High specificity of human secretory class II phospholipase A_2 for phosphatidic acid

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Lysophosphatidic acid (LPA) is a potent lipid second messenger which stimulates platelet aggregation, cell proliferation and smooth-muscle contraction. The phospholipase A_2 (PLA_2)-catalysed hydrolysis of phosphatidic acid (PA) is thought to be a primary synthetic route for LPA. Of the multiple forms of PLA_2 present in human tissues, human secretory class-II PLA_2 (hs-PLA_2) has been implicated in the production of LPA from platelets and whole blood cells challenged with inflammatory stimuli. To explore further the possibility that hs-PLA_2 is involved in the production of LPA, we rigorously measured the phospholipid head group specificity of hs-PLA_2 by a novel PLA_2 kinetic system using polymerized mixed liposomes. Kinetic analysis of recombinant hs-PLA_2 demonstrates that hs-PLA_2 strongly prefers PA as substrate over other phospholipids found in the mammalian plasma membrane including phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The order of preference is PA $\gg$ PE $\approx$ PS $>$ PC. To identify amino acid residues of hs-PLA_2 that are involved in its unique substrate specificity, we mutated two residues, Glu-56 and Lys-69, which were shown to interact with the phospholipid head group in the X-ray-crystallographic structure of the hs-PLA_2–transition-state-analogue complex. The K69Y mutant showed selective inactivation toward PA whereas the E56K mutant displayed a most pronounced inactivation to PE. Thus it appears that Lys-69 is at least partially involved in the PA specificity of hs-PLA_2 and Glu-56 in the distinction between PE and PC. In conjunction with a recent cell study [Fourcade, Simon, Viodé, Rugani, Leballe, Ragab, Fournié, Sarda and Chap (1995) Cell **80**, 919–927], these studies suggest that hs-PLA_2 can rapidly hydrolyse PA molecules exposed to the outer layer of cell-derived microvesicles and thereby produce LPA.

INTRODUCTION

Phospholipase A_2 (PLA_2; EC 3.1.1.4) catalyses the hydrolysis of the fatty acid ester in the 2-position of 3-*sn*-phospholipids (for recent reviews, see refs. [1–4]). PLA_2 is thought to play key roles in inflammation and cell signalling because the products of PLA_2 reaction, i.e. fatty acid and lysophospholipid, function either as precursors of pro-inflammatory metabolites or as lipid second messengers. Multiple forms of PLA_2 have been identified from human tissues, of which 85 kDa cytosolic PLA_2 and 14 kDa secretory class-II PLA_2 have been most extensively characterized as a putative pro-inflammatory PLA_2. Human secretory class-II PLA_2 (hs-PLA_2) is synthesized and secreted by a variety of cells including platelets [5], neutrophils [6] and mast cells [7]. Although its presence in inflammatory fluids, tissue exudates and the serum has implicated it in inflammation [8], its physiological substrates and functions are not fully understood. Recently, it has been suggested that it might be involved in the production of lysophosphatidic acid (LPA) from platelets and whole blood cells challenged with inflammatory stimuli [9]. LPA is a potent lipid second messenger which stimulates platelet aggregation, cell proliferation and smooth-muscle contraction [10]. The PLA_2-catalysed hydrolysis of newly generated phosphatidic acid (PA) is thought to be a primary synthetic route for LPA, at least for platelet-derived LPA [11]. Although PA-specific PLA_2 activities have been identified [12,13], the nature of PA-specific PLA_2 is not fully elucidated. To explore further the possibility that hs-PLA_2 is responsible for the production of LPA from PA, we have thoroughly examined the phospholipid head group specificity of hs-PLA_2 by a novel PLA_2 kinetic system using polymerized mixed liposomes [14,15]. Herein, we present kinetic data demonstrating that hs-PLA_2 strongly prefers PA as substrate over other phospholipids found in the mammalian plasma membrane including phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Furthermore we report site-specific mutagenesis studies of hs-PLA_2 leading to identification of protein residues that are involved in its unique substrate specificity.

EXPERIMENTAL

Materials

1-Hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (pyrene-PC), -ethanolamine (pyrene-PE) and -glycerol (pyrene-PG) were purchased from Molecular Probes (Eugene, OR, U.S.A.). 1-Hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphatidic acid (pyrene-PA) and -phosphoserine (pyrene-PS) were synthesized by the phospholipase D-catalysed trans-phosphatidylation of pyrene-PC and purified as described [16]. Phospholipase D was purified from Savoy cabbage as described [17]. 1,2-Bis-[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphoglycerol (BLPG) was prepared as described elsewhere [14]. Large unilamellar liposomes of BLPG were prepared by multiple extrusion of phospholipid dispersion in 10 mM

Abbreviations used: BLPG, 1,2-bis-[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphoglycerol; hs-PLA_2, human secretory class-II PLA_2; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PLA_2, phospholipase A_2; PS, phosphatidylserine; pyrene-PA, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphate; pyrene-PC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; pyrene-PE, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoethanolamine; pyrene-PG, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol; pyrene-PS, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoserine.

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Tris/HCl buffer, pH 8.4, through 0.1 μm polycarbonate filter (Millipore) in a microextruder Liposofast (Avestin, Ottawa, Ontario, Canada) and then polymerized in the presence of 10 mM dithiothreitol as described [15]. Phospholipid concentrations were determined by phosphate analysis [18]. Fatty acid-free BSA was from Miles Inc. All the restriction enzymes, T4 ligase and T4 polynucleotide kinase were obtained from Boehringer-Mannheim Biochemicals. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, U.S.A.).

Construction of mutant PLA_2 genes

The synthetic hs-PLA_2 gene was a gift from Dr. David Wilton of the University of Southampton (Southampton, Hants., U.K.). The coding region for hs-PLA_2 was subcloned into the pET-21-a vector (Novagen) and designated pYS. This synthetic gene carries the Asn to Ala mutation (N1A) at the N-terminus to facilitate the removal of the initiator methionine by the endogenous methionine aminopeptidase [19]. The mutagenesis was performed using a Sculptor *in vitro* mutagenesis kit from Amersham by the method of Nakamaye and Eckstein [20] with modifications. In this method, a phagemid DNA prepared from pYS vector in the presence of helper phage R408 (Promega) was used as a template for mutagenesis. The oligonucleotides used for the construction of mutants were 5′ CA ACC ACG TTT CTT GAG ACG TTT GTA 3′ (E56K), 5′ TTT GTA AGA CAG GAA GTA GGT ACC GCA ACC ACG 3′ (K63Y), 5′ TTT GTA AGA CAG GAA ACG GGT ACC GCA ACC ACG 3′ (K63R) in which underlined bases indicate the location of the mutation. After DNA sequences of entire coding regions of mutants had been verified by the sequencing analysis using a Sequenase 2.0 kit (Amersham), individual recombinant pYS vectors were transformed into *Escherichia coli* strain BL21(DE3) (Novagen) for protein expression.

Bacterial protein expression and purification

N1A-hs-PLA_2 and mutant proteins were expressed in BL21(DE3) harbouring corresponding pYS vectors. A 2-litre culture of Luria broth containing 100 μg/ml ampicillin was grown at 37 °C, and protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside when the absorbance of the medium at 600 nm reached 0.6–0.8. After cells had been incubated for an additional 4 h at 37 °C, they were harvested and frozen at −20 °C. The cells were resuspended in 50 ml of buffer A (50 mM Tris/HCl, pH 8.0, containing 1 mM EDTA, 50 mM NaCl, 0.5 mM PMSF) containing 0.4 % (v/v) Triton X-100 and 0.4 % (w/v) sodium deoxycholate and stirred at 4 °C for 20 min. The sonication was performed on ice using a Sonifier 450 (Branson) by a pulse mode for 10×15 s. The inclusion-body pellet was obtained by centrifugation for 20 min at 12000 ***g*** (4 °C). The pellet was resuspended in 0.8 % (v/v) Triton X-100/0.8 % (w/v) sodium deoxycholate, sonicated as above and centrifuged. The pellet was resuspended and washed by stirring in 50 ml of buffer A containing 1 % (v/v) Triton X-100 for 20 min at room temperature. The pellet was collected by centrifugation as above and washed in 50 ml of buffer A, and centrifuged. Inclusion bodies were solubilized in 50 ml of buffer A, containing 6 M guanidinium chloride and 5 % β-mercaptoethanol, and stirred overnight at 4 °C. Solubilized protein was obtained by centrifugation at 70000 ***g*** for 40 min at 4 °C. The denatured protein was refolded by dialysing twice against 4 litres of 25 mM Tris/HCl buffer, pH 7.5, containing 5 mM $CaCl_2$, 5 mM L-cysteine and 0.9 M guanidinium chloride for 48 h at 4 °C. Any precipitate was removed by filtration through a 0.45 μm filter. Guanidinium chloride was removed by dialysis against 2×4 litres of 25 mM Tris/HCl, pH 8.0. To remove any precipitates the solution was centrifuged for 20 min at 9000 ***g*** and filtered through a small (2.5 cm × 5 cm) Sephadex G-25 column equilibrated with 25 mM Hepes buffer, pH 8.0. The clear solution was loaded on to a Pharmacia CM-Sepharose column (2.5 cm × 20 cm) equilibrated with 25 mM Hepes buffer, pH 8.0. The column was washed extensively with the same buffer until no further peak emerged and then the folded protein was eluted with a gradient of NaCl from 0 to 2 M in the same buffer. N1A-hs-PLA_2 was eluted at about 1 M NaCl. The protein peak was collected, dialysed against water, lyophilized and stored at −20 °C. Protein purity was confirmed by SDS/PAGE. Protein concentrations were determined by the micro bicinchoninic acid (Pierce) method [21]. The CD spectra of proteins were recorded in 10 mM phosphate buffer, pH 7.4, at 25 °C using a JASCO J-600 spectropolarimeter. Each spectrum was obtained at wavelengths between 195 and 300 nm and averaged from ten separate scans.

Expression of hs-PLA_2 in baculovirus-infected Sf9 cells

Wild-type hs-PLA_2 was produced from baculovirus-infected Sf9 cells. The cDNA of hs-PLA_2 kindly provided by Dr. L. Lin of the Genetics Institute was subcloned into the pVL1392 baculovirus transfer vector (Pharmingen) and designated pRD. Transfection of Sf9 cells (Invitrogen) was performed using the BaculoGold™ Transfection Kit from Pharmingen. This procedure makes use of baculovirus DNA containing a lethal deletion. Co-transfection of the viral DNA with a complementing plasmid pRD reconstituted viable virus inside insect cells. Cells were incubated for 4 days at 27 °C and the supernatant was collected and used to infect more cells for the amplification of virus. After three cycles of amplification, high-titre virus stock solution was used for protein expression. Sf9 cells were maintained as monolayer cultures in serum-free TNM-FH medium (Invitrogen). For protein expression, 1×10^7 Sf9 cells were seeded on to 10 cm culture plates (total volume of medium 200 ml) and infected with a multiplicity of infection of 10. The cells were then incubated for 3 days at 27 °C. Because the hs-PLA_2 gene contained the signal sequence, the expressed protein was secreted into the medium. After the supernatant had been collected and any dislodged cells removed by centrifugation at 2000 ***g***, it was dialysed against deionized water (3×4 litres) and then against 10 mM borate buffer, pH 9 (4 litres) at room temperature. To enrich highly basic hs-PLA_2 (isoelectric point > 10), the solution was passed through a CM-Sepharose column (5 cm × 10 cm) equilibrated with 10 mM borate buffer, pH 9. After a wash of the column with 1 column volume of the same buffer, the bound protein was eluted with 1 M NaCl in the same buffer. The eluted solution was dialysed against 25 mM Hepes buffer, pH 8.0, and loaded on to a Mono S column (Pharmacia), equilibrated with 25 mM Hepes buffer, pH 8.0. The protein was eluted with a gradient of NaCl from 0 to 2 M in the same buffer.

Kinetic measurements

PLA_2-catalysed hydrolysis of polymerized mixed liposomes was carried out at 37 °C in 2 ml of 10 mM Hepes buffer, pH 7.4, containing 0.1 μM pyrene-containing phospholipids (1 mol %) inserted in 9.9 μM BLPG, 2 μM BSA, 0.16 M NaCl and 10 mM $CaCl_2$. Enzyme concentrations were adjusted to keep the half-life of the reaction below 5 min. The progress of the hydrolysis was monitored as an increase in fluorescence emission at 378 nm using a Hitachi F4500 fluorescence spectrometer with the excitation wavelength set at 345 nm. The spectral bandwidth was

set at 5 nm for both excitation and emission. hs-PLA_2 and all the mutants exhibited the same kinetic pattern toward the various polymerized mixed liposomes used here, therefore kinetic analysis was performed by the method described previously [14,15,22]. The apparent second-order constant, $(k_{cat}/K_m)_{app}$, was calculated by dividing by enzyme concentration the pseudo-first-order rate constant which was calculated from the non-linear least-squares analysis of the reaction progress curves. Although $(k_{cat}/K_m)_{app}$ has no obvious physical meaning, it is a useful parameter for comparing overall interfacial activity of wild-type and mutants [22,23].

RESULTS

PLA_2 catalysis involves two kinetically distinct steps [24,25]: adsorption of PLA_2 to the interface and subsequent substrate binding to the active site. Thus kinetic measurements using aggregates of different phospholipids as substrate often yield inaccurate information about the substrate specificity of PLA_2 because its activity is sensitive not only to the chemical nature of the phospholipids but also to the physical state of the aggregate they form. To solve this problem, we have recently developed a well-defined PLA_2 kinetic system using polymerized mixed liposomes [14]. In this system, it is possible to distinguish unambiguously between phospholipids interacting with the active site of PLA_2 (i.e. hydrolysable insert) and ones interacting with the interfacial binding site of PLA_2 (i.e. polymerized matrix). Thus one can accurately determine the phospholipid head group specificity of PLA_2 by varying the chemical structure of the hydrolysable phospholipids in an inert polymerized matrix. Using this approach, we rigorously determined the head group specificity of hs-PLA_2. Two zwitterionic phospholipids, pyrene-PC and pyrene-PE, and three anionic phospholipids, pyrene-PG, pyrene-PS and pyrene-PA, were used as inserts in BLPG polymerized matrix. The anionic BLPG was used as a polymerized matrix because hs-PLA_2 strongly prefers anionic surfaces to electrically neutral ones [15]. The apparent second-order constants, $(k_{cat}/K_m)_{app}$, determined for various polymerized mixed liposomes are summarized in Table 1. To establish that N1A-hs-PLA_2 expressed in *E. coli* is indistinguishable from native hs-PLA_2, we compared its activity toward various polymerized mixed liposomes with that of hs-PLA_2 expressed in baculovirus-infected insect cells. As shown in Table 1, the two enzymes showed essentially the same activity toward all the polymerized mixed liposomes. The bacterially expressed N1A-hs-PLA_2 was therefore used for further kinetic and structure–function studies. Two unique features were noted from the activity of N1A-hs-PLA_2 toward various polymerized mixed liposomes. First, it strongly preferred as substrate two anionic phospholipids, pyrene-PA and pyrene-PG, to zwitterionic ones. Of the two, PA was slightly more favoured, being hydrolysed 33% faster than PG. Interestingly, however, N1A-hs-PLA_2 hydrolysed pyrene-PS 20-fold more slowly than pyrene-PA, indicating that the serine head group of PS does not interact with its substrate-binding site as favourably as the phosphate anion of PA or the glycerol head group of PG. Second, N1A-hs-PLA_2 distinguished between the two zwitterionic phospholipids to a larger degree; PE was hydrolysed about ten times faster than PC. Taken together, hs-PLA_2 preferentially hydrolyses the phospholipids in the order PA > PG $\gg$ PE $\approx$ PS > PC and its preference for PA over PE (PA/PE in Table 1) is about 17.

To identify the amino acid residues that are responsible for the observed substrate specificity of hs-PLA_2, we mutated two residues, Glu-56 and Lys-69, that were shown to interact with substrate in the X-ray-crystallographic structure of the hs-PLA_2–transition-state-analogue complex (Figure 1) [26]. Glu-56 makes a direct stabilizing contact with the ammonium ion of the

Table 1 Kinetic parameters of hs-PLA_2 and mutants

See the Experimental section for experimental conditions and methods to calculate rate constants. Values of $(k_{cat}/K_m)_{app}$ are means ± S.D. determined from a minimum of three measurements. Enzyme concentrations were adjusted from 0.1 nM to 0.1 μM to keep the half-life of the reaction below 5 min.

	$10^6 \times (k_{cat}/K_m)_{app}$					
hs-PLA_2	Pyrene-PC	Pyrene-PE	Pyrene-PG	Pyrene-PA	Pyrene-PS	PA/PE
Wild-type	0.20 ± 0.03	2.0 ± 0.3	28 ± 4	36 ± 5	1.9 ± 0.4	18
N1A	0.22 ± 0.04	2.4 ± 0.3	30 ± 5	40 ± 7	2.0 ± 0.3	17
N1A/E56K	0.09 ± 0.01	0.53 ± 0.06	25 ± 3	35 ± 4	4.3 ± 1.0	66
N1A/K69Y	0.26 ± 0.04	2.3 ± 0.4	20 ± 4	21 ± 5	1.7 ± 0.3	9
N1A/K69R	0.047 ± 0.007	0.45 ± 0.12	6.0 ± 1.0	7.6 ± 1.1	0.42 ± 0.10	17

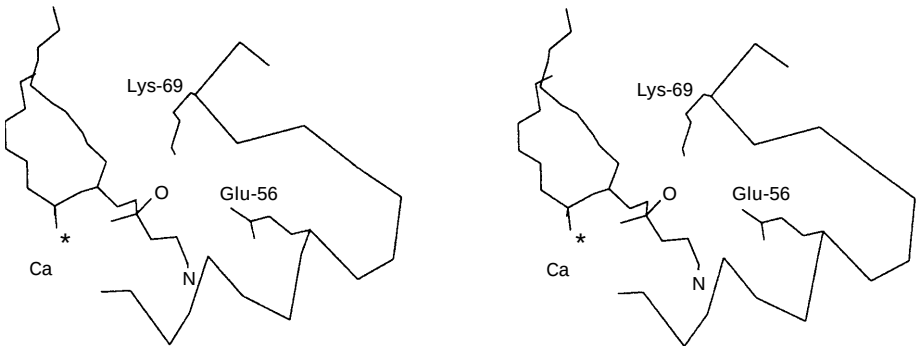

Figure 1 A stereodiagram describing the interaction of a transition-state analogue (L-1-*O*-octyl-2-heptylphosphonyl-*sn*-glycero-3-phosphoethanolamine) with the active site of hs-PLA_2 [23]

The ammonium ion of the PE head group and the *pro-S* non-bridging oxygen of *sn*-3 phosphate, which interact with Glu-56 and Lys-69 respectively, are indicated as N and O. The asterisk indicates the active-site-bound Ca^{2+}. Class-II PLA_2s, including hs-PLA_2, substitute a lysine residue for the tyrosine at position 69. The K69Y mutant has essentially the same activity as the wild-type toward PC, PE and PS substrates but shows 50 and 30% lower activity toward PA and PG respectively. One explanation for this finding is that the ϵ-ammonium group of Lys-69 forms additional ion-pair or hydrogen bonds with PA and PG head groups, which would not be achievable by the phenolic oxygen of Tyr-69 or with PC and PE as substrate.

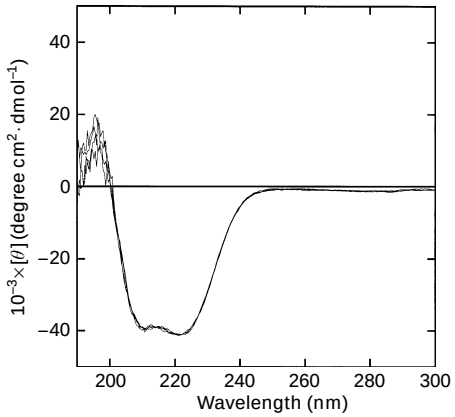

Figure 2 CD spectra of N1A-hs-PLA$_2$ and mutants including E56K, K69Y and K69R

The spectra are essentially indistinguishable. Enzyme concentrations were 20 μM in 0.01 M phosphate buffer, pH 7.4.

PE head group whereas Lys-69 forms a hydrogen bond with the pro-S non-bridging oxygen of the *sn*-3 phosphate. Based on this structural information, we reasoned that PC is less favoured as a substrate than PE because its bulky substituents interfere with the electrostatic interaction between Glu-56 and the choline head group. We also presumed that Lys-69, with its ability to form an extra hydrogen bond or an ion-pair with PA and PG, might be involved in the anionic phospholipid specificity. Because Glu-56 is a non-conserved surface-exposed residue, we introduced a charge reversal at this position (E56K) to maximize the effect of the mutation. On the other hand, Lys-69 is highly conserved among class-II secretory PLA$_2$s and is replaced by Tyr in all class-I secretory PLA$_2$s. Taking this into account, we made two conservative mutations, K69Y and K69R. All mutant proteins were expressed and refolded as efficiently as N1A-hs-PLA$_2$, indicating similar thermodynamic stability (results not shown). Also, the CD spectra of these mutants were indistinguishable, indicating that the mutations did not induce any gross structural change in protein conformation (Figure 2). When Glu-56 was mutated to Lys, N1A-hs-PLA$_2$ showed modest 17 and 13 % falls in activity toward pyrene-PG and -PA respectively and a twofold decrease toward pyrene-PC. The E56K mutation, however, caused a pronounced fivefold decrease in activity toward pyrene-PE and, surprisingly, a twofold increase toward pyrene-PS. As a result, the PE/PC ratio was reduced to 5 for N1A/E56K-hs-PLA$_2$ whereas the PA/PE ratio increased to 66. Thus it is evident that the E56K mutation removes the stabilizing electrostatic interaction of Glu-56 with the ethanolamine head group and with the choline head group although to a lesser degree. It also seems that PS is a poorer substrate than PA or PG at least partly because of the destabilizing interaction between the Glu-56 side chain and the α-carboxylate of the serine head group, which is relieved by the E56K mutation. The K69Y mutation had little effect on the activity toward pyrene-PC, -PE and -PS, indicating that Tyr can effectively replace Lys in binding the substrate. On the other hand, the same mutation caused 50 and 30 % decreases in activity toward pyrene-PA and -PG respectively. Thus Lys-69 appears to be involved at least partially in the stabilizing interaction with PA and PG. As a result, the PA/PE ratio was reduced to 9 for N1A/K69Y-hs-PLA$_2$. Unlike the K69Y mutation, the K69R mutation uniformly (about fivefold) decreased the activity of N1A-hs-PLA$_2$ toward all substrates. This indicates that the arginine side chain is much less effective than the lysine side chain in forming a hydrogen bond with the pro-S non-bridging oxygen of the *sn*-3 phosphate presumably because of less favourable geometry. The arginine side chain can, however, still play the same role of preferentially binding the PA and PG head groups.

DISCUSSION

This report describes a rigorous determination of the phospholipid head group specificity of hs-PLA$_2$. Although it was previously reported that hs-PLA$_2$ prefers anionic phospholipids to zwitterionic ones [27,28], the phospholipid head group specificity of hs-PLA$_2$ and its molecular origin have never been systematically investigated. In this study, bacterially expressed N1A-hs-PLA$_2$ was used, which proved to be indistinguishable from hs-PLA$_2$ expressed in insect cells but allowed easier mutagenesis and higher efficiency of protein expression. It is well established that the transmembrane phospholipid distribution is asymmetrical in biological membranes [29]. For instance, PE and PS are the major components of the inner monolayer of human red blood cell membrane whereas PC and sphingomyelin are found enriched in the outer monolayer [30]. PA is thought to be synthesized only as a minor component on the inner plasma membrane but accumulates on cell activation [31,32]. Our results demonstrate that hs-PLA$_2$ strongly prefers PA to other major phospholipids found in mammalian plasma membranes. The lowest activity was observed with PC. In conjunction with the inability of hs-PLA$_2$ to bind tightly to electrically neutral membrane surfaces, this accounts for its failure to act on intact cells exposing PC on their external membrane surface. Under normal physiological conditions, extracellular hs-PLA$_2$ would not interact with internal anionic phospholipids because of the transmembrane phospholipid asymmetry. It has been shown, however, that inflammatory stimuli promote shedding of microvesicles from blood cells including platelets which display a progressive loss of transmembrane asymmetry with surface exposure of PE, PS and PA [33,34]. It was shown that hs-PLA$_2$ hydrolysed PA in the microvesicles and produced LPA which in turn induced platelet aggregation [9]. Our substrate-specificity data provide an explanation for how hs-PLA$_2$ preferentially hydrolyses PA over PE and PS. hs-PLA$_2$ hydrolyses PA 17–20 times faster than PE and PS respectively under conditions in which each phospholipid is distributed homogeneously on the anionic polymerized BLPG matrix. Under physiological conditions, hs-PLA$_2$ would probably exhibit more pronounced PA specificity because the accumulation of PA would probably form highly anionic microdomains on the vesicle surface for which hs-PLA$_2$ has high affinity. We have shown that hs-PLA$_2$ binds anionic interfaces more tightly than electrically neutral ones by three orders of magnitude [15]. Thus it is expected that the exposure of PA molecules to the outer layer of microvesicles will result in the rapid production of LPA by hs-PLA$_2$.

Kinetic analysis of hs-PLA$_2$ mutants provides a new insight into the mode of protein–substrate interaction in the active site of hs-PLA$_2$. Most secretory PLA$_2$s including mammalian pancreatic PLA$_2$s show comparable activities toward PC and PE. The pronounced distinction between reactivity towards PE and PC is therefore a unique kinetic property of hs-PLA$_2$, which could be due to either electrostatic or steric factors or a combination of the two. It is evident from the fivefold inactivation of hs-PLA$_2$ toward PE caused by the E56K mutation that the electrostatic interaction between the γ-carboxylate of Glu-56 and the ammonium ion of PE makes a significant contribution to the energetics of hs-PLA$_2$-PE binding. However, the twofold in-

activation toward PC caused by the same mutation indicates that Glu-56 also interacts favourably with the choline head group, although to a lesser degree. This, in conjunction with the finding that N1A/E56K-hs-PLA_2 still hydrolyses PE five times faster than PC, indicates that the distinction between reactivity toward PE and PC stems largely from the inability of hs-PLA_2 to accommodate the bulkier choline head group in the active site. On the other hand, the comparable activity of hs-PLA_2 toward PE and PS suggests that the γ-carboxylate of Glu-56 might be able to interact favourably with the α-ammonium ion of PS. The twofold increase in activity caused by the E56K mutation, however, shows that this interaction can be more effectively replaced by the interaction between the ϵ-ammonium ion of Lys-56 and the α-carboxylate of PS, thereby revealing the complex nature of the interaction between the PS head group and the active site of hs-PLA_2. Unlike PE, PC and PS, PA lacks a head group and instead has an extra oxyanion. Thus the fact that PA is the preferred substrate for hs-PLA_2 points to the existence of a strong stabilizing interaction between the phosphate anion and the protein residue(s). We reasoned that Lys-69 was the most likely candidate to be involved in this interaction based on its proximity to the phosphate anion. Although crystal structures have shown that both Tyr-69 of class-I PLA_2s [35] and Lys-69 of class-II PLA_2s [26] form a hydrogen bond with the *pro-S* non-bridging oxygen of the *sn*-3 phosphate, the ϵ-ammonium group of Lys-69 might be able to make an extra electrostatic contact with the phosphate anion of PA or form extra hydrogen bonds with the PG head group. Also, our recent mutagenesis study on another class-II PLA_2 from the venom of *Agkistrodon piscivorus piscivorus* showed that Lys-69 is involved in its preference for PG as substrate [36]. Indeed, the K69Y mutation causes the selective inactivation of hs-PLA_2 toward PA and PG. The degree of inactivation is, however, marginal and cannot fully account for the pronounced PA specificity of hs-PLA_2. Although PA is the smallest with regard to head group size, its specificity is probably not due to the steric factor because another favoured phospholipid, PG, has a head group that is as large as that of PE. Thus it appears that the PA specificity of hs-PLA_2 derives from the stabilizing interactions involving multiple amino acid side chains. Further structure–function studies are in progress to identify protein residues that are involved in these interactions.

This work was supported by a grant from the National Institutes of Health (CGM 52598), a Biomedical Science Grant from the Arthritis Foundation and a Grant-in-Aid (AHA 95006280) from the American Heart Association.

Received 12 August 1996; accepted 1 October 1996

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