Phospholipase A_2 Engineering. Structural and Functional Roles of the Highly Conserved Active Site Residue Aspartate-49[†]

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ABSTRACT: Site-directed mutagenesis and high-resolution two-dimensional (2D) proton nuclear magnetic resonance (NMR) were used to probe the structural and functional roles of a highly conserved residue, Asp-49, in the interfacial catalysis by bovine pancreatic phospholipase A_2 (PLA2, overexpressed in *Escherichia coli*). According to crystal structures, the side chain carboxylate of Asp-49, along with the carbonyl oxygens of Tyr-28, Gly-30, and Gly-32, and two water molecules, provides the necessary ligands for Ca^{2+} which is essential for the enzymatic activity. The Asp-49 of PLA2 was changed to Asn, Glu, Gln, Lys, and Ala; the resulting mutants are named D49N, D49E, D49Q, D49K, and D49A, respectively. The conformational stabilities of all five mutants are similar to that of WT as judged by guanidine hydrochloride-induced denaturation. The structural analyses by NMR indicated no global perturbations upon substitutions, although localized conformational perturbations can be observed for less conserved replacements. Direct Ca^{2+} binding studies showed no specific binding for D49A, D49N, D49Q, and D49K; however, D49E retains a 12-fold weaker calcium binding affinity ($K_{d,Ca}$ = 23 mM). The specific activities of all five mutant enzymes decrease significantly, ranging from 5.4×10^2- to 5.8×10^5-fold in comparison with that of the wild-type enzyme. The observed activities of mutants require the presence of Ca^{2+}. This demonstrates the functional importance of Asp-49 in the catalytic mechanism of PLA2, presumably by helping to bind and properly orient Ca^{2+}. Fluorescence binding studies showed that the mutants fully retain the affinity for binding to the surfaces of zwitterionic micelles and anionic vesicles (i.e., the E to E* step), even though the spectroscopic property has been perturbed. On the other hand, D49A cannot bind active site-directed ligands at the interface (the E* to E*L step), apparently due to its inability to bind Ca^{2+}. The catalytically inactive D49A was also used to study the binding of PLA2 to the interface of substrates, and confirmed the previous results obtained from ether analogs.

Phospholipase A_2 (PLA2)[1] catalyzes the hydrolysis of the *sn*-2-ester bond of 3-*sn*-phosphoglycerides. This enzyme uses a catalytic machinery similar to the catalytic triad of serine proteases, except that the serine residue is replaced by water. In addition, Ca^{2+} is absolutely essential for the catalytic activity. According to the X-ray crystal structure of PLA2 in the resting state (Dijkstra et al., 1981), Ca^{2+} is liganded to the side chain carboxylate of Asp-49, the carbonyl oxygens of Tyr-28, Gly-30, and Gly-32, and two water molecules (Figure 1). It has been suggested that during the catalytic cycle the two water ligands are replaced by the carbonyl oxygen of the *sn*-2-ester group and the *pro-S* oxygen of the phosphodiester group from the substrate (Dijkstra et al., 1981; Tsai et al., 1985; Rosario-Jansen et al., 1987; Scott et al., 1990), as shown in Figure 2. The coordination with the carboxylate of Asp-49 persists throughout the catalytic cycle. Thus, Asp-49 should be an important residue for the catalysis by PLA2. It is also conserved in all PLA2 variants with catalytic activities (Davidson & Dennis, 1990).

The focus of this work is to use site-directed mutagenesis to evaluate the quantitative contribution of Asp-49 to the structure and function of bovine pancreatic PLA2 overexpressed in *Escherichia coli* (Deng et al., 1990). The assessment of the conformational stability of the enzyme was carried out by Gdn-HCl-induced denaturation experiments. One- and two-dimensional NMR were used to analyze the structural differences between WT and mutant enzymes both with and without Ca^{2+}. Direct calcium binding study of the enzyme was conducted with a difference UV spectroscopic method. Interfacial catalysis by specific D49 mutant en-

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[1] Abbreviations: 1D, one-dimensional; 2D, two-dimensional; CD, circular dichroism; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; DC_8PC, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine; deoxy-LPC, 1-hexadecylpropanediol 3-phosphocholine; $DC_{14}PC$, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; $DC_{14}PM$, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol; DQF-COSY, double quantum filtered correlated spectroscopy; EDTA, ethylenediaminetetraacetate; Gdn-HCl, guanidine hydrochloride; GPE, *sn*-glycero-3-phosphoethanolamine; MG14, 1-octyl-2-phosphonoheptyl-*sn*-glycero-3-phosphoethanolamine; MJ33, 1-hexadecyl-3-trifluoroethyl-*rac*-glycero-2-phosphomethanol; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; PAGE, polyacrylamide gel electrophoresis; PLA2, phospholipase A_2; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SDS, sodium dodecyl sulfate; TMSP, sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; UV, ultraviolet; WT, wild type.

