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THE ROLE OF LYSYL RESIDUES OF PHOSPHOLIPASES A_2 IN THE FORMATION OF THE CATALYTIC COMPLEX

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The aspartyl residue at position 49 in phospholipases A2 (PLA) has been viewed as a component of the catalytic apparatus because of its involvement in binding the essential cofactor, calcium. We recently discovered a new class of PLA's in which, among other changes in highly invariant residues, Asp-49 is replaced by a lysine (Maraganore et al. (1984) J. Biol. Chem. 259, 13839) . These Lys-49 PLA's are also calcium-dependent, but, in contrast to the Asp- 49 enzymes, they bind phospholipid strongly in the absence of calcium. Lys-49 PLA's are, therefore, ideal for studying structural and mechanistic aspects of these enzymes. Attempts to modify Lys-49 with the amino group-specific reagent, trinitrobenzenesulfonic acid (TNBS) led to the inactivation of the PLA, but reaction occured not as expected at position 49, but at Lys-53. These findings lead us to propose a model, applicable to PLA's in general, in which cationic side chains at position 53 in these enzymes participate in phospholipid binding on the path to formation of the catalytic complex. This model serves to explain a number of unresolved observations in the current literature relating to enzyme-substrate interactions in the PLA's.

Phospholipases A2 (PLA) are calcium-requiring enzymes that catalyze hydrolysis of the 2-position ester of 3-sn-phosphoglycerides. These enzymes are prevalent throughout nature, structurally homologous, and are generally believed to share the same catalytic mechanism (for reviews, see 1, 2, and 3). The PLA's can be classified on the basis of several criteria, one being mechanistic differences relative to formation of the catalytic complex of enzyme, phospholipid and calcium. The Asp-49 PLA's (4) from venom sources have an ordered addition to the enzyme of, first, calcium, and, second, phospholipid

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(5). Asp-49 enzymes from some pancreatic sources appear to have a random addition of calcium and phospholipid in the formation of the catalytic complex (6). The Lys-49 PLA's show an ordered addition of, first, phospholipid, and, second, calcium (1).

In an attempt to demonstrate the location of Lys-49 in the binding pocket of the Lys-49 PLA's, we reacted a Lys-49 enzyme and a closely-related Asp-49 PLA, with trinitrobenzenesulfonic acid, TNBS (7). We were gratified to note that this reagent inactivated the Lys-49 PLA but had no such effect on the Asp-49 enzyme. We were surprised to discover, however, that modification actually took place not at Lys-49 but Lys-53. This led us to examine the literature regarding analyses of other PLA's with cationic residues at 53 relative to substrate binding properties and sensitivity to group-specific reagents. We present herein a unified model of the PLA-phospholipid interaction wherein residue 53 plays a crucial role.

MATERIALS

MATERIALS- The basic, monomeric Asp-49 (App-D-49) and Lys-49 (App-K-49) PLA's from Agkistrodon piscivorus piscivorus were purified as described earlier (1). TNBS was purchased from Pierce Chemical Co., 1,2-dioctanoyl phosphatidylcholine and 1,2-dihexanoyl phosphatidylcholine were purchased from Serdary Co., and TLCK-trypsin was purchased from Boehringer-Mannheim, Co..

ANALYTICAL PROCEDURES- Amino acid and sequence analyses were performed as described earlier (1). PLA activity was measured towards monolayers of 1,2-dioctanoyl phosphatidylcholine as described by Cohen, et al. (8).

CHEMICAL MODIFICATION OF APP-D-49 AND APP-K-49 WITH TNBS- App-D-49 or App-K-49 was dissolved in a solution (2 ml) of 0.05 M sodium bicarbonate, pH 10.1, and was reacted, at room temperature, with a 40- fold molar excess of TNBS to enzyme or a 3-fold molar excess of enzyme to TNBS. Similar conditions applied to the modification of either PLA at pH 7.32 except for the use of 0.05 M sodium phosphate. The modification was monitored with a spectrophotometer (Perkin-Elmer Lambda 5) at 340 nm. Determination of the stoichiometry of the reaction was based upon an extinction coefficient of $1.0 \times 10^{4}\ M^{-1}\ cm^{-1}$ at 340 nm for trinitrophenyllysine generated. The modification of App-K-49 and App-D-49 by TNBS was also studied in the presence of 1,2-dihexanoyl phosphatidylcholine, 1 mM, at a concentration below the CMC.

