Photo-regulation of Hydrolysis Activity of Semisynthetic Mutant Phospholipases A_2 Replaced by Non-natural Aromatic Amino Acids

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Trp^3 of phospholipase A_2 (PLA2) was replaced by non-natural aromatic amino acids, 3-(2-naphthyl)-L-alanine (Nap), 3-(9-anthryl)-DL-alanine (Ant), and *p*-phenylazo-L-phenylalanine (AzoF), by a semisynthetic method. A fully ε-amidinated PLA2 (AMPA) was subjected to three cycles of Edman degradation to obtain des-Ala^1,Leu^2,Trp^3-AMPA [des-(Ala^1-Trp^3)-AMPA](DES3). Subsequently, the tripeptide having a non-natural amino acid at the third position was connected to DES3 to obtain [Nap^3]AMPA (Nap-AMPA), [Ant^3]AMPA (Ant-AMPA), and [AzoF^3]AMPA (AzoF-AMPA). Nap-AMPA and Ant-AMPA partially retained hydrolysis activity for phospholipid membranes, while AzoF-AMPA having a *trans*(*E*) configuration of the AzoF unit lost this activity. UV irradiation during the hydrolysis reduced the activities of Nap-AMPA and Ant-AMPA. However, AzoF-AMPA with a *cis*(*Z*) configuration of the AzoF unit showed, on UV irradiation, hydrolytic activity. The change of enzymic activity induced by UV irradiation is ascribed to a conformational change of the mutant proteins.

A lipolytic enzyme, phospholipase A_2 (PLA2) cleaves (specifically) the 2-acyl linkage of phosphoglycerides. The reaction proceeds faster with substrates in aggregated states such as micelles and vesicles than in a molecularly dispersed state.[1] In contrast, the precursor of PLA2, prophospholipase A_2, hydrolyses monomeric and aggregated substrates with similar rates. This difference between prophospholipase A_2 and PLA2 is explained by the presence of an interface-recognition site (IRS) in PLA2 but not in the precursor.[2,3] The IRS is composed of amino acid residues involved in the N-terminal hydrophobic part of PLA2, namely Ala^1, Leu^2, Trp^3, Arg^6, Leu^{19}, Met^{20}, Leu^{31}, and Try^{69}, and its function is to immobilize the enzyme at the lipid–water interface.[4] It has been shown that the N-terminal fragment of pancreatic PLA_2 takes an α-helical conformation, playing the key role in the activity of the IRS.[5,6]

It has been reported that selective replacement of amino acid residues in the IRS region resulted in altered hydrolytic activity due to conformational changes.[7] In the present study, we introduced a non-natural aromatic amino acid into the IRS region for the purpose of developing a photosensitive mutant PLA2. A number of mutant PLA2s, which show high affinity for bilayer membranes,[8] high activity,[9,10] altered substrate specificity,[11] and low specificity,[12] have been prepared by site-directed mutagenesis and chemical modification. We employed a semisynthetic method for the preparation of mutant proteins, because this method seemed to be more suitable for the incorporation of non-natural amino acids into proteins than did gene technology. Several mutant proteins modified at the N-terminal region of the proteins, such as trypsinogen,[13] ferredoxin,[14,15] and PLA2,[7] have been successfully prepared by semisynthetic methods.

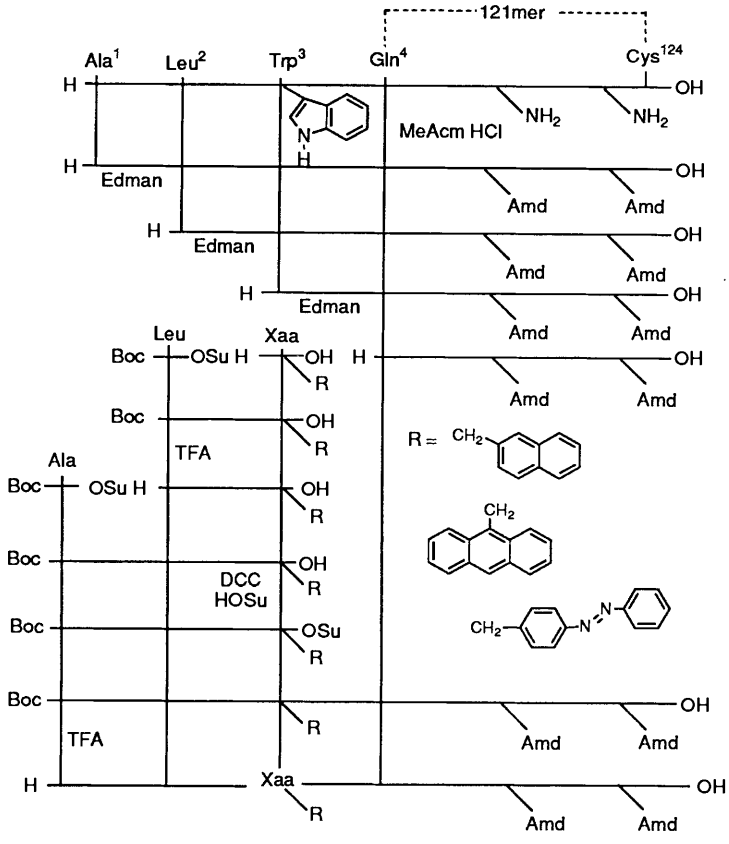
Three kinds of mutant PLA2s, which contain 3-(2-naphthyl)-L-alanine (Nap-AMPA), 3-(9-anthryl)-DL-alanine (Ant-AMPA), or *p*-phenylazo-L-phenylalanine (AzoF-AMPA) in place of Trp^3, were synthesized by the semisynthetic method. Two different ways of developing photosensitive enzymes were employed for incorporation of these non-natural aromatic amino acids into the protein. One is the adoption of naphthylalanine (Nap) and anthrylalanine (Ant) for the replacement. These amino acid residues might affect protein conformation upon UV irradiation to give the polar photo-excited state. The other is the use of azophenylalanine (AzoF). This amino acid residue might affect protein conformation due to variable steric situations induced by *cis*/*trans* (*Z*/*E*) photoisomerization. Either way, the mutant protein is expected to show a photosensitive hydrolytic activity.

