Activities of Native and Tyrosine-69 Mutant Phospholipases A2 on Phospholipid Analogues. A Reevaluation of the Minimal Substrate Requirements†

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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 \( R_\text{o} \) isomer of thionophospholipids more efficiently than the wild-type enzyme, but the \( S_\text{o} \) thiono isomer is hydrolyzed too, although at a low (\( \approx 4\% \)) 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 \( \epsilon \)-NH\(_2\) group can play a role similar to that of the Tyr OH group in native PLA\(_2\). 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 A\(_2\) (PLA\(_2\)) 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 PLA\(_2\)s 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 PLA\(_2\)s 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 (Donnë-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 PLA\(_2\) 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 et al., 1981; Dijkstra et al., 1983). Although several di- and trivalent cations bind stoichiometrically to PLA\(_2\)s 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 A\(_2\), the Ca\(^{2+}\) 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 Ca\(^{2+}\) ion was merely suggested by intuition, until Tsai et al. (1985) showed that the Ca\(^{2+}\) ion is probably in contact with the \( \text{oxygen} \)
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 A$_2$. We decided to study these interactions in more detail by making use of native porcine pancreatic PLA$_2$ 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 A$_2$ (de Haas et al., 1968).

**Materials and Methods**

**Construction of Mutant Phospholipases.** Mutant Y69F$^1$ 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). A*terisks denote the places of base mutations in the mutagenic primer, relative to the wild-type sequence. The cDNA encoding the mutant PLA$_2$ species was sequenced, and a BstXI-BglII fragment containing the wild-type sequence. The cDNA encoding the mutant PLA$_2$ 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 PLA$_2$ species was sequenced, and a BstXI-BglII fragment containing the wild-type sequence. The cDNA encoding the mutant PLA$_2$, was ligated into the expression vector pOIK3. After transformation and expression in *Escherichia coli* K-12 strain MC4100 (Silhavy et al., 1984), containing plasmid pC1857, the mutant PLA$_2$ 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-diacly-sn-glycerols used in this study were prepared by acylation of 3-0-benzyl-sn-glycerol (Bonsen et al., 1972a) followed by catalytic hydrogenolysis. Phosphorylation with an excess of phosphorus-oxy-trichloride and subsequent reaction with methanol yielded the dimethyl ester phosphatic acid, which was purified by silicic acid chromatography with hexane-ether mixtures as eluants. The 1,2-sn-diclyllecithin was prepared from the diglyceride by phosphorylation with 2-chloro-2-oxo-1,3,2-dioxaphospholane described by Chandrakumar and Hajdu (1983). The thion-olecithin 1,2-didodecanoyl-sn-glycero-3-thionophosphocholine was synthesized from the corresponding diglyceride as described by Nifant’ev et al. (1978). The R$_g$ and S$_p$ isomers were prepared from the mixture by degradation with phospholipase A$_2$ from *Crotalus adamanteus* venom, separation of the products, and reacylation of the lyso R$_g$ derivative, essentially as described by Bruzik et al. (1983).

The synthesis of rac-1-tetradecanoyl-2-dodecanoyl-phosphonolecithin was carried out, starting from the barium salt of rac-3-deoxyglycerol 3-phosphonate (Baer & Basu, 1969). After conversion of the barium salt to the free acid with sulfuric acid, the starting compound was converted into the dibenzyl ester with phenyldiazomethane. This compound was monosubstituted in dry toluene at 0 °C with 1 equiv of tetradecanoyl chloride in the presence of pyridine. The reaction mixture was purified by silicic acid column chromatography with hexane-diethyl ether mixtures as eluants. Pure rac-1-tetradecanoyl-3-deoxyglycerol 3-phosphonate dibenzyl ester (R$_g$ 0.35 in CHCl$_3$/methanol = 97:3) was obtained, free from traces of the corresponding 2-acyl derivative (R$_g$ 0.31). The 1-acyl compound was acylated with a small excess of dodecanoyl chloride. The triester was converted to the acid by catalytic hydrogenolysis with palladium as catalyst, and this compound was allowed to react with choline tosylate in trichloroacetonitrile and pyridine as solvent (Rosenthal, 1966). The final product was purified by isocratic silicic acid chromatography with CHCl$_3$/methanol/water = 65/15/2 as solvent. The purity and identity of the product and the intermediates were verified by 'H NMR. The racemic phosphonolecithin was separated into the sn-1 and sn-3 isomers with the aid of phospholipase A$_2$ essentially as was described for the thionolecithin (Bruzik et al., 1983). The steps include a short incubation until about 40% hydrolysis was reached, followed by separation of the lysolecithin and the diacylecithin by silicic acid chromatography. The lyso compound was reacylated by standard procedures to yield 1-tetradecanoyl-2-dodecanoyl-sn-3-phosphonolecithin. The diacylecithin that was recovered after the first phospholipase incubation contained the sn-1 isomer, contaminated with the sn-3 isomer. A second prolonged phospholipase A$_2$ digestion removed the contaminating sn-3 isomer, and the pure sn-1 isomer was isolated by silicic acid chromatography.

