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The Role of Asp-49 and Other Conserved Amino Acids in Phospholipases A2 and Their Importance for Enzymatic Activity

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The role of aspartic acid-49 (Asp-49) in the active site of porcine pancreatic phospholipase A2 was studied by recombinant DNA techniques: two mutant proteins were constructed containing either glutamic acid (Glu) or lysine (Lys) at position 49. Enzymatic characterization indicated that the presence of Asp-49 is essential for effective hydrolysis of phospholipids. Conversion of Asp-49 to either Glu or Lys strongly reduces the binding of Ca^{2+} ions, in particular for the lysine mutant, but the affinity for substrate analogues is hardly affected. Extensive purification of naturally occurring Lys-49 phospholipase A2 from the venom of *Agkistrodon piscivorus piscivorus* yielded a protein that was nearly inactive. Inhibition studies showed that this residual activity was due to a small amount of contaminating enzyme and that the Lys-49 homologue itself has no enzymatic activity. Our results indicate that Asp-49 is essential for the catalytic action of phospholipase A2. The importance of Asp-49 was further evaluated by comparison of the primary sequences of 53 phospholipases A2 and phospholipase homologues showing that substitutions at position 49 are accompanied by structural variations of otherwise conserved residues. The occurrence of several nonconserved substitutions appeared to be a general characteristic of nonactive phospholipase A2 homologues.

Key words: phospholipase A2, site-directed mutagenesis, sequence homology of phospholipase A2, calcium binding

Phospholipase A2 (EC 3.1.1.4; PLA) catalyzes the hydrolysis of fatty acid ester bonds at the *sn*-2-position of 3-*sn*-phospholipids. The enzyme is abundant in mammalian pancreatic tissue and also in snake and bee venom. It is a small protein (14 kDa) with a high stability against denaturing conditions. The enzymatic activity of phospholipase A2 is absolutely dependent on the presence of calcium ions. From differential labelling experiments [1] and crystallographic data [2] of bovine pancreatic PLA, it became clear that aspartic acid (Asp-49) is the residue that controls calcium binding. From these and other experiments, a possible mechanism for the

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catalytic action of PLA has been proposed [3]. In this model (Fig. 1), the catalytic unit comprises His-48, Asp-99, and a water molecule. Their function is believed to be analogous to the catalytic residues in serine proteases. The catalytically important calcium ion is liganded by three carbonyl oxygens of Tyr-28, Gly-30, Gly-32, two water molecules, and Asp-49 [2]. The role of the calcium ion is probably the stabilization of the formed tetrahedral intermediate.

Irrespective of their origin, mammalian pancreas or snake venom, the primary structure of 53 phospholipases shows a high degree of homology. In snake venom and in pancreatic PLAses, the catalytic triad (His-48, Asp-99, H_2O) is conserved. In all but two of the PLA sequences, the residues that form the calcium-binding pocket are conserved as well. Therefore, it seemed likely that the proposed catalytic mechanism is a general mode of action for all PLA species. Recently, phospholipases A2 from the venom of *Agkistrodon piscivorus piscivorus* (App-PLA) and *Bothrops atrox*, which contain a lysine residue at position 49, were described, and these proteins were reported to have considerable enzymatic activity [4–6]. These findings prompted us to study the role of Asp-49 in more detail.

In the present paper, we describe the introduction of a glutamic acid and a lysine at position 49 in porcine pancreatic PLA (D49E-PLA and D49K-PLA, respectively) using recombinant DNA techniques. The mutant enzymes were purified and characterized. Results of these studies were compared with the properties of extensively purified Lys-49 App-PLA. The differences between active and inactive phospholipases A2 were analyzed on the basis of a homology comparison of 53 phospholipases.

EXPERIMENTAL PROCEDURE

Strains and Culture Conditions

Escherichia coli K-12 strain PC 2494 (Δ(lac-pro), supE, thi/F′ $traD_{36}$, $proA^+$ B^+ $lacI^q$, lacZΔM15; Phabagen Collection, Utrecht) was used for plasmid constructions and as a host for M13-derived vectors. HB 2154 (ara, Δ(lac-pro), thi/F'$proA^+$ B^+, $lacI^q$, lacZΔM15, mutL::Tn10)[7] was used as recipient strain in the mutagenesis experiments. *Sacchazomyces cerevisiae* strain GRF18 and yeast culturing conditions have been described earlier[8].

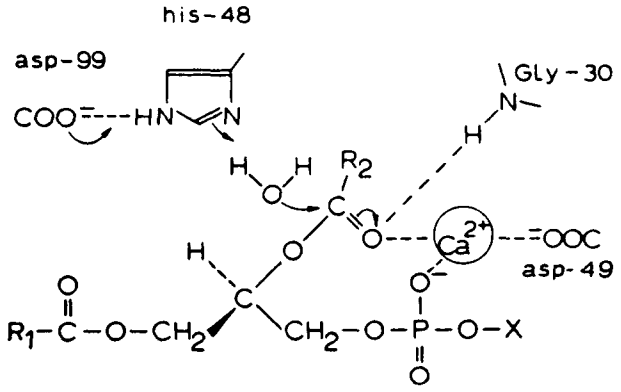


Fig. 1. Schematic representation of a proposed catalytic mechanism of phospholipase A2[3].