Experimental

Materials.—Bovine pancreatic phospholipase A_2 was purchased from Sigma Chemical Co., St. Louis, USA. 3-(2-Naphthyl)-L-alanine (Nap) and *p*-nitro-L-phenylalanine (pNO_2F) were purchased from Aldrich Chemical Co., Milwaukee, WI, USA. 3-(9-Anthryl)-DL-alanine (Ant) was synthesized by the method reported by Egusa *et al.*[16] *N*-(*tert*-Butoxycarbonyl)-L-leucine hydroxysuccinimide ester (Boc-Leu-OSu) and *N*-(*tert*-butoxycarbonyl)-L-alanine hydroxysuccinimide ester (Boc-Ala-OSu) were purchased from Kokusan Chemical Works, Ltd., Tokyo, Japan. Dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (HOSu) were obtained from Peptide Institute, Inc., Osaka, Japan. Methyl acetimidate hydrochloride was prepared by the method reported by Hunter and Ludwig.[17] 1-Palmitoyl-2-[1-^{14}C]palmitoylphosphatidylcholine ([^{14}C]-DPPC) was purchased from New England Nuclear Co., Boston, USA.

Synthesis.—The mutant proteins were prepared by the semisynthetic method as shown in Scheme 1.

Amidination of PLA2. ε-Amidinated PLA2 (AMPA) was prepared by principally the same method as those reported by Slotboom and De Haas[7] and Hunter and Ludwig.[17] PLA2 (5.5 mg) was dissolved in distilled water (8 cm^3) and the pH of the solution was adjusted to 9.8 (measured by a pH meter) with 5 mol dm^{-3} NaOH. Methyl acetimidate hydrochloride (125 mg, freshly prepared) was added over a period of 2 h at room temperature and the mixture was stirred for another 2 h while the pH of the solution was maintained between 9.8 and 10.2. Excesses of reagents were then removed by gel chromatography on Sephadex G-25 with a Tris-HCl buffer solution (0.1 mol dm^{-3}, pH 9.5) as eluent. Fractions containing ε-amidinated PLA2 were collected and concentrated to a small volume. The pH of the solution was adjusted to 6.9 with 1 mol dm^{-3} hydrochloric acid, and the solution was desalted by a Sephadex



Scheme 1 Preparation scheme of semisynthetic mutant PLA2

G-25 column with deionized water as eluent. The collected fraction was lyophilized to obtain AMPA.

DES3. Ala[1], Leu[2], and Trp[3] in the N-terminal region of AMPA were eliminated by three cycles of Edman degradation.[18] Amino acid residues cleaved were identified by TLC analysis using two different solvent systems [chloroform–methanol (9:1) and chloroform–formic acid (20:1)]. The product was purified on a Sephadex G-50 column to obtain DES3.

Tripeptide. Non-natural amino acids (Xaa) were treated with a 3-fold molar excess of Boc-Leu-OSu and one mol equiv. of triethylamine in dimethylformamide (DMF) for 1 day. The product, Boc-Leu-Xaa-OH, was solidified from an ethyl acetate–diethyl ether mixture. The solid product was subjected to gel-filtration chromatography on a Sephadex LH-20 column with methanol as eluent. The dipeptides were treated with trifluoroacetic acid (TFA) in the presence of 1 vol% anisole at 0 °C for 30 min. Upon addition of diethyl ether, the dipeptides were obtained as their respective solid TFA salts. Subsequently, the dipeptide salts were treated with a 3-fold molar excess of Boc-Ala-OSu and 2 mol equiv. of triethylamine in DMF for 1 day. Boc-Ala-Leu-Ant-OH was solidified from a diethyl ether–hexane solution, and Boc-Ala-Leu-Nap-OH and Boc-Ala-Leu-pNO_2F-OH from a methanol–diethyl ether solution. The obtained solid was subjected to gel-filtration chromatography on a Sephadex LH-20 column with DMF as eluent. Boc-Ala-Leu-pNO_2F-OH was converted into Boc-Ala-Leu-AzoF-OH by the method reported by Goodman and Kossoy.[19] The carboxy group of the tripeptides containing a non-natural amino acid was esterified with HOSu by using DCC as coupling reagent.

