contributes clearly to the lipid binding domain of porcine [Asp[1](OBu^t)]AMPA. Therefore, one would expect that a phospholipase A_2 with an N-terminal amino acid residue having a more hydrophobic side chain than Ala or Asn will show improved lipid binding properties compared to the native enzyme. Surprisingly, the substitution of Ala[1] by Phe, Nle, and Leu resulted in enzymes unable to bind to neutral lipid–water interfaces and consequently to hydrolyze efficiently micellar lipids. Most likely, the interaction of the α-NH_3^+ function of these mutants within the protein structures is prevented by the presence of the hydrophobic side chains of the N-terminal amino acid residues. Consequently, the N-terminal tertiary structures, necessary for a functional lipid binding domain, are absent. It seems unlikely that the steric constraints of these hydrophobic side chains of Leu[1], Phe[1], and Nle[1] prevent the hydrogen-bonded systems around the α-amino groups of these mutants. The linear chain of Nle will cause less steric hindrance than, for instance, the side chain of Asp(OBu^t). A more plausible explanation would be that the hydrophobic side chains of Leu[1], Phe[1], and Nle[1] interact with another hydrophobic amino acid residue, e.g., the nearby Trp[3] of the lipid binding domain, thereby disrupting the desired tertiary structures of these enzymes.

The N-terminus of [Gly[1]]AMPA exists in two conformations, which are in slow exchange on the time scale of NMR (Figure 9). The "pro-D" configuration of the Gly[1] residue

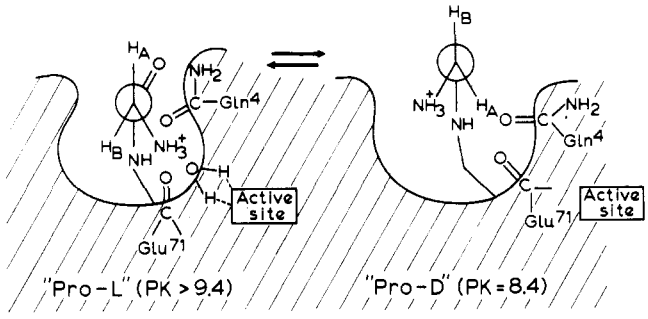

FIGURE 9: Schematic drawing showing the Newman projections of the two configurations of Gly[1] in porcine [Gly[1]]AMPA.

is expected to have the rather low pK value of 8.4 found for the α-NH_3^+ form of a part of [Gly[1]]AMPA. This population will not possess an N-terminal conformation favorable for binding to neutral lipid–water interfaces. The "pro-L" configuration of the N-terminal glycine will have a pK value >9.4. The absence of the methyl group of Ala[1] in the case of Gly[1] permits an even stronger interaction of the α-NH_3^+ function of [Gly[1]]AMPA, within the protein structure. The Gly[1] in the pro-L form will induce an N-terminal conformation necessary for a functional lipid binding domain. The intensities of the two ^{13}C resonances of the [1-^{13}C]Gly[1] of the porcine mutant indicate that at pH values below 8.5 most of the Gly[1] is in the pro-L configuration. Also, the CIDNP ^1H NMR difference spectrum of [Gly[1]]AMPA recorded at pH 5.5 indicates the presence of a lipid binding domain that will be induced by a pro-L form of Gly[1]. In contrast, the fluorescence intensity of Trp[3] of [Gly[1]]AMPA suggests the absence of a lipid binding domain also at pH 5.0 or 6.0, which most likely is the result of the presence of the pro-D form of Gly[1]. The high concentrations of 1 mM [Gly[1]]AMPA necessary to obtain the ^{13}C and ^1H NMR spectra as compared to the concentration of 0.01 mM used for the fluorescence intensity measurements might be the reason for the apparently contradictory results. In contrast to most pancreatic phospholipases, the porcine phospholipase A_2 has an enhanced tendency to aggregate at high protein concentrations (Aguiar et al., 1979) at pH values above 5. Porcine [Gly[1]]AMPA will probably have a similar tendency to form protein aggregates at high protein concentrations, if a functional lipid binding domain is present. This tendency to aggregate at high protein concentrations and neutral pH values will favor the pro-L form of Gly[1] essential for optimal lipid binding properties at the expense of the pro-D form of Gly[1]. Direct binding studies showed that [Gly[1]]-AMPA binds to $C_{16}PN$ micelles in the presence of Ca^{2+} with a dissociation constant of 2.0 mM. According to the pH titration behavior of [[1-^{13}C]Gly[1]]AMPA in the presence of Ca^{2+} and excess $C_{16}PN$ (Figure 5), all the Gly enzyme with a protonated α-NH_3^+ is present in lipid–protein complexes. Binding occurs only when the Gly[1] adopts the pro-L configuration, necessary for a functional lipid binding domain. The presence of micelles apparently shifts the equilibrium between the pro-L and pro-D form of Gly[1] completely to the pro-L configuration. The binding of [Gly[1]]AMPA to the micellar substrate analogue in the presence of Ca^{2+} is lost at pH values $\gg$9. The slow exchange between the ^{13}C resonances, due to α-NH_2 and α-NH_3^+ forms of [Gly[1]]AMPA, both present at fixed chemical shifts at every pH value indicates that only deprotonation of the α-amino group of [Gly[1]]AMPA is responsible for the loss of affinity toward neutral lipid–water interfaces.

The [Gly[1]]AMPA seems to combine the properties of [L-Ala[1]]- and [D-Ala[1]]AMPA. The pro-L configuration is

strongly favored by the substitution of proton H_A by a methyl group (Figure 9), thus changing [Gly[1]]- into [L-Ala[1]]AMPA. The methyl group of L-Ala[1] must be responsible for the fact that the α-NH_3^+ group of the native enzyme is always in a buried position in contrast to the α-NH_3^+ function of [Gly[1]]AMPA. The presence of the hydrophobic side chain of Trp[3] in the direct vicinity of this methyl group of L-Ala[1], when the α-NH_3^+ group is buried within the protein structure, might be an important factor for the fixation of L-Ala[1] in native AMPA. If the proton H_B of Gly[1] (Figure 9) is substituted by a methyl group, [D-Ala[1]]AMPA is obtained, and only for the lipid binding properties undesired pro-D conformation seems possible. Also [AibU[1]]AMPA in which both the protons H_A and H_B are replaced by methyl groups does not possess a functional lipid binding domain (Table II). These findings indicate that in the pro-L configuration it is energetically very unfavorable or sterically impossible to occupy the position of the H_B proton by a methyl group. Which conformation at the N-terminal Gly residue of the porcine mutant is favored depends on the protein concentration, the pH, the presence of substrate, and Ca^{2+} ions.

Acknowledgments

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Registry No. PLA, 9001-84-7; C_{18}PN, 65956-63-0; bis(thiohexanoyl)lecithin, 76622-79-2; dioctanoyllecithin, 4539-70-2.

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