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Evidence for the involvement of tyrosine-69 in the control of stereospecificity of porcine pancreatic phospholipase A₂

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We have studied the role of Tyr-69 of porcine pancreatic phospholipase A₂ in catalysis and substrate binding, using site-directed mutagenesis. A mutant was constructed containing Phe at position 69. Kinetic characterization revealed that the Phe-69 mutant has retained enzymatic activity on monomeric and micellar substrates, and that the mutation has only minor effects on k_{cat} and K_m . This shows that Tyr-69 plays no role in the true catalytic events during substrate hydrolysis. In contrast, the mutation has a profound influence on the stereospecificity of the enzyme. Whereas the wild-type phospholipase A₂ is only able to catalyse the degradation of *sn*-3 phospholipids, the Phe-69 mutant hydrolyses both the *sn*-3 isomers and, at a low (1–2%) rate, the *sn*-1 isomers. Despite the fact that the stereospecificity of the mutant phospholipase has been altered, Phe-69 phospholipase still requires Ca²⁺ ions as a cofactor and also retains its specificity for the *sn*-2 ester bond. Our data suggest that in porcine pancreatic phospholipase A₂ the hydroxyl group of Tyr-69 serves to fix and orient the phosphate group of phospholipid monomers by hydrogen bonding. Because no such interaction can occur between the Phe-69 side-chain and the phosphate moiety of the substrate monomer, the mutant enzyme loses part of its stereospecificity but not its positional specificity.

Key words: phospholipase A₂/site-directed mutagenesis/stereospecificity/substrate specificity/Tyr-69

Introduction

Phospholipase A₂ (PLA₂, EC 3.1.1.4) catalyses the hydrolysis of the fatty acid ester bond at the 2 position of 1,2-diacyl-*sn*-3-phosphoglycerides (L- α -phospholipids). The stereoisomeric 2,3-diacyl-*sn*-1-phosphoglycerides (D- α -phospholipids) bind with an equal affinity to the catalytic site but are not hydrolysed (Bonsen *et al.*, 1972a). In mammals the enzyme occurs both extracellularly, serving digestive functions, and intracellularly, serving several regulatory functions. Phospholipase A₂ is also found in bee and snake venoms. The enzymes from mammalian pancreas and snake venoms exhibit a high degree of homology in their primary structure (for a review see Waite, 1987). The three-dimensional structures of (pro) PLA₂ from bovine and porcine pancreas and from *Crotalus atrox* venom have been determined and were shown to be very similar (Dijkstra *et al.*, 1981, 1983; Brunie *et al.*, 1985; Renetseder *et al.*, 1985). From an analysis of the three-dimensional structure of PLA₂ and from chemical modification studies on porcine and bovine PLA₂, it was proposed that the binding site for lipid aggregates consists of a hydrophobic surface region, formed by the side-chains of residues Leu-2, Trp-3, Arg-6, Leu-19, Met-20, Leu-31 and Tyr-69 (Volwerk and de Haas, 1982).

Which residues in particular are important for the interaction with monomeric substrate molecules remains speculative, since no enzyme–substrate complex has been crystallized yet. Using mononitrated PLA₂ from porcine, bovine and equine pancreas, Meyer *et al.* (1979) found that NO₂-Tyr-69 is strongly perturbed by both micellar and monomeric substrate analogues. At pH 6 the affinity of NO₂-Tyr-69 PLA₂ for monomeric substrate analogues was close to that of the native enzyme, but at pH 8 the affinity was severely diminished for NO₂-Tyr-69 PLA₂ but not for native PLA₂. The authors concluded that a non-ionized tyrosyl-69 hydroxyl group is important for the binding of substrate monomers. From an analysis of the three-dimensional structure of bovine PLA₂ inhibited by *p*-bromophenacylbromide (Renetseder *et al.*, 1988) it appears conceivable that the Tyr-69 side-chain will interact with the phosphate moiety of the substrate. To gain a better understanding of the role of Tyr-69 in porcine pancreatic phospholipase A₂, we have substituted phenylalanine for Tyr-69, using site-directed mutagenesis. The effect of this substitution on enzymatic activity, substrate specificity and the affinity for substrate (analogues) is discussed.

