Modification of the Head-Group Selectivity of Porcine Pancreatic Phospholipase A_2 by Protein Engineering

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ABSTRACT: On the basis of the three-dimensional structures of phospholipid and porcine pancreatic phospholipase A_2 (pla_2), it was predicted that the removal of a negative charge in the hydrophilic region of the phospholipid binding site would influence the head-group selectivity of porcine pancreatic pla_2. To test this prediction, glutamic acid 46 was changed to leucine by site-directed mutagenesis. The E46L mutant, expressed in *Escherichia coli*, was purified and characterized. The mutation did not affect the activity toward the mixed micellar substrate, but the activity of E46L toward DiC_{12}-P, which has two negative charges on the head group, was three times higher than that of DiC_{12}-PC, which carries no net charge in the head group. The native pla_2 was inhibited by the product(s) released from DiC_{12}-P but not the mutant enzyme. Kinetic analysis revealed that the E46L mutant and the native pla_2 had comparable affinities (K_m) toward monomeric and micellar phospholipids of zwitterionic type while the activity (k_{cat}) of E46L, toward the same substrates, was approximately 50% lower compared to that of native pla_2. When micellar DiC_{12}-P was used as a substrate, the K_m^{app} value for E46L was four times lower and the k_{cat}^{app}/K_m^{app} was 5-fold higher than those of native pla_2. However, the kinetic parameters of mutant and native pla_2s remained unchanged for monomeric HEPG, with one negative charge in the head group. Thus, we have modified the head-group selectivity of porcine pancreatic pla_2 by protein engineering.

Phospholipase A_2 (EC 3.1.1.4) (pla_2)[1] are a family of lipolytic enzymes, which catalyze the hydrolysis of fatty acid ester bonds at the *sn*-2-position of *sn*-3-phospholipids in a calcium-dependent reaction (Waite, 1987). The enzyme is wide spread in nature and is both intra- and extracellular in origin (Verheij et al., 1981). The intracellular pla_2, which plays an important role in inflammation, blood platelet aggregation, and many other physiologically important cellular processes, is present in almost all mammalian cells but only in small quantities (Holland et al., 1990; Hazen et al., 1991). In contrast, the extracellular pla_2 serves mainly in the digestion of food and is abundant in mammalian pancreatic secretion as well as in snake and bee venom (Drenth et al., 1987). However, sequence comparison and immunological studies revealed that there is considerable homology between intra- and extracellular pla_2 (Hayakawa et al., 1988; Seilhamer et al., 1989; Lai & Wada, 1988; Okamoto et al., 1985). Thus, the understanding of the structure–function relationships of an extracellular pla_2 will not only be useful for answering some of the fundamental questions on the mechanism of action of intracellular pla_2 but also help in the design of therapeutic drugs.

The three-dimensional structures of several pla_2 molecules together with the mechanism of catalysis revealed the presence of a catalytic diad with His 48 and Asp 99 at the active site which is similar to many proteolytic enzymes (Brunie et al., 1985; Drenth et al., 1976; Dijkstra et al., 1981, 1982; Verheij et al., 1980; Kuipers et al., 1989). However, unlike the serine proteases, in pla_2, a water molecule present near the active site histidine acts as the nucleophile. When this water molecule attacks the carbonyl carbon of the substrate, the imidazole ring of His 48 picks up a proton from the water molecule and thereby facilitates the cleavage of an ester bond. Using the crystal structure of porcine pancreatic pla_2, we have reexamined the active site organization of this enzyme and modeled DiC_{10}-PC at the active site cavity (Jones et al., 1993). This has revealed that the hydrophobic region of the phospholipid fits to the hydrophobic region of the protein while the hydrophilic head region lies in the hydrophilic region of the protein molecule (Jones et al., 1993). Although no persistent close contacts were observed between the native pla_2 and the head group of the phosphocholine substrate, during molecular simulation, the adjacent charged groups are expected to influence the binding of the head group by longer range electrostatic interactions. By using an amide inhibitor (2-(dodecanoylamino)-1-hexanoylphosphoglycol), Thunnissen et al. (1990) have demonstrated the contacts between the phosphate group of the inhibitor and Tyr 69 and Asp 49 of the mutant porcine pancreatic pla_2, but the contact with the latter residue is via Ca^{2+} ion. Furthermore, the two separate protein-engineering studies with bovine (Noel et al., 1990, 1991) and porcine (Lugtigheid et al., 1993) pancreatic pla_2 have recently demonstrated the importance of Lys 56 and Arg 53 in the head-group specificity of pla_2. In addition, the high positive potential of the active site Ca^{2+} is also expected to influence the interaction between the head group and various charged amino acids. All this information indicates that the

