Activities of Native and Tyrosine-69 Mutant Phospholipases A2 on Phospholipid Analogues. A Reevaluation of the Minimal Substrate Requirements
Kuipers, Oscar P.; Dekker, Nicolaas; Verheij, Hubertus M.; Haas, Gerard H. de

Published in:
Biochemistry

DOI:
10.1021/bi00477a029

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1990

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Activities of Native and Tyrosine-69 Mutant Phospholipases A2 on Phospholipid Analogues. A Reevaluation of the Minimal Substrate Requirements†

Oscar P. Kuipers, Nicolaas Dekker, Hubertus M. Verheij,* and Gerard H. de Haas
Department of Biochemistry, University of Utrecht, CBLE, P.O. Box 80.054, NL-3508 TB Utrecht, The Netherlands
Received November 27, 1989; Revised Manuscript Received February 26, 1990

ABSTRACT: The role of Tyr-69 of porcine pancreatic phospholipase A2 in substrate binding was studied with the help of proteins modified by site-directed mutagenesis and phospholipid analogues with a changed head-group geometry. Two mutants were used containing Phe and Lys, respectively, at position 69. Modifications in the phospholipids included introduction of a sulfur at the phosphorus (thionophospholipids), removal of the negative charge at phosphorus (phosphatidic acid dimethyl ester), and reduction (phosphonolipids) or extension (diacylbutanetriol choline phosphate) of the distance between the phosphorus and the acyl ester bond. Replacement of Tyr-69 by Lys reduces enzymatic activity, but the mutant enzyme retains both the stereospecificity and positional specificity of native phospholipase A2. The Phe-69 mutant not only hydrolyzes the R, isomer of thionophospholipids more efficiently than the wild-type enzyme, but the S, thiono isomer is hydrolyzed too, although at a low (~4%) rate. Phosphonolipids are hydrolyzed by native phospholipase A2 about 7 times more slowly than natural phospholipids, with retention of positional specificity and a (partial) loss of stereospecificity. The dimethyl ester of phosphatidic acid is degraded efficiently in a calcium-dependent and positional-specific way by native phospholipase A2 and by the mutants, indicating that a negative charge at phosphorus is not an absolute substrate requirement. The activities on the phosphatidic acid dimethyl ester of native enzyme and the Lys-69 mutant are lower than those on the corresponding lecithin, in contrast to the Phe-69 mutant, which has equal activities on both substrates. Our data suggest that in porcine pancreatic phospholipase A2 fixation of the phosphate group is achieved both by an interaction with the phenolic OH of Tyr-69 and by an interaction with the calcium ion. In the mutant Y69K the e-NH2 group can play a role similar to that of the Tyr OH group in native PLA2. The smaller side chain of the Y69F mutant can interact with more bulky head groups, allowing for relatively high enzymatic activities on modified phospholipids. On the basis of these results, a reevaluation of the minimal substrate requirements of phospholipase A2 is presented.

The lipolytic enzyme phospholipase A2 (PLA2) specifically cleaves the 2-acyl ester bond of phosphoglycerides in a calcium-dependent reaction. The mechanism by which the calcium ion activates this enzyme is in debate. For some phospholipases from snake venoms it has been suggested that the enzyme binds calcium ions and substrate in an ordered way [for a review, see Verheij et al. (1981a)]. For PLA2s isolated from mammalian pancreatic tissue the situation is more complex. At pH values of 6 or lower, all pancreatic phospholipases readily bind to micelles of substrates or substrate analogues in the absence of calcium ions, although no hydrolysis occurs as long as no calcium ion is bound in the active site. Above pH 6 the binding of some PLA2s to lipid aggregates becomes calcium dependent (van Dam-Mieras et al., 1975). Comparison of naturally occurring isozymes as well as site-specific mutagenesis studies revealed that two aspartate residues, i.e., Asp-66 and Asp-71, are involved in the binding of a second calcium ion (Donné-Op den Kelder et al., 1983; van den Bergh et al., 1989a). Hence, it was assumed that the role of this second calcium ion is to improve the binding of PLA2 to micelles and thereby to increase the turnover number of the enzyme (van den Bergh et al., 1989a).

Chemical modification studies as well as X-ray analyses have shown that the "catalytic" calcium ion is bound to Asp-49 in the vicinity of the active site histidine-48 (Fleer el al., 1981; Dijkstra et al., 1983). Although several di- and trivalent cations bind stoichiometrically to PLA2s from pancreatic tissue or snake venom, the enzymatic activity in these cases is only a few percent at the most. These observations suggested (Verheij et al., 1980) that the calcium ion in the active site serves two functions: the fixation of the phosphate of the polar head group and the polarization of the carbonyl of the susceptible ester bond. An X-ray structure of the ternary complex between phospholipase A2, the Ca2+ ion, and a substrate analogue molecule could elucidate the relative position of these molecules. Unfortunately, the crystallization of such a complex has not yet been achieved. There is, however, experimental evidence accumulating that sheds more light on these interactions. The fixation of the phosphate by the Ca2+ ion was merely suggested by intuition, until Tsai et al. (1985) showed that the Ca2+ ion is probably in contact with the pro-R oxygen.
of this phosphate. More recently, Kuipers et al. (1989a) suggested that the hydroxyl of Tyr-69 is also in contact with the phosphate function and that this interaction contributes to the stereospecificity of phospholipase A₂. We decided to study these interactions in more detail by making use of native porcine pancreatic PLA₂ and two mutants in which Tyr-69 was replaced by a Phe (no hydrogen-bond donor) and a Lys (a hydrogen-bond donor), respectively, and substrate molecules in which the polar head group was changed. Thus, molecules lacking the negative charge on phosphorus and lecithin analogues with an increased or decreased distance between the phosphorus and the susceptible ester bond were included. The results are discussed in terms of the proposed catalytic mechanism (Verheij et al., 1980) and the minimal substrate requirements of phospholipase A₂ (de Haas et al., 1968).