Materials and methods

Construction of mutant phospholipase

Escherichia coli K-12 strain PC2494 [Δ (lac-pro), sup E, thi/F' tra D₃₆, pro A⁺B⁺, lac I^q, lacZ Δ M15, Phabagen collection, Utrecht] was used for plasmid constructions as a host for M13-derived vectors. HB2154 [ara, Δ (lac-pro), thi/F' pro A⁺B⁺, lac I^q, lacZ Δ M25, mut L::Tn10 (Carter *et al.*, 1985)] was used as the recipient strain in the mutagenesis experiments.

Substitutions in the proPLA₂-cDNA (de Geus *et al.*, 1987) were introduced by the gapped duplex procedure, using amber selection (Kramer *et al.*, 1984). The proPLA₂-cDNA (de Geus *et al.*, 1987) was provided with three unique restriction sites (SacII, KpnI and BglII) to facilitate the cloning of small PLA₂-cDNA fragments in future experiments. The applied mutations were silent except in the case of the SacII site, where the Ser^V-Ser^{VI}-Arg^{VII} sequence of the pro-peptide was changed into a Ser^V-Pro^{VI}-Arg^{VII} sequence, which slightly improved the processing rate of the pro-peptide by trypsin (data not shown). The mutagenic oligonucleotides 5' CATAATGCCCGCG GACTGATGCCTT3' (SacII) and 5' CACAGGGGTACCTGA TCC3' (KpnI) were used for site-directed mutagenesis. The BglII site was introduced by ligation of an adaptor consisting of the oligonucleotides 5'AGCTTAGATCTAACAGT 3' and 5'ACTG TTAGATCTA 3' into the ScaI–HindIII vector fragment of M13mp8 containing the proPLA₂-cDNA (de Geus *et al.*, 1987). All oligonucleotides were synthesized on a Biosearch 6800 DNA synthesizer. The sites of mutation in the sequences of the oligonucleotides are underlined. The resulting proPLA₂-cDNA was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) and a BamHI–HindIII fragment containing the entire proPLA₂-cDNA was cloned into the expression vector described by de Geus *et al.* (1987). The new construction,

containing the three additional restriction sites, was designated pOK13. With the use of the proPLA₂-cDNA described above, the Phe-69 mutation was applied with the oligonucleotide 5' GCT.TTC.GGT.GAA.GGG.ATT.G 3'. The cDNA encoding the mutant PLA₂ species was sequenced, and a *Bst*XI-*Bgl*II fragment containing the mutation was ligated into expression vector pOK13. After transformation and expression in *E. coli* K-12 strain MC4100 (Silhavy *et al.*, 1984) containing plasmid pCI857, the mutant phospholipase was 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 8.0.

Phospholipids

The 1,2-diacyl-*sn*-glycero-3-phosphocholines used in this study were obtained after reacylation of *sn*-glycerol-3-phosphocholine. Mixed acid lecithins were prepared by degradation of the parent compound with PLA₂, followed by reacylation of the resulting lysophospholipid. The 1,2-diacyl-*sn*-glycero-3-sulphates were synthesized as described before (van Oort *et al.*, 1985). The 2,3-diacyl isomers of lecithins and sulphates were isolated after extensive degradation of the racemic compounds with PLA₂. Both isomers of the ether analogue 1-tetradecyl-2-hexadecanoyl-3-phosphocholine were prepared from the corresponding 'diglycerides' by standard procedures (Bonsen *et al.*, 1972b). The β -lecithin 1-hexadecanoyl-3-tetradecanoyl-*sn*-glycero-2-phosphocholine was prepared from 1,3-ditetradecanoyl-*sn*-glycero-3-phosphocholine by degradation with PLA₂ and reacylation of the resulting lysophospholipid with palmitoylchloride. DL-2-Nonanoylpropanediol-1-phosphocholine was synthesized by acylation of the mono-tritylated compound, followed by detritylation and phosphorylation with 2-chloro-2-oxo-1,3,2-dioxaphospholane (Chandrakumar and Hajdu, 1983). The triester was subsequently converted to the lecithin by treatment with trimethylamine. 1,2-Didodecanoyl-*sn*-glycero-3-thiophosphocholine was synthesized from 3-*O*-benzyl-*sn*-glycerol as described by Nifant'ev *et al.* (1978). The R_p- and S_p-isomers were isolated by degradation with phospholipase A₂

and reacylation of the R_p-lysophospholipid, essentially as described before (Bruzik *et al.*, 1983).