The purity of synthetic peptides was checked by TLC with a solvent system of chloroform–methanol–acetic acid (85:15:5 v/v/v). All the products were identified by ^1H NMR spectroscopy.

Xaa-AMPS. DES3 (2.0 mg) was dissolved in 0.1 mol dm^{-3} Hepes buffer solution (0.4 cm^3; pH 8.0)–DMF (4 cm^3), and was treated with a 50-fold molar excess of Boc-Ala-Leu-Xaa-OSu for 4 h at room temperature. An insoluble solid was removed by centrifugation, and the supernatant was subjected to a Sephadex G-25 column to obtain Boc-[Xaa³]AMPA. The Boc group was removed by treatment with TFA (20 cm^3) at room temperature under nitrogen for 20 min. A cold mixture (6 cm^3) of DMF–Tris-HCl buffer solution (10 mmol dm^{-3}, pH 7.8; 1:1 v/v) was added, and the solution was subjected to chromatography on a Sephadex G-25 column and elution with a Tris-HCl buffer solution (10 mmol dm^{-3}, pH 7.8). Fractions containing the mutant protein were collected, and desalted by gel filtration on a Sephadex G-50 column to obtain Xaa-AMPA.

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 7.5% separation gel with a 4% stacking gel. An aliquot of the semisynthesized protein, after separation on a Sephadex G-25 column, was mixed with a buffer solution (50 mmol dm^{-3} Tris, pH 6.9; 10% glycerol, 0.05% Bromophenol Blue, 10% SDS, 5% 2-mercaptoethanol). After the sample had been heated at 95 °C for 5 min, it was applied to the SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250. AzoF-AMPA showed a band at a position nearly the same as that of the native phospholipase A_2, indicating that the mutant protein has virtually the same relative molecular mass as the native one.

Measurements.—UV and fluorescence spectra were recorded on a JASCO Ubest-50 UV–VIS spectrophotometer and a Hitachi MPF-4 spectrofluorometer, respectively. Circular dichroism (CD) spectra were recorded on a JASCO J-600 CD spectropolarimeter in a cell with 0.1 cm optical path length. The data are shown as the mean residue ellipticity, $[\theta]$.

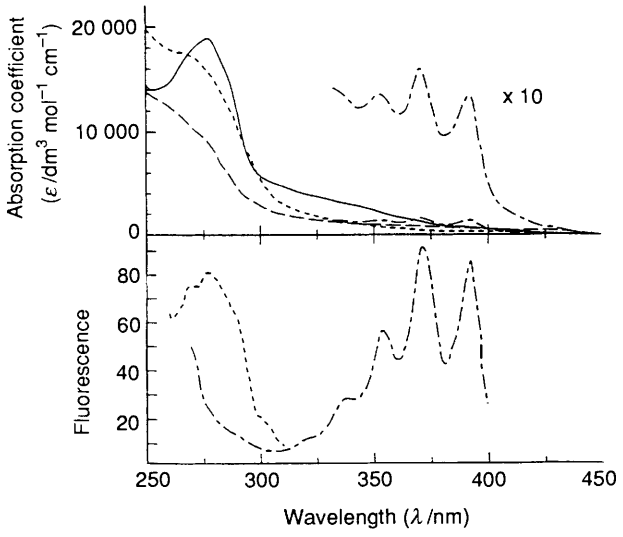


Fig. 1 UV (upper) and fluorescence excitation (lower) spectra of AMPA (——), DES3 (– – –), Nap-AMPA (· · · · ·), and Ant-AMPA (– · –). The UV spectrum of Ant-AMPA is magnified by 10 in the wavelength range 330–450 nm. Fluorescence excitation spectra of Nap-AMPA and Ant-AMPA were monitored at 335 nm and 418 nm, respectively. All samples were measured in Tris-HCl buffer (10 mmol dm^{-3}, pH 7.8)