**Materials and Methods**

**Construction of Mutant Phospholipases.** Mutant Y69K¹ was constructed essentially as described for mutant Y69F (Kuipers et al., 1989a). Briefly, the mutagenic oligonucleotide 5'-GCT TTC GGT C*TT* GGG ATT G-3' was used in the gapped duplex procedure, for which amber selection was employed (Kramer et al., 1984). Asterisks denote the places of base mutations in the mutagenic primer, relative to the wild-type sequence. The cDNA encoding the mutant PLA₂ species was sequenced, and a BstXI–BglII fragment containing the mutation was ligated into the expression vector pOK13. After transformation and expression in *Escherichia coli* K-12 strain MC4100 (Silhavy et al., 1984), containing plasmid pCI857, the mutant PLA₂ was obtained by tryptic cleavage of reoxidized fusion protein (de Geus et al., 1987). Purification was achieved by CM-cellulose chromatography at pH 5 and 6, followed by chromatography on DEAE-cellulose at pH 8.

**Phospholipids.** The 1,2-diacyl-sn-glycerols used in this study were prepared by acylation of 3-O-benzyl-sn-glycerol (Bonsen et al., 1972a) followed by catalytic hydrogenolysis. Phosphorylation with an excess of phosphorusoxy-trichloride and subsequent reaction with methanol yielded the dimethyl ester phosphaticid acid, which was purified by silicic acid chromatography with hexane–ether mixtures as eluants. The 1,2-sn-diacyllecithin was prepared from the diglyceride by phosphorylation with 2-chloro-2-oxo-1,3,2-dioxaphospholane followed by ring opening with trimethylamine essentially as described by Chandrakumar and Hajdu (1983). The thionolecithin 1,2-diacyllecithin–sn-glycerol–3-thionophosphocholine was synthesized from the corresponding diglyceride as described by Nifant’ev et al. (1978). The **Rₙ** and **Sₙ** isomers were prepared from the mixture by degradation with phospholipase A₂ from *Crotalus adamanteus* venom, separation of the contaminants the excitation wavelength was 280 nm. The binding of the lyso PLA₂ to monomers and micelles of phospholipids was determined by following the increase of tryptophan fluorescence or by ultraviolet difference spectroscopy upon addition of increasing concentrations of the nonhydrolyzable substrate analogues choline n-dodecyl phosphate (cmc 1.3 mM) for monomer binding and choline n-hexadecyl phosphate (cmc 10 μM) for micelle binding. Assays were performed in a buffer containing 100 mM NaNO₃, 50 mM CaCl₂, and 100 mM NaCl at pH 6.0. From saturation curves, obtained with lipid monomers, a **Kₘ** value can directly be derived. The data concerning micelle binding were analyzed in terms of the binding of the enzyme to a theoretical lipid particle consisting of N monomers with a dissociation constant **K₆**. The **K₆** value, discussed extensively by de Araujo et al. (1979), is the experimental concentration at which 50% of the enzyme is saturated with micelles. Experimental conditions were as indicated before (van Dam-Mierse et al., 1975), except that in the fluorescence experiments the excitation wavelength was 280 nm. The binding of PLA₂ to micelles of choline n-hexadecyl phosphate (Cl6PN) was determined also by gel filtration on Sephadex G-75 columns as described before (de Araujo et al., 1979).

**NMR Studies.** ³¹P NMR spectra were recorded at 81.015 MHz on a Bruker WP-200 WB spectrometer equipped with a multinuclear 10-mm probe. To samples of 2.5 mL containing...
of these product analogues at concentrations below the cmc of glycero-3-phosphocholine visualized by ultraviolet difference spectroscopy. The addition of phosphates (Figure 2) produces a tyrosine perturbation. From a series of such spectra at varying lipid concentrations a dissociation constant of 0.3 μM (34.2 μM) with 2.54 mM C16PN; (curve 6) wild-type PLA2 (34.2 μM) with 2.54 mM C16PN. For details of the experiments, see Materials and Methods.

RESULTS

Enzymatic Activities of Tyr-69 Mutant Phospholipases. In a previous study (Kuiipers et al., 1989a) it was shown that replacement of tyrosine-69 by phenylalanine results in an active enzyme with a changed stereospecificity. This substitution removes a possible hydrogen bridge contact, and hence, the substitution of lysine for tyrosine-69 was also of interest. In Table I the activities of this mutant phospholipase are given, and for reasons of comparison the data of native and Y69F PLA2s are also included. It is clear that the Y69K mutant PLA2 has rather low activity on aggregated substrates as compared to native and Y69F PLA2s. This was somewhat unexpected, since a lysyl residue is often encountered at this position in many venom PLA2s. When monomeric substrates are used, all three PLA2s show comparable kcat/Km values.

Direct Binding Studies. The addition of choline n-alkyl phosphates (Figure 1, analogue 4) to native porcine pancreatic phospholipase A2 causes spectral perturbations, which can be visualized by ultraviolet difference spectroscopy. The addition of these product analogues at concentrations below the cmc induces a perturbation spectrum that is characteristic of (a) perturbed tyrosine residue(s). Above the cmc in the presence of micelles not only is this tyrosine perturbation observed but also a perturbation of the single tryptophan-3 of PLA2. It has been suggested by Meijer et al. (1979) that Tyr-69 causes the spectral changes after addition of monomeric substrate analogues, and it was therefore of interest to analyze the spectra of Y69F and Y69K after addition of choline alkyl phosphates at concentrations both below and above the cmc. As can be seen from Figure 2A the addition of monomeric concentrations of choline n-dodecyl phosphate (C12PN) to native PLA2 induces a tyrosine perturbation. From a series of such spectra at varying lipid concentrations a dissociation constant of 0.3

