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Function of the fully conserved residues Asp99, Tyr52 and Tyr73 in phospholipase A₂

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In the active centre of pancreatic phospholipase A₂ His48 is at hydrogen-bonding distance to Asp99. This Asp-His couple is assumed to act together with a water molecule as a catalytic triad. Asp99 is also linked via an extended hydrogen bonding system to the side chains of Tyr52 and Tyr73. To probe the function of the fully conserved Asp99, Tyr52 and Tyr73 residues in phospholipase A₂, the Asp99 residue was replaced by Asn, and each of the two tyrosines was separately replaced by either a Phe or a Gln. The catalytic and binding properties of the Phe52 and Phe73 mutants did not change significantly relative to the wild-type enzyme. The results suggest a structural role rather than a catalytic function of Tyr52 and Tyr73. Substitution of asparagine for aspartate hardly affects the binding constants for both monomeric and micellar substrate analogues. Kinetic characterization revealed that the Asn99 mutant has retained no less than 65% of its enzymatic activity on the monomeric substrate rac 1,2-dihexanoylthio-propyl-3-phosphocholine, probably due to the fact that during hydrolysis of monomeric substrate by phospholipase A₂ proton transfer is not the rate-limiting step. The Asp to Asn substitution decreases the catalytic rate on micellar 1,2-dioctanoyl-sn-glycero-3-phosphocholine 25-fold. To explain this remaining activity we suggest that in the mutant the Asn99 orients His48 in the same way as Asp99 orients His48 in native phospholipase A₂ and that the lowered activity is caused by a reduced stabilization of the transition state. Key words: catalytic mechanism/phospholipase A₂/protein folding/site-directed mutagenesis

Introduction

The lipolytic enzyme phospholipase A₂ (PLA₂) catalyses the hydrolysis of the 2-acyl ester linkage of 1,2-diacyl-sn-3-phosphoglycerides in a calcium-dependent reaction (van Deenen and de Haas, 1964). In the pancreas the enzyme occurs as a zymogen which is activated by trypsin upon secretion into the duodenal tract, where it serves a digestive function (de Haas et al., 1968; Dutjih et al., 1975). The catalytic properties of precursor and activated PLA₂, with respect to monomeric substrates, are quite similar. However, when the substrate is present in a aggregated form, such as micelles, there is a large increase in enzymatic activity of the mature enzyme, but not of its zymogen (Pietersen et al., 1974). Chemical modification and X-ray crystallography studies (Verheij et al., 1980; Fleer et al., 1981; Dijkstra et al., 1983) have indicated the importance of His48, Asp99 and Asp49, the latter residue as a Ca²⁺-binding ligand, for the enzymatic activity of pancreatic PLA₂. Based on these data a general catalytic mechanism has been proposed (Verheij et al., 1980), in which the aspartate-histidine couple and a water molecule serve as a catalytic triad (Figure 1). The presence of the Asp-His couple in the active site of PLA₂ suggests a similarity to the proton relay system of the serine esterases (Stamato et al., 1986). This comparison is not unrealistic, because both phospholipase and the proteolytic serine esterases are able to hydrolyse carboxylic acid esters. In the widely studied serine proteases, the active site histidine of the pair is coupled to the nucleophilic serine residue and stabilizes the oxyanionic form of the serine by acting as a base. In the so-called charge relay system the catalytic Asp residue accepts a proton from the His, in this way stabilizing the monoprotonated His. Recently it was suggested that the active site aspartate contributes to the catalytic efficiency by stabilizing the positively charged form of the histidine residue in the transition state (Warshel et al., 1989) and by anchoring it in the most favourable orientation for acid/base catalysis (Sprang et al., 1987). In the three-dimensional structure of PLA₂ no serine is present near His48, but a water molecule, 3.1 Å from the N₁ nitrogen of His48, which lies in the plane of the imidazole ring, could perform the nucleophilic function. Thus, the ester hydrolysis by PLA₂ seems to be comparable with the deacylation step in the serine proteases, the difference being that in the latter case the substrate is covalently bound to the enzyme.