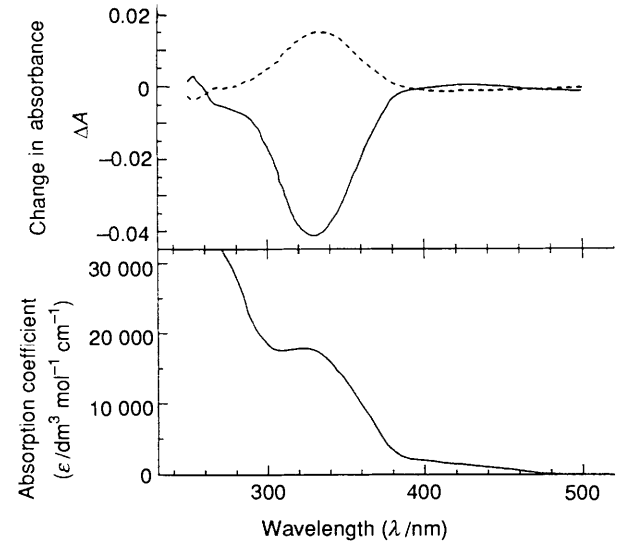


Fig. 2 Photoisomerization of AzoF-AMPA monitored by UV spectroscopy. The bottom spectrum is AzoF-AMPA in Tris-HCl buffer (10 mmol dm^{-3}, pH 7.8). Difference spectra between after and before UV irradiation (——) and between after and before visible-light irradiation (· · · · ·) are shown in the top figure.

Photoisomerization from *E*- to *Z*-configuration was induced by UV irradiation using a UV31 optical filter and a solution filter of nickel(II) sulfate. Visible-light irradiation using an L39 optical filter was used for the *Z* to *E* isomerization.

Concentration of AzoF-AMPA was 1.13 μmol dm^{-3}.

The helix content of samples was calculated by $-[\theta]_{222}/40\,000 \times 100$ (%).

Determination of concentration. The concentrations of PLA2 were determined from the absorbance at 280 nm by using a value for ε of 18 200 dm^3 mol^{-1} cm^{-1}, unless otherwise stated. Concentrations of mutant proteins were determined by the method reported by Lowry *et al.*,[20] taking bovine serum albumin as a standard protein. Amounts of free amino groups were determined by a colorimetric method using 2,4,6-trinitrobenzenesulfonic acid (TNBS).[21]

Hydrolytic activity of mutant PLA2s with Quin 2-encapsulated vesicles as substrate. The hydrolytic activity of mutant PLA2s was assayed by the method reported by Nam *et al.*[22] DPPC vesicles encapsulating 2-{[2-bis(carboxymethyl)amino-5-methylphenoxy]methyl}-6-methoxy-8-bis(carboxymethyl)-aminoquinoline (Quin 2) were prepared and dispersed in a Hepes buffer solution (10 mmol dm^{-3}, pH 7.4; 100 mmol dm^{-3} NaCl) containing $CaCl_2$ (10 mmol dm^{-3}). Xaa-AMPA was added to give a final concentration of 1.65 μmol dm^{-3}. The increase in fluorescence upon addition of mutant PLA2 was monitored.

Hydrolytic activity of mutant PLA2s with vesicles containing radiolabelled lipid as substrate. The activity was determined by principally the same method as reported by Zhang and Dennis.[23] A chloroform solution of DPPC (4.05 μmol) and [^{14}C]DPPC (1 μCi; 18.18 nmol) was dried under a stream of nitrogen. The film was dispersed in a Tris-HCl buffer solution (23.1 cm^3; 10 mmol dm^{-3}, pH 7.8; 5 mmol dm^{-3} $CaCl_2$), and was sonicated at 50 °C. The reaction was started by addition of mutant proteins to 0.4 cm^3 of the dispersion (the final concentration was 21.4 μg cm^{-3} for Nap-AMPA and 34.6 μg cm^{-3} for the other two), and was continued for 60 min at 22 °C. The solution was irradiated with a high-pressure mercury lamp (150 W) through an L-39 optical cut-off filter for Ant-AMPA and a UV-29 filter for the other two. The reaction was stopped by the addition of 100 mmol dm^{-3} aq. EDTA (100 mm^{-3}). A chloroform–methanol (9:1 v/v) mixture (1.3 cm^3) was added and the two phases were completely separated by centrifugation. The lower phase was dried under a nitrogen stream, and the residue was subjected to TLC analysis using a solvent system of chloroform–methanol–water (65:25:4, v/v/v). The radioactivity of the plate was counted by a scanning scintillation counter (Radiochromatoscanner).

Results and Discussion

Semisynthesis of Mutant PLA2.—PLA2 was amidinated on ε-amino groups to a conversion of 83%. AMPA thus obtained was shown to be homogeneous electrophoretically. The average yield for one complete Edman cycle was ~ 80%. The amino acid, which was cleaved at each Edman degradation operation, was identified. The elimination of three amino acid residues was confirmed by the absence of the Trp absorption in a UV spectrum of DES3 (Fig. 1). On the other hand, Nap-AMPA and Ant-AMPA showed the absorption of Nap and Ant, respectively, in a UV spectrum (Fig. 1), indicating coupling of the tripeptide with DES3.