Table I: Enzymatic Activities and Binding of Wild-Type and Two Mutant Phospholipases A2 Using Monomeric and Micellar Substances and Substrate Analogues

<table>
<thead>
<tr>
<th>Substances and Substrate Analogues</th>
<th>diC6dithioPC</th>
<th>diC8PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>enzyme kcat/Km (s⁻¹M⁻¹)</td>
<td>C12PN Kd (mM)</td>
<td>Vmax (μmol/min⁻¹mg⁻¹)</td>
</tr>
<tr>
<td>kcat/Km (M⁻¹min⁻¹)</td>
<td>C16PN N/Kd (μM)</td>
<td></td>
</tr>
<tr>
<td>wild type</td>
<td>900</td>
<td>0.3</td>
</tr>
<tr>
<td>Lys-69</td>
<td>1350</td>
<td>0.3</td>
</tr>
<tr>
<td>Phe-69</td>
<td>1300</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*Monomeric substrates (analogue): diC6dithioPC, rac-1,2-dihexanoyldithioleicithin; C12PN, choline n-dodecyl phosphate; Micellar substrates (analogue): diC8PC, 1,2-diacyl-sn-glycero-3-phosphocholine; C16PN, choline n-hexadecyl phosphate. Statistical errors did not exceed 10% in each case. For details, see Materials and Methods.

A 5 mM solution of R1 or S1 isomer was added 10% D2O for deuterium lock. Chemical shifts are referenced to external 85% H3PO4 (0 ppm), a positive signal indicating a downfield shift. Spectra were recorded at 293 K with a sweep width of 2000 Hz with 8K data points, usually 128 transients with 90° pulses, and a cycle time of 2 s. Broad-band proton noise decoupling was employed. Prior to Fourier transformation the data were multiplied by a Gaussian window function (LB-2, GB 0.2).

![Figure 1: Schematic structures of several phospholipid analogues. R1 and R2 represent alkyl chains of variable lengths. (1) 1,2-diacyl-sn-glycerol-3-phosphocholine (lecithin); (2) 1,2-diacyl-sn-glycerol-3-thionosphosphocholine (thionolecithin); (3) 1,2-diacyl-sn-glycerol-3-(dimethyl phosphate); (4) choline n-alkyl phosphate; (5) choline 1,2-bis(acetylxy)-3-propyl-phosphonate (phosphono analogue of lecithin); (6) 1,2-diacyl-1,2,4-butanetriolcholine phosphate.](image1)

![Figure 2: (Panel A) UV difference spectroscopy spectra obtained upon binding of wild-type and Y69F PLA2s to the monomeric product analogue choline n-dodecyl phosphate: (curve 1) Y69F PLA2 (27.7 μM) with 0.7 mM C12PN; (curve 2) wild-type PLA2 (31.3 μM) with 0.7 mM C12PN. (Panel B) UV difference spectroscopy spectra obtained upon binding of wild-type, Y69K, and Y69F PLA2s to the micellar product analogue choline n-hexadecyl phosphate: (curve 1) Y69K PLA2 (27.4 μM) with 0.57 mM C16PN; (curve 2) Y69K PLA2 (27.4 μM) with 2.54 mM C16PN; (curve 3) Y69F PLA2 (34.3 μM) with 0.57 mM C16PN; (curve 4) wild-type PLA2 (34.2 μM) with 0.57 mM C16PN; (curve 5) Y69F PLA2 (34.3 μM) with 2.54 mM C16PN; (curve 6) wild-type PLA2 (34.2 μM) with 2.54 mM C16PN. For details of the experiments, see Materials and Methods.](image2)
mM was calculated, in agreement with previously reported values (Meijer et al., 1979). In contrast, no such spectral changes were observed after addition of this detergent to both mutant phospholipases. The Y69F mutant PL2A lacked the tyrosine perturbation peak at 289 nm, and instead, two smaller peaks were visible (Figure 2A). These peaks could be due to a weak tryptophan perturbation, in agreement with the fact that tryptophan fluorescence increases slightly upon addition of choline n-dodecyl phosphate (data not shown). From the increase of the fluorescence signal, dissociation constants of 0.3 mM were obtained both for native PL2A and for Y69F PL2A. Thus Tyr-69 seems indeed to be one of the chromophores involved in the interaction of monomeric substrate (analogues) with native porcine pancreatic phospholipase A2.

When the titrations were carried out with the Y69K mutant, signals were observed neither with ultraviolet nor with fluorescence spectroscopy. This could mean that this mutant PL2A does not bind monomeric choline alkyl phosphates, but in view of the kinetic data of Y69K PL2A with monomeric substrates we consider this possibility unlikely, and we suppose that the lack of signal is due to intramolecular quenching.

The addition of the product analogue choline n-hexadecyl phosphate at concentrations above its cmc induced spectral changes in native and the Y69F mutant that were dominated by an absorption at 294 nm (Figure 2B). Such a spectrum is characteristic of a tryptophan perturbation as has been concluded before to be the case for native PL2A (van Damm-Mieras et al., 1975). From the spectral changes the dissociation constants (NKd) of native and Y69F PL2A were calculated to be 380 and 480 μM, respectively (Table I). The fact that these values are similar to the binding constants for monomeric substrate analogues is just a coincidence. The shape of the spectra (Figure 2B and the gel filtration studies that were carried out (see below) indicate that we are dealing with the formation of a lipid–enzyme complex of high aggregation number. Small signals were observed when the titration was carried out with the Y69K mutant.

