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Evidence for Functional Involvement of Asparagine 67 in Substrate Recognition by Snake Venom Phospholipases A_2

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Site-directed mutagenesis studies of recombinant *Trimeresurus flavoviridis* venom gland phospholipase A_2 (PLA_2) showed that the Asn residue at position 67 takes part in recognition of the substrate 2-arachidonoyl *sn*-glycero-3-phosphocholine in both monomeric and micellar states. The amount of arachidonate released from phosphatidylcholine mixed micelles was reduced to 30% for N67D and N67K mutants, and to 70% for N67G mutant, but remained unchanged for N67S mutant. In contrast, for monomeric substrate, the activity was decreased to 40% for N67D and N67G and to 60% for N67K but remained unchanged for N67S. These results suggest that the properties of the side chain of residue 67 exert a significant influence on recognition of 2-arachidonoyl *sn*-glycero-3-phosphocholine.

Key words: arachidonoyl phosphatidylcholine, phospholipase A_2, site-directed mutagenesis, snake venom.

Phospholipase A_2 (PLA_2; phosphatidylcholine 2-acyl hydrolase, EC 3.1.1.4) catalyzes the hydrolysis of the 2-acyl ester bond of 3-*sn*-phosphoglycerides, producing free fatty acids and lysophospholipids. PLA_2s are classified into two major groups (I and II) based on their structural characteristics (*1*). Group I PLA_2s are found in elapidae snake venoms and mammalian pancreatic juice, whereas group II PLA_2s are found in viperidae snake venoms, mammalian platelets, and rheumatoid arthritic synovial fluid. Snake venom PLA_2 isozymes manifest diverse physiological activities such as myotoxicity, presynaptic and postsynaptic neurotoxicity, cardiotoxicity, and hemolytic, anticoagulant, and edema-inducing effects which were probably generated *via* accelerated evolution (*2-4*). *Trimeresurus flavoviridis* (*Tf*; Habu, viperinae) snake venom contains a variety of PLA_2 isozymes with different physiological activities. Among them, three major PLA_2 isozymes occur abundantly in *Tf* venom: Asp-49-PLA_2 (*5, 6*) and two Lys-49-PLA_2s called basic protein I (BP-I) and basic protein II (BP-II) (*7, 8*) (Fig. 1). BP-I and BP-II possess a shared amino acid sequence except for one amino acid at position 67 (numbered according to the aligned numbering of PLA_2s from various sources). BP-I possesses Asp-67 while BP-II contains Asn-67. It was found that this Asp/Asn substitution is due to independent mRNAs, not modification after translation or an artifact during the purification step (*11*). Recently, it became evident that BP-II is much more potent than BP-I in terms of contractile activity on guinea pig ileum (GPI) (*12*) and release of arachidonic acid from 2-arachidonoyl-1-stearoyl-L-phosphatidylcholine (ASPC) liposomes (*12, 13*). These phenomena imply that the amino acid at position 67 of Lys-49-PLA_2s is involved in the discriminatory recognition of 2-arachidonoyl-phospholipid membranes. However, Lys-49-PLA_2s, BP-I and BP-II, are structurally different from Asp-49-PLA_2 in the Ca^{2+} binding site, that is, Tyr-28, Trp-31, Gly-33, and Asp-49 in Asp-49-PLA_2 are replaced by Asn, Val, Arg, and Lys for BP-I and BP-II, respectively, and they have extremely low activity towards monomers and micelles of phospholipids, including ASPC, when compared with Asp-49-PLA_2. These results imply that Asp-49-PLA_2 is different from Lys-49-PLA_2 in substrate recognition of monomers and micelles of phospholipids. We previously demonstrated that *T. gramineus* PLA_2-II (Asp-49-PLA_2), which possesses Asn-67, has considerably stronger contractile activity than *Tg* PLA_2-I (Asp-49-PLA_2), which possesses Asp-67 (*14*). This result suggests that the residue at position 67 of Asp-49-PLA_2s is also crucial for GPI contraction and thus for recognition of 2-arachidonoyl phospholipids. However, the involvement of the residue at position 67 in substrate recognition of Asp-49-PLA_2s is not clear because these isozymes are different in amino acid sequence, with only 79% homology.