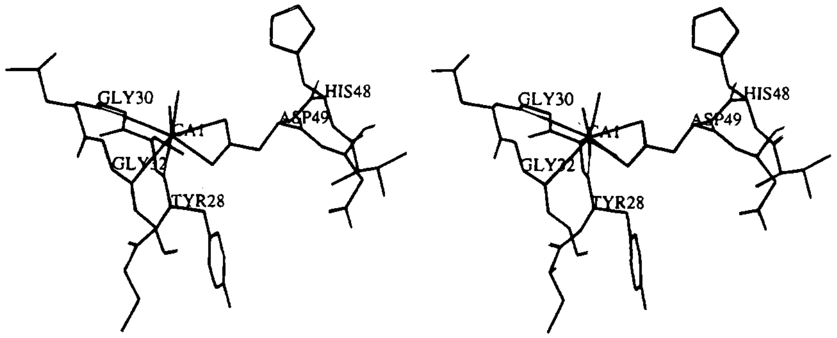

FIGURE 1: Stereo view of the Ca^{2+} binding site of bovine pancreatic PLA2 from its X-ray crystal structure (Dijkstra et al., 1981).

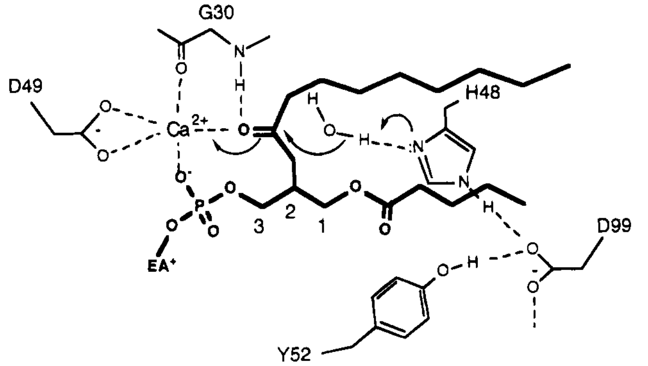

FIGURE 2: Schematic diagram of a proposed catalytic mechanism of PLA2 [adapted from Scott et al. (1990)].

zymes was characterized through the use of micellar and vesicle forms of substrates. Fluorescence spectroscopic methods were used to analyze the E to E* step and the E* to E*L step. The results show that Asp-49 does not play an important role in the conformational stability or the global structure of the enzyme. However, both oxygen atoms of the carboxyl group of Asp-49 are essential for the Ca^{2+} binding and catalysis. Detailed binding studies indicated that Ca^{2+} binding is not essential for the E to E* step; however, it is critically important for the binding of active site-directed ligand to the active site. The catalytically inactive D49A was then used to study the binding of PLA2 to the interface of substrates, and confirmed the previous results obtained from ether analogs.

MATERIALS AND METHODS

Materials and Routine Procedures. Oligonucleotides were obtained from Bio-Synthesis (Lewisville, TX) and used without further purification. Mutagenesis and sequencing kits were obtained from Bio-Rad and United States Biochemicals, respectively. DC_8PC, $DC_{14}PC$, DOPC, and POPC were purchased from Avanti Polar Lipids (Birmingham, AL). The following lipids used in this study were prepared as previously described: $DC_{14}PM$ (Jain & Gelb, 1991); deoxy-LPC and MJ33 (Jain et al., 1991c); DTPM (Jain et al., 1986a); MG14 was provided by Professor Michael Gelb at the University of Washington (Seattle). Ultrapure guanidine hydrochloride (Gdn-HCl) was obtained from ICN Biochemical; 99.9 atom % D D_2O, "100%" D_2O, and sodium 3-(trimethylsilyl)propionate-*2,2,3,3*-d_4 (TMSP) were purchased from MSD Isotopes. DCl was purchased from Cambridge Isotopes. The Fast Flow Sepharose-S and -Q resins (cation and anion exchangers, respectively) were obtained from Pharmacia–LKB. Other chemicals and biochemicals were of the highest quality available commercially. FPLC experiments were performed on a Pharmacia–LKB FPLC system. SDS–PAGE analyses of proteins were performed with the Phast System (Pharmacia–LKB) on 20% acrylamide gels.