AzoF-AMPA showed a broad absorption at λ ~325 nm due to the *E* configuration of the AzoF residue (Fig. 2). UV irradiation of AzoF-AMPA decreased the intensity at 325 nm, indicating that photoisomerization of the phenylazophenyl group from *E*- to *Z*-configuration was occurring in the mutant protein. It took nearly 20 min to reach the minimum absorbance at 325 nm under these conditions. On the other hand, visible-light irradiation of the mutant protein in the *Z* configuration increased the absorption at 325 nm. This isomerization from *Z*- to *E*-configuration was complete within 20 s of the commencement of irradiation.

Conformation.—CD spectra of AMPA, DES3, and mutant PLA2s are shown in Fig. 3. AMPA and DES3 showed negative Cotton effects at 208 and 222 nm with similar intensitites, indicating the presence of a partial α-helical conformation in these proteins. Since the N-terminal region of PLA2 is known to take the α-helical conformation, the lower intensity of the Cotton effect for DES3 compared with that for AMPA should be due to removal of three amino acids from the N-terminal region, resulting in destabilization of the N-terminal α-helical

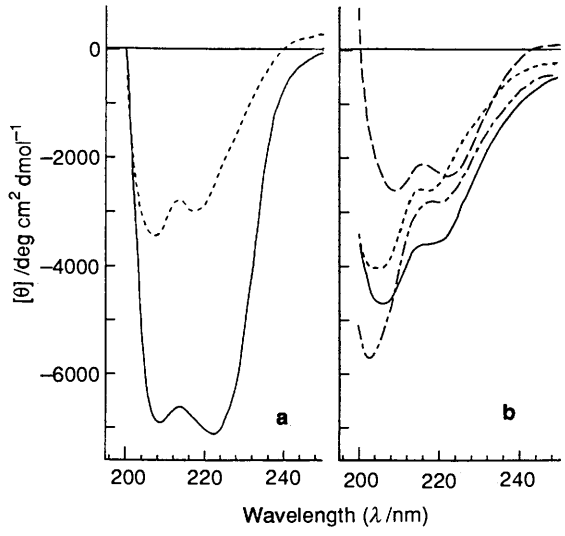


Fig. 3 CD spectra of (a) AMPA (——), DES3 (· · · · ·) and (b) Nap-AMPA (– – –), Ant-AMPA (– · –), AzoF-AMPA in the *Z* form (——), and AzoF-AMPA in the *E* form (· · · · ·) in Tris-HCl buffer (10 mmol dm^{-3}, pH 7.8) at a concentration of 1.13 μmol dm^{-3}

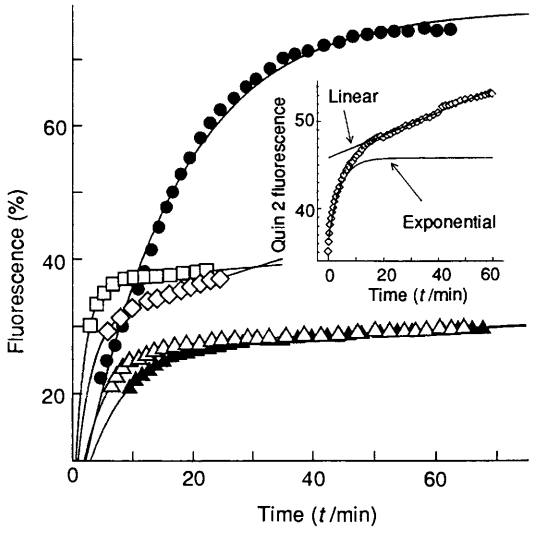


Fig. 4 Change of fluorescence intensity of Quin 2-encapsulated DPPC vesicles on addition of AMPA (●), DES3 (□), Nap-AMPA (◇), AzoF-AMPA in the *Z* form (△), and AzoF-AMPA in the *E* form (▲). Concentrations of mutant PLA2s were 0.64 mg cm^{-3} for AMPA and AzoF-AMPA and 1.4 mg cm^{-3} for DES3 and Nap-AMPA, respectively. The solid lines represent the results of curve-fitting calculations according to equation (1). Linear and exponential components are shown in the inset.

structure. α-Helix content was calculated for AMPA and DES3 to be 17.8 and 6.5%, respectively, from the Cotton effect at 222 nm.

α-Helix content of each mutant PLA2 was as low as it was in DES3. However, Nap-AMPA is considered to take a secondary structure similar to that of PLA2, because the Cotton effect at 200 nm is positive (probably due to a positive Cotton effect at the shorter wavelength for the α-helix), which is similar to that for PLA2, but opposite to that found for other mutant proteins. In particular, the Cotton effects at 208 and 222 nm for AzoF-AMPA increased in negative intensity upon UV irradiation, indicating that the α-helix content of AzoF-AMPA in the *Z* form is higher than that in the *E* form.