Construction of Mutant-proPLA Expression Vectors

Restriction enzymes, T4 DNA ligase, and Klenow fragment of DNA polymerase I were obtained from Pharmacia and used according to the manufacturer's instructions. The synthetic oligonucleotides were synthesized on a Biosearch 6800 DNA synthesizer.

To introduce oligonucleotide-directed mutations, the cDNA coding for porcine pancreatic prophospholipase A2 (proPLA)[8] was subcloned in M13mp8-RF DNA to provide single-stranded template DNA. Substitutions in the proPLA-gene were accomplished by the gapped duplex procedure, using amber selection [9]. The mutagenic oligonucleotide 5' GTAGCAGTTCTC,TGTGTGTCTC 3' introduces simultaneously a lysine or a glutamic acid at position 49. The cDNA's encoding the mutant proPLA-species were sequenced by the dideoxy chain termination method [10]. Construction of the D49E-proPLA and D49K-proPLA encoding yeast expression plasmids was similar to that described for pCB08T [8]. The resulting plasmids were called pCE49 and pCK49, respectively.

Expression and Purification of D49K-PLA and D49E-PLA

To obtain expression, plasmids pCE49 and pCK49 were introduced into yeast strain GRF18, and large-scale cultures were grown. The purification procedure was essentially the same as already described [8]. The purified mutant proPLAses were activated by limited tryptic proteolysis essentially as described earlier [11]. After acidification and lyophilisation, the preparation was desalted on a Sephadex G-50 fine column eluting in 5 mM NaAc pH 5. The gel filtration step was immediately followed by carboxy methyl (CM)-cellulose chromatography at pH 5, using a linear salt gradient reaching 0.5 M NaCl.

Direct Binding Studies

Direct binding of calcium and substrate were measured with ultraviolet absorbance spectroscopy. Difference spectra were recorded on an Aminco Model DW-2a spectrophotometer equipped with a Midan data analyser, coupled to an Apple II desktop computer. Measurements were carried out as described earlier[12–14]. The enzymes were dissolved in a buffer containing 20 mM NaAc, pH 6.0, 0.1 M NaCl. Calcium chloride was titrated up to 150 mM. Substrate binding was studied by titrating monomeric or micellar substrate analogues: *n*-decylphosphocholine (up to 10 mM) and *n*-hexadecylphosphocholine (up to 5 mM), respectively. Protein concentrations used were 0.25 mg/ml for Ca^{2+} binding experiments and 0.5 mg/ml for lipid binding studies. All titrations were carried out in tandem cuvettes.

Purification of K49-PLA and D49-PLA From *Agkistrodon piscivorus piscivorus*

Lyophilized venom from *Agkistrodon piscivorus piscivorus* was obtained from Latoxan (France). The K49 App-PLA and D49 App-PLA were purified following the procedure of Maraganore et al.[4]. Further purification of the K49-enzyme was done on a CM-cellulose column at pH 8.5 (20 mM $Na_2B_4O_7$), developed with a linear salt gradient (0.1–0.5 M NaCl).

p-Bromophenacyl Bromide Inactivation

Rates of modification of App-PLAses by *p*-bromophenacyl bromide were determined at 15°C or occasionally at 0°C. A 0.1 M cacodylate buffer (pH 6.0) containing

0.1 M NaCl was used. Inactivation rates were determined both in the absence and in the presence of 50 mM $CaCl_2$. Protein concentrations were 1 mg/ml, and the reaction was started by the addition of 1 mg reagent in 20 μl acetone to a final volume of 1 ml. The inactivation rate was determined by taking samples and analyzing them for residual activity. Half-time values for the reaction were determined from the slopes of semilogarithmic plots of residual activity vs. time as described before[15]. To determine the extent of modification of histidine residues 200 μg samples were withdrawn, and the reaction was quenched with acetic acid. Salts and reagent were removed by gel filtration on small (5 × 0.4 cm) columns filled with Sephadex G-25 fine in 10 mM acetic acid. After lyophilization, samples were hydrolyzed in vacuo for 24 h at 110°C in 6 N HCl. Amino acid analyses were carried out on an LKB 4151 alpha plus analyzer. Half-time values were in this case determined from the slopes of semilogarithmic plots of the amount of histidine lost vs. time.

Phospholipase Assay

Activities of phospholipases were measured with the titrimetric assay using 1,2-dioctanoyl-3-*sn*-glycerophosphocholine as a substrate in 1 mM sodium acetate buffer (pH 6.0) in the presence of 25 mM $CaCl_2$ at 25°C[16].

Sequence Analysis

The sequence of K49 App-PLA was established by automated sequence analysis with a Beckman sequencer model 890C, using a quadrol programme no. 122974. The phenylthiohydantoin derivatives of the amino acids were identified by high-performance liquid chromatography.