According to the three-dimensional structure, the Asp99 residue forms four hydrogen bonds in the interior of the enzyme. The O₃ atoms can accept hydrogen bonds from Tyr73 and from a water molecule, which in turn is H-bonded to the NH₃⁺ group of Ala1 and to Tyr52. The O₂ atom of Asp99 can accept hydrogen bonds of the H atom of the N₃ of His48 and of Tyr52 (Figure 1). The two tyrosines at positions 52 and 73 are fully

**Fig. 1.** Hydrogen-bonding network around the active site and the N-terminus of porcine PLA₂ (from Renetseder, 1986).
conserved in all 79 known sequences of pancreatic, snake venom and intracellular mammalian PLA2 (for a comparison of 50 sequences see van den Bergh et al., 1989). The conservation of these two tyrosines and their location close to His48 and Asp99 has prompted Brunie et al. (1985) to propose that these residues belong to the active site and could serve a catalytic function as an acyl acceptor or as a general acid. However, experimental evidence for such a function has not been obtained yet. Our purpose is to probe the functions of Asp99 and the two conserved Tyr residues by site-directed mutagenesis. All mutations are applied in a PLA2 mutant, in which a surface loop was deleted; this mutant is denoted as Δ62–66 PLA2. In contrast to native porcine pancreatic PLA2, this Δ62–66 PLA2 mutant readily crystallizes and shows increased catalytic activities compared with native enzyme (Kuipers et al., 1989a). In this way the kinetic and structural characterization of the mutant enzymes, which are anticipated to have considerably lower catalytic activities than the Δ62–66 enzyme, can be facilitated. The mutant enzymes will be denoted by a ‘Δ’, followed by the amino acid residue substitution.

To provide an empirical test for the contribution of the Asp99 residue to PLA2 catalysis, this residue was mutated to the isosteric asparagine. The Tyr52 and Tyr73 residues were each replaced separately by either a Phe or a Gln. The Phe mutation will retain the aromatic character of the residue, but remove possible hydrogen bonds to Asp99 and the water molecule. The mutants with Gln at positions 52 or 73 are still able to form hydrogen bonds, but have lost their aromatic characteristics. To characterize these PLA2 mutants their affinities for monomeric and micellar substrate analogues were determined, and their catalytic properties towards monomeric and micellar substrates were investigated. The results of the characterization of these mutant enzymes will be discussed in relation to a possible role in catalysis, folding and/or stability.

Materials and methods

Construction of mutant phospholipases

Escherichia coli K-12 strain PC 2494 [Δ(lac-pro), supE, thi/F' traD36, proA A+, lacZ ΔM15, Phabagen collection, Utrecht] was used for plasmid constructions and as a host for M13-derived vectors. HB 2154 [ara, Δ(lac-pro), thi/F', proA A+, lacZ ΔM15, mutL::Tn10] (Carter et al., 1985) was used as recipient strain in mutagenesis experiments. Substitutions were introduced in porcine pancreatic PLA2 lacking a surface loop (Δ62–66) (Kuipers et al., 1989a). After subcloning the pro-Δ62–66 DNA into the M13 vector mp8, mutagenesis was performed according to the gapped-duplex method (Kramer et al., 1984). The mutant oligonucleotides 5' CATTCCGTGT*ACAGTTACAT*A TAGAAGGC 3' (Asn99), 5' GGCATCTCTA*AGGAGTTGTCG 3' (Phe52), 5' GGC- ATCTCTT*TG*GCAGTTGTCG 3' (Gln52), 5' GAGTAGGAGA*AGCCTTCCGTG 3' (Phe73) and 5' TGAGTACGAT* TG*GCCTTGCTG 3' (Gln73), were used for site-directed mutagenesis. In these oligonucleotides, synthesized on a Biosearch 6800 DNA synthesizer, an asterisk denotes a change of the preceding nucleotide compared with the cDNA sequence of the wild-type PLA2. Screening of mutants was performed by dyeoxy-sequencing after plaque purification. From the sequenced DNA a Km = HindIII fragment (or a Km = BglII fragment in the case of Phe73) containing the mutated Δ62–66 DNA, was cloned into the expression vector pOK13 (de Geus et al., 1987; Kuipers et al., 1989b). After transformation and expression in E.coli K-12 strain MC4100 (Casadaban, 1976), containing plasmid pCI857, mutant phospholipases were obtained by tryptic cleavage of reoxidized fusion protein (de Geus et al., 1987) and purified by CM—cellulose chromatography at pH 5 and 6. Final purification to homogeneity was achieved on DEAE—cellulose at pH 8. After purification residue substitutions were confirmed by amino acid analyses. For amino acid analyses, samples were hydrolysed in vacuo for 24 h at 110°C in 6 N HCl. Analyses were carried out on an LKB 4151 alpha plus analyser. The values for Thr and Ser were corrected for 5 and 10% loss respectively. Protein concentrations were calculated from the absorbance at 280 nm, for which were used E1% values of 13.0 for ΔY52F, ΔY52Q and ΔY73F and 14.0 for Δ62–66 and ΔD99N PLA2.