In the work reported here, we studied the functional involvement of residue 67 of Asp-49-PLA_2s in recognition of 2-arachidonoyl *sn*-glycero-3-phosphocholine in monomeric and micellar states. Examination of 50 amino acid sequences of group II PLA_2s so far determined showed that there are five different amino acids at position 67, Asn, Asp, Lys, Ser, and Gly (Table I). For our purpose, recombinant mutants of *Tf* Asp-49-PLA_2 in which Asn at position 67 was replaced by Asp, Lys, Ser, and Gly were prepared by site-directed mutagenesis and overexpressed in *Escherichia coli*.

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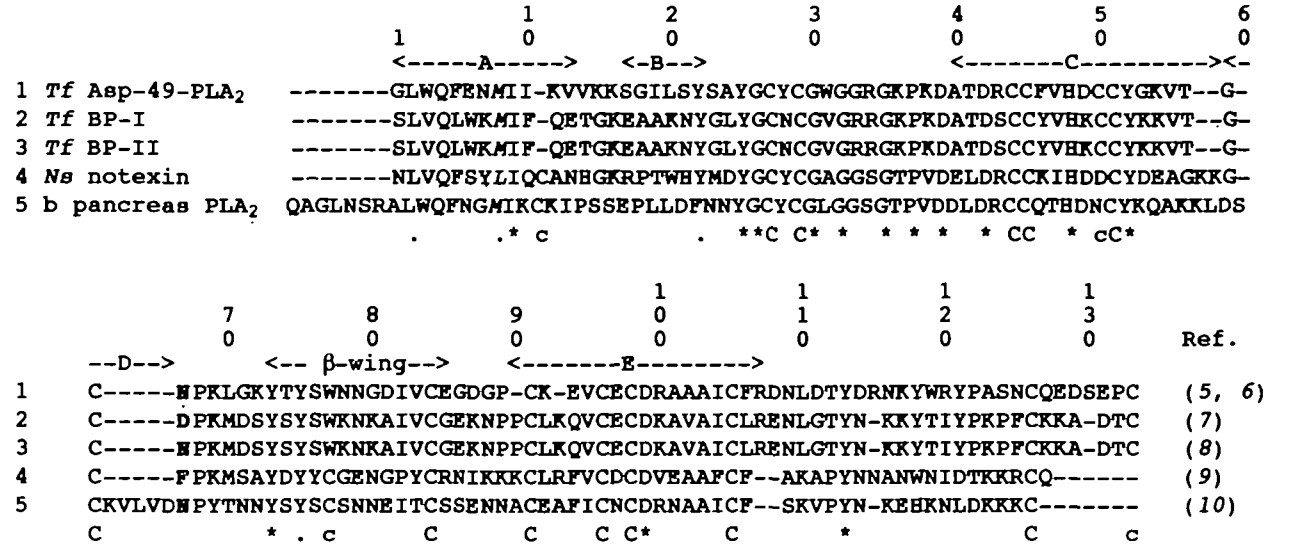
Abbreviations: PLA_2, phospholipase A_2; GPI, guinea pig ileum; ASPC, 2-arachidonoyl-1-stearoyl-L-phosphatidylcholine; PCR, polymerase chain reaction; DTT, dithiothreitol; PTH, phenylthiohydantoin.