Results

To study the role of aspartic acid 49 in phospholipase A2, two mutants of porcine pancreatic PLA were constructed: one in which the charge was retained albeit shifted away by one methylene group (D49E-PLA) and one in which a positively charged lysine residue was introduced (D49K-PLA). The corresponding proPLA mutants were expressed and secreted in *S. cerevisiae*, using the leader sequence of the yeast α-mating factor, and mutant precursor proteins were purified from the culture medium [8]. Limited trypsinolysis, followed by CM-cellulose chromatography yielded pure proteins as judged by single bands on sodium dodecyl sulfate polyacrylamide gels. Wild-type PLA isolated from yeast was indistinguishable from native porcine PLA with respect to enzymatic activity. The activities of the mutant proteins (Table I) were very low: D49E-PLA had only 0.1% activity relative to native PLA, whereas D49K-PLA showed less than 0.004% activity, which is in the order of the nonenzymatic hydrolysis of this substrate. Using ultraviolet difference spectroscopy, the binding capacity of the proteins for Ca^{2+} ions was determined. Following the addition of calcium ions, wild-type PLA showed a large increase in optical density at 242 nm. When this increase is plotted as a function of the Ca^{2+} concentration, a saturation curve (Fig. 2) was obtained, from which a dissociation constant of 2.8 mM could be calculated. This value is in agreement with previously reported results[12]. With D49E-PLA, a slow monotonic increase in optical density was obtained that might be interpreted as the binding of calcium ions with a very low affinity (K_D > 100 mM). The fact that this increase in absorbance does not occur with D49K-PLA

TABLE I. Kinetic and Direct Binding Parameters of Native and Mutant Porcine Pancreatic Phospholipases A2*

Enzyme	V_{max} (U/mg)	K_{Ca} (mM)	K_{C10PN} (mM)	K_{C16PN}[a] (mM)
Native PLA	1840 ± 100	2.8 ± 0.3	2.1 ± 0.2	0.65 ± 0.07
D49E-PLA	1.6 ± 0.4	> 100	4.0 ± 0.4	0.88 ± 0.09
D49K-PLA	0.07 ± 0.07	-[b]	5.5 ± 0.5	0.74 ± 0.07

*The activity on dioctanoyllecithin was determined by varying amounts of substrate from a stock solution of 35 mg/ml to a final concentration of maximally 12 mM. Direct binding experiments were carried out as described in Experimental section. C10PN: *n*-decylphosphocholine; C16PN: *n*-hexadecylphosphocholine. N represents the number of lipid molecules complexed to phospholipase in the micellar complex. $N \times K_D$ can be defined [16] as the experimentally observed concentration where half of the protein is bound to micelles.

[a]K_{C16PN} represents the value $N \times K_D$.

[b]No detectable binding.

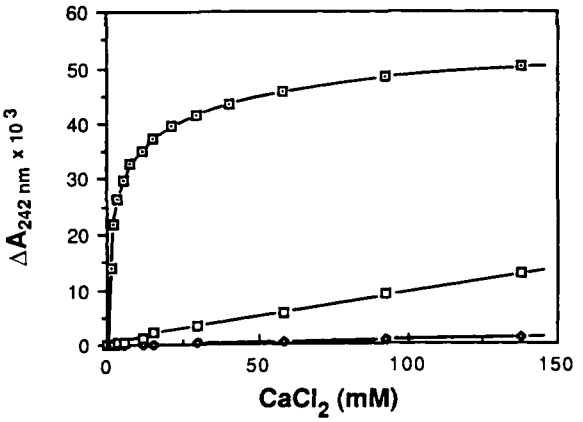


Fig. 2. Binding of calcium ions to native and recombinant porcine pancreatic phospholipases A2. Optical densities at 242 nm from ultraviolet absorbance difference spectra at various concentrations of calcium chloride were determined for porcine pancreatic phospholipase A2 (⊡), D49E-PLA (□) and D49K-PLA (◇), respectively. For conditions see Experimental section.

suggests that the signal that is observed with D49E-PLA represents a weak but specific binding of Ca^{2+} ions to the latter mutant; furthermore the present authors conclude that calcium ions do not bind to the D49K-PLA.

A characteristic of pancreatic phospholipases A2 is their ability to bind monomeric substrate analogues below the critical micellar concentration (CMC) and to form complexes composed of two protein molecules and about 80 lipid molecules above the CMC [14]. Both processes can be studied by following the increase in optical density at 289 nm (monomeric substrate analogues) or at 293 nm (micellar binding) after the addition of the substrate analogues *n*-decylphosphocholine (CMC = 10 mM) or *n*-hexadecylphosphocholine (CMC = 0.01 mM) [13]. Both mutant PLAses bind to micelles with the same affinity as wild-type PLA: half-saturation ($N \times K_D$) occurs at about 0.8 mM *n*-hexadecylphosphocholine. A slight difference in the affinity for monomeric *n*-decylphosphocholine was observed (Table I).