Phospholipase assays

The activities of phospholipases were determined qualitatively using TLC on silica gel plates developed in chloroform-methanol-water (65-25-4, by vol.) mixtures. Lipids (10-20 mg/ml) were solubilized in the presence of sodium deoxycholate (2.5 mol/mol phospholipid) in buffer (50 mM borate, 5 mM CaCl₂, pH 8.0). Spots were visualized after spraying the plates with phosphorus-reagent (Dittmer and Lester, 1964) or spraying with 30% sulphuric acid and charring. For GLC analysis spots were visualized with I₂-vapour and fatty acids were analysed as their methyl-esters on a Perkin-Elmer 8500 Gas Chromatograph.

Quantitative measurements 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. The burette of the Radiometer ABU was filled with 10 mM sodium hydroxide. For substrates containing fatty acyl chains of >8 carbon atoms, sodium deoxycholate was included in the test at a 2.5-fold molar ratio to substrate. Routinely the activities were measured on an egg yolk suspension in the presence of deoxycholate (Nieuwenhuizen *et al.*, 1974). Activities on monomeric racemic 1,2-dihexanoyldithiolecithin were determined at pH 8 in the presence of 100 mM Tris, 100 mM NaCl and 100 mM CaCl₂, as described previously (Volwerk *et al.*, 1979).

Direct binding of PLA₂ to monomers and micelles

The affinity of PLA₂ for monomers and micelles was determined by following the increase of tryptophan fluorescence upon addition of increasing concentrations of the non-hydrolysable substrate analogues *n*-dodecanoylphosphocholine (CMC 1.3 mM) for monomer binding and *n*-hexadecylphosphocholine (CMC 10 μ M) for binding to micelles. Assays were performed in a buffer containing 100 mM NaAc, 50 mM CaCl₂ and 100 mM NaCl at pH 6.0. From saturation curves obtained with lipid monomers, a K_d -value can directly be derived. 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 K_d . As has been discussed extensively by de Araujo *et al.* (1979), the $N.K_d$ value is the experimental concentration at which 50% of the enzyme is saturated with micelles.

Results

Enzymatic activities of Phe-69 phospholipase

The enzymatic activities and binding properties of the mutant and the native enzyme were determined on monomeric 1,2-dihexanoyldithiolecithin and with the substrate analogue *n*-dodecanoylphosphocholine. The activities and binding properties with substrates present in aggregates were determined with

Table I. Enzymatic activity of native and Y69F PLA₂ on monomeric substrate of diC6dithioPC and binding to the substrate analogue C12PN

Enzyme	diC6dithioPC			C12PN K_d (mM)
	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ M ⁻¹)	
Native	0.7	0.8	900	0.3
Y69F	1.4	1.0	1400	0.3

Monomeric substrate was *rac* 1,2-dihexanoyldithiolecithin. C12PN stands for *n*-dodecanoylphosphocholine. Accuracy was ~10% for each given value. For details see Materials and methods.

Table II. Enzymatic activity of native and Y69F PLA₂ on micellar substrates and binding to the substrate analogue C16PN

Enzyme	L-diC8-PC		C16PN $N.K_d$ (μ M)	Egg yolk activity (μ mol min ⁻¹ mg ⁻¹)	L-diC8-GS activity (μ mol min ⁻¹ mg ⁻¹)
	V_{max} (μ mol min ⁻¹ mg ⁻¹)	K_m^{app} (mM)			
Native	2000	3.7	70	1300	1470
Y69F	440	9.5	240	305	930

The substrate concentration of 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine (L-diC8-PC) was varied between 0.5 and 10 mM. C16PN stands for *n*-hexadecylphosphocholine. The activity on 1,2-dioctanoyl-*sn*-glycero-3-substrate (L-diC8-GS) was measured at a fixed substrate concentration of 2 mM. Accuracy was ~10% for each given value. For details see Materials and methods.

micelles of various substrates and substrate analogues. The results of these determinations are summarized in Tables I and II. It is clear that the mutation Y69F has only limited effects on the binding and the specificity constant for monomeric substrates. Thus Tyr-69 is not involved in the true catalytic steps. Using aggregated substrates either in the form of mixed micelles (egg yolk), as neutral lecithin micelles or as negatively charged micelles, it is evident that the mutant is a somewhat less effective catalyst than the wild-type enzyme. The apparent affinity of the mutant for aggregated lecithins was reduced with regard to that of the wild-type PLA₂. Direct binding experiments also showed the reduced affinity of this mutant PLA₂ for micelles of the non-hydrolysable substrate analogue *n*-hexadecylphosphorylcholine. These data stress the importance of Tyr-69, specifically its phenolic OH moiety, for productive interaction of the enzyme with micelles.