To test whether this mutant still binds to micelles, we carried out direct binding experiments on Sephadex G-75 columns (see Materials and Methods). On such columns, PL2A bound to the C16PN micelles (molecular mass ~70 kDa) will coelute near the void volume of the column, in contrast to unbound PL2A, which will elute at a position corresponding to a molecular mass of 14 kDa. When we carried out this experiment, the mutant Y69K comigrated with choline n-hexadecyl phosphate micelles in the void volume of Sephadex G-75 (data not shown). We therefore conclude that the low signal in the spectroscopic experiment cannot be due to a lack of binding.

**Activities on Thionolecithins.** The Rg isomer of thionolecithins (Figure 1, analogue 2) has been shown to be a substrate for PL2A from various sources (Tsai et al., 1985). The activities of native and of mutant PL2A were determined on 1,2-didodecanoyl-sn-glycero-3-phosphocholine (Figure 1, analogue 1) and on its Rg and Sg thionoanalogues. It is known that the Rg and Sg isomers of dipalmitoylthionolecithin have different phase transitions (Chang et al., 1986). Therefore, we tested the medium-chain phospholipids in the presence of tuarodeoxycholate in a 2-fold excess to obtain clear mixed micellar solutions. Such micelles are to be preferred over the use of bilayer structures where the activity of phospholipases is strongly influenced by physicochemical parameters (Jain & Berg, 1989). From the results presented in Table II it can be seen that, with the normal phospholipid as a substrate, the native enzyme is more active than the Phe and Lys mutants. The ratios between the activities are, however, comparable to the ratios with diocanoyllecithin as a substrate, demonstrating that the presence of detergents does not give rise to conflicting results. The activity of the native PL2A on the Rg thionolecithin is about 4 times lower than that on the normal lecithin. More striking is the observation that the two mutant PL2A respond quite differently to the introduction of a sulfur in the phosphate moiety. The Y69K mutant has a reduced activity; the Y69F mutant has an increased activity on the thiono analogue compared to regular lecithin. Also, with Sg isomer there is a clear distinction: both native PL2A and the Y69K mutant show very low activities on this compound, whereas the Y69F mutant has about 4% activity on the Sg compared to the Rg isomer.

Although the titration experiments were carried out under a nitrogen atmosphere, the possibility that a slow oxidation took place could not be excluded a priori. Such an oxidation would generate normal lecithin that could be hydrolyzed by PL2A instead of the thiono analogue itself. Thus, the low hydrolysis rates that are given in Table II could be too high because of oxidation. Therefore, the hydrolysis was also followed by 31P NMR, a technique that allows the simultaneous observation of hydrolysis and of a possible oxidation. Figure 3 (panel A) shows the hydrolysis of the Rg isomer by native PL2A and the concomitant appearance of the corresponding lyso derivative. With both mutant PL2A similar results were obtained (data not shown). When the Sg isomer was incubated in the presence of the Y69F mutant, a slow hydrolysis occurred (Figure 3, panel B). From these data a specific activity of 1.6 was calculated, a value that agrees well with the activity found in the titrator (Table I). In agreement with this observation was the fact that inspection of the spectral range around 0 ppm after overnight incubation did not reveal the presence of oxidation products that could be expected to show up around this position. Overnight incubation of native PL2A and of Y69K mutant revealed the presence of traces of lyso derivative (data not shown) from which specific activities of less than 0.06 and 0.008, respectively, were calculated. Thus, whereas the Y69F mutant is only 2.3 times more active than native PL2A, the Rg isomer, this ratio has increased to at least 27 for the Sg isomer.

**Activities on Neutral Phospholipid Analogues.** Another modification that was made in the polar head group of the substrate molecule was the removal of the negative charge of the phosphate (Figure 1, analogue 3). This modification makes the analogue insoluble; in water it forms emulsions with the size of the droplets depending on the degree of agitation. Under these conditions no hydrolysis by PL2A occurs (data not shown). The triester droplets are readily soluble in diethyl ether, but in the presence of deoxycholate the system remains biphasic. It was known already that, neither in diethyl ether/buffer systems nor in the presence of deoxycholate, enzymatic hydrolysis occurred with dibenzyl esters of 1,2-

<table>
<thead>
<tr>
<th>enzyme</th>
<th>diC12PC</th>
<th>Rg isomer</th>
<th>Sg isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>55</td>
<td>13</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Lys-69</td>
<td>11</td>
<td>2</td>
<td>0.02</td>
</tr>
<tr>
<td>Phe-69</td>
<td>14</td>
<td>30</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Table II: Activities of Wild-Type and Mutant PL2A on Normal Lecithins and on the Two Isomers of Thionolecithins

**Biochemistry, Vol. 29, No. 25, 1990 6097**
or taurodeoxycholate was degradation of this substrate by PLA2 or by the mutant enzymes observed by us (data not shown). In the presence of relatively high concentrations of EDTA no hydrolysis occurs. Second, thin-layer chromatography in ether–hexane (95:5 v/v) of the hydrolysis products of the dimethyl ester of 1-hexadecanoyl-2-tetradecanoyl-sn-glycerol 3-phosphate after incubation with porcine pancreatic PLA2 shows two spots with RF values of 0.87 and 0.26 corresponding to fatty acid and dimethyl ester of 1-acyl-sn-glycerol phosphate, respectively. In this solvent system (lyso)phospholipids with a negative charge on the phosphorus remain at the origin, and hence, removal of a methyl group prior to hydrolysis can be excluded. Gas chromatographic analysis showed that in accordance with the known specificity of phospholipases A2 exclusively myristic acid was released, whereas the lyso derivative contained palmitic acid only (data not shown).