The absence of activity observed for D49K-PLA is in sharp contrast with reports [4–6] that have described App-PLA with Lys-49 as enzymatically active. This dis-

crepancy prompted us to examine in more detail the basic Lys-49 PLA from the venom of *Agkistrodon piscivorus piscivorus*. The enzyme was isolated from the venom by the same procedure as described earlier by Maraganore et al.[4]. This purification, which includes a gel filtration step and a fractionation on sulfopropyl (SP)-Sephadex at pH 6.5, yielded two protein peaks corresponding to D49 App-PLA and K49 App-PLA, respectively. Amino acid analyses revealed an amino acid composition in full aggreement with the sequence data [5] of both enzymes. The amino terminal sequence of the first eight residues of K49 App-PLA also agreed with the sequence reported for this protein. The specific activity on dioctanoyllecithin of the proteins was 12,000 (U/mg) for D49 App-PLA, whereas it was only 39 (U/mg) for K49 App-PLA. The K49 enzyme then was further purified on a CM-cellulose column at pH 8.5 (Fig. 3). Several minor protein peaks with enzymatic activity elute before the bulk of the protein. The latter K49 App-PLA has an extremely low specific activity of 3 U/mg, which is less than 0.03% that of D49 App-PLA. Thus, the enzymatic activity in the K49 protein after SP-Sephadex chromatography is due mainly to the presence of small amounts of contaminating PLAses which can be removed, to a large extent, by rechromatography on CM-cellulose at pH 8.5.

To determine whether the very low activity found in the K49 App-PLA is an intrinsic property of this PLA species or is due to the presence of an impurity that was not removed at pH 8.5, we performed inhibition experiments with *p*-bromophenacyl bromide, a reagent known to modify the active-site histidine in PLAses [15]. The present study simultaneously followed the loss in enzymatic activity and the loss in histidine content as determined by amino acid analyses (Table II). For D49 App-PLA the loss of enzymatic activity coincides with the loss in histidine content ($t_{1/2} \sim 7.5$ min). For the K49 App-PLA, no such correlation was found: the enzymatic activity disappeared much more slowly ($t_{1/2} \sim 9$ min) than the histidine content ($t_{1/2} < 2$ min). When the latter inhibition experiments were repeated at 0°C, a qualitatively similar picture was obtained: the modification of one single histidine occurred more rapidly ($t_{1/2} \sim 22$ min) than the decrease of enzymatic activity ($t_{1/2} \sim 99$ min).

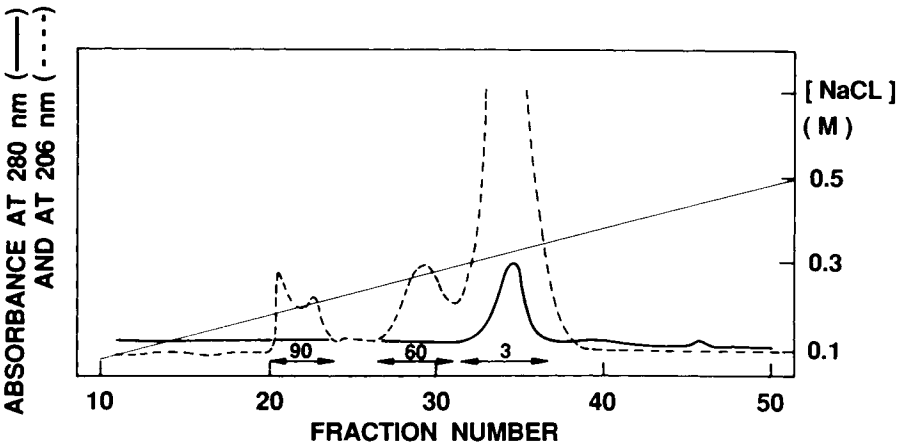


Fig. 3. Purification of K49 App-PLA by CM-cellulose chromatography. Partially purified K49 App-PLA (see Experimental section) was purified further on a CM-cellulose column at pH 8.5, developed with a linear gradient of NaCl. The specific activities of the eluting protein fractions (in U/mg) are as indicated below each peak.

TABLE II. Half-Time Values of Inactivation of D49 App-PLA and of K49 App-PLA With *p*-Bromophenacyl Bromide*

	$-CaCl_2$		$+CaCl_2$	
Enzyme	Activity $t_{½}$ (min)	Histidine $t_{½}$ (min)	Activity $t_{½}$ (min)	Histidine $t_{½}$ (min)
D49 App-PLA[a]	7.3 ± 0.7	7.9 ± 0.8	39 ± 4	40 ± 4
K49 App-PLA[a]	9.2 ± 0.9	<2	89 ± 9	<2
K49 App-PLA[b]	99 ± 10	22 ± 2	~15 h	21 ± 2

*Inactivation was carried out at 15°C or 0°C (at pH 6.0) in the presence of a 70-fold excess of reagent, either in the absence or in the presence of 50 mM $CaCl_2$. Activity was measured titrimetrically with dioctanoyllecithin as a substrate. Histidine contents were determined by amino acid analyses. For details see Experimental section.
[a]At 15°C.
[b]At 0°C.