Effect of substitution on stereo- and positional-specificity

Phospholipases A₂ are highly stereo- and position-specific. Although the exact nature of the interaction between enzyme and substrate is unknown, it has been suggested (Renetseder *et al.*, 1988) that the hydroxyl group of Tyr-69 is involved in phosphate binding. We therefore tested the mutant enzyme for its ability to hydrolyse lipids other than *sn*-3 phospholipids. In initial experiments, using TLC as a qualitative assay, we found in fact that the Y69F mutant behaved differently from the wild-type enzyme. The Y69F mutant appeared to be capable of catalysing the hydrolysis of several phospholipids that are not hydrolysed by the wild-type enzyme. This different behaviour on both isomers of didecanoyllecithin is illustrated in Figure 1.

To obtain quantitative data we repeated these experiments in the pH-stat. In order to minimize kinetic differences due to physico-chemical properties of aggregated lipids, we used sodium deoxycholate to get uniformly dispersed lecithins (Table III). The mutant Y69F hydrolysed D-lecithins and D-glycerosulphates at low rates but the difference with the wild-type enzyme is significant. Because the Y69F mutant still keeps its preference for L-phospholipids, the loss of stereospecificity is partial and not absolute. The fact that 1-tetradecyl-,2-hexadecanoyl-*sn*-glycero-3-phosphocholine, containing an ether bond at *sn*-1, is hydrolysed by the Y69F mutant indicates that this mutant, although it has partially lost its stereospecificity, still retains its

positional specificity for the secondary ester bond. This retention of positional specificity was confirmed with DL-2-nonanoyl-propanediol-1-phosphocholine, a phospholipid with only a secondary acyl ester bond. This compound is still being degraded by the wild-type enzyme, with retention of stereospecificity, since only the L-compound is hydrolysed. The positional specificity was further investigated on mixed-acid diacyl phospholipids. Incubation of 1-tetradecanoyl-2-hexadecanoyl-*sn*-glycero-3-phosphocholine with wild-type and mutant phospholipases, and gas-chromatographic analysis of the liberated fatty acid (palmitic acid) and the resulting lysolecithin, showed that the products of hydrolysis of wild-type and mutant phospholipases were indistinguishable, in agreement with the known 2-specificity of PLA₂. The positional specificity was also verified with the β -lecithin 1-hexadecanoyl-3-tetradecanoyl-*sn*-glycero-2-phosphocholine as a substrate. Again no difference between wild-type and mutant phospholipase was observed, as from this lecithin only hexadecanoic acid was removed. To further investigate the interactions of Tyr-69 and Phe-69 in the wild-type and mutant enzymes respectively with the phosphate moiety of the substrate, we tested these enzymes on substrates modified at phosphorus. The R_p- and S_p-isomers of lecithins with a sulphur present on the phosphate (Bruzik *et al.*, 1983) were synthesized, and incubated with wild-type and Phe-69 PLA₂. TLC analyses showed that the R_p-1,2-didecanoyl-*sn*-glycero-3-thiophosphocholine was hydrolysed rapidly by both the wild-type and the mutant enzymes, whereas the S_p-isomer was resistant to the action of the wild-type enzyme. The Phe-69 mutant, however, slowly degraded the S_p-isomer to completion. Since the mutant Y69F had lost part of its stereospecificity it was of interest to test whether this mutant still required Ca²⁺ as a cofactor. We assayed the Y69F PLA₂ in the presence of 5 mM EDTA and found that the hydrolysis of both D- and L-lecithins was completely blocked.