1	C-----HPKLGKYTYSWNNGDIVCEGDGP-CK-EVCECDRAAAICFRDNLDTYDRNKYWRYPASNCQEDSEPC	(*5*, *6*)
2	C-----DPKMDSYSYSWKNKAIVCGEKNPPCLKQVCECDKAVAICLRENLGTYN-KKYTIYPKPFCKKA-DTC	(*7*)
3	C-----HPKMDSYSYSWKNKAIVCGEKNPPCLKQVCECDKAVAICLRENLGTYN-KKYTIYPKPFCKKA-DTC	(*8*)
4	C-----FPKMSAYDYYCGENGPYCRNIKKKCLRFVCDCDVEAAFCF--AKAPYNNANWNIDTKKRCQ------	(*9*)
5	CKVLVDHPYTNNYSYSCSNNEITCSSENNACEAFICNCDRNAAICF--SKVPYN-KEHKNLDKKKC-------	(*10*)
	C * . c C C C C* C * C c	

Fig. 1. **Amino acid sequences of PLA_2s from *T. flavoviridis* venom and bovine pancreas.** Five helices (A to E) and a β-wing are indicated on the top of the sequences. Amino acid residues at position 67 are indicated by bold face.

TABLE I. **Amino acid residues at position 67.**

Asn	
Trimeresurus	
T. flavoviridis	Asp-49-PLA_2, basic protein II, Thr-37-PLA_2
T. okinavensis	DE-I isozyme
T. gramineus	PLA_2 II, IV
Agkistrodon	
A. halys pallas	agkistrotoxin
A. piscivorus piscivorus	APP-K49
Crotalus	
C. durisus terrificus	crotoxin B1, B2
C. scutulatus scutulatus	Mojave toxin
C. atrox	PLA_2
C. adamanteus	PLA_2
Bothrops	
B. asper	p3
Vipera	
V. ammodytes ammodytes	vipoxin, inhibitor
Cerastes	
C. cerates	PLA_2
Eristocophis	
E. macmahoni	PLA_2
Asp	
Trimeresurus	
T. flavoviridis	PLX, PLX', basic protein I
T. gramineus	PLA_2 I, V, VI
T. macrosquamatus	Lys-49
Agkistrodon	
A. halys blomohoffi	PA2-II
Crotalus	
C. durisus terrificus	crotoxin A
Bitis	
B. nasicoris	isozyme CM-II
B. gabonica	PLA_2
Lys	
Agkistrodon	
A. halys blomohoffi	PA2-I
Bothrops	
B. asper	p2
Ser	
Vipera	
V. ammodytes ammodytes	ammodytoxin A, B, C, ammodytin L
Bilis	
B. caudalis	caudoxin
Gly	
Human synovial fluid, platelets	

MATERIALS AND METHODS

Materials—Restriction endonucleases and other modifying enzymes were obtained from Takara Shuzo (Kyoto) and *Taq* DNA polymerase was from Perkin Elmer Japan (Chiba). Oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems Model 370A). Heparin Sepharose CL6B was from Pharmacia (Sweden). Phospholipids such as 2-arachidonoyl-1-stearoyl-L-phosphatidylcholine (ASPC), L-α-phosphatidylcholine from egg yolk (Type XI-E) (EYPC), and L-α-phosphatidyle-DL-glycerol from egg yolk (EYPG) were obtained from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade. Purified native *Tf* Asp-49-PLA_2 was prepared as described previously (*5*).

Construction of Expression Plasmids for Recombinant Tf Asp-49-PLA_2s—To introduce Met at the -1 position and to replace Met-8 by Leu, and to introduce an *Eco*RI site at the 5' end for subcloning into the expression vector pBluescript II SK+, polymerase chain reaction (PCR) was carried out for a *Tf* Asp-49-PLA_2 cDNA fragment subcloned into the *Eco*RI/*Pst*I site of pUC119 (*6*) as a template, using the 5'-primer OG-1 (GCAGGAATTCGATGGGCCTGTGGCAATTCGAGAATCTGATC) and the M13 forward sequencing primer as the 3'-primer. After digestion with *Eco*RI and *Hin*dIII restriction enzymes and purification using a Gene Clean kit II (Bio 101, USA), DNA fragments were ligated into pBluescript II SK+ plasmid. The nature of the resulting recombinant plasmids was confirmed by sequencing. The position 67 mutants of Asp-49-PLA_2, N67D (Asn-67 to Asp), N67K, N67S, and N67G were constructed with the mutagenic oligonucleotides, 5'-CCGGCTGCGACCCCAAACTCG-3', 5'-CCGGCTGCAAACCCAAACTCG-3', 5'-CCGGCTGCAGCCCCAAACTCG-3', and 5'-CCGGCTGCGGCCCCAAACTCG-3', respectively, using a three-step PCR method (*15*) with 5' primer (OG-1), primer B (5'-GAGTGCAAAGCTGGCACCTGCGTAATACGACTCACTATAG-3') and primer D (5'-GAGTGCAAAGCTGGCACCTG-3').