These results are consistent with an inactive K49 App-PLA that rapidly reacts with *p*-bromophenacyl bromide ($t_{1/2}$ ~ 22 min, at 0°C), whereas this PLA species is contaminated with a small amount (< 1%) of some kind of active PLA that reacts slower ($t_{1/2}$ ~ 99 min, at 0°C) with *p*-bromophenacyl bromide. The inhibition experiments were repeated in the presence of Ca^{2+} ions, which are known [15] to protect the active site histidine from modification. Indeed, the D49 App-PLA was protected by the presence of Ca^{2+} ions as was the enzymatic activity in the K49 App-PLA. Modification of histidine as determined by amino acid analyses was retarded for the same extent in the case of D49 App-PLA, whereas the rate of modification in the case of K49 App-PLA was unaffected (Table II). This is consistent with the fact that K49 App-PLA does not bind calcium ions.

DISCUSSION

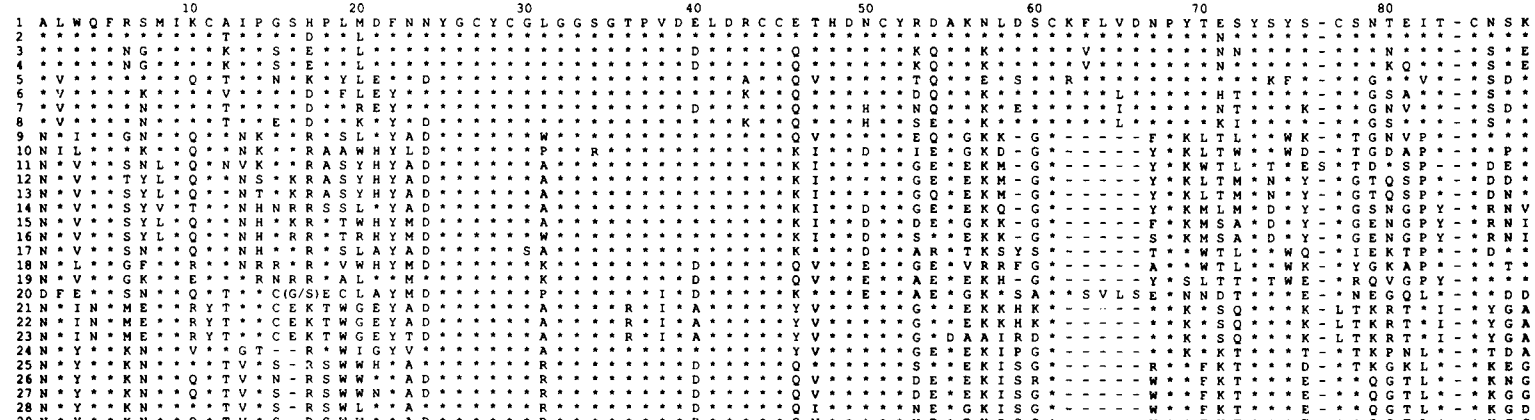
The presence of Asp-49 in the active site has been demonstrated to have a crucial role in the catalytic action of phospholipase A2. A chemical modification study showed modification of the side chain of Asp-49 to be accompanied by a loss in binding of the essential cofactor Ca^{2+} and concomitant loss of enzymatic activity [1]. Crystallographic analysis [2] showed that the Ca^{2+} ion was liganded not only by Asp-49 but also by Tyr-28, Gly-30, and Gly-32, residues that are highly conserved in all phospholipases A2 except in two proteins isolated from the venom of *Agkistrodon piscivorus piscivorus* and *Bothrops atrox* in which Asp-49 is replaced by lysine. These Lys-49 PLAses were reported to have considerable enzymatic activity: k_{cat}/K_m values as high as 30–40% of related Asp-49 phospholipases from the same venom [4–6]. The different properties of these D49 and K49 PLAses have been explained exclusively on the basis of this substitution [5]. Both homologues, however, contain different amino acids at 62 positions (more than 50%) of the sequence. Therefore, in the present study, it was decided to replace by a point mutation Asp-49 in porcine pancreatic phospholipase A2 and to substitute it with either a lysine or a glutamic acid. The mutant containing Glu was about 1,000 times less active than native PLA. This low but reproducible activity may be caused by the fact that the Ca^{2+} ion, which is bound to the carboxylate of Glu, instead of Asp, is too far away from the susceptible ester bond of the substrate to polarize effectively the carbonyl of the ester bond and stabilize the tetrahedral intermediate, as has been suggested to be the case for the

native enzyme (Fig. 1). The mutant in which Asp-49 is replaced by lysine shows an activity 25,000 times lower than native phospholipase A2 even in the presence of Ca^{2+} concentrations as high as 250 mM. In fact, activity may be even lower, because under the assay conditions, the addition of 500 μg of mutant enzyme results in hydrolysis rates about equal to the non-enzymatic hydrolysis of the substrate. Because the D49K-PLA still binds to monomeric and to micellar substrate analogues the loss of activity must be due to the loss of affinity of the mutant protein for calcium ions.