**Phospholipase Assays.** The activities of phospholipases were determined quantitatively in a titrimetric assay at pH 8 in the presence of 1 mM borate, 25 mM CaCl$_2$, and 100 mM NaCl at 25 °C with a 10 mM sodium hydroxide solution. The solution was flushed with nitrogen prior to the addition of substrate, and during the experiment the reaction vessel was kept under a nitrogen atmosphere. Lecithins and analogues were solubilized with sodium taurodeoxycholate (2 mol/mol of phospholipid) to form mixed micelles. The phosphorus triester was solubilized with the aid of either deoxycholate, taurodeoxycholate, or choline n-hexadecyl phosphate. Activities on monomeric rac-1,2-diheptanoylphospholipid were determined at pH 8 in the presence of 200 mM Tris, 100 mM NaCl, and 100 mM CaCl$_2$, as described previously by Volwerk et al. (1979).

**Direct Binding of PLA$_2$ to Monomers and Micelles of Phospholipids.** The affinity of phospholipase A$_2$ for monomers and micelles 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 NaOAc, 50 mM CaCl$_2$, and 100 mM NaCl at pH 6.0. From saturation curves, obtained with lipid monomers, a Kd 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 Kd. The NKd 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-Mieras et al., 1975), except that in the fluorescence experiments the excitation wavelength was 280 nm. The binding of PLA$_2$ to micelles of choline n-hexadecyl phosphate (C16PN) 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

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1 Abbreviations: C12PN, choline n-dodecyl phosphate; C16PN, choline n-hexadecyl phosphate; cmc, critical micellar concentration; F, phenylalanine; K, lysine; Y, tyrosine.
a 5 mM solution of R<sub>d</sub> or S<sub>d</sub> isomer was added 10% D<sub>2</sub>O for deuterium lock. Chemical shifts are referenced to external 85% H<sub>2</sub>PO<sub>4</sub> (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).

RESULTS

Enzymatic Activities of Tyr-69 Mutant Phospholipases. In a previous study (Kuipers 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 1 the activities of this mutant phospholipase are given, and for reasons of comparison the data of native and Y69F PLA<sub>2</sub> are also included. It is clear that the Y69K mutant PLA<sub>2</sub> has rather low activity on aggregated substrates as compared to native and Y69F PLA<sub>2</sub>. This was somewhat unexpected, since a lysyl residue is often encountered at this position in many venom PLA<sub>2</sub>s. When monomeric substrates are used, all three PLA<sub>2</sub>s show comparable k<sub>cat</sub>/K<sub>m</sub> values.

Direct Binding Studies. The addition of choline n-alkyl phosphates (Figure 1, analogue 4) to native porcine pancreatic phospholipase A<sub>2</sub> causes spectral perturbations, which can be visualized by ultraviolet difference spectroscopy. The addition of these product analogues at concentrations below and above the cmc induces a perturbation spectrum that is characteristic of (a) perturbed tyrosine residue(s). Above the cmc in the presence

Table I: Enzymatic Activities and Binding of Wild-Type and Two Mutant Phospholipases A<sub>2</sub> Using Monomeric and Micellar Substances and Substrate Analogues<sup>a</sup>