Expression and Purification of Recombinant and Mutant Asp-49-PLA_2s—Recombinant and mutant Asp-49-PLA_2s were isolated as fusion proteins from the *E. coli* host, JM109, with pBluescript II-(M8L)PLA_2 or its mutated plasmids at position 67, as described elsewhere. The fusion proteins were produced as inclusion bodies, which were cleaved with cyanogen bromide in 70% HCOOH. The protein was dissolved in 50 mM Tris·HCl, pH 8.0, containing 6 M Gdn·HCl and 10 mM dithiothreitol (DTT). Refolding of recombinant Asp-49-PLA_2s was achieved by dilution (final concentration of protein 100 μg/ml) into a solution of 50 mM Tris·HCl, pH 8.0 containing 0.9 M Gdn·HCl as described in a previous report (*16*) with minor modifications. The solution was allowed to stand for 2 h at 4°C, then 5 mM $CaCl_2$ and 5 mM cysteine were added into the solution. After standing for 72 h at 4°C, the refolding mixture was concentrated and desalted by ultrafiltration using a YM-10 membrane (Amicon, USA). This solution was loaded onto a heparin Sepharose CL6B column (Pharmacia, Sweden) and eluted with a linear gradient of 0 to 0.5 M KCl in 50 mM Tris·HCl (pH 8.0). The protein solution was desalted using a YM-10 membrane. Generation of active recombinant Asp-49-PLA_2s was monitored by measurement of PLA_2 activity with an egg-yolk emulsion as the substrate.

SDS-PAGE and HPLC—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (*17*). The gels were stained by Coomassie Blue R 350. HPLC for proteins was performed on a Hitachi 638-30 liquid chromatograph equipped with a Hitachi 638-0410 UV detector and a Hitachi 056 recorder.

Amino-Terminal Protein Sequence Analysis—For N-terminal sequence determination of recombinant PLA_2s, purified recombinant (M8L)PLA_2 was analyzed on an Applied Biosystems 470A gas-phase sequencer equipped with a model 120A phenylthiohydantoin (PTH) analyzer for the on-line detection of PTH-amino acids.