IDENTIFICATION OF THE TRINITROPHENYLATED LYSINE IN APP-K-49- App-K-49 (13.2 mg) was reacted with an excess of TNBS as described above except that the reaction was stopped after the derivatization of < 2 moles of lysine per mole of enzyme. The trinitrophenylated enzyme (TNP-App-K-49) was reduced and S-carboxymethylated. This product, RCM-TNP-App-K-49, was dialyzed against 5% acetic acid and,

then, water and lyophilized. RCM-TNP-App-K-49 was dissolved in 0.2 M N-ethylmorpholine acetate (1 ml), pH 8.0, and digested with TLCK-trypsin (1%, w/w). The tryptic peptides were lyophilized, redissolved in 1.0 ml of 0.15% trifluoroacetic acid (TFA) and 200 ul was subjected to reverse-phase HPLC on a Beckman Ultrasphere ODS column (25 X 0.46 cm). Peptides were separated with a linear gradient of increasing acetonitrile concentration from 0 to 60% in the same aqueous TFA solvent. The effluent was monitored at 340 nm.

RESULTS

Trinitrophenylation of App-K-49 and App-D-49:

Rate constants determined under several conditions for the reaction of TNBS with App-K-49 and App-D-49 are given in Table 1.

Reaction of App-D-49 at pH 10.1 with an excess of TNBS (Figure 1) resulted in the modification of all lysyl residues and this reaction followed pseudo first-order kinetics. The rate constant determined at this pH (using an excess of PLA to TNBS) and at pH 7.3 (using an excess of reagent) revealed a 15-fold difference in rate. Although one would expect a much slower reaction at the lower pH, our results are consistent with other reported applications of TNBS toward protein substrates (9). The presence of 1,2-dihexanoyl phosphatidylcholine in the reaction mixture had no measurable effect on the reactivity of TNBS for the lysyl residues of the Asp-49 PLA.

TABLE 1 - The Modification of Lysyl Residues in App-K-49 and App-D-49 with TNBS

CONDITIONS	RATE CONSTANT, M^{-1} SEC^{-1} App-K-49	App-D-49	TOTAL # AMINO GROUPS	# AMINO GROUPS MODIFIED
pH 10.10, Enzyme in excess	fast- 146		20	1
	slow- 4.6		20	14
		6.0	13	13
pH 7.32, TNBS in excess	fast- 5.5		20	1
	slow- 0.4		20	6
		0.4	13	5
pH 7.32, TNBS in excess, presence of 1mM dihexanoyl lecithin	fast- 9.8		20	1
	slow- 0.3		20	6
		0.4	13	5

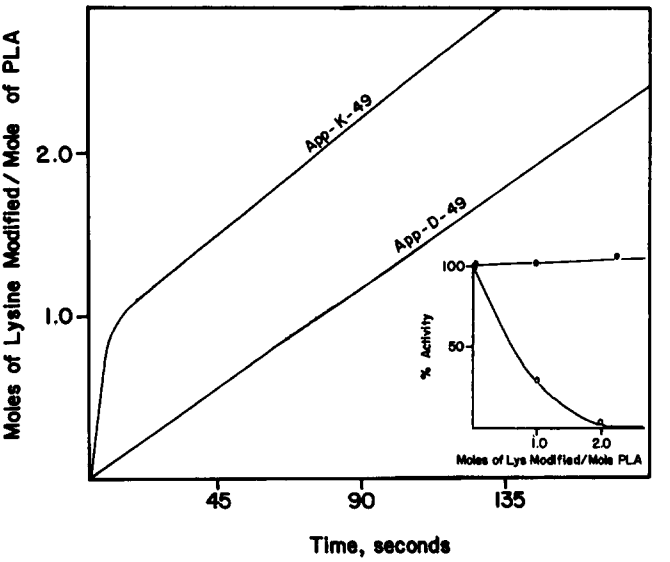

Figure 1- Time course of the modification at pH 10.1 of App-K-49 and App-D-49 with a 40-fold molar excess of TNBS. Inset- The effect on the enzymatic activity of App-D-49 and App-K-49 towards monolayers of short-chain lecithin upon derivatization of lysyl residues with TNBS.