Site-Directed Mutagenesis and Protein Purification. Site-directed mutants were generated with oligonucleotides 5′ATAGCAATTATTATGTGTTTG3′ (Asp-49 to Asn), 5′ATAGCAATTTTCATGTGTTTG3′ (Asp-49 to Glu), 5′ATAGCAATTTTGATGTGTTTG3′ (Asp-49 to Gln), 5′ATAGCAATTAGCATGTGTTTG3′ (Asp-49 to Ala), and 5′ATAGCAATTTTTATGTGTTTG3′ (Asp-49 to Lys) by using a mutagenesis kit according to the manual provided by the manufacturer. Since the mutation efficiency was very high, the mutants were selected by DNA sequencing according to Sanger's dideoxy method using a DNA sequencing kit. The recombinant PLA2 and mutants were isolated from the *E. coli* expression host, BL21(DE3)[plysS], carrying the pTO-pla2 plasmid (Deng et al., 1990). The procedure for purification is similar to those described elsewhere (Noel et al., 1991; Li & Tsai, 1993).

CD Spectroscopy and $\Delta G_d^{H_2O}$ Measurements. CD spectra were recorded on a JASCO J-500C spectropolarimeter, and the data were processed with DP-500/AT system (version 1.29) software. Stock solutions of 5 mM enzyme and Gdn-HCl at 8.6 M were prepared in a buffer containing 10 mM borate and 0.1 mM EDTA at pH 8.0. The precise concentrations of the enzyme and Gdn-HCl were determined spectrophotometrically and by the refractive index method (Nozaki, 1972), respectively. Typical CD samples contained, in the borate buffer mentioned above, 0.08 mg/mL enzyme and Gdn-HCl concentration varying from 0 to 8.5 M. The spectra were recorded at 30 °C with a spectral width from 200 to 250 nm and 5 scans. After the corresponding background was subtracted, the ellipticity at 222 nm of each CD sample was recorded and used to calculate the Gibbs free energy of unfolding.

NMR Methods. All proton NMR experiments were conducted in D_2O at 37 °C and pH 4.0–4.1 (uncorrected pH from pH meter reading) on a Bruker AM-500 spectrometer. Typical NMR sample preparation is described as follows. The enzyme sample was dissolved in D_2O (99.9 atom % D) and mixed with stock solutions of $CaCl_2$ and NaCl, both in D_2O. The pH of the solution was adjusted to 4.1–4.3 (uncorrected) with DCl and NaOD stock solutions. The solution was then kept at room temperature for 8 h to allow for deuterium exchange and lyophilized, and the procedure was repeated. The lyophilized sample was dissolved in 0.5 mL of "100%" D_2O, and the pH was adjusted to 4.0–4.1 (uncorrected) with the same DCl and NaOD stock solutions. The final NMR samples without Ca^{2+} contained 1.5−2.0 mM enzyme and 200 mM NaCl (300 mM NaCl for WT). The final NMR samples with Ca^{2+} contained 50 mM $CaCl_2$ in addition to the same amounts of enzyme and NaCl. TMSP was used as an internal chemical shift reference.

Standard pulse sequences and phase cycling were used for 2D NMR experiments: DQF-COSY (Rance et al., 1983) and NOESY (Bodenhausen et al., 1984). The mixing time for NOESY experiments was 200 ms. All spectra were obtained in the phase-sensitive mode with quadrature detection in the *f*1 dimension by time-proportional incrementation (Marion & Wüthrich, 1983). A 2048 × 512 matrix in the time domain was recorded and zero-filled to a 4096 × 2048 matrix prior to multiplication by an unshifted sine bell function (SSB1 = SSB2 = 0) for COSY, and by a Gaussian function (LB2 = −3, GB2 = 0.1) in the *f*1 dimension and a shifted sine bell function (SSB1 = 12) in the *f*2 dimension for NOESY.