<table>
<thead>
<tr>
<th>enzyme</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;·M&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (µmol·min&lt;sup&gt;-1&lt;/sup&gt;·mg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (mM)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;&lt;sup&gt;ph&lt;/sup&gt; (mM)</th>
<th>N/Kd</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>900</td>
<td>0.3</td>
<td>2000</td>
<td>3.7</td>
<td>380</td>
</tr>
<tr>
<td>Lys-69</td>
<td>1350</td>
<td>0.3</td>
<td>230</td>
<td>10.6</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phe-69</td>
<td>1300</td>
<td>0.3</td>
<td>440</td>
<td>9.5</td>
<td>480</td>
</tr>
</tbody>
</table>

<sup>a</sup>Monomeric substrates (analogue): diC6<sub>8</sub>PC, rac-1,2-dihexanoylthiolethanol; C12PN, choline n-dodecyl phosphate; Micellar substrates (analogue): diC8<sub>PC</sub>, 1,2-diacyloxy-sn-glycero-3-phosphocholine; C16PN, choline n-hexadecyl phosphate. Standard errors did not exceed 10% in each case. For details, see Materials and Methods.

<sup>b</sup>Not possible to determine; see text.

FIGURE 1: Schematic structures of several phospholipid analogues. R<sub>d</sub> and R<sub<f</sub> represent alkyl chains of variable lengths. (1) 1,2-diacyl-sn-glycero-3-phosphocholine (lecithin); (2) 1,2-diacyl-sn-glycero-3-thionophosphocholine (thionolecithin); (3) 1,2-diacyl-sn-glycero-3-(dimethyl phosphate); (4) choline n-alkyl phosphate; (5) choline 1,2-bis(acyloxy)-3-propyl-phosphonate (phosphono analogue of lecithin); (6) 1,2-diacyl-1,2,4-butanetriolcholine phosphate.
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 PLA₂ 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 PLA₂ and for Y69F PLA₂. 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 PLA₂ does not bind monomeric choline alkyl phosphates, but in view of the kinetic data of Y69K PLA₂ 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 PLA₂ (van Dam-Mieras et al., 1975). From the spectral changes the dissociation constants (NK₄) of native and Y69F PLA₂ 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 a just 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, PLA₂ bound to the C16PN micelles (molecular mass ~ 70 kDa) will coelute near the void volume of the column, in contrast to unbound PLA₂, 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 R₆ isomer of thionolecithins (Figure 1, analogue 2) has been shown to be a substrate for PLA₂ from various sources (Tsai et al., 1985). The activities of native and of mutant PLA₂ were determined on 1,2-didodecanoyl-sn-glycero-3-phosphocholine; 1,2-didodecanoyl-sn-glycero-3-phosphocholine; 1,2-didodecanoyl-sn-glycero-3-thionolecithin. Kinetic assays were performed with a pH-stat, as described under Materials and Methods. Standard errors did not exceed 10% of the reported value.

<table>
<thead>
<tr>
<th>Table II: Activities of Wild-Type and Mutant PLA₂s on Normal Lecithins and on the Two Isomers of Thionolecithins*</th>
<th>Activity (μmol-min⁻¹·mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>enzyme</td>
<td>diC12PC</td>
</tr>
<tr>
<td>wild type</td>
<td>55</td>
</tr>
<tr>
<td>Lys-69</td>
<td>11</td>
</tr>
<tr>
<td>Phe-69</td>
<td>14</td>
</tr>
</tbody>
</table>

* diC₁₂PC, 1,2-didodecanoyl-sn-glycero-3-phosphocholine; diC₁₂thionoPC, 1,2-didodecanoyl-sn-glycero-3-thionolecithin. The triester droplets are readily soluble in diethyl ether/buffer systems and in water; in water it forms emulsions with the size of the droplets depending on the degree of agitation. Under these conditions no hydrolysis by PLA₂ 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-
or taurodeoxycholate was degradation of this substrate by experimental details, see Materials and Methods.