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[1] Abbreviations: pla_2, phospholipase A_2; CMC, critical micellar concentration; HEPC, 2-(hexadecanoylthio)-1-ethylphosphocholine; HEPG, 2-(hexadecanoylthio)-1-ethylphosphoglycerol; DiC_{12}-P, 1,2-dilauroyl-*sn*-glycero-3-phosphatidic acid; DiC_{12}-PC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DiC_{10}-PC, 1,2-didecanoyl-*sn*-glycero-3-phosphocholine; DiC_8-S, 1,2-dioctanoyl-*sn*-glycero-3-sulfate; K_m and k_{cat}, Michaelis–Menten constants at monomeric substrate concentration; K_m^{app} and k_{cat}^{app}, Michaelis–Menten constants at micellar substrate concentration.

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head-group specificity of pla_2 can be modified by engineering the charge(s) in the region where the head group of the phospholipid binds to the protein molecule.

In the present study, we have predicted that the removal of a negative charge in the head-group binding region of the protein would change the head-group selectivity of porcine pla_2. In order to test this hypothesis, we have changed glutamic acid 46 to leucine by site-directed mutagenesis and studied the effect of this mutation on the catalytic properties of pla_2 using both monomeric and micellar phospholipids with zwitterionic and negative head groups. The data presented in this paper show that the removal of a negative charge in the hydrophilic region of pla_2, where the head group binds, does indeed modify the head-group selectivity of this enzyme.

MATERIALS AND METHODS

Materials. The pla_2 expression system, comprising *Escherichia coli* strain AB1157, pCI857 (a source of the temperature variant of the λ repressor CI), and pOK13.2 (containing the pla_2 cDNA, cloned downstream from a portion of the β-galactosidase gene and expressed as a fusion protein under the control of the λ pR promoter), was a gift from Dr. H. M. Verheij, Department of Enzymology and Protein Engineering, University of Utrecht, The Netherlands. The monomeric substrates (HEPC and HEPG) were synthesized as described below. Polybuffer-ion-exchanger-94 and polybuffer-74 were from Pharmacia, Uppsala, Sweden, while acrylagel and bisacrylagel were from National Diagnositics, Aylesbury, Berks, U.K. All the remaining chemicals were analytical grade and purchased either from BDH Chemicals, Poole, Dorset, U.K., or from Sigma Chemicals, Poole, Dorset, U.K.

Modeling and Design of the E46L Mutant. The graphics program FRODO (Jones, 1978) was used to model the structure of DiC_{10}-PC and the interaction of this substrate with porcine pla_2. The structure of DiC_{10}-PC was modeled as described (Jones et al., 1993) with reference to the conformation of other phospholipids (Hauser et al., 1981). The GROMOS programs (van Gunsteren & Berendsen, 1987) were used for energy minimizations and molecular dynamics simulation. Using the model structures of DiC_{20}-PC, porcine pla_2 and Ca^{2+} ion, we studied the interaction between the substrate, DiC_{10}-PC, and various amino acids around the active site of pla_2 and Ca^{2+} as described (Jones et al., 1993). On the basis of this study, the negatively charged glutamic acid 46 was identified as a residue which could influence the binding of the phospholipid head group. Therefore, this negatively charged amino acid was changed to a neutral amino acid, leucine.

Construction of the E46L Mutant. Site-directed mutagenesis was used to construct the E46L gene using wild-type pla_2 DNA subcloned as a *Bam*HI–*Pst*I fragment into the M13mp18 phage. This was accomplished with single-stranded phage DNA using the Amersham International plc kit (Reference No. RPN 1523) and the oligonucleotide described below which was prepared on a DuPont DNA synthesizer using phosphoramidites chemistry (Sinha et al., 1983). The DNA sequence of the mutagenic oligonucleotide primer corresponding to the noncoding strand, with codon 46 underlined, is as follows: 5′-GTC GTG TGT CAG GCA GCA CCT-3′.