Reaction of App-K-49 (with TNBS in excess) at pH 10.1 revealed two distinct reactions: a stoichiometric, fast reaction followed by a slow reaction (Figure 1). The completion of the stoichiometric reaction corresponded to the derivatization of one mole of lysine per mole of enzyme. To determine the rate constant of the fast reaction at pH 10.1 it was necessary to conduct the modification under conditions in which the enzyme was in excess. The fast reaction was found to proceed with a rate approximately 32 times greater than the steady-state reaction. The slow reaction, in the case of App-K-49, was found to proceed with a rate which was identical to that for the modification of all lysyl residues in App-D-49. In contrast to App-D-49, reaction of App-K-49 with a 40-fold excess of TNBS resulted in the modification of all but four amino groups. At pH 7.3, the modification of App-K-49 (with TNBS in excess) resulted in the decrease in rate for both fast and slow reactions to a comparable extent. The presence of monomolecular phospholipid in the reaction

mixture increased the rate of the fast reaction by nearly two-fold while the rate of the slow reaction was relatively unaffected.

Modification of App-D-49 and App-K-49 at pH 10.1 with an excess of TNBS was also studied with respect to the effect of this treatment upon enzymatic activity toward monolayers of short-chain lecithins at the air-water interface (Figure 1, inset). In the case of App-D-49 modification of up to 6 lysyl residues had no effect upon the enzymatic activity toward this substrate. In contrast, modification of a single lysine in App-K-49 resulted in the near-complete inactivation of the enzyme. The inactivation of App-K-49 occured both in the absence and presence of phospholipid.

Identification of Lys-53 as the Major Site of Trinitrophenylation in App-K-49:

Tryptic peptides from RCM-TNP-App-K-49 were separated by reverse-phase HPLC (Figure 2) and the effluent was monitored at 340 nm to facilitate identification of TNP-peptides. Although some minor

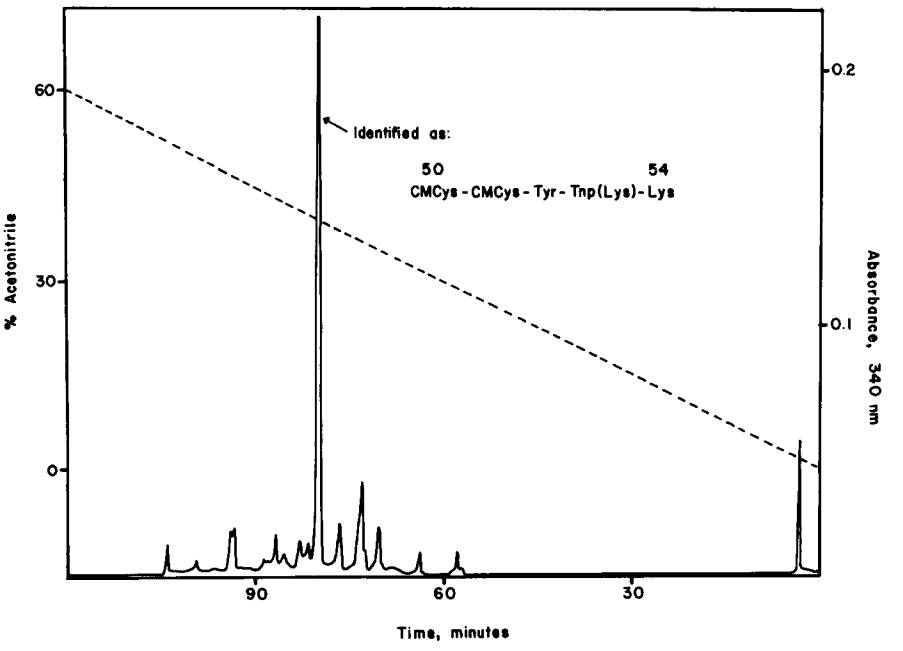

Figure 2- HPLC separation of the tryptic peptides from RCM-TNP-App-K-49.