Direct Calcium Binding Measurements. The affinity of the enzyme toward Ca^{2+} was measured at room temperature with a Kontron Uvikon 930 spectrophotometer interfaced with a Samsung computer. The final solution for this measurement includes 0.7 mg/mL enzyme, 50 mM Tris, 100 mM NaCl, and 0−150 mM $CaCl_2$ at pH 8.0. The ultraviolet difference spectra at 242 nm were recorded at various Ca^{2+} concentrations and used to calculate the dissociation constant ($K_{d,Ca}$) and binding site number (n) as previously described (Pieterson et al., 1974; Tsai et al., 1985). The maximal absorption from calcium binding to the enzyme (ΔA_{max}) can be obtained from a double-reciprocal plot of absorption (ΔA) *vs* Ca^{2+} concentration ($[Ca^{2+}]$). The saturation degree (γ) of the enzyme by Ca^{2+} equals $\Delta A/\Delta A_{max}$, and a plot of $\gamma/[Ca^{2+}]$ *vs* γ gives the values of $K_{d,Ca}$ and n.

Kinetic Measurements. The activities toward the micellar substrate DC_8PC were measured at 45 °C with a Radiometer RTSS Titration System as previously described (Noel et al., 1991). The final reaction solution for kinetic analysis contained 1 mM borate, 25 mM $CaCl_2$, 100 mM NaCl, 0.1 mM EDTA, and 1−5 mM DC_8PC at pH 8.0. The apparent $V_{max,app}$ and $K_{m,app}$ were determined using Eadie−Hofstee plots (Atkins & Nimmo, 1975) of v *vs* $v/[S]$. On the basis of the PLA2 molecular mass of 13 500 daltons, the apparent $k_{cat,app}$ was calculated from $V_{max,app}$. Specific activities of the enzyme were measured under the same reaction conditions except the DC_8PC concentration was fixed at 6 mM. Kinetic analysis of PLA2 in the scooting mode on $DC_{14}PM$ vesicles was conducted with a pH-stat method under first-order conditions as previously described (Jain & Gelb, 1991; Berg et al., 1991). The initial velocity of hydrolysis of $DC_{14}PM$ vesicles (v_0)[2] was obtained in 1 mM Ca^{2+}, 5 μg/mL polymyxin B, and 1 mM NaCl solution at pH 8.0 (Berg et al., 1991).

Table 1: Free Energy of Unfolding Induced by Gdn-HCl for WT and D49 Mutants[a]

enzyme	$\Delta G_d^{H_2O}$ (kcal/mol)	$D_{1/2}$ (M)	m [kcal/(mol·M)]
WT	9.5	6.8	1.41
D49N	9.5	6.6	1.45
D49E	10.2	6.8	1.49
D49Q	9.9	6.8	1.47
D49K	10.3	6.9	1.50
D49A	9.7	6.9	1.40

[a] Calculated from $\Delta G = \Delta G_d^{H_2O} - m[\text{Gdn-HCl}]$, at 30 °C, pH 8.0, in 10 mM borate, 0.1 mM EDTA, and no Ca^{2+}. The error limit for $\Delta G_d^{H_2O}$ is estimated to be ±0.3 kcal/mol.

Spectroscopic Methods for Binding Studies at the Interface. Binding of PLA2 to aqueous dispersions of phospholipids is accompanied by characteristic changes in the fluorescence and absorbance properties of the tryptophan residue present in position 3 from the N-terminus. The dissociation constant of the enzyme at the interface for the E to E* step (K_d) was obtained by monitoring the increase in the fluorescence intensity of PLA2 at 333 nm as a function of the concentration of a neutral diluent, deoxy-LPC, which has no affinity for the active site. If the same protocol is carried out for a mixture of the enzyme with a saturating concentration of an active site-directed ligand (such as soluble competitive inhibitor), the deoxy-LPC concentration gives the value of the dissociation constant of the enzyme at the interface for the EL to E*L step (K_d^I). The basis for the spectroscopic change (Jain & Maliwal, 1993) and that for the analytical interpretation and the corrections (Jain et al., 1993) have been established previously.