Expression and Purification of Recombinant and Mutant pla_2. An *E. coli* strain, AB1157, carrying both pCI857 and either a negative or a mutated form of the pOK13.2, was used to express the pla_2 cDNA as a cro-lacZ fusion protein (de Geus et al., 1987). All conditions used for the growth of *E. coli* and for the induction of the pla_2-fusion protein were the same as reported previously (Bhat et al., 1991) except the PYG broth which yields 110–125 g (wet weight) of *E. coli* cells per 10 L of culture. Similarly, the conditions used for cell harvesting and isolation of inclusion bodies as well as for purification of mutant pla_2 were also the same as described (Bhat et al., 1991; Goodenough et al., 1991). Recombinant pla_2 was also produced using the above expression system and purified under identical conditions for comparison.

Synthesis of HEPC and HEPG. HEPC was synthesized as described (Bhat et al., 1993) while the following procedure was used for the synthesis of HEPG. Firstly, mercaptoethanol (11.5 g; 147 mmol) was acylated with palmitoylchloride (8.4 g; 31 mmol) as described (Bhat et al., 1993). The product, 2-(hexadecanoylthio)-1-ethanol, was purified by flash column chromatography on silica gel using petroleum ether (40–60 °C)/diethyl ether (7:3 v/v). To the above sample (1.5 g; 4.75 mmol), dissolved in 30 mL of anhydrous tetrahydrofuran and triethylamine (662 μL; 4.75 mmol), was added a solution of 2-chloro-2-oxo-1,2,3-dioxaphospholane (433 μL; 4.75 mmol) in a 5 mL of dry tetrahydrofuran in drops at 0 °C. After the addition was complete, the ice bath was removed and the solution left stirring at room temperature overnight. The precipitated triethylamine hydrochloride was filtered off under argon using oven-dried glassware to which acetic acid/water (1 mL; 8.6 mmol; 1:1 v/v) was added, and the reaction mixture was stirred overnight. The tetrahydrofuran solution containing the phosphate ester was added to aqueous barium acetate, and the product was purified as its barium salt by repeated flash column chromatography on silica gel using chloroform/methanol (9:1 v/v) as the first eluent followed by chloroform/methanol/water (65:25:4 v/v) as the second eluent. The barium salt (875 mg) was converted to its sodium salt by dissolving it in 500 mL of chloroform/methanol/water (65:25:4 v/v) and adding Na_2SO_4 (675 mg) in water (10 mL). The $BaSO_4$ was removed by filtration through Celite. After phase separation, the chloroform phase was evaporated in vacuo and the resulting oil was dried by removing the water azeotropically with dry dichloromethane. The yield was 718 mg (33%). IR: 3426 (OH), 1692 (S—C=O), 1253, 1230, 1124, 1052 nm (PO_2). ^1H NMR ($CDCl_3$): δ 0.88 (CH_3), 1.26 (CH_2), 1.64 (β-CH_2), 2.56 (α-CH_2), 3.15 (CH_2–S), 3.36 (OH), 3.72 (CH_2–O), 3.94 (O–CH_2CH_2–O). ^{13}C NMR: δ d at 62.3, 64.4, 67.4 (^{13}C–^{31}P coupling in CH_2–O–P).

Determination of the Critical Micellar Concentration (CMC) for HEPC, HEPG, DiC_{12}-P, and DiC_{12}-PC Using Rhodamine-6G. This was determined as described (Bonsen et al., 1972). A 1-mL sample containing 0.5 mL of Tris–HCl buffer (100 mL; pH 7.0) with 0.2 M NaCl, 0.1 mL of Rhodamine-6G (20 mg/L), and 0.4 mL of distilled water plus phospholipid (0.001–2.0 mM) was mixed in a cuvette and the absorbance measured at 542 nm against the reagent blank. The change in absorbance was then plotted vs the phospholipid concentration, and the concentration at which the maximal change in absorption was observed was taken as the CMC of that particular phospholipid. The CMC value of HEPC and HEPG was in the range of 0.3–0.4 mM while that of DiC_{12}-PC and DiC_{12}-P gave a value of 0.2–0.25 mM.