**Activities on Substrates with Modified Ester–Phosphate Distance.** The next change that was made in the structure of the substrate was a modification of the distance between the phosphate and the sn-2 ester bond. Thus, when the phosphorus was linked to the glycerol via a direct carbon–phosphorus bond (Figure 1, analogue 5), the distance between the negative charge and the susceptible ester bond was reduced. Despite this change in the structure rac-1-tetradecanoyl-2-dodecanoylphosphonolecithin turned out to be a rather good substrate for phospholipase A2; in the presence of a 2-fold excess of taurodeoxycholate, this analogue was degraded by native PLA2 with about 16% of the rate on normal rac-didodecanoyllecithin. Also the Phe-69 mutant was able to hydrolyze these substrates, but only with about 6% of the rate observed with normal rac-didodecanoyllecithins. In order to determine which ester bond was cleaved, we analyzed the lyso derivative and the liberated fatty acid, which were isolated after hydrolysis of the phosphonolecithin by native PLA2, with gas–liquid chromatography. The results indicated that the enzyme had retained full positional specificity, since only dodecanoic acid was released. To our surprise, however, we found that native porcine pancreatic PLA2 was able to hydrolyze this racemic phospholipid to completion. Also after 100% hydrolysis the only fatty acid that was released was dodecanoic acid. Using the pure L and D isomers, we were able to quantitatively determine the activities of PLA2 on these isomeric phospholipids with the pH-stat. The D isomer was hydrolyzed about 100 times more slowly than the L isomer. Although the rate of hydrolysis of the D isomer is quite low, the difference with nonenzymatic hydrolysis is large. Qualitatively, this is illustrated by the fact that in racemic mixtures of phosphonolipids the phosphonolipid is hydrolyzed 100% by native and the two mutant PLA2s. In contrast, racemic mixtures of normal phospholipids were degraded 50% only by

**Activities of Wild-Type, Lys-69, and Phe-69 PLA2s on diC12PC and Its Dimethyl Ester Derivative in the Presence of the Substrate Analogue C16PN**

<table>
<thead>
<tr>
<th>enzyme</th>
<th>diC12PC</th>
<th>diC12dimethylPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>12</td>
<td>1.4</td>
</tr>
<tr>
<td>Lys-69</td>
<td>4.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Phe-69</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

*diC12PC, 1,2-didodecanoyl-sn-glycerol-3-phosphocholine; diC12dimethylPA, 1,2-didodecanoyl-sn-glycerol-3-(dimethyl phosphate). Assays were performed with a pH-stat, as described under Materials and Methods. Accuracy was about 10% for each given value.*

---

**Diacyl-sn-glycerol 3-phosphate and with dimethyl esters of 1,3-diacyl-sn-glycerol 2-phosphates (Slotboom et al., 1970). In the present study we obtained similar results with the dimethyl ester of 1,2-diacyl-sn-glycerol 3-phosphate.** Neither in the presence of ether nor in the presence of deoxycholate or taurodeoxycholate was degradation of this substrate by PLA2 or by the mutant enzymes observed by us (data not shown). In the presence of relatively high concentrations of the product analogue choline n-hexadecyl phosphate (Figure 1, analogue 4), however, the dimethyl ester gives clear solutions, probably mixed micelles, which are readily attacked by native pancreatic PLA2 as well as by the Y69F and Y69K mutants (Table III). Again, as seen with the thionolecithin, the relative high activity of the Y69F phospholipase as compared to the native and Y69K enzymes on this modified substrate is worth mentioning. That this hydrolysis is due to the action of PLA2 was shown by the following experiments. First, the reaction is calcium dependent: in the presence of EDTA no hydrolysis occurs. Second, thin-layer chromatography in ether–hexane (95:5 v/v) of the hydrolysis products of the dimethyl ester of 1-hexadecanoyl-2-tetradecanoyl-sn-glycerol 3-phosphate after incubation with porcine pancreatic PLA2 shows two spots with RF values of 0.87 and 0.26 corresponding to fatty acid and dimethyl ester of 1-acyl-sn-glycerol phosphate, respectively. In this solvent system (lyso)phospholipids with a negative charge on the phosphorus remain at the origin, and hence, removal of a methyl group prior to hydrolysis can be excluded. Gas chromatographic analysis showed that in accordance with the known specificity of phospholipases A2 exclusively myristic acid was released, whereas the lyso derivative contained palmitic acid only (data not shown).
Substrate Requirements of Phospholipase A,

![Diagram](image)

### FIGURE 4: Degradation of rac-1,2-didodecanoyllecithins and rac-1-myristoyl-2-dodecanoylphosphonolecithins by wild-type, Y69K, and Y69F PLA2s. Incubations were performed in the presence of sodium taurodeoxycholate (2 mol/mol of phospholipid) in 50 mM borate buffer containing 5 mM CaCl2 at pH 8.0 in a total volume of 500 μL for 8 h at 37 °C. Lanes a–c show the degradation products of rac-1,2-didodecanoyllecithins by wild-type (25 μg), Y69K (100 μg), and Y69F (100 μg) PLA2s, respectively. Lanes d–f show the degradation products of rac-1-myristoyl-2-dodecanoylphosphonolecithins by wild-type (25 μg), Y69K (100 μg), and Y69F (100 μg) PLA2s, respectively. The TLC plate was developed in CHCl3-MeOH-H2O (65:35:8 by volume). Spots were visualized by spraying with phosphorus reagent. The upper spots represent nondegraded lecithin or phosphonolecithin, whereas the lower spots represent the lyso derivatives of these compounds.

native and the Y69K mutant but 100% by the Y69F mutant (Figure 4). From these data we conclude that, with the phosphonolecithin as a substrate, the enzyme retains its positional specificity but loses part of its stereospecificity.

Increasing the distance between the phosphate and the ester bonds as in rac-1-hexadecanoyl-2-tetradecanoyl-1,2,4-butanetriol phosphate (Figure 1, analogue 6) completely abolished the activity of both native and the mutant phospholipases.