These results emphasize the essential role of Asp-49 for phospholipase A2 activity but conflict with the activities reported for naturally occurring Lys-49 phospholipase homologues. Purification of K49 App-PLA following the two-step procedure described by Maraganore et al. [4] yielded a protein with a low enzymatic activity. Additional separation at a different pH value removed traces of active phospholipase A2 contaminants, which were not revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis or amino acid analyses. The resulting K49 App-PLA has a specific activity of 3 U/mg, which is about 4,000 times less active than the related Asp-49 enzyme isolated from the same venom, but still high compared with recombinant pancreatic D49K PLA, which had a specific activity as low as 0.07 U/mg on dioctanoyllecithin, the substrate on which pancreatic phospholipase is most active [16]. To determine if the low activity of the Lys-49 venom PLA was an intrinsic activity or if it was due to the presence of a contaminating active phospholipase A2 species, irreversible inhibition experiments were conducted, which clearly showed that the enzymatically active component of the Lys-49 App-PLA reacts much slower with *p*-bromophenacyl bromide than with the bulk of the protein (Table II). Moreover, in the presence of calcium ions, the modification reaction leading to the loss in enzymatic activity is slowed down a factor ten, as expected for Asp-49 phospholipases A2 [15]. In line with the lack of affinity for calcium ions of venom Lys-49 PLA, the bulk of the protein is modified with the same velocity both in the presence and absence of Ca^{2+} ions. Thus, the present authors conclude that the residual activity of venom Lys-49 PLA must be due to a (small) contamination with (an) active phospholipase A2 species.

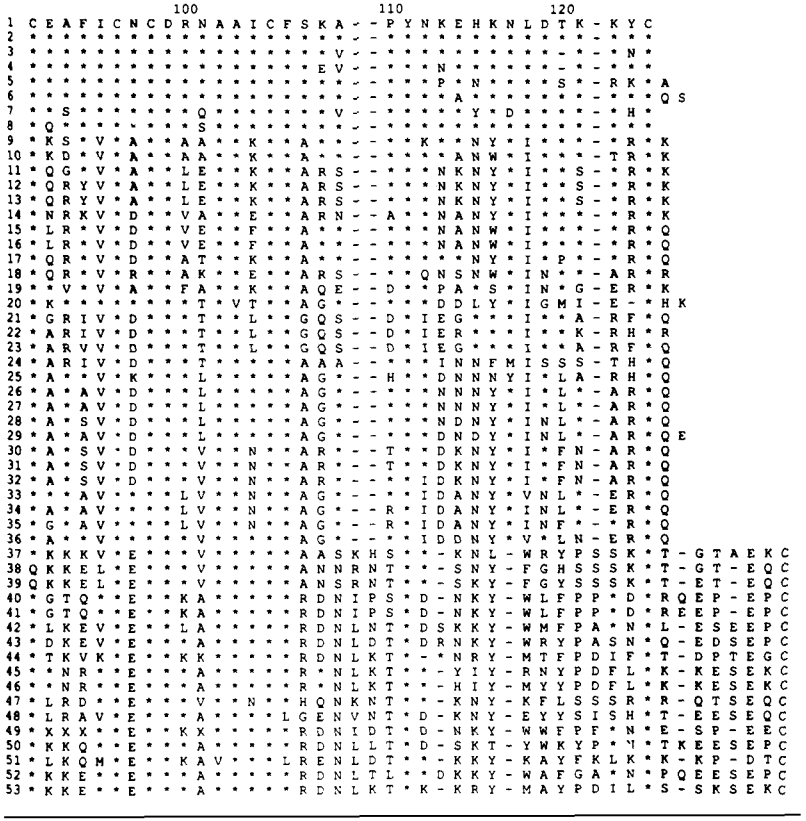
Extracellular phospholipases A2 show a high degree of homology. On the basis of a comparison of the sequence of 32 phospholipases from mammalian pancreas and from snake venom, it has been concluded [17,18] that these enzymes have developed through a divergent evolutionary process from a common ancestor. Table III shows a sequence comparison of 53 phospholipases and phospholipase homologues. Most of the proteins listed in this table have phospholipase A2 activity; there are, however, exceptions of species that have a very low activity or do not display any enzymatic activity at all: sequences 10, 17, 19, 20, 48, and, according to this study, also 51. As shown in Table III, it appears that 19 amino acids are completely conserved. Of these residues, ten are half-cystines corresponding with the fact that all phospholipases have five conserved disulfide bridges. The half-cystines 61 and 91 form a disulfide bridge in most phospholipases, but this bridge is lacking in two venom enzymes (sequences 41 and 42). Amino acids, which are nearly invariant, are possibly of more interest for the analysis of the structural elements that distinguish active phospholipases from inactive homologues. Examples of such nearly invariant residues are: Phe-5, Tyr-28, Gly-30, Gly-33, Gly-35, His-48, Asp-49, Ala-102, Ala-103, and Phe-106. The hydrophobic residues Phe-5 and Phe-106 are buried and form a hydrophobic wall that shields the active site from the aqueous environment. The x-ray structures of bovine