Activity Measurement Using Egg Yolk Lecithin and Synthetic Phospholipids. This was measured titrimetrically using a Radiometer SBR titrigraph as described (Nieuwenhuizen et al., 1974). The egg yolk assay was carried out using a 4-mL reaction mixture containing 8 mg of freeze-dried egg yolk lecithin, $CaCl_2$ (10 mM, final concentration), deoxycholic

acid (3 mM, final concentration), and pla_2 (0.5–1.0 μg) at 37 °C and pH 8.5. The released fatty acid was titrated against 5 mM NaOH. From the linear slope, the amount of fatty acid released per minute was calculated, and the activity was expressed as μmoles of fatty acid released per minute mg of protein.

For measuring activity toward synthetic phospholipids, DiC_{12}-PC and DiC_{12}-P were used at 5 mM (micellar) concentration. A 3.6-mL reaction mixture containing 1.2 mL of NaCl (100 mM, final concentration), 0.6 mL of $CaCl_2$ (25 mM, final concentration), 0.6 mL of deoxycholic acid (6 mM, final concentration), 0.6 mL of sodium tetraborate (1 mM, final concentration), and 0.5 mL of substrate was adjusted to pH 8.5 and preincubated for 2–3 min at 37 °C before the addition of 0.1 mL of diluted pla_2 (0.25–0.5 μg). The released fatty acid was then titrated against 5 mM NaOH. From the linear titration slope, the activity was calculated and expressed as described above.

Kinetic Studies Using Monomeric and Micellar Phospholipids. For determining the kinetic constants at monomeric concentration, HEPC and HEPG were used as respective zwitterionic and negative head-group substrates. The assay was done as described (Bhat et al., 1993) using various concentrations of HEPC or HEPG over the range 0.01–0.25 mM. The plot of activity vs substrate concentration followed the Michaelis–Menten equation from which the K_m and k_{cat} values were calculated.

DiC_{12}-PC and DiC_{12}-P were used as model substrates for determining the kinetic constants at micellar concentration. The assay was followed by the titration method as described above using the substrate over the range 0.25–5.0 mM. The titration curves were linear at least up to 5 min in all the cases except with native porcine pancreatic pla_2 vs DiC_{12}-P. The activity obtained at 0.25 mM concentration was taken as the activity toward monomeric substrate, and this activity was subtracted from the activities obtained at different substrate concentrations. Similarly, the monomeric substrate concentration was deducted from all substrate concentrations before determining the kinetic constants using the Michaelis–Menten equation. Therefore, the K_m and k_{cat} values were designated as apparent values in the results.

RESULTS

Design- and Structure-Based Predictions. Model building and molecular dynamics were used to predict the binding of a single phospholipid molecule to the active site of porcine pancreatic pla_2 (Jones et al., 1993). Theoretical calculation of the overall binding was simplified by a clear distinction between hydrophilic and hydrophobic regions of the binding surface corresponding to the head group and the acyl chains of the phospholipid together with the biochemical evidence on catalysis (Verheij et al., 1980). The conformation of the phospholipid molecule bound to pla_2 is similar to that in the aggregated substrate as in the crystal. This was evident when attempts to modify the conformation to fit the shape of the active site were reversed by molecular simulation (Jones et al., 1993). The head group of the substrate binds to the hydrophilic region of the protein, and although no persistent interactions with the enzyme were observed in molecular simulation, the head group is likely to be influenced by the adjacent charged residues. Some of these changes (e.g., E46, R43, R53, or K56; Figure 1) are distal to the flat surface and are believed to interact with the aggregated substrates which could influence the binding and the activity of pla_2. Removing one of the negative charges (e.g., E46; Figure 1) might change

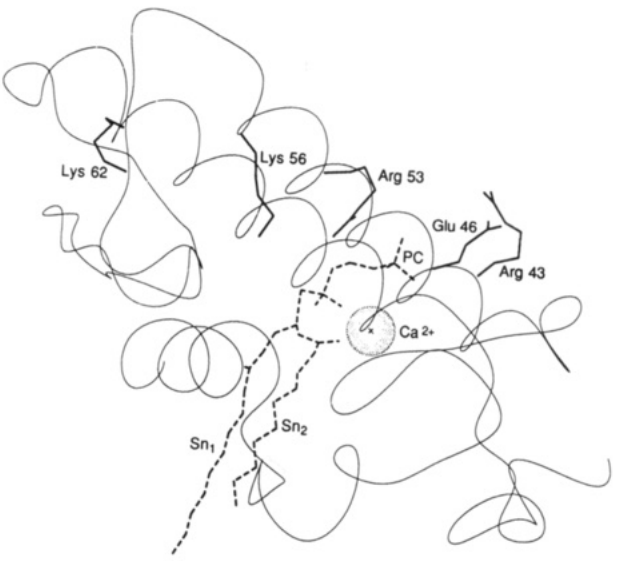

FIGURE 1: Skeleton diagram of the porcine pancreatic phospholipase A_2–phospholipid (DiC_{10}-PC) complex showing the active site Ca^{2+} and some of the charged amino acid residues which interact with the head group.