peaks were observed at this wavelength, a major peak, accounting for > 70% of the measured absorbance at 340nm, eluted at 42% acetonitrile in the gradient. This material was collected, dried, and subjected to amino acid analysis and automated Edman degradation. The peak was shown to contain a pure peptide corresponding to that from Cys-50 to Lys-54 (Cys-Cys-Tyr-(TNPLys)-Lys). The phenylthiohydantoin of the TNP-Lys-53 was identified by its absorbance at 340 nm.

DISCUSSION

We have used TNBS in the selective modification of an active site lysyl residue, Lys-53, in a Lys-49 PLA. Using a 40-fold molar excess of reagent, all 13 amino groups in App-D-49. Interestingly, only 15/20 amino groups react in App-K-49. and App-K-49. Reaction of TNBS with these two classes of PLA's reveals different kinetics: the modification of App-D-49 proceeds with pseudo first-order kinetics while the modification of App-K-49 reveals biphasic kinetics with a fast, stoichiometric reaction followed by a slow reaction. The fast reaction involves the derivatization of one lysine residue which is 32 times more reactive than all other reactive lysines in the Lys-49 PLA and, as established by the peptide chemistry, this residue is Lys-53.

Modification of App-D-49 with TNBS has no effect upon this enzyme's activity towards monolayers of short-chain lecithin. This is consistent with findings in this laboratory (John M. Maraganore and Robert L. Heinrikson, unpublished results) in which carbamylation of up to 5 lysyl residues has no measurable effect on the activity of App-D-49. These results support the conclusion that such modifications result in the derivatization of lysyl residues of this Asp-49 PLA which play no significant functional role. On the contrary, modification of App-K-49 with TNBS and derivatization of a single lysine results in the near-complete inactivation of this enzyme towards monolayers of short-chain lecithins. Interestingly, carbamylation of as many as six lysyl residues in this Lys-49 PLA has no

effect upon catalytic activity (John M. Maraganore and Robert L. Heinrikson, unpublished results). Therefore, modification of the Lys-49 PLA's with TNBS results in the specific alteration of a lysine in or near the active site of the enzyme. This specificity may be due to anchiomeric facilitation through interactions of TNBS with the hydrophobic active center of this enzyme.

While the specificity of TNBS for a single lysine residue in the active center of App-K-49 is apparent from the kinetic data presented above, it is even more evident in the HPLC pattern of tryptic TNP-peptides generated from RCM-TNP-App-K-49 (Figure 2). The major peak at 340 nm corresponded to the pentapeptide Cys-50 to Lys-54, and the site of reaction was established as Lys-53 by automated Edman degradation. No measurable modification of Lys-49 was detected; moreover, the specificity of the reagent for Lys-53 was remarkable, especially as App-K-49 has 19 lysines / 121 amino acid residues (John M. Maraganore and Robert L. Heinrikson, unpublished results).

These results provide the basis for conclusions that serve to unify certain ideas regarding substrate binding in the PLA's. First, it would appear that, as Lys-53 is a positively-charged residue which resides in the hydrophobic active center of the Lys-49 PLA's and as it is only one turn of the alpha-helix away from Lys-49, this group could be another of several apparent compensatory changes in the Lys-49 PLA's. Together with the change at position 49, Lys-53 may account for the reversed order of binding of calcium and/or phospholipid in the formation of the catalytic complex. To test this hypothesis, we studied the effects of monomolecular substrates on the modification of Lys-53. In fact, such compounds activate the pre-steady-state reaction of App-K-49 with TNBS (Table 1). It was of interest to find an effect upon the modification of Lys-53 with TNBS in the presence of monomolecular substrate in the reaction mixture. Indeed, this

result demonstrates that Lys-53 is near the phospholipid binding site of the Lys-49 PLA's. It is tempting to speculate that this residue may be involved in the binding of a second, non-substrate phospholipid molecule involved in the recognition of surfaces by all PLA's: kinetic evidence generated by several investigators (10) points to the requirement of a "helper" molecule in the PLA-catalyzed hydrolysis of organized substrate.