Hydrolytic Activity.—The hydrolytic activity of mutant PLA2 was measured without removal of the protecting groups of ε-amino groups, since AMPA retained ~60% of the activity of the native enzyme.[7] The hydrolytic activity was assayed by using Quin 2-encapsulated vesicles. Quin 2 fluoresces strongly upon chelation with Ca^{2+}, and the extent of hydrolysis of lipid molecules by PLA2 can be determined by measuring the fluorescence intensity.[22] The time course of increasing fluorescence intensity induced by AMPA is expressed by a single exponential curve (Fig. 4). On the other hand, the increase of fluorescence intensity induced by Nap-AMPA and AzoF-AMPA was resolved into two components. One is slow and linearly related with time, and the other is fast and exponentially related with time as shown in the inset to Fig. 4. The reaction profile (change of fluorescence intensity, *F*) was analysed in terms of equation (1). The solid lines in

$$F = A_{fast}[1 - \exp(-k_{fast}t)] + A_{slow}t \quad (1)$$

Fig. 4 were obtained by the curve fitting of the experimental data according to equation (1). Optimization of the three parameters was achieved by a nonlinear regression analysis. The standard deviation between the observed and calculated values was less than 1%.

Jain and Berg have proposed the following equation [equation (2)] for the time-course of hydrolysis by PLA2[24]

$$P_t/P_{max} = [k_i/(k_i + k_d)][k_d t + k_i/(k_i + k_d) \times \{1 - \exp(-k_i t - k_d t)\}] \quad (2)$$

where P_t and P_{max} are the amounts of hydrolysed lipid molecules at time *t* and at infinite time, respectively. The rate constant for the dissociation of PLA2 from the vesicle is k_d. The rate constant k_i is for the first-order relaxation that describes the exponential curve of reaction progress, and is proportional to the maximal velocity per enzyme molecule, k_{cat}/K_m.[24,25] Since equations (1) and (2) are in the same form, the following relations (3–5) are obtained.

$$kA_{fast} = [k_i/(k_i + k_d)]^2 C \quad (3)$$

$$k_{fast} = k_i + k_d \quad (4)$$

$$A_{slow} = k_i k_d/(k_i + k_d)C \quad (5)$$

The immediate change of fluorescence intensity on addition of PLA2 is related to initial binding and catalysis by PLA2 on lipid membranes. The subsequent slow increase of fluorescence represents hydrolysis in a stationary state, where association and dissociation of PLA2 to the membrane are equilibrated. The rate constants, k_i and k_d, were calculated according to the following equations (6 and 7) and are summarized in Table 1.

$$k_i = k_{fast}^2/(k_{fast} + A_{slow}/A_{fast}) \quad (6)$$

$$k_d = k_{fast}A_{slow}/(A_{fast}k_{fast} + A_{slow}) \quad (7)$$

The hydrolysis rate, k_i, decreased in the order of DES3 > Nap-AMPA > AzoF-AMPA in the *Z*-form > AzoF-AMPA in the *E*-form. On the other hand, residency times of these proteins ($1/k_d$), which represents the rate of protein transfer from one site of a membrane to the other, were nearly the same with DES3 and AzoF-AMPA in the *Z*- or *E*-form, but 4–5-fold that of Nap-AMPA. However, the residency time of Nap-AMPA is still much longer than that of the native PLA2 (20 min).[26] This long residency time may be one of the reasons for the fairly low activities of these mutant enzymes. The IRS region of the mutant proteins might be highly hydrophobic as a result of incorporation of the hydrophobic aromatic amino acid residue. This mutation should make it difficult for the mutant protein to dissociate from the lipid membrane.

Table 1 Interfacial catalysis by mutant PLA2s

	Concn.[a] (μg cm^{-3})	Exponential[b] A_{fast}	Exponential[b] k_{fast}	Linear[b] A_{slow}	Kinetics k_d	Kinetics k_i	Residency time[c] (t/min)
AMPA[d]	0.64	77.4[d]	0.062 9[d]				
DES3	1.4	36.5	0.581	0.076 0	0.002 07	0.579	483
Nap-AMPA	1.4	30.5	0.415	0.272	0.008 73	0.263	115
AzoF-AMPA (*Z*)	0.64	26.8	0.242	0.046 3	0.001 72	0.240	581
AzoF-AMPA (*E*)	0.64	26.4	0.158	0.049 0	0.001 83	0.156	546

[a] Concentration is based on the amount of mutant PLA2 per total volume of assay cuvette. [b] $F = A_{fast}(1 - e^{-k_{fast}t}) + A_{slow}t$. [c] Residency time is given by the reciprocal of the dissociation rate constant, $1/k_d$. [d] The fluorescence increase of AMPA showed a single exponential curve, probably due to the formation of large pores.