TABLE III. Aligned Amino Acid Sequences of 53 Phospholipases A_2*



(continued)

TABLE III. Aligned Amino Acid Sequences of 53 Phospholipases A_2* (continued)



*The IUPAC one-letter amino acid code [Eur. J. Biochem. 5:151–1531, 1968] is used. Gaps (−) have been introduced in order to get maximal alignments of half-cystines and maximal homology. Residues identical to the corresponding residue in porcine pancreatic PLA are indicated with an *asterisk*. Note that gaps do not contribute to numbering and that the numbers here do not necessarily correspond to those in the original publications. Key: 1, porcine pancreas [17]; 2, porcine pancreas, iso-enzyme [19]; 3, bovine pancreas [17]; a recently described DNA sequence [Nucleic Acids Res, 15 (1987) 3178] is identical to the sequence given here except for a substitution Asn-123→Lys and probably corresponds to the more basic iso-enzyme found in bovine pancreas [Eur. J. Biochem. 53:92–97, 1975]; 4, ovine pancreas [20]; 5, equine pancreas [17,21]; 6, human pancreas [21,22]; 7, rat pancreas [23]; 8, canine pancreas [23]; 9, *Pseudechis australis*: Pa11 [24]; 10, *P. australis*: Pa13 [24]; 11–13, *Laticauda semifasciata*: fractions I, III and IV, respectively [17]; 14, *Enhydrina schistosa*: myotoxin [17]; 15–17, *Notechis scutatus*: fractions notexin, II.5 and II.1, respectively [17]; 18–20, *Oxyuranus scutellatus*: taipoxins α, β and γ, respectively [17]; 21–23, *Bungarus multicinctus*: β-bungarotoxins A1, A2 and A3 respectively [17]; 24, *B. multicinctus*: PLA [17]; 25, *Haemachatus hemachatus* [17]; 26, *Naja naja kaouthia*: CMII [17]; 27, *N. n. kaouthia*: CMIII [17]; 28, *N. n. oxiana* [17]; 29, *N. n. atra* [17]; 30–32, *N. melanoleuca*: DEI,DEII and DEIII, respectively [17]; 33–35, *N. mossambica:* CMI, CMII and CMIII, respectively [17]; 36, *Aspidelaps scutatus* [25]; 37, *Bitis caudalis* [17]; 38, *B. gabonica* [17]; 39, *B. nasicornis* [26]; 40, *Crotalus adamanteus* [17]; 41, *C. atrox* [17]; 42, *Trimeresurus okinavensis* [17]; 43, *T. flavoviridis* [27]; 44, *T. flavoviridis*: PLX [28]; 45, *Vipera ammodytes ammodytes*: ammodytoxin A [29]; 46, *V. a. ammodytes*: ammodytoxin B [30]; 47, *V. a. ammodytes*: Vipoxin PLA [31]; 48, *V. a. ammodytes*: Vipoxin inhibitor [31,32]; 49, *Agkistrodon halys blomhoffii*: a rearranged version of the original sequence [see 33]; 50, *A. piscivorus piscivorus*: D49 PLA [5]; 51, *A. p. piscivorus:* K49 PLA [5]; 52, *A. halys pallas*: acidic PLA [34]; 53, *A. h. blomhoffii*: basic PLA [35].

pancreatic [2] and *Crotalus atrox* [36] phospholipases A2 suggest that replacement of these residues would severely distort the structure. Ala-102 and Ala-103 are close to the entrance of the active site, and the presence of larger, bulky side chains might interfere with the productive binding of substrate near the active site residues His-48 and Asp-49 (see also Fig. 1). Finally, residues 25–35 constitute to the calcium-binding loop, which together with Asp-49, keep the cofactor calcium in position. It is clear, as shown in Table III, that all inactive phospholipase A2 homologues have in common that one or more substitutions in these nearly invariant residues have occurred. In addition, some substitutions disturb functional rather than absolute conservation, as exemplified by sequences 10 and 20, which contain at position 31 a proline; this leads undoubtedly to severe damage of the conformation of the calcium-binding loop. Another interesting example is the presence of Ser-69 and Asn-69, respectively in sequences 19 and 20, in which active phospholipases contain either a lysine or tyrosine residue. Although the precise function of Lys-69 and Tyr-69 is not known, it is tempting to assume that the phenolic OH or ϵ-amino group are involved in the interaction with substrate.

The naturally occurring Lys-49 App-PLA is characterized by several substitutions of nearly invariant residues: Phe-5 → Leu, Tyr-28 → Asn, Gly-33 → His, Asp-49 → Lys, Ala-102 → Val, and Phe-106 → Leu. Although the individual effect of all these substitutions is unknown, each of these changes alone may be sufficient to cause a severe decrease in enzymatic activity. Therefore, all results based on the activity of Lys-49 *Agkistrodon piscivorus piscivorus* "phospholipase" and their implications for the mechanism of action of phospholipases A2 should be therefore considered invalid.