Phospholipids

The 1,2-dioctanoyl-sn-glycerol-3-phosphocholine (DiC8PC) used in this study was obtained after reacylation of sn-glycerol-3-phosphocholine (Cubero Robles and van den Berg, 1969). Racemic 1,2-dihexanoyldithio-propyl-3-phosphocholine (diCdithioPC) was synthesized from the corresponding mercaptan by acylation of the SH-groups with acyl chloride and phosphorylation of the hydroxyl group (Volwerk et al., 1979). n-Dodecylphosphocholine (C12PN) and n-hexadecylphosphocholine (C16PN) were prepared as described by van Dam-Mieras et al. (1975).

Phospholipase assays

Quantitative measurements with micelles of diC8PC as the substrate were carried out with a titrimetric assay at pH 8 in the presence of 1 mM borate, 25 mM CaCl2 and 100 mM NaCl at 25°C. Activities on monomeric diCdithioPC were determined at pH 8, with 5,5′-dithiobis-[2-nitrobenzoic acid] (DTNB) as a chromogenic reagent in the presence of 100 mM NaCl, 100 mM CaCl2 and 100 mM Tris, as described previously (Volwerk et al., 1979).

Direct binding of PLA2 to monomers and micelles

The affinities of PLA2 for monomers and micelles were determined by following the increase of tryptophan fluorescence upon addition of increasing concentrations of the non-hydrolysable substrate analogues n-dodecylphosphocholine (CMC 1.3 mM) for monomer binding and n-hexadecylphosphocholine (CMC 10 µM) for binding to micelles. Spectra were recorded at 25°C with a Perkin-Elmer LS-5 Luminescence Spectrometer. The excitation was performed at 280 nm and the emission spectra were recorded from 310 to 370 nm; slit widths were 5 nm. Assays were performed in a buffer containing 100 mM NaAc, 50 mM CaCl2 and 100 mM NaCl at pH 6.0. Protein concentrations used were 7–8 µM. From saturation curves obtained with lipid monomers, a Kd value can be derived directly. The data concerning micelle binding were analysed in terms of the binding of the enzyme to a theoretical lipid particle consisting of N monomers with a dissociation constant Ka. As has been discussed extensively by de Araujo et al. (1979), the N·Kd value is the experimental concentration at which 50% of the enzyme is saturated with micelles.

Results

Production of PLA2 with mutations at positions 52, 73 or 99

For the expression of (mutant) phospholipases use was made of an expression system in which the protein is produced in the form of a fusion protein with cro-LacZ (de Geus et al., 1987). Subsequently the fusion protein is folded and the disulphide
bridges of PLA₂ are formed in vitro. In the next step PLA₂ is liberated from the resulting reoxidized hybrid protein by limited trypsinolysis. For native PLA₂ this procedure yields the amount obtained before with native PLA₂. The yield of the ΔD99N and the ΔY52Q mutants after cleavage of reoxidized fusion protein by trypsin was only 20% of the usual amount, whereas the ΔY73Q mutant could not be obtained at all, despite the fact that the yield of fusion protein was similar for all mutants. A possible explanation for the low yield of the ΔD99N, ΔY52Q and ΔY73Q mutant enzymes could be that the in vitro refolding of the ΔD99N and ΔY52Q mutants was seriously affected. The presence of mismatched disulfide bonds would make the scrambled phospholipase-like structures susceptible to degradation by trypsin, whereas correctly folded (native) PLA₂ is quite stable in the presence of trypsin. Indeed SDS–PAGE analyses of trypsin-incubated reoxidized fusion proteins of wild-type, ΔE62–66, ΔD99N, ΔY52Q and ΔY73Q PLA₂ indicated the susceptibility of the latter three proteins. After incubation with trypsin the amounts of released ΔD99N and ΔY52Q PLA₂ were lower than normal, whereas ΔY73Q PLA₂ was virtually undetectable (data not shown).