Phospholipase A2 Activity—PLA_2 activity was measured titrimetrically with egg-yolk emulsion, EYPC, and EYPG as substrates on a Radiometer RTS-5 titration assembly (pH 8.0, 37°C). Substrates were prepared as micelles mixed with deoxycholate for egg-yolk and Triton X-100 for EYPC and EYPG. Enzymatically released fatty acids were titrated with 10 mM NaOH, and the specific activity of the enzyme for egg-yolk emulsion was calculated from the alkali uptake (μmol/min) per mg of protein. The apparent kinetic constants k_{cat} and K_m were determined from v *vs.* v/[S] plot by linear regression analysis (*18*). The k_{cat} was calculated from apparent V_{max} on the basis of the molecular weight of 14,000. Hydrolysis of monomeric or micellar 2-arachidonoyl-1-stearoyl-L-phosphatidylcholine (ASPC) was analyzed by HPLC as described (*12, 13*) with minor modifications. ASPC was dissolved in chloroform and the solution was allowed to evaporate under nitrogen to form a thin film in a conical glass vessel. The dried lipid was allowed to stand under vacuum overnight with shielding from light and was then vortex-mixed and sonicated in a mixture of 10 mM Tris·HCl (pH 8.0), 100 mM NaCl, 10 mM $CaCl_2$, and 1 mM Triton X-100. Enzyme solution (0.25 μM final concentration) was added to a solution of ASPC-Triton X-100 mixed micelles. The reaction mixture was incubated at 37°C for 3 h. The solvent was evaporated off and the residue was dissolved in ethanol (150 μl), then an aliquot (100 μl) was subjected to HPLC. Liberated arachidonic acid was estimated, using authentic arachidonic acid as a standard (Sigma), by HPLC on a Hitachi 655A-11 liquid chromatograph with a reversed-phase Wakosil 5C18-200 column (4.6 mm × 150 mm) (Wako, Osaka). In the assay for monomer, a solution (50 μl) of PLA_2s (0.25 μM final concentration) dissolved in 10 mM Tris·HCl (pH 8.0) containing 10 mM $CaCl_2$ was added to a solution of ASPC (260 μg) in a mixture of diethyl ether and ethanol (95 : 5 v/v) (150 μl). The mixture was incubated at room temperature for 3 h and arachidonic acid liberated was analyzed as described above. The concentration of phospholipid was determined by using Phospholipid Test-Wako (Wako, Osaka). Protein concentration was determined by measuring the absorbance at 280 nm [$E^{1cm}_{1\%}$ (280 nm) = 25.6] or by the Micro BCA method (*19*).

RESULTS AND DISCUSSION

All mutant enzymes in this study were produced as insoluble fusion proteins which include a part of the β-galactosidase derived from the pBluescript II vector. Modifications were made to *Tf* Asp-49-PLA_2 cDNA (*6*) to introduce a cleavage site (Met) for cyanogen bromide at position −1 and to replace the unique Met residue at position 8 with Leu, in addition to the introduction of an *Eco*RI cloning site. The average yield of recombinant mutant PLA_2s (about 80% pure in SDS-PAGE) from 0.25 liter of growth medium was about 50 mg. Renaturation of mutant PLA_2s was conducted at the concentration of 50 μg/ml in 50 mM Tris·HCl (pH 8.0) containing 0.9 M Gdn·HCl. After refolding at 4°C for 72 h, followed by deionization and concentration, mutant PLA_2s were purified on a heparin affinity column. The yields of active recombinant PLA_2s were 0.2 to 0.8 mg (4-16%) from 5 mg of unfolded proteins. Recombinant PLA_2 mutants appeared homogeneous in SDS-PAGE (Fig. 2a) and reversed phase-HPLC (Fig. 2b). The N-terminal amino acid sequence down to the 10th residue was consistent with that of native PLA_2 except for Met at position 8, which was replaced with Leu. The details of expression and renaturation of *Tf* Asp-49-PLA_2 have been reported elsewhere.

To elucidate the structure-function relationship of the 67th amino acid residue of *Tf* Asp-49-PLA_2, Asn-67 was replaced by Asp, Lys, Ser, and Gly, which were found at position 67 of various group II PLA_2 isozymes isolated from viperinae snake venoms and mammalian platelet and rheumatoid arthritic synovial fluid (Table I and Fig. 1).

The lipolytic activities of native and mutant enzymes were analyzed using egg-yolk emulsion (micelles), EYPC and EYPG (micelles), and synthetic phospholipid, ASPC (monomer and micelles). For egg-yolk assay, lipids were prepared as micelles mixed with deoxycholate, and the activity was determined from the rate of alkali uptake against liberated fatty acids. In this assay, the activity levels of the mutants at position 67, (M8L/N67D)PLA_2 and (M8L/N67K)PLA_2, were comparable to those of native *Tf* PLA_2 and (M8L)PLA_2. On the other hand, the activity levels of (M8L/N67S)PLA_2 and (M8L/N67G)PLA_2 decreased to 17-21% of those of native PLA_2 and (M8L)PLA_2 (Table II).