Table I: Activities of Native, Recombinant, and Mutant Phospholipases A_2 toward Natural and Synthetic Phospholipids[a]

	activity (μmol min^{-1} mg of protein^{-1})		
phospholipase A_2	egg yolk	DiC_{12}-PC	DiC_{12}-P
native	529 ± 12	462 ± 38	492 ± 20
recombinant	504 ± 21	385 ± 24	409 ± 27
mutant (E46L)	532 ± 27	220 ± 35	619 ± 33

[a] All values are the average and standard deviation of four determinations with two protein concentrations.

the head-group selectivity of the enzyme if our model of substrate binding is correct. For instance, the binding and the activity on phospholipids with negative head groups (e.g., phosphatidic acid) might increase relative to the activity on phospholpids with positive or zwitterionic head groups due to medium-range electrostatic effects. There might also be subtle effects on activity, particularly if there is a change in geometry on going from the ground state to the tetrahedral intermediate, which affects the binding of the head group to this part of the protein.

Expression and Purification of the Recombinant and E46L Mutant pla_2. The recombinant and mutant pla_2 were expressed and purified as described (Bhat et al., 1991; Goodenough et al., 1992). However, in recent purification, CM-sepharose column chromatography at pH 5 was introduced before chromatofocusing the sample over the pH region 7.4–4.0, in order to remove the major contaminating proteins as well as to improve the reproducibility of separation and pH gradient during chromatofocusing. Typical yields of 3–5 mg of the pure recombinant or mutant pla_2 were obtained from 100 g of *E. coli* cells. The purified enzyme, when stored frozen in 10 mM sodium acetate buffer, pH 5.0, retained its full activity for several months.

Activity toward Egg Yolk Lecithin and Synthetic Phospholipids. The mutant E46L showed the same level of activity as the native and recombinant pla_2 when egg yolk lecithin (mixed micelle) was used as a substrate (Table I). However, the specific activity of the native and recombinant pla_2 toward zwitterionic phospholipid was 2-fold higher than that of the E46L mutant (Table I). In contrast, the E46L mutant showed 30–50% higher specific activity toward DiC_{12}-P, with two negative charges in the head group, than the native and

Table II: Kinetic Constants of Native, Recombinant, and E46L Mutant Phospholipases A_2 toward Monomeric HEPC and HEPG[a]

	HEPC			HEPG		
PLA_2	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
native	0.056	15.1	270	0.042	18.2	433
recombinant	0.051	14.4	282	0.053	22.4	423
mutant (E46L)	0.041	8.4	205	0.049	18.9	386

[a] All values are from two separate determinations done in triplicates.

Table III: Kinetic Constants of Native and E46L Mutant Phospholipases A_2 toward Micellar DiC_{12}-PC and CiC_{12}-P[a]

	DiC_{12}-PC			DiC_{12}-P		
pla_2	K_m^{app} (mM)	k_{cat}^{app} (min^{-1})	k_{cat}^{app}/K_m^{app} (min^{-1} mM^{-1})	K_m^{app} (mM)	k_{cat}^{app} (min^{-1})	k_{cat}^{app}/K_m^{app} (min^{-1} mM^{-1})
native	4.0 ± 0.8	8580 ± 960	2145 ± 480	10.9 ± 2.3	10 200 ± 1500	935 ± 130
E46L	3.9 ± 1.2	4440 ± 900	1138 ± 320	2.6 ± 0.7	10 980 ± 900	4223 ± 1359

[a] All values are the average from two experiments done as described in the methods section. The covariance of k_{cat}/K_m has been calculated using the functional variance method (Davies & Goldsmith, 1977).

recombinant pla_2 (Table I). Table I also shows that the activity of E46L toward DiC_{12}-P was approximately three times higher compared to its activity against DiC_{12}-PC. These results clearly show that the E46L mutant preferentially hydrolyzes phospholipids which are negatively charged.

Kinetics of Native, Recombinant, and E46L Mutant pla_2s. Table II shows the kinetic constants for native, recombinant, and E46L mutant pla_2s determined using monomeric glycol thiophospholipids with zwitterionic (HEPC) and negative (HEPG) head groups as substrates. The K_m values of all three pla_2 molecules, for monomeric HEPC and HEPG, were comparable, while the k_{cat} value of the E46L mutant toward HEPC was noticeably lower (about 55%) than that of the native and recombinant pla_2. In contrast, with HEPG, all three pla_2s showed comparable k_{cat} values although the comparison of the k_{cat}/K_m ratios toward the above substrates indicated that the mutant pla_2 hydrolyzes the negatively charged glycol thiophospholipid, HEPG, more efficiently than the corresponding zwitterionic thiophospholipid.

Further kinetic analysis of mutant and native pla_2s using micellar phospholipids with zwitterionic (DiC_{12}-PC) and negatively charged (DiC_{12}-P) head groups provided some interesting results (Table III). Both the E46L mutant and the native pla_2 showed comparable K_m^{app} values for the micellar DiC_{12}-PC, but the k_{cat}^{app} value of the E46L mutant was only half that of the native pla_2. In contrast, the K_m^{app} of E46L for the micellar DiC_{12}-P was at least four times lower than that of native pla_2. However, comparison of the k_{cat}^{app} values shows that both the native and the E46L mutant hydrolyzed the double negatively charged phospholipid, DiC_{12}-P, preferentially to the zwitterionic DiC_{12}-PC. Furthermore, an approximately 3-fold increase in the k_{cat}^{app} value of the E46L mutant toward DiC_{12}-P as compared to that against DiC_{12}-PC (Table III) again shows that the mutation has shifted the substrate preference of pla_2 from zwitterionic to negatively charged phospholipid. The calculation of the k_{cat}^{app}/K_m^{app} ratio also revealed that the E46L mutant hydrolyzed DiC_{12}-P five times more efficiently than the native pla_2, although the efficiency of the E46L mutant pla_2 is only half as much as that of the native toward DiC_{12}-PC. On the basis of these results, it can be concluded that the removal of a negative charge in the head-group binding region of porcine pancreatic pla_2 significantly increases its affinity toward micellar phospholipids with two negative charges on the head group without altering its affinity toward phospholipids of zwitterionic type.

Another interesting observation was that the activity of the E46L mutant toward DiC_{12}-P increased linearly up to 5–6 min while the increase of native pla_2 activity against the same substrate was linear only up to 1–2 min, and thereafter, the release of fatty acid was inhibited (Figure 2A). In contrast, both native and E46L mutant pla_2 hydrolyzed DiC_{12}-PC continuously at least up to 4 min under identical conditions (Figure 2B). These results indicate that the mutation has changed the properties of porcine pla_2 and enabled it to overcome some type of product inhibition, although the nature and type of inhibition are not yet clearly understood. However, the data presented in Tables I and III do not reflect the significant difference observed in Figure 2A between E46L and native pla_2. This is because the calculated activity is based on the linear part of slope.

DISCUSSION

Modeling of the phospholipid–pla_2 complex has led to the identification of charged residues which could interact differentially with different phospholipid head groups. The model (Figure 1) presented in this paper is in good agreement with the major features of the pla_2–inhibitor complex studied by Thunnissen et al. (1990). The molecular simulation provided little evidence for short-range interactions between the charged residues and the phospholipid head group. However, Figure 1 reveals that the head group of the phospholipid lies between two positively charged amino acids, Arg 53 and Arg 43, which are expected to interact more favorably with the negatively charged head group than with either the positively charged or zwitterionic head groups. However, the presence of negatively charged Glu 46, adjacent to Arg 43, is likely to influence the interaction between the positively charged Arg 43 and the head group of the phospholipid. Thus, the removal of a negative charge at position 46 was predicted to enhance the positive interaction between pla_2 and the negatively charged phospholipid. However, this mutation was not expected to reduce the interaction of the enzyme with the zwitterionic phospholipid.

Two positively charged amino acids, Lys 56 and Lys 62, were also reported to interact with the head group of phospholipids (Kuipers et al., 1989; Noel et al., 1992). In a protein-engineering study with bovine pla_2, Noel et al. (1992) have shown that the change of Lys 56 to Glu reduces the activity toward negatively charged DiC_{12}-PG. Similarly, Kuipers et al. (1989) have demonstrated that the removal of Lys 62 decreases the activity of porcine pancreatic pla_2 toward negatively charged phospholipid by four to five times, although the deletion of a loop, 62–66, increased the activity toward micellar DiC_6-PC by 16 times. On the basis of these results

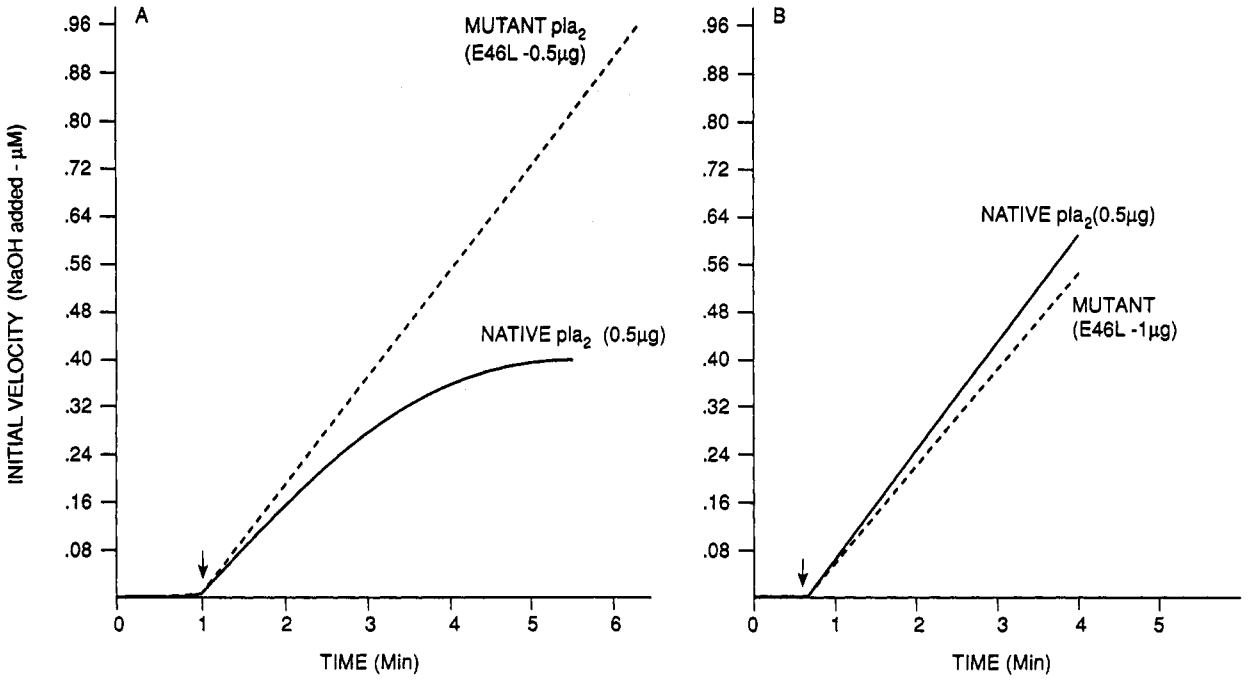

FIGURE 2: Activity curves of native and mutant (E46L) pla_2 with DiC_{12}-P (A) and DiC_{12}-PC (B). The substrate concentration was 2 mM, and all other assay conditions were as described in the methods section.

together with our modeling studies, it was predicted that the negatively charged Glu 46 would interact unfavorably with the negatively charged phospholipid, while neutralizing this charge would enhance the interaction between the negatively charged head group and Arg 53 and Arg 43. Furthermore, the high positive potential of the active site Ca^{2+} together with lysines 56 and 62 is also expected to interact more favorably with the negatively charged phospholipids than with the zwitterionic and positively charged phospholipids. Thus, the E46L mutant was predicted to show increased activity and affinity toward negatively charged phospholipids.

The data presented in Table I revealed that the native, recombinant, and E46L mutant pla_2s have comparable activities toward egg yolk lecithin but all three pla_2s hydrolyzed the double negatively charged DiC_{12}-P more efficiently than the corresponding zwitterionic phospholipid. This observation is consistent with the previous reports, where it has been shown that pancreatic pla_2, unlike snake venom enzymes, possesses a strong preference for negatively charged phospholipids (Van Den et al., 1962; de Haas & van Deenen, 1963; Hendrickson et al., 1981). Furthermore, it has been reported that at alkaline pH and above the CMC, the anionic sulfates are better substrates than the corresponding phosphorylcholine detergents (van Oort et al., 1985). Although the present results are consistent with the above observations, the removal of a negative charge at position 46 increased the activity of porcine pla_2 toward negatively charged DiC_{12}-P by about 50–80% and decreased the activity toward zwitterionic phospholipid by 2-fold compared to that of native pla_2. As this mutation was expected to increase the interaction between pla_2 and the negatively charged head group, the former results were as predicted. However, the latter results were rather unexpected, as the negative effects of this mutation on the interaction between pla_2 and zwitterionic phospholipid was predicted to be minimal.

The kinetic data (Tables II and III) showed no appreciable change in the K_m and K_m^{app} values of native and E46L mutant pla_2 toward monomeric and micellar phospholipids of zwitterionic type, but the k_{cat} and k_{cat}^{app} values of the E46L mutant against the same substrates were about 2-fold lower than those of the native and recombinant pla_2. This revealed that this mutation has not changed the affinity toward monomeric and micellar zwitterionic phospholipids but has decreased the catalytic efficiency of porcine pla_2. Wells (1971) has convincingly demonstrated that the rate-limiting step in the venom-pla_2-catalyzed hydrolysis of lecithin is the decomposition of the tetrahedral intermediate with fatty acid release first followed by that of lysolecithin. Because porcine pla_2 is believed to follow the same mechanism as snake venom pla_2 (Verheij et al., 1980) and the E46L mutant and the native pla_2 showed similar K_m values toward the above substrates, the decreased activity of the E46L mutant toward the zwitterionic phospholipid could be due to the slow decomposition of the tetrahedral intermediate.

Kinetic results presented in Table II also showed that there was no significant difference between native, recombinant, and mutant pla_2s with respect to the K_m and k_{cat} values, when single negatively charged monomeric phospholipid, HEPG, was used as a substrate. However, k_{cat} and the k_{cat}/K_m ratios of E46L toward the above substrate were 2-fold higher than those against the monomeric HEPC, which is consistent with our prediction. More interestingly, with double negatively charged micelles of DiC_{12}-P, the K_m^{app} value for the E46L mutant was four times lower and the k_{cat}^{app} value was marginally higher than those of the native pla_2. These results indeed showed that the removal of a negative charge near the hydrophilic region had significantly increased the affinity of pla_2 toward micellar DiC_{12}-P but increased the catalytic efficiency by only 10–15%.

Why did the E46L mutant, which showed similar K_m and k_{cat} values as the native pla_2 with single negatively charged monomeric glycol phospholipid, exhibit a 4-fold decrease in K_m^{app} and a marginal increase in k_{cat}^{app} when double negatively charged phospholipid, DiC_{12}-P, was used as a substrate? Answering this question is hampered by the lack of structural knowledge of the E46L mutant pla_2–micellar phospholipid complex. However, four possible reasons for the observed discrepancy are as follows: (a) the interaction between pla_2

and glycol-based phospholipids may be different than that with glycerol-based phospholipids, (b) the interaction of two negative charges with pla_2 is increased compared to that of a single negative charge, (c) the interaction of E46L mutant pla_2 with monomeric glycol phospholipid may be different than that with micellar glycerol phospholipid, and (d) the interaction of the smaller phosphate head group may be different than that of the glycerol head group.

Another interesting question to be addressed is why the activity of native pla_2 toward micellar DiC_{12}-P stops within such a short time (1–2 min) while that of E46L continues linearly at least up to 5–6 min (Figure 2A). It has been reported that the activity of native pla_2 toward negatively charged DiC_8-S decreases above the CMC, which is believed to be due to the inhibition of pla_2 by the released lyso compound (van Oort et al., 1985). As native pla_2 has four times lower affinity toward DiC_{12}-P, it is likely that the released lysophosphatidic acid may compete to the active site and cause inhibition. However, the present results and the mutation we introduced may be useful for commercial purposes in preparing lysophosphatidic acid from negatively charged phospholipids.

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