Interaction of ΔE62–66, ΔD99N, ΔY52F, ΔY73F and ΔY52Q mutant PLA₂ with monomeric and micellar substrate analogues

After production and purification of the mutant enzymes, it was of interest to investigate the influence of the mutations on the affinities for monomeric and micellar substrate analogues. The three-dimensional structure of PLA₂ indicates that the residues at positions 73 and 99 are buried in the interior of the protein. Residue 52 is somewhat more exposed and might be able to have an interaction with a monomeric substrate (analogue) molecule. Because of their buried character none of these residues is expected to be involved directly in binding to lipid–water interfaces. The results of the direct binding experiments of the four mutant enzymes and ΔE62–66 with C12PN monomers and with C16PN micelles are given in Table I. The ΔD99N mutant displayed an affinity twice as high for C12PN monomers as the parent ΔE62–66. Since the main effect of this mutation is the removal of one negative charge in the active site, a diminished electrostatic repulsion between this residue and the phosphate of the C12PN monomer could be responsible for the slightly increased affinity of the Asn99 mutant for monomers. The affinity for monomers was not changed in the case of the ΔY52F and ΔY73F mutants, whereas the ΔY52Q mutant exhibited a slightly lower affinity for monomers. These results indicate that the residues at positions 52, 73 and 99 are probably not directly involved in substrate binding via hydrogen bonding, although, given the lower affinity of ΔY52Q PLA₂ for monomers, a contribution of Tyr52 or Phe52 via hydrophobic interaction with the monomeric substrate molecule cannot be excluded. The replacement of Tyr52 or Tyr73 by a Phe hardly affected the enzymes affinity for C16PN micelles. Only the affinities of the ΔY52Q and the ΔD99N mutants were moderately diminished, relative to the ΔE62–66 mutant. It can be concluded that the binding capacities both for monomeric and for micellar substrate analogues are preserved in all mutants.

### Table I. Affinities of ΔE62–66 and four mutant PLA₂s for a monomeric and a micellar substrate analogue

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>C12PN (monomers)</th>
<th>C16PN (micelles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kᵢ (mM)</td>
<td>N'/Kᵢ (µM)</td>
</tr>
<tr>
<td>ΔE62–66</td>
<td>0.8</td>
<td>140</td>
</tr>
<tr>
<td>ΔD99N</td>
<td>0.4</td>
<td>320</td>
</tr>
<tr>
<td>ΔY52F</td>
<td>0.8</td>
<td>172</td>
</tr>
<tr>
<td>ΔY73F</td>
<td>0.8</td>
<td>118</td>
</tr>
<tr>
<td>ΔY52Q</td>
<td>2.1</td>
<td>396</td>
</tr>
</tbody>
</table>

C12N stands for n-dodecylphosphocholine and C16N represents n-hexadecylphosphocholine. Accuracy was ~10% for each given value, except for the Kᵢ of ΔY52Q on C12PN, which exceeds the CMC of C12N considerably, yielding an accuracy of ~40%.

### Table II. Enzymatic activities of ΔE62–66 and four mutant PLA₂s on a monomeric and on a micellar substrate

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>diC6dithioPC (monomers)</th>
<th>diC8PC (micelles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k₉/Kᵢ (s/M)10⁻³</td>
<td>vₘₐₓ (µmol/min/mg)</td>
</tr>
<tr>
<td>ΔE62–66</td>
<td>1.64</td>
<td>4310</td>
</tr>
<tr>
<td>ΔD99N</td>
<td>1.07</td>
<td>172</td>
</tr>
<tr>
<td>ΔY52F</td>
<td>1.61</td>
<td>4750</td>
</tr>
<tr>
<td>ΔY73F</td>
<td>1.31</td>
<td>4360</td>
</tr>
<tr>
<td>ΔY52Q</td>
<td>0.26</td>
<td>39</td>
</tr>
</tbody>
</table>

Accuracy was ~10% for each given value.

### Enzymatic activities of ΔE62–66, ΔD99N, ΔY52F, ΔY73F and ΔY52Q mutant PLA₂s on monomeric and micellar substrates

The enzymatic activities of the mutant enzymes were determined with the monomeric substrate diC6dithioPC (Table II). The specificity constants for ΔE62–66, ΔY52F and ΔY73F PLA₂s fall in the same range. For the ΔY52Q mutant the specificity constant is significantly lower, whereas the substitution of asparagine for aspartic acid has only limited effects on the specificity constant for the monomeric substrate.