### DISCUSSION

From results obtained with phospholipases in which Tyr-69 was nitrated, Meijer et al. (1979) concluded that the side chain of this residue is involved in the interaction of PLA2 with monomeric and with micellar substrate analogues. Our results support this conclusion for the monomers, but the role of Tyr-69 in the interaction with micelles is less clear. Addition of monomeric choline phosphate n-dodecyl to the Y69F mutant does not give rise to the spectral changes, characteristic of tyrosine perturbations (Donovan, 1969), that are observed with the native PLA2. The small signal that is observed with the Y69F PLA2 has the characteristics of a tryptophan perturbation. The fact that this venom PLA2 contains three Trp residues in the interaction with micelles is less clear. Addition of calcium ions to the C. atrox enzyme does not produce spectral changes (Purdon et al., 1977). The quenching of lipid-induced spectra in native PLA2 by Lys-69 is not a general phenomenon. Native C. atrox PLA2, which contains a lysyl residue at position 69, produces large spectral changes in the presence of choline n-hexadecyl phosphate [data not shown; see also Verheij et al. (1981b)]. The fact that this venom PLA2 contains three Trp residues at positions 21, 31, and 117 but no aromatic residue at position 3 makes it difficult to draw definite conclusions from these experiments.

From the spectral data no quantitative affinities of the Y69K mutant for micelles can be obtained. Qualitatively, the use of gel filtration showed that the Y69K mutant has affinity for micelles of choline n-hexadecyl phosphate. This conclusion is supported by the kinetic data of native and mutant PLA2s (Table 1) with several aggregated substrates, since without binding to these aggregates one would not observe activity. The Kmapp values that were obtained with dioctanoyllecithin show that no large changes have occurred in the affinity for aggregated substrate molecules as a result of the mutation of Tyr-69 into phenylalanine or lysine. Meijer et al. (1979) based their conclusions about the role of Tyr-69 in the interaction with aggregated lipids on nitrated and on dansylated PLA2. The fact that nitrated tyrosine is a stronger acid than tyrosine implies that at pH 8 nitrotyrosine carries a negative charge. It has been discussed before (Verheij et al., 1981a) that a negative charge at the lipid binding domain interferes with binding. On the other hand, the introduction of a large hydrophobic group like the dansyl moiety obviously improves hydrophobic interactions.

In the 56 phospholipases A2 that have been sequenced at present (van den Berg et al., 1989b), tyrosine and lysine are found with about equal frequency at position 69, although in pancreatic enzymes invariably a tyrosine is present. In active PLA2s only once was a different residue, i.e., a phenylalanine, reported in the enzyme from Laticauda colubrina venom (Takasaki et al., 1988). Given the observation that in naturally occurring PLA2s lysine and tyrosine both can be present at this position, the low activity of the Y69K mutant relative to native porcine pancreatic PLA2 was unexpected. It must be realized, however, that Tyr-69 is adjacent to a surface loop of variable conformation in pancreatic enzymes, whereas this loop is absent in all venom PLA2s. It has been shown recently (Kuipers et al., 1989b) that deletion of this loop in pancreatic PLA2 markedly increases activity on short-chain lecithins. The greater flexibility of a lysyl side chain compared to that of tyrosine in combination with the presence of the mobile surface loop might explain the low catalytic power of the Y69K pancreatic PLA2 mutant.

For many years phospholipase A2 has been regarded as an enzyme that degrades phospholipids in a calcium-dependent reaction with high stereospecificity and positional specificity. In a recent paper (Kuipers et al., 1989a) it was shown that replacement of Tyr-69 by a phenylalanine resulted in a partial loss of the stereospecificity. In the present study we also included the substitution of Tyr-69 by Lys, and the results obtained with this mutant confirm our previous notion that the stereospecificity at sn-2 of the glycerol backbone is dependent on the presence of a hydrophobic bridge donor in the side chain.
chain of residue 69 of porcine pancreatic PLA$_2$. Also, the fact that Y69K PLA$_2$, like native PLA$_2$, only degrades the $R_p$ isomer of thionolecithins while the Y69F mutant can degrade both $R_p$ and $S_p$ isomers (Table II) strongly suggests that it is indeed the hydrogen bridge from either Tyr-69 or Lys-69 to phosphate that determines to a large extent stereospecificity both at sn-2 and at phosphorus. Because the Y69F mutant is able to degrade both $\Delta$ and $\Lambda$ phospholipids, detailed kinetic analyses of racemic compounds like dihexanoylithiolecithin (Table I) is strictly impossible. However, because the activity of the Y69F mutant on $\Delta$-lecithins is about 50 times lower than that on $\Lambda$-lecithins (Kuipers et al., 1989), the resulting error is small compared to standard deviations caused by the test system itself. The same reasoning applies to the hydrolysis of the racemic phosphonolecithin by native and mutant phospholipases.