Discussion

Using chemically modified pancreatic PLA₂, Meyer *et al.* (1979) concluded that Tyr-69 in PLA₂ is involved in the binding of both monomeric substrates and aggregated substrates. The introduction of a large hydrophobic group like the dansyl group on amino Tyr-69 increased the affinity of the modified PLA₂ for

Fig. 1. Degradation of L- and D-didecanoyllecithins by native and Y69F mutant PLA₂. Incubations of didecanoyllecithins (12.5 mM) were performed in the presence of deoxycholate (12.5 mM) in 50 mM borate buffer, containing 5 mM CaCl₂ at pH 8.0 in a total volume of 300 μ l. To the L-lecithins were added 5 μ g native (lane a) and 20 μ g Y69F mutant enzyme (lane b); to the D-lecithins were added 25 μ g native (lane c) and 100 μ g Y69F mutant enzyme (lane d). Incubations were for 8 h at 37°C. The TLC plate was developed in CHCl₃-MeOH-H₂O (65-35-8 by vol.), and spots were visualized by spraying with phosphorus reagent. Only the lower half of the plate has been photographed.

Table III. Enzymatic activity of native and Y69F PLA₂ measured on both *sn*-3 and *sn*-1 species of three different micellar substrates

Substrate	Activities (μ mol min ⁻¹ mg ⁻¹)	
	Native	Y69F
L-diC ₁₂ -GS ^a	66	51
D-diC ₁₂ -GS	— ^b	1
L-diC ₁₀ -PC ^a	106	28
D-diC ₁₀ -PC	— ^b	0.21
L-14,16-PC ^a	46	17
D-14,16-PC	— ^b	0.23

Activities were determined at pH 8.0 and 25°C in the presence of 25 mM CaCl₂ 100 mM NaCl and 1 mM borate and substrate concentrations of 1 mM. The lecithin analogues were assayed in the presence of 2.5 mM sodium deoxycholate. Accuracy was ~10% for each given value.

^aL- and D-diC₁₂-GS: both stereo-isomers of

1,2-didodecanoylglycero-sulphate; L- and D-di C₁₀-PC: both stereo-isomers of 1,2-didecanoylphosphocholine; L- and D-14,16-PC: both stereo-isomers of 1-tetradecyl-2-hexadecanoylphosphocholine.

^bNot detectable in the pH-state; TLC after overnight incubation, under conditions as described in the legend of Figure 1, did not show any hydrolysis of the substrate.

Fig. 2. Structure of a phospholipid molecule. X represents a hydrogen atom or an alcohol.

micelles about 10-fold. In the present study we find that substitution of Tyr-69 by Phe reduces the affinity for micelles more than 3-fold. The side-chain of Tyr-69, specifically its phenolic OH moiety, might thus be involved in a first recognition of and interaction with aggregated substrates. The fact that this residue is located at the surface of native PLA₂ and sticks into the solvent in the three-dimensional structure of the bovine enzyme (Dijkstra *et al.*, 1981) but points more inward in the three-dimensional structure of the porcine enzyme (Dijkstra *et al.*, 1983), already indicates the flexibility of this residue. The question then arises what the orientation of this side-chain will be in the enzyme–monomer substrate complex.

Interestingly, Renetseder *et al.* (1988) observed that in the crystal structure of bovine PLA₂ inhibited by *p*-bromophenacyl-bromide the Tyr-69 side-chain is pointing inward instead of outward. This notable change in the position of the phenolic OH resembles the 120° rotation observed for Tyr-248 in carboxypeptidase A (Rees and Lipscomb, 1982) upon binding of a substrate molecule. If the phenolic OH of Tyr-69 in PLA₂ is actually hydrogen-bonded to a hydrogen bond acceptor in the substrate molecule then its energetic content must be low. The Y69F mutant binds monomeric substrate molecules and their analogues about equally efficiently as the wild-type enzyme. This low energy content may be explained by the fact that formation of the hydrogen bond between the phenolic OH and the putative acceptor requires removal of bound water molecules from both acceptor and donor groups. Small changes in binding energy have also been observed for the interaction between hydrogen-bridged partners in trysoyl-tRNA synthetase (Wells and Fersht, 1985). Likewise in carboxypeptidase A, mutation of Tyr-248 to Phe hardly affects the *K_m* for ester substrates (Gardell *et al.*, 1985). The fact that the mutation Y69F does not significantly change the affinity for monomeric substrates once more underlines that this affinity is dictated mainly by hydrophobic interactions with the acyl chains of the substrate: the enzyme binds monomers of dioctanoylleicithins ~ 100 times more tightly than dibutyrylleicithin molecules (Verheij *et al.*, 1981). The data presented here imply that the major role of Tyr-69 is not to increase affinity for the monomeric substrate but, rather, to orient the substrate in a specific way.