The above observations were further confirmed by

kinetic studies with micellar EYPC and EYPG (Table III). For micellar EYPC, the K_m values of recombinant PLA_2s, including N67G mutant, were at the same level except for N67D mutant, while the k_{cat} value of N67G mutant was noticeably smaller than those of other recombinant PLA_2s.

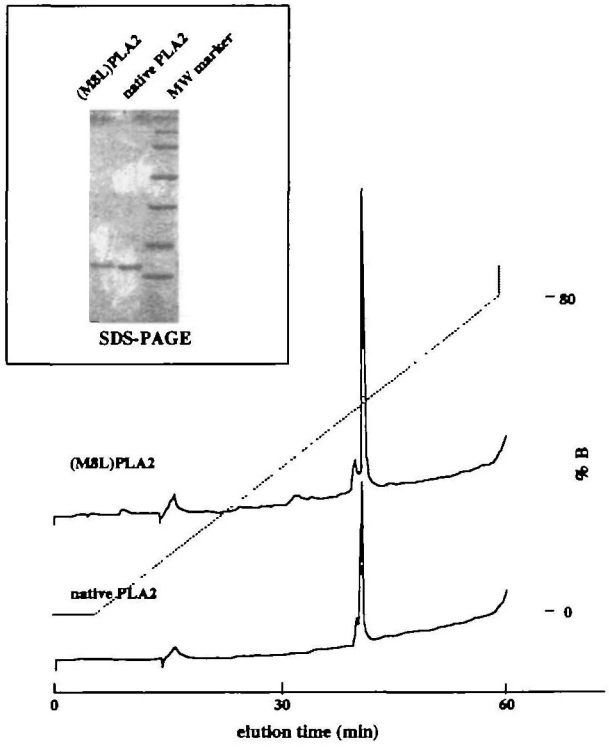


Fig. 2. **SDS-PAGE (a) and HPLC profiles (b) of purified *T. flavoviridis* recombinant and native PLA_2s.** HPLC conditions were as follows: column, TSK-gel ODS 120T column (4.6 mm×250 mm); solvent, 0.1% trifluoroacetic acid (A)-acetonitrile containing 20% A (B); gradient, linear gradient of B (0-80% for 60 min); flow rate, 1 ml per min.

In contrast, for micellar EYPG, the N67G mutant gave smaller k_{cat} and K_m values, but the k_{cat}/K_m value was comparable to those of the others. The N67S mutant showed no hydrolytic activity toward micellar EYPG even with 30 μg of protein. The K_m value of this mutant toward micellar EYPG was at the same level as those of the others, but the k_{cat} value was very much smaller than those of the others. Those observations indicate that N67G and N67S mutants have reduced lipolytic activity toward zwitterionic phospholid (EYPC) but moderate reactivity toward negatively charged phospholipid (EYPG). A recent preliminary molecular dynamics study showed that substitution of Asn-67 to Gly or Ser causes slight changes in the conformation of helix C involving the active site His-48, while no appreciable change was seen for N67D and N67K mutants (data not shown). It is likely that the conformational change in the active site results in the decreased catalytic activities of N67G and N67S mutants.

Marked differences were noted in lipolytic activity toward ASPC. Figure 3 shows HPLC elution profiles of arachidonic acid released from ASPC micelles incubated with various mutant *Tf* PLA_2s for 3 h. Liberation of arachidonic acid was 96% for native *Tf* PLA_2 (data not shown). The relative activities of mutant enzymes for liberation of arachidonic acid from ASPC micelles with respect to that of native *Tf* PLA_2 were 93% for (M8L) PLA_2, 31% for (M8L/N67D)PLA_2, 25% for (M8L/N67K) PLA_2, 106% for (M8L/N67S)PLA_2, and 54% for (M8L/N67G)PLA_2 (Table II). When ASPC monomer was incubated with mutant PLA_2s for 3 h, low lipolytic activities were noted for (M8L/N67D)PLA_2 and (M8L/N67G)PLA_2 (both 18%) and (M8L/N67K)PLA_2 (41%), while (M8L/N67S)PLA_2 (70%) was almost as active as (M8L)PLA_2 (77%) (Table II). (M8L/N67S) mutant, which shows low lipolytic activity on egg-yolk assay (17%), possessed full activity toward ASPC, indicating that the relative specificity of the enzyme for ASPC increases upon substitution of Asn-67 to Ser. These results suggest that the amino acid residue at position 67 of PLA_2s plays a significant role in