The activity of native and Y69K pancreatic PLA$_{2S}$ on 1,2-didodecanoylthionolecithin is about 4-fold lower than that on the regular 1,2-didodecanoyllecithin. This reduction in activity is low compared to the 24-fold reduction reported by Tsai et al. (1985) for the bee venom PLA$_2$-catalyzed hydrolysis of 1,2-dihexadecanoylthionolecithin. This difference might be explained by the detergent used: taurodeoxycholate in this study and Triton X-100 by Tsai and co-workers, and/or by the different fatty acyl chain lengths. Another explanation could be that bee venom phospholipase A$_2$, despite its functional homology with other extracellular PLA$_{2S}$, might be structurally distinct in its phosphate binding pocket. The structural homology of bee venom phospholipases and pancreatic or snake venom phospholipases has indeed been questioned before (Verheij et al., 1981a; Maraganore et al., 1987). That substitutions at the entrance to the active site can change the preference of a given PLA$_2$ from regular phospholipids to thiono analogues is illustrated by the Y69F mutant which, in contrast to native PLA$_2$, prefers thionophospholipids to regular ones. Since this mutant, in contrast to native and Y69K PLA$_{2S}$, also has measurable activity on the $S_p$ isomer, we conclude that the hydrogen bridge that can be formed between phosphate and Tyr-69 or Lys-69 is of major importance for stereospecificity at phosphorus. A question that remains to be answered is whether this hydrogen bridge occurs between the amino acyl side chain and the P oxygen or the P sulfur when the $R_p$ isomer is bound to the active site. Several arguments seem to support an interaction with the sulfur. Due to the hydrogen-bonding capacities and the size, a substitution of sulfur for oxygen would reduce the interaction with Tyr-69 or Lys-69 on account of a weaker hydrogen bond and for steric reasons. Thus the introduction of a sulfur on phosphorus would make the thiono analogue a weaker substrate than the parent lecithin. In the mutant Y69F no such effects are evident, and the fit with sulfur could even be favored by hydrophobic interactions. Another argument that fits the proposed orientation of the sulfur is the observation by Tsai et al. (1985) that replacement of calcium by cadmium ions causes to a large extent the reversal in the preference of the enzyme for the $S_p$ and $R_p$ isomers. Given the preference of cadmium for sulfur over oxygen as a ligand, this also supports an orientation with the sulfur in the $R_p$ isomer in proximity of Tyr-69 or Lys-69.

As early as 1968 it was shown (de Haas et al., 1968) that the presence of a negative charge on phosphorus is essential for phospholipase A$_2$ activity in the presence of deoxycholate or diethyl ester. The results presented in the present study show that this conclusion has to be revised to some extent. Phosphorus triesters like didodecanoylphosphatidic acid di-methyl ester are practically insoluble in aqueous buffers and are hence not degraded by PLA$_2$. Addition of diethyl ether, yielding two clear phases, does not promote hydrolysis, probably because the triester is hardly surface active and, therefore, does not accumulate at the ether-water interface. Deoxycholate apparently is not readily incorporated into the triester droplets, and formation of mixed micelles does not seem to occur readily: even at a 10-fold molar excess triester droplets are still visible, and hydrolysis is negligible. In the presence of the product analogue choline $n$-hexadecyl phosphate, clear solutions of presumably mixed micelles are formed. In these micelles the triester is degraded at significant rates compared to lecithin although the latter is degraded more slowly in this detergent than in the presence of deoxycholate (compare Tables II and III). Since choline $n$-alkyl phosphates are product analogues and hence probably competitive inhibitors, these low hydrolysis rates were not unexpected. More striking is the fact that the two mutant PLA$_{2S}$ behave quite differently. Whereas the Y69K mutant has the same relative preference as native PLA$_2$ for lecithin over triester, the Y69F mutant has equal activities on both surfaces. An attractive explanation is that upon introduction of two methyl groups in the phosphate moiety the interaction with the hydrogen-bond donors Tyr and Lys is weakened, whereas the methyl group(s) still can have a hydrophobic interaction with Phe. In all cases the Ca$^{2+}$ ion could still be liganded by the one free oxygen atom at the phosphorus. As a result of both interactions the latter mutant can still have a rather high affinity for the triester. Unfortunately, direct verification of this affinity is experimentally difficult because of the low solubility of the triester in water. Estimations of this affinity after codispersion of the triester in micelles is impossible, since under these conditions several equilibria are involved (Verger & de Haas, 1976).

The finding that the phosphonolecithin (Figure 1, analogue 5) is a substrate, while the butanetriol lecithin (Figure 1, analogue 6) is not degraded at all, was not completely unexpected. It has been reported by Bonsen et al. (1972b) that the dihexanoyl- and diocanoylphosphonolecithins are degraded with 15% and 5%, respectively, of the rates observed with the corresponding phospholipids. The same authors reported that the dihexanoylbutanetriol analogue was completely resistant to the action of PLA$_2$. Our results with the phosphonolecithin show that this compound is hydrolyzed by PLA$_2$ with retention of the positional specificity but with a loss of the stereospecificity of the enzyme. This furthermore suggests that the results that were obtained by Bonsen et al. (1972b) with the short-chain derivatives were not caused by the aspecific hydrolysis of loosely bound substrate.

Because the phosphonolecithin analogue is degraded more slowly than the normal lecithin, the question arises whether this reduction in hydrolysis rate is caused by an improper orientation of either the sn-2 ester bond or of the phosphate in the phosphonolecithin. If the susceptible ester bond had been shifted over a significant distance in the active site, a large effect on the rate of catalysis would have been expected. Since the reduction in the rate of hydrolysis is only moderate and because the enzyme retains its positional specificity, we presume that, upon binding of the phosphono substrate in the active site, the acyl ester bond remains in place. Consequently, we also presume that, as a result of the reduced distance between the acyl ester bond and the phosphate moiety in the phosphono substrate, this phosphonate occupies a somewhat different position in the active site than the phosphate of normal phospholipids. For this reason it is not clear whether
Substrate Requirements of Phospholipase A

![Diagram](57x610)

**Figure 5:** Schematic representation of the proposed relative positions of the Ca\(^{2+}\) ion, residue 69 of wild-type and mutant PLA\(_2\), and the phosphate moiety of lecithin and phosphonolecithin: (Panel A) interactions of lecithin with wild-type PLA\(_2\); (Panel B) interaction of lecithin with Phe-69 mutant PLA\(_2\); and (Panels C and D) these interactions with a phospholipid. The charges of the Ca\(^{2+}\) ion and of the phosphate are not indicated for reasons of clarity.

...an interaction of the phosphonate can still exist with Ca\(^{2+}\) and/or with the side chain of Tyr-69.

In considering the possibility of an interaction between the phosphonate and the Ca\(^{2+}\) ion, one has to keep in mind that the Ca\(^{2+}\) ion is relatively small and is kept in a fixed position by the side chain of Asp-49 and the backbone carbonyl oxygens of residues Tyr-28, Gly-30, and Gly-32. Therefore, we assume that it is highly improbable that the Ca\(^{2+}\) ion is able to shift even over a small distance in the enzyme, and we consider an interaction between phosphonate and the Ca\(^{2+}\) ion in phosphonolipids improbable. As to the possibility of an interaction between Tyr-69 and the phosphonate moiety of the phosphonolipid, it should be noticed that rotation of the side chain around the \(\alpha\)-carbon atom can change the position of the phenolic hydroxyl group considerably. A displacement as large as 12 Å has indeed been reported for Tyr-248 in carboxypeptidase A, to accommodate the binding of a substrate molecule (Rees \& Lipscomb, 1982). Also for bovine PLA\(_2\), a shift of the position of Tyr-69 has been reported. This residue occupies an exposed position in native PLA\(_2\), but it moves inside the substrate binding pocket in a PLA\(_2\) modified by the introduction of a \(p\)-bromophenacycl group, covalently attached to the active-site histidine (Renetseder et al., 1988). These two observations are indicative of a high mobility of surface tyrosine residues in general and of Tyr-69 in PLA\(_2\) in particular.

With phospholipids, it was shown that a hydrogen bond between Tyr-69 and phosphate is crucial for stereospecificity, which suggests that Tyr-69 indeed shifts toward the inside of the enzyme upon binding of a substrate molecule. In analogy, we assume that Tyr-69, by shifting even further toward the inside of the enzyme, is still able to interact with the phosphonate moiety of a phosphonolipid.

In Figure 5 a schematic representation of the orientation of a phospholipid and of a phosphonolipid in the active site of PLA\(_2\) is given. Although direct proof for the proposed hydrogen bond between Tyr-69 and the phosphonate of phosphonolipids is lacking, there are some kinetic data which are consistent with such an interaction. When Tyr-69 was replaced by Phe, the activity of the enzyme was reduced about 3-fold, not only the phospholipids but also with phosphonolipids as substrate. This suggests that the mutation Tyr-69 to Phe prevents a hydrogen bond with both the phosphate and the phosphonate. For the Y69F mutant acting on glycerophospholipids and for native phospholipase acting on phosphonolipids, the effects on stereospecificity are qualitatively and quantitatively similar. Thus it seems that a sole interaction either between calcium and phosphate (wild-type PLA\(_2\) and phosphonolecithin) is not enough for the maintenance of stereospecificity.

Considering the function of the Ca\(^{2+}\) ion in PLA\(_2\), in 1980 a double role for the Ca\(^{2+}\) ion was proposed (Verheij et al., 1980). First, Ca\(^{2+}\) was supposed to bind the negative charge of the phosphate, and second, Ca\(^{2+}\) was supposed to enhance the polarization of the carbonyl oxygen of the ester at the 2-position. Our results underline and extend this previous notion, by showing that Ca\(^{2+}\) and Tyr-69 together can fix the position of the phosphate moiety of phospholipids, thereby securing stereospecificity. With some substrates (phosphonolecithin, phosphatidic acid dimethyl ester), where a strong interaction of Ca\(^{2+}\) with phosphate is less obvious, the Ca\(^{2+}\) ion is still indispensable for enzymatic activity. This observation is in agreement with the proposed catalytic role of Ca\(^{2+}\).

Whether this role is a direct one, such as the polarization of the carbonyl oxygen of the 2-acyl ester bond, or an indirect one like the stabilization of the active conformation of PLA\(_2\), suggested by Tsai et al. (1985), remains a subject of further studies.

**Acknowledgments**

We are grateful to Ruud Dijkman for the synthesis of many of the phospholipids, Jana Kerver and Cornelieke Pals for expert technical assistance, and Dr. J. J. M. van den Berg for gas chromatographic analyses.

**References**


Steady-State and Laser Flash Induced Photoreduction of Yeast Glutathione Reductase by 5-Deazariboflavin and by a Viologen Analogue: Stabilization of Flavin Adenine Dinucleotide Semiquinone Species by Complexation†

José A. Navarro, Mercedes Roncel, and Gordon Tollin*
Department of Biochemistry, University of Arizona, Tucson, Arizona 85721
Received September 29, 1989; Revised Manuscript Received March 27, 1990

ABSTRACT: Steady-state and laser flash photolysis techniques have been used to examine the photoreduction of yeast glutathione reductase by the one-electron reduction products of 5-deazariboflavin and the viologen analogue 1,1'-propylene-2,2'-bipyridyl. Steady-state photoreduction of the enzyme with the viologen generates the two-electron-reduced form, whereas photoreduction with deazaflavin generates the anion semiquinone. This reduction is apparently inhibited when deazaflavin is the photoreductant, perhaps due to complexation of the two-electron-reduced enzyme with deazaflavin. Steady-state experiments demonstrate that complexation of the anion semiquinone with deazaflavin stabilizes the enzyme.

Glutathione reductase (GR) catalyzes the reduction of the disulfide bond of oxidized glutathione (GSSG), using NADPH as the source of reducing equivalents and an FAD cofactor and a protein disulfide as intermediate electron carriers. (Williams, 1976). The enzyme isolated from human erythrocytes has been extensively characterized, and a refined X-ray structure at 1.54-Å resolution has been reported (Karplus & Schultz, 1987a). It is a dimer (total MW = 105 000) consisting of two identical subunits, each containing one FAD molecule (Krauth-Siegel et al., 1982). In the first part of the catalytic reaction, the enzyme is reduced to its stable EH2 form by NADPH, from which NADP* dissociates. This species has an open disulfide (Cys-58-Cys-63 in the human enzyme; Pai & Schulz, 1983) resulting from electron transfer via the flavin, and spectroscopic data indicate the existence of a charge-transfer complex between a thiolate anion (the proximal...