Lys-53 is not absolutely conserved in the PLA's, nor is it unique to the Lys-49 enzymes . In fact, a lysine at position 53 exists in the bovine pancreatic PLA and an arginine exists at this position in the porcine enzymes. Interestingly, both the bovine and porcine enzymes are proposed to have a random addition of calcium and substrate in the formation of their catalytic complex (6). We would propose that this affinity for the phospholipid substrate in the absence of calcium is mediated by the presence in the active-site pocket of a cationic side chain at residue 53. In the case of the Lys-49 PLA's, the presence in the active site of two positively-charged side chains (Lys-49 and 53) assures an ordered binding of, first, phospholipid, and, second, calcium. The presence of cationic side chains at position 53 in the active sites of the bovine and porcine PLA's could account for a weak affinity of these enzymes for phospholipid in the absence of calcium and, perhaps, their random mechanism of formation of the catalytic complex. Other Asp-49 PLA's that have been characterized to date usually have a glycine at residue 53, and we suggest that the absence of positively-charged contributions to the active center of these enzymes accounts for an ordered binding of, first, calcium, and, second, phospholipid. These structural and mechanistic differences between the three classes of PLA's are schematically represented in Figure 3.

Our proposed role for Arg-53 in the porcine pancreatic PLA is supported by modification studies by Vensel and Kantrowitz (11) where phenylglyoxal was used to modify an "essential" arginine residue in

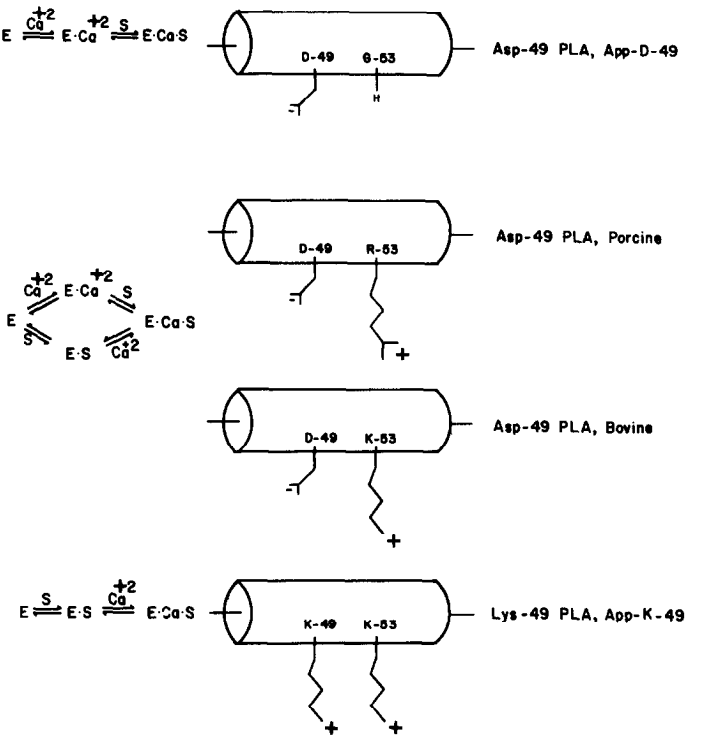

Figure 3- Schematic representation of the proposed relationship between cationic and/or anionic side chains at positions 49 and 53 and the ordered or random formation of the catalytic complex.

this enzyme. They found that this derivatization of a single arginyl residue resulted in the inactivation of the enzyme. Also, the inactivation was blocked by n-alkylphosphorylcholine inhibitors. These investigators were unable to identify the arginine modified, but we suspect, based upon the present findings, that it was Arg-53. Furthermore, we predict that trinitrophenylation of the bovine enzyme will occur at Lys-53 and that such treatment will render the enzyme inactive.

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