When looking at the data for the hydrolysis of micelles of dioctanoyllecithin (Table II) it is clear that the mutations Y52F and Y73F do not exert any significant effect on the activities, nor on the Kᵢ values. Again a significant drop in activity and in substrate affinity was observed for the mutation Y52Q. These observations underline the importance of the presence of an aromatic residue at position 52 and show that a phenolic hydroxyl at positions 52 and 73 is not a requirement for enzymatic activity. Although the Kᵢ values of ΔD99N and of ΔE62–66 PLA₂ differ only slightly, the ΔD99N has a 25-fold decreased activity compared with ΔE62–66 PLA₂. These results suggest that in the ΔD99N mutant the loss in activity is due to a reduced stabilization of the transition state, but that the active site histidine remains oriented, leaving itsimidazol ring in the correct tautomeric state for acid/base catalysis.

### Discussion

Previous papers from this laboratory have dealt with PLA₂ mutants which had in common that point mutations, or even a loop deletion of five amino acids in combination with three additional mutations, were applied at the surface of the enzyme (Kuipers et al., 1989a, b, 1990). With these mutants no serious problems in their production, refolding and purification were ever present. The present paper enzymes are described that have...
mutations in the interior of the protein, and, perhaps due to this location, difficulties were encountered with the production of some of these mutants. Instability of proteins with mutations of internal residues is not unprecedented in protein engineering literature. For instance, it was reported recently that R35K and R87K mutations in the active site of staphylococcal nuclease disrupt the conformation of the active site, thereby reducing the catalytic efficiencies by a factor of $10^4$ to $10^5$ respectively (Pourmotabbed et al., 1990). Furthermore, it has been shown that the interior packing and hydrophobicity of amino acid residues has a great influence on the stability of a protein (Sandberg and Terwilliger, 1989). In another study it was shown that weakly polar interactions between aromatic side chains of oligopeptides can stabilize their folding. The aromatic groups of globular proteins display a similar behaviour and thereby contribute to the stability of the three-dimensional structure of proteins (Burley and Petsko, 1989). In our case, the expression of all mutant proteins as fusion proteins in E.coli K-12 did not give rise to any difficulties, as the yield of inclusion bodies was equal for all mutants. However, after reoxidation and tryptic activation, the yields of $\Delta$D99N and $\Delta$Y52Q were considerably lower than those of $\Delta$Y52F and $\Delta$Y73F, while mutant $\Delta$Y73Q could not be obtained at all. It was shown by SDS-PAGE that, following limited trypsinolysis, a significant fraction of the reoxidized $\Delta$Y52Q and $\Delta$Y73Q mutant fusion proteins were degraded to peptides smaller than PLA2, and that the remaining PLA2 band is about as stable as native PLA2. The most straightforward explanation of this increased susceptibility to trypsin is that these mutants were only in part correctly folded. The incorrectly folded fraction is destroyed by the action of trypsin and the correctly folded fraction remains intact during prolonged periods of trypsinolysis.

The substitution in the $\Delta$62-66 PLA2 has only limited effects on the affinity for monomeric and for micellar substrates and the water molecule could help in the stabilization of the enzyme by substituting the much smaller Gln residue for Tyr. To elucidate the role of the highly conserved aspartate residue in the active centre of porcine pancreatic PLA2, we replaced Asp99 by Asn. As could be expected for such an internal residue, the substitution in the $\Delta$62-66 PLA2 has only limited effects on the affinity for monomeric and for micellar substrates and
substrate analogues. The effect of the mutation on the enzymatic hydrolysis of monomeric substrates is surprisingly small, since the ΔD99N enzyme still possesses 65% of the Δ62–66 activity (Table II) which seems to be in conflict with the proposed role in catalysis of Asp99 (cf. Figure 1). Besides the enzymatic activities on monomeric substrates, the activities of the Δ62–66 and the ΔD99N PLA2 on zwitterionic aggregated phospholipids were determined. The substitution of asparagine for aspartate decreased the hydrolysis rate of dioctanoylcellulithin by the mutant enzyme to ~4% compared with the Δ62–66 PLA2. The latter reduction in catalytic efficiency is of the magnitude which has been predicted for such a substitution in the catalytic Asp-His-Ser triad of serine esterases (Fersht, 1985; Warshel et al., 1989). In evaluating the discrepancy in these percentages of monomeric and micellar activities one should keep in mind that the 4% value still represents a turnover number of 41/s, whereas the 65% monomer hydrolysis corresponds to a turnover number of 1.3/s only. The latter value is so low that proton transfer from the attacking water molecule to His48 and from His48 to the leaving glycerol might not be rate limiting. For the hydrolysis of monomeric substrates it has indeed been observed that the reaction occurs as quickly in D2O as in H2O (J.J. Volwerk and H.M. Verheij, unpublished results). For the hydrolysis of micellar dioctanoylcellulithin by Δ62–66 the turnover number is as high as 1600/s and proton transfer to and from His48 may be an important factor in catalysis, thus explaining the sensitivity of the rate constant for the substitution of Asn for Asp.