TABLE II. **Activities of *T. flavoviridis* Asp-49-PLA_2 and its mutants at position 67 toward egg-yolk emulsion and monomeric and micellar ASPC.**

Enzyme	Specific activity toward egg-yolk emulsion (μmol/min·mg)	Relative activity toward ASPC[a]					
		Mixed micelles[b]			Monomeric		
Native Asp-49-PLA_2	1,160	1.0		(n=4)[c]	1.0		(n=4)
(M8L)PLA_2	1,090	0.93	(1.0)	(n=5)	0.77	(1.0)	(n=4)
(M8L/N67D)PLA_2	1,150	0.31	(0.33)	(n=3)	0.18	(0.23)	(n=2)
(M8L/N67K)PLA_2	1,010	0.25	(0.27)	(n=3)	0.41	(0.53)	(n=2)
(M8L/N67S)PLA_2	190	1.06	(1.14)	(n=2)	0.70	(0.91)	(n=2)
(M8L/N67G)PLA_2	230	0.54	(0.58)	(n=3)	0.18	(0.23)	(n=2)

[a]Estimated from the amount of arachidonate released. [b]Together with Triton X-100. [c]n means the number of experiments.

TABLE III. **Kinetic parameters of *T. flavoviridis* Asp-49-PLA_2 and its mutants at position 67 on EYPC and ETPG micellar substrates.**

Enzyme	EYPC-Triton X-100 mixed micelles			EYPG-Triton X-100 mixed micelles		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
Native Asp-49-PLA_2	2.05	233	1.14×10^6	19.5	392	2.0×10^4
(M8L)PLA_2	2.00	100	0.50×10^5	5.64	142	2.5×10^4
(M8L/N67D)PLA_2	0.37	60.6	1.64×10^6	2.34	76.9	3.2×10^4
(M8L/N67K)PLA_2	1.91	110	0.58×10^5	12.6	12.6	1.4×10^4
(M8L/N67S)PLA_2	[a]	[a]	[a]	2.37	3.1	1.3×10^3
(M8L/N67G)PLA_2	2.22	11.1	5.0×10^3	0.84	18.7	2.2×10^4

[a]Could not be determined.