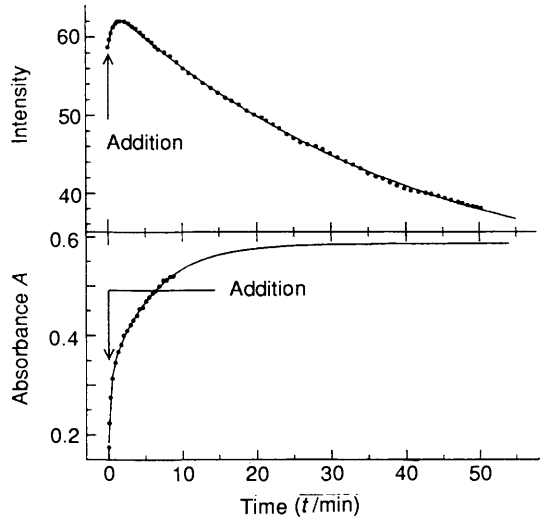


Fig. 5 Changes of fluorescence intensity of Quin 2-encapsulated DPPC vesicles (top) and of absorption at 290 nm (bottom) on addition of Ant-AMPA (1.65 μmol dm^{-3})

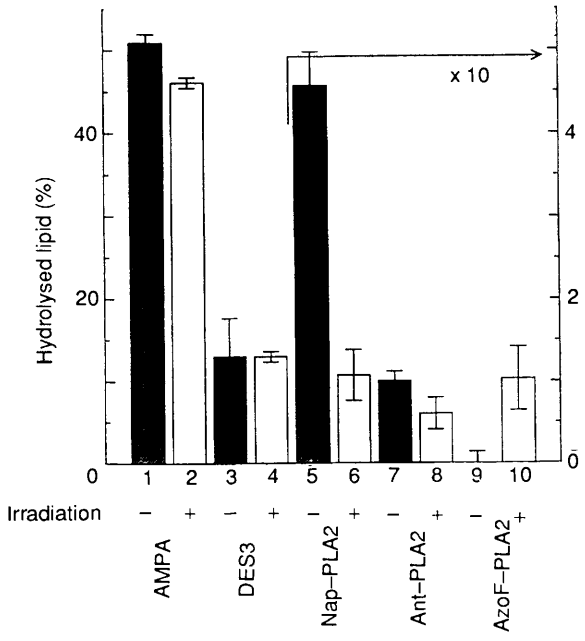


Fig. 6 Effect of UV irradiation on hydrolytic activity of AMPA, DES3, and mutant PLA2s. The ordinate represents the amount of hydrolysed lipid molecules after 60 min. Without UV irradiation, ■. With UV irradiation, □. Standard errors of the mean (SEMs) are shown as I-bars.

The change of fluorescence intensity of Ant-AMPA differs from others as shown in Fig. 5. The intensity increased sharply immediately after the addition of Ant-AMPA (lifetime, reciprocal of kinetic constant, = 0.613 min), and decreased slowly (lifetime = 34 min). The turbidity of the disperson also changed upon addition of Ant-AMPA with a lifetime of 0.29 min, indicating aggregation of vesicles induced by bound Ant-AMPA. The aggregation obstructed our determination of kinetic constants for Ant-AMPA.

Effect of UV Irradiation on Hydrolytic Activity.—Hydrolytic activity was assayed by using radiolabelled lipid molecules, and the effect of UV irradiation on hydrolytic activity was investigated. Fig. 6 shows the experimental results for AMPA, DES3, and three mutant PLA2s. The hydrolytic activity of DES3 decreased to 20% of that of AMPA. The reduced activity may be a result of distortion of the IRS of DES3. Nap-AMPA and Ant-AMPA were of considerably lower activity, that is 10 and 2% of that of AMPA, respectively. Hydrolysis by AzoF-AMPA in the *E* form did not exceed spontaneous hydrolysis under the same conditions. These results are consistent with those obtained by using Quin 2-encapsulated vesicles.

Although the primary sequence of the mutant proteins is nearly the same as that of PLA2, the hydrolytic activities of the mutant proteins are much lower than that of PLA2. This is probably because the tertiary structure of the IRS region of the mutant proteins changed considerably from the native PLA2, which is reflected in the CD spectra (Fig. 3). The catalytic activity of Nap-AMPA is the highest among the mutant proteins, but is only 10% of that of AMPA. The naphthylalanine residue is considered to replace Trp[3] without disrupting the IRS structure so much as do the others, because the size of the side group is not much different from those of natural amino acids.

UV irradiation showed very little effect on hydrolysis catalysed by AMPA or DES3. On the other hand, the activities of Nap-AMPA and Ant-AMPA were reduced by UV irradiation during hydrolysis. In particular, the activity of Nap-AMPA decreased to 25% of that before UV irradiation. Since the naphthyl group in the excited state becomes more polar than in the ground state, the structure of the IRS may be distorted by dislocation of the excited naphthyl group to a more polar environment during UV irradiation. This explanation for the photoregulation of the enzymic activity, however, should be verified by mutant enzymes having a much higher activity than the present mutant proteins.

The reduced activity of Ant-AMPA upon UV irradiation may be explained by the same reasoning as for Nap-AMPA. The extent of the decrease in activity of Ant-AMPA by UV irradiation was less than that of Nap-AMPA. The anthryl group is so bulky that the structure of the IRS should be significantly distorted even in the ground state, resulting in a smaller effect, upon photoirradiation, on the tertiary structure.