Although our data for the substitution of asparagine for aspartate fit the theoretical predictions, the observed 25-fold reduction is low compared with values of ~25 000 for analogous substitutions in trypsin and in rat pancreatic trypsin (Carter and Wells, 1988; Craik et al., 1987). For the D102N trypsin mutant X-ray crystallographic studies (Sprang et al., 1987) showed that the imidazole ring of His57 is free to rotate to the more favoured trans conformation, away from the catalytic site. So it may be assumed that the decreased serine nucleophilicity in D102N trypsin results from the lack of a base in the active site to accept the serine hydroxyl proton. His57 does not act as a base in this mutant, because it exists in the incorrect tautomer. In order to try to understand better the different effects of the Asp to Asn mutation in trypsin and in PLA2, a careful comparison of the positions of the amino acid side chains in the active centres of both trypsin and PLA2 was made. In PLA2, one of the carboxylate oxygen atoms of Asp99 accepts hydrogen bonds from both the hydroxyl group of Tyr73 and a water molecule (Figure 2A), whereas in trypsin this hydrogen bonding results from the main-chain amide groups of residues 56 and 57. The other carboxylate oxygen atom of Asp99 in PLA2 accepts hydrogen bonds from the N3 atom of His48 and the hydroxyl group of Tyr52, comparable with the hydrogen bonding by the N1 atom of His57 and the hydroxyl group of Ser214 in trypsin. Whereas in trypsin the introduction of an amide disrupts hydrogen bonding, the situation in ΔD99N PLA2 is such that introduction of the amide function of Asn99 requires only minimal structural changes to create hydrogen bonds where the amide functions as the donor and the hydroxyl group of Tyr73 and a water molecule as acceptors. Therefore, we propose that the hydrogen bond between the N3 atom of His48 and the carbonyl oxygen of Asn99 could remain intact in ΔD99N PLA2 (Figure 2B). In this model possible steric conflicts are negligible since only minor rearrangements of two hydrogen atoms are required. If this model is correct it means that the Asn99 residue in the ΔD99N mutant is still able to stabilize the imidazole of His48 in the correct tautomeric state for acid/base catalysis. This might explain why the decrease in enzymatic activity of the ΔD99N PLA2 is far less pronounced than the >104 times reduced rate of the D102N trypsin mutant. The loss of enzymatic activity of ΔD99N PLA2 on micellar substrates may be ascribed partially to differences in the proximity of the reactive groups of the substrate and the enzyme, due to a slightly different active site architecture resulting from the removal of a negative charge from the interior of the enzyme. However, a more likely explanation is that the replacement of Asp99 by a noncharged Asn residue leads to a reduction of the negative potential on His48, which could destabilize the transition state.

A structural analysis of the mutants described in this paper might help to understand better side-chain interactions of these mutants. Therefore, attempts at crystallization of these mutants are being made currently. Awaiting these structures also, experiments which combine the mutations at positions 52, 73 and 99 might provide a means to shed more light on a possible cooperativity between these residues. The orienting capacity of Asn99, which is assisted by hydrogen bonds from Tyr52 and Tyr73 might be lost in mutants such as ΔD99N/Y52F or ΔD99N/Y73F, which makes their future production desirable.

Note added in proof
After this manuscript was accepted for publication a paper appeared [Dupureur, C.M., Deng, T., Kwak, J.-G., Noel, J.P. and Tsai, M.-D. (1980) J. Am. Chem. Soc., 112, 7074–7076] describing substitutions of Tyr52, Tyr73 and Asp99 in bovine pancreatic phospholipase A2. The conclusions of these authors concerning the role of both tyrosine residues are in line with our conclusions. The substitution of Asn for Asp99 decreased the kcat/Km ratio for dioctanoylcellulithin micelles 1000-fold in the bovine enzyme, as opposed to the 25-fold decrease observed by us with the same substrate. The high residual activity (65%) described in this paper for hydrolysis of monomeric substrates excludes the possibility that the 4% residual activity on micelles is caused by a contamination of the D99N mutant with Asp99 phospholipase A2. This suggests an interesting difference in the active site stabilization of the bovine pancreatic phospholipase A2 used by Dupureur et al. and the porcine pancreatic phospholipase A2 used by us, where a surface loop of five amino acids was also deleted.

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