Neither methyl ester of 1,2-diacyl-sn-glycerol 3-phosphate. Neither in the presence of ether nor in the presence of deoxycholate 1,3-diacyl-sn-glycerol 2-phosphates (Slotboom et al., 1970). In the presence of relatively high concentrations of diacyl-sn-glycerol 3-phosphate and with dimethyl esters of thiono phosphocholine the hydrolysis of the 

%Rf 0.87 and 0.26 corresponding to fatty acid and dimethyl ester of 1-acyllyso-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-dido-decanoyllecithin. 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 phosphonolipids 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

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 was seen with the thionole-

### Table III: Activities of Wild-Type, Lys-69, and Phe-69 PLA$_2$s 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-glycero-3-phosphocholine; diC12dimethylPA, 1,2-didodecanoyl-sn-glycero-3-(dimethyl phosphate). Assays were performed with a pH-stat, as described under Materials and Methods. Accuracy was about 10% for each given value.
Substrate Requirements of Phospholipase $A_2$

**FIGURE 4:** Degradation of rac-1,2-didodecanoyllecithins and rac-1-myristoyl-2-dodecanoylphosphonolecithins by wild-type, Y69K, and Y69F PLA$_{A_2}$. Incubations were performed in the presence of sodium taurodeoxycholate (2 mol/mol of phospholipid) in 50 mM borate buffer containing 5 mM CaCl$_2$ 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) PLA$_{A_2}$, 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) PLA$_{A_2}$, respectively. The TLC plate was developed in CHCl$_3$-MeOH-H$_2$O (65:35:8 by volume), and spots were visualized by spraying with phosphorus reagent. The upper spots represent nondegraded lecithin of this residue is involved in the interaction of PLA$_2$ with the activity of both native and the mutant phospholipases.

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 PLA$_2$ 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 PLA$_2$. The small signal that is observed with the Y69F PLA$_2$ has the characteristics of a tryptophan perturbation. On the other hand, the introduction of a large hydrophobic group like the dansyl moiety obviously improves hydrophobic interactions.

In the 56 phospholipases $A_2$ that have been sequenced at present (van den Bergh 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 PLA$_{A_2}$s 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 PLA$_{A_2}$ s lysine and tyrosine both can be present at this position, the low activity of the Y69K mutant relative to native porcine pancreatic PLA$_2$ 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 PLA$_{A_2}$s. It has been shown recently (Kuipers et al., 1989b) that deletion of this loop in pancreatic PLA$_2$ 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 PLA$_2$ mutant.

For many years phospholipase $A_2$ 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 hydrogen bridge donor in the side
that Y69K PLA2, like native PLA2, only degrades the Rp isomer of thionolecithins while the Y69F mutant can degrade both Rp and Ss 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 D and L phospholipids, detailed kinetic analyses of racemic compounds like dihexanoyl diithioleceithin (Table I) is strictly impossible. However, because the activity of the Y69F mutant on Δ-lecithins is about 50 times lower than that on L-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 phospho-lecithin by native and mutant phospholipases.

The activity of native and Y69K pancreatic PLA2s 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 PLA2-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 A2, despite its functional homology with other extracellular PLA2s, 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 PLA2 from regular phospholipids to thiono analogues is illustrated by the Y69F mutant which, in contrast to native PLA2, prefers thiono-phospholipids to regular ones. Since this mutant, in contrast to native and Y69K PLA2s, also has measurable activity on the Ss 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 Rp 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 Ss and Rp isomers. Given the preference of cadmium for sulfur over oxygen as a ligand, this also supports an orientation with the sulfur in the Rp 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 A2 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-
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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 α-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 $p$-bromophenacyl 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 phosphatidic acid 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 phospholipids, 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 phosphonolochitin) 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 (phosphonolochitin, 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.

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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†

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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 anion semiquinone with deazaflavin. Steady-state experiments demonstrate that complexation of the anion semiquinone with NADP⁺ also inhibits further reduction. Both one-electron reduction reactions of oxidized glutathione reductase proceed at close to diffusion-controlled rates (second-order rate constants \( k > 8000 \) s⁻¹), despite the relatively buried nature of the FAD cofactor. Addition of NADP⁺ and oxidized glutathione produced no effects on the kinetics of the initial entry of the electron into the enzyme. Thus, if this reaction occurs in the semiquinone, it must be quite rapid (\( k > 8000 \) s⁻¹).

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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-A 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...