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REFERENCES

1. Fleer EAM., Verheij HM, de Haas GH: Eur J Biochem 113:283–288, 1981.
2. Dijkstra BW, Kalk KH, Hol WGJ, Drenth J: J Mol Biol 147:97–123, 1981.
3. Verheij HM, Volwerk JJ, Jansen EHJM, Puijk WC, Dijkstra BW, Drenth J, de Haas GH: Biochemistry 19:743–750, 1980.
4. Maraganore JM, Merutka G, Cho W, Welches W, Kézdy FJ, Heinrikson RL: J Biol Chem 259:13839–13843, 1984.
5. Maraganore JM, Heinrikson RL: J Biol Chem 261:4797–4804, 1986.
6. Maraganore JM, Poorman RA, Heinrikson RL: J Protein Chem 6:173-189, 1987.
7. Carter P, Bedouelle H, Winter G: Nucleic Acids Res 13:4431–4443, 1985.
8. van den Bergh CJ, Bekkers ACAPA, de Geus P, Verheij HM, de Haas GH: Eur J Biochem 170:241–246, 1987.
9. Kramer W, Drutsa V, Jansen H-W, Kramer B, Pflugfelder M, Fritz H-J: Nucleic Acids Res 12:9441–9457, 1984.
10. Sanger F, Nicklen S, Coulson AR: Proc Natl Acad Sci USA 74:5463–5467, 1977.
11. Nieuwenhuizen W, Kunze H, de Haas GH: Methods Enzymol 32B:147-154, 1974.

12. Pieterson WA, Volwerk JJ, de Haas GH: Biochemistry 13:1439–1445, 1974.
13. van Dam-Mieras MCE, Slotboom AJ, Pieterson WA, de Haas GH:Biochemistry 14:5387–5394, 1975.
14. de Araujo PS, Rosseneu MY, Kremer JMH, van Zoelen EJJ, de Haas GH: Biochemistry 18:580–586, 1979.
15. Volwerk JJ, Pieterson WA, de Haas GH: Biochemistry 13:1446–1454, 1974.
16. de Haas GH, Bonsen PPM, Pieterson WA, van Deenen LLM: Biochim Biophys Acta 239:252-266, 1971.
17. Dufton MJ, Eaker D, Hider RC: Eur J Biochem 137:537–544, 1983.
18. Dufton MJ, Eaker D, Hider RC: Eur J Biochem 137:544–551, 1983.
19. Puijk WC, Verheij HM, Wietzes P, de Haas GH: Biochim Biophys Acta 580:411–415, 1979.
20. Verheij HM, Raykova D, Dijkman R, Lenting H, de Haas GH: Recl Trav Chim Pays-Bas 107:203–207, 1988.
21. Verheij HM, Westerman J, Sternby B, de Haas GH: Biochim Biophys Acta 747:93–99, 1983.
22. Seilhammer JJ, Randall TL, Yamanaka M, Johnson LK: DNA 5:519–527, 1986.
23. Ohara O, Tamaki M, Nakamura E, Tsuruta Y, Fujii Y, Shin M, Teraoka H, Okamoto M: J Biochem 99:733–739, 1986.
24. Nishida S, Terashima M, Tamiya N: Toxicon 23:87–104, 1985.
25. Joubert FJ: Biol Chem Hoppe-Seyler 368:1597–1602, 1987.
26. Joubert FJ, Townshend GS, Botes DP: Hoppe-Seyler Z Physiol Chem 364:1717–1726, 1983.
27. Tanaka S, Mohri N, Kihara H, Ohno M: J Biochem 99:281–289, 1986.
28. Kini RM, Kawabata S-I, Iwanaga S: Toxicon 24:1117–1130, 1986.
29. Ritonja A, Gubenšek F: Biochim Biophys Acta 828: 306–312, 1985.
30. Ritonja A, Machleidt W, Turk V, Gubenšek F: Biol Chem Hoppe-Seyler 367:919–923, 1986.
31. Mancheva I, Kleinschmidt T, Aleksiev B, Braunitzer G: Hoppe-Seyler's Z Physiol Chem 365:885–894, 1984.
32. Mancheva I, Kleinschmidt T, Aleksiev B, Braunitzer G: Biol Chem Hoppe-Seyler 368:343–352, 1987.
33. Heinrikson RL, Krueger ET, Keim PS: J Biol Chem 252:4913-4921, 1977.
34. Chen, Y-C, Maraganore JM, Reardon I, Heinrikson RL: Toxicon 25:401–409, 1987.
35. Forst S, Weiss J, Blackburn P, Fangione B, Goni F, Elsbach P: Biochemistry 25:4309–4314, 1986.
36. Brunie S, Bolin J, Gewirth D, Sigler PB: J Biol Chem 260:9742–9747, 1985.