AzoF-AMPA in the *Z* form is a little more active than in

the *E* form. This result is in parallel with the result obtained by using Quin 2-encapsulated vesicles. Since the α-helix content increases by photoisomerization of AzoF from *E* to *Z* form as revealed by CD spectroscopy, the hydrolytic activity of AzoF-AMPA in the *Z* form is ascribable to a partial recovery of the native helical structure of the IRS in the mutant protein. However, AzoF in the *E* form cannot be accommodated into the native IRS because of its bulk along the longer molecular axis.

By incorporation of the bulky aromatic group into the IRS, the mutant PLA2s changed their hydrolytic activity in response to UV irradiation. It is believed that the protein structure is changed by UV irradiation, resulting in altered interactions of proteins with the phospholipid membrane.

References

1 G. H. De Haas, P. P. M. Bonsen, W. A. Pieterson and L. L. M. Van Deenen, *Biochim. Biophys. Acta*, 1971, **239**, 252.
2 R. Verger, M. C. E. Mieras and G. H. De Haas, *J. Biol. Chem.*, 1973, **248**, 4023.
3 W. A. Pieterson, J. C. Vidal, J. J. Volwerk and G. H. De Haas, *Biochemistry*, 1974, **13**, 1455.
4 M. C. E. Van Dam-Mieras, A. J. Slotboom, W. A. Pieterson and G. H. De Haas, *Biochemistry*, 1975, **14**, 5387.
5 B. W. Dijkstra, J. Drenth and K. H. Kalk, *Nature*, 1981, **289**, 604.
6 J. P. Abita, M. Lazdunski, P. P. M. Bonsen, W. A. Pieterson and G. H. De Haas, *Eur. J. Biochem.*, 1972, **30**, 37.
7 P. A. J. Slotboom and G. H. De Haas, *Biochemistry*, 1975, **14**, 5394.
8 F. C. Van der Wiele, W. Atsma, B. Roelofsen, M. V. Linde, J. V. Binsbergen, F. Radvanyi, D. Raykova, A. J. Slotboom and G. H. De Haas, *Biochemistry*, 1988, **27**, 1688.
9 O. P. Kuipers, M. M. G. M. Thunnissen, P. D. Geus, B. W. Dijkstra, J. Drenth, H. M. Verheij and G. H. De Haas, *Science*, 1989, **244**, 82.
10 J. P. Noel, C. A. Bingham, T. Deng, C. M. Dupureur, K. J. Hamilton, R.-T. Jiang, J.-G. Kwak, C. Sekharudu, M. Sundaralingam and M.-D. Tsai, *Biochemistry*, 1991, **30**, 11801.
11 J. P. Noel, T. Deng, K. J. Hamilton and M.-D. Tsai, *J. Am. Chem. Soc.*, 1990, **112**, 3704.
12 C. M. Dupureur, Y. Li and M.-D. Tsai, *J. Am. Chem. Soc.*, 1992, **114**, 2748.
13 N. C. Robinson, H. Neurath and K. A. Walsh, *Biochemistry*, 1973, **12**, 420.
14 E. T. Lode, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 1973, **32**, 542.
15 E. T. Lode, C. L. Murray, W. V. Sweeney and J. C. Rabinowitz, *Proc. Natl. Acad. Sci. U.S.A.*, 1974, **71**, 1361.
16 S. Egusa, M. Sisido and Y. Imanishi, *Bull. Chem. Soc. Jpn.*, 1986, **59**, 3175.
17 M. J. Hunter and M. L. Ludwig, *J. Am. Chem. Soc.*, 1962, **84**, 3491.
18 G. W. Anderson, I. E. Zimmerman and F. M. Callahan, *J. Am. Chem. Soc.*, 1964, **86**, 1839.
19 M. Goodman and A. Kossoy, *J. Am. Chem. Soc.*, 1966, **88**, 5010.
20 D. H. Lowry, N. J. Rosehrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265.
21 J. N. Van Eijk, H. M. Verheij, R. Dijkman and G. H. De Haas, *Eur. J. Biochem.*, 1983, **132**, 183.
22 K.-Y. Nam, A. Morino, S. Kimura, H. Fujiki and Y. Imanishi, *Biochem. J.* 1990, **268**, 169.
23 Y. Zhang and E. A. Dennis, *J. Biol. Chem.*, 1988, **263**, 9965.
24 M. K. Jain and O. G. Berg, *Biochim. Biophys. Acta*, 1989, **1002**, 127.
25 O. G. Berg, B.-Z. Yu, J. Rogers and M. K. Jain, *Biochemistry*, 1991, **30**, 7283.
26 M. K. Jain, J. Rogers, O. Berg and M. H. Gelb, *Biochemistry*, 1991, **30**, 7340.

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