All spectroscopic measurements were carried out in 10 mM Tris and 5 mM NaCl at pH 8.0 and 23 °C. Concentrations of other species were as indicated in the text. The concentration of protein for the fluorescence measurements was 3−10 μM, whereas the concentration for the UV spectroscopic measurements was 30 μM. The fluorescence measurements were carried out on an SLM4800S or SLM AB2 instrument with excitation at 292 nm and emission at 335 nm with excitation and emission slit widths of 4 nm. The UV absorbance measurements were carried out on a Hewlett Packard Model 8452 spectrophotometer equipped with a diode array detector. Data manipulation was carried out by the standard software supplied with the instrument.

RESULTS

Structural Properties of Ca^{2+}-Free Enzymes. (1) Conformational Stability. Cooperative reversible unfolding was observed upon systematic addition of Gdn-HCl in an apparent two-state unfolding mechanism. The conformational stability of mutants was determined by Gdn-HCl-induced denaturation according to the standard equation:

$$\Delta G_d = \Delta G_d^{H_2O} - m[\text{Gdn-HCl}] \quad (1)$$

where ΔG_d is the Gibbs free energy of denaturation at various concentrations of Gdn-HCl, $\Delta G_d^{H_2O}$ is that extrapolated to zero concentration of Gdn-HCl, and m is a constant related to the susceptibility of the protein toward denaturation by the denaturant (Pace, 1986). The $\Delta G_d^{H_2O}$, m value, and the midpoint of the denaturation curve ($D_{1/2}$) of each mutant, along with those of the WT, are listed in Table 1. The replacement of Asp-49 by Asn, Glu, Gln, Lys, and Ala did

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[2] Definition of kinetic parameters at the *interface*: K_d, dissociation constant of enzyme at the interface; K_d^I, dissociation constant of enzyme at the interface containing a saturating concentration of an active site directed ligand; v_0, turnover number at $X_s = 1$, where X_s is the mole fraction of substrate; k_i, first-order relaxation constant; N_s, number of substrate molecules in the outer monolayer of the target vesicle; $K_{Ca}^*(DC_{14}PM)$, effective kinetic dissociation constant of Ca^{2+} on DC_{14}-PM vesicles; K_{Ca}^*, dissociation constant of Ca^{2+} determined by the protection method; K_I^*, dissociation constant of inhibitor determined by the protection method.

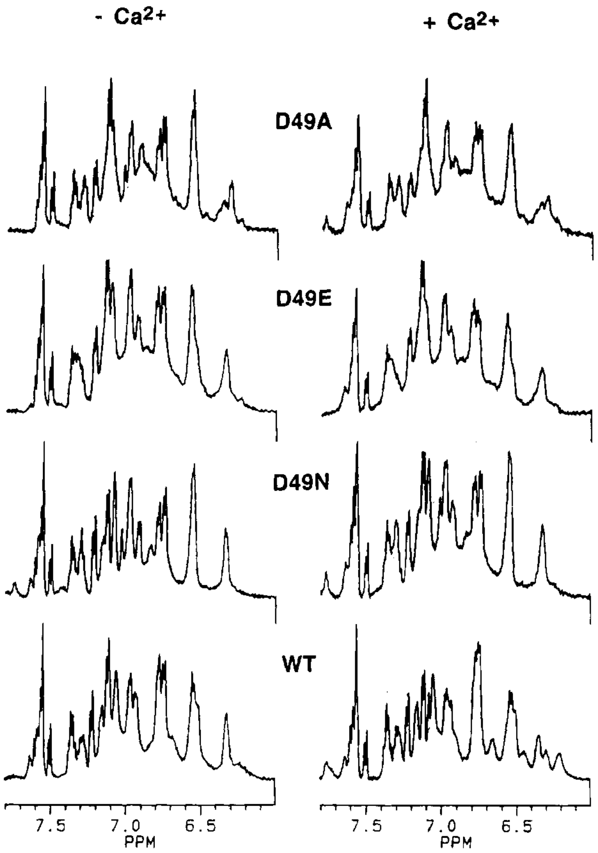

FIGURE 3: One-dimensional proton NMR spectra (showing the aromatic region) of WT, D49N, D49E, and D49A in the absence (left) and presence (right) of Ca^{2+}. Sample conditions were described under Materials and Methods. The FID were processed with Gaussian multiplication (LB = −5, GB = 0.1) prior to Fourier transformation.

not have any significant effect on the conformational stability of the enzyme. This suggests that, despite the fact that this residue is highly conserved