The question remains which group in the substrate molecule acts as the hydrogen bond acceptor for the phenolic OH of Tyr-69. Inspection of the structure of a phospholipid (Figure 2) shows that three groups could function as hydrogen bond acceptors for the OH moiety of Tyr-69: the carbonyl- and ester oxygens of the susceptible ester bond, the oxygens of the ester bond at the 1-position and the phosphate group. As to the first possibility: if the phenolic OH of Tyr-69 were hydrogen-bonded

to the carbonyl oxygen of the susceptible ester bond, its role could be to stabilize the transition state. Alternatively the phenolic OH could interact with the ester oxygen, where it might act as a general acid in the reaction. We believe that neither of these roles for Tyr-69 is very likely, since the mutation Y69F would result in a dramatic loss of enzymatic activity. In fact we find that the mutant's activity on monomeric substrates has hardly changed. As to the second possibility: the fact that DL-2-nonanoylpropanediol-1-phosphocholine molecules are hydrolysed in a stereospecific way by the wild-type enzyme indicates that the presence of a hydrogen bond acceptor at the 1-position is not required for the maintenance of stereospecificity. The third possibility, the specific interaction of Tyr-69 with the phosphate (or sulphate) group of the substrate, seems more likely. The fact that the S_p-isomer of 1,2-didecanoyl-*sn*-glycero-3-thiophosphocholine can only be degraded by the Phe-69 PLA₂ strongly suggests the existence of a hydrogen bridge between the hydroxyl group of Tyr-69 and the phosphate moiety of the substrate molecule. Such an interaction would explain that in native PLA₂ the affinity for monomeric substrates is constant between pH 5 and 9 whereas in NO₂-Tyr-69 PLA₂ this affinity becomes immeasurably small when NO₂-Tyr-69 (pK_a 7.1) becomes deprotonated, thus inducing a strong charge repulsion between enzyme and substrate (Meyer *et al.*, 1979).

Using synthetic phospholipids, de Haas *et al.* (1968) showed that PLA₂ has high stereo- and positional-specificity. It was concluded that stereospecificity, fulfilling the three-point interaction as defined by Ogston (1948), must reside in fixation of the susceptible ester bond, the 2-acyl chain and the phosphate moiety. Since no three-dimensional structure of an enzyme–substrate or enzyme–inhibitor complex has been determined yet, the exact position and orientation of the acyl chains and the phosphate group of the substrate relative to the enzyme's active site and the bound Ca²⁺ ion remains obscure. It has been proposed, however, that the liganded Ca²⁺ ion has two functions: polarization of the carbonyl of the scissile ester bond and fixation of the phosphate moiety (Verheij *et al.*, 1980). Mutant Y69F still has an absolute requirement for Ca²⁺ ions, but has lost part of its stereospecificity. This suggests that the hydroxyl of Tyr-69 is more important than the Ca²⁺ ion in the fixation and orientation of the phosphate moiety. Thus the major role of Ca²⁺ seems to be the polarization of the scissile ester bond.

The data in the present paper show that in wild-type porcine PLA₂ the phosphate group most likely interacts with the phenolic hydroxyl of Tyr-69. Replacement of tyrosine by phenylalanine results in a partial loss of stereospecificity at C2 and at phosphorus. To the best of our knowledge this is the first report of the alteration of stereospecificity of an enzyme by protein engineering. Considering the possible orientation of the substrate in the active site, it is plausible that during hydrolysis of substrates with the D-configuration the position of the phosphate group and the hydrogen atom at the 2-position are interchanged as compared to their position during hydrolysis of L-phospholipids. Such an interchange of positions would explain that the mutant PLA₂ has lost part of its stereospecificity but has retained its positional specificity. The fact that the Y69F mutant hydrolyses D-phospholipids ~ 50–100 times more slowly than L-phospholipids indicates that such a reverse orientation of the phosphate moiety might be less favourable than the correct orientation. Because PLA₂ is obviously not perfectly shaped for the hydrolysis of D-phospholipids, it would be attractive to optimize the binding pocket for the phosphate of these phospholipids by protein engineering. A rational approach for

such an enterprise will, in our opinion, require the determination of the three-dimensional structure of an enzyme–substrate (analogue) complex.

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