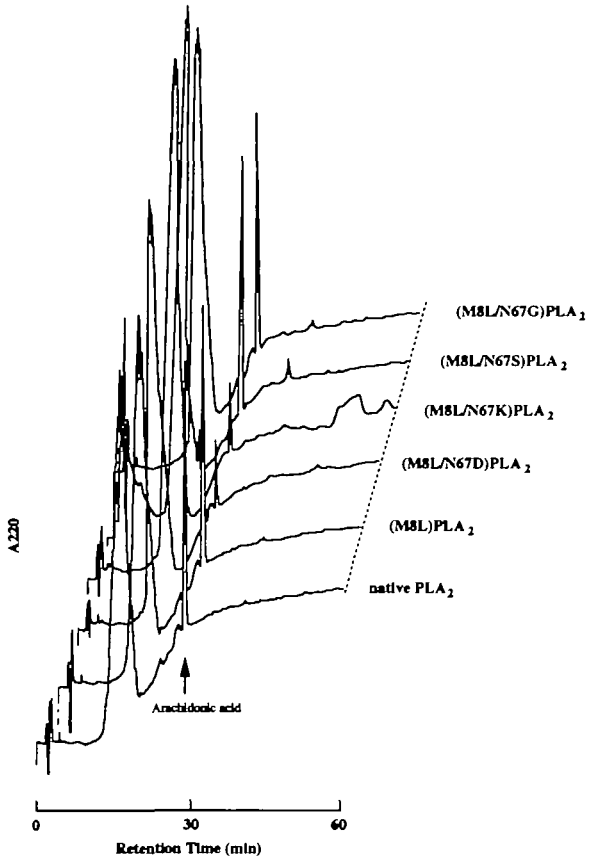


Fig. 3. **HPLC elution profiles of arachidonic acid released from 2-arachidonoyl phosphatidylcholine micelles incubated with *T. flavoviridis* Asp-49-PLA_2 and its mutants.** HPLC conditions were as follows: column, Wakosil 5C18-200 column (4.6 mm × 150 mm); gradient, linear gradient of acetonitrile (50% for initial 5 min, 50-85% for 20 min, and then 85-90% for 30 min) in 0.1% trifluoroacetic acid; flow rate, 1 ml per min.

recognition of the arachidonoyl group of ASPC.

The most remarkable feature found in the present study is that mutant PLA_2s at position 67 show different lipolytic activities toward arachidonic acid-containing phosphatidylcholine. In the assay with ASPC-Triton X-100 mixed micelles, (M8L/N67D)PLA_2 and (M8L/N67K)PLA_2 were less active than (M8L)PLA_2 and (M8L/N67S)PLA_2, indicating that a charged amino acid residue at position 67 is unfavorable for recognition of the 2-arachidonoyl group of micellar phospholipids. The 67th residue is located in a part of the micellar lipid-water interface recognition site (*20, 21*). We have shown in the previous report that the amino acid at position 67 of Lys-49-PLA_2s, BP-I and BP-II, participates in discriminatory recognition of 2-arachidonoyl-phospholipid membranes (*12, 13*). The disadvantage of having a charged amino acid at residue 67 for 2-arachidonate recognition was also observed for monomeric substrate as exemplified by (M8L/N67D)PLA_2 and (M8L/N67K)PLA_2 (Table II). A negatively charged mutant (M8L/N67D)PLA_2 showed particularly low activity (18%). If 2-arachidonate interacts with the 67th residue of PLA_2s, the π electron-rich tail of arachidonate may destabilize the interaction with Asp-67. Furthermore, (M8L/N67G)PLA_2 had relatively low activity in egg-yolk assay and for both monomeric and micellar ASPC. These results suggest that the length of the side chain and the charged state of the 67th amino acid both affect the recognition of substrate phospholipids, including 2-arachidonate of ASPC. Mammalian platelet secretory PLA_2s contain a Gly residue at this position. It was reported that platelet secretory PLA_2s are unable to induce rabbit platelet activation or to release arachidonic acid from platelets, in contrast to snake venom PLA_2s (*22*). These facts seem to be in accord with the findings in the present study.

Examination of the structure-activity relationships of PLA_2s by means of site-directed mutagenesis have been reported mainly for bovine and porcine pancreatic type (group I) PLA_2s. Thunnissen *et al.* demonstrated, based on the X-ray structure of a mutant PLA_2 complexed with an amide inhibitor, that there are contacts between phosphate of the inhibitor and Tyr-69 of PLA_2 (*23*). Verheij *et al.* showed the contribution of Tyr-69 as a stereospecificity determinant of the enzyme (*24, 25*). Furthermore, a mutant PLA_2, which lacks a surface loop (D helix, residues 62-66), manifested altered polar head group specificity (*26, 27*). These observations further suggest that the region between helix C and the β-wing consisting of the 59th to 72nd residues in group I and II PLA_2s is functionally important for the recognition of substrates.

In conclusion, the present mutagenesis study suggests that the amino acid residue at position 67 plays a significant role in the recognition of the 2-arachidonoyl moiety of phospholipids. This finding should be useful for designing PLA_2 with specific recognition ability for the 2-alkyl part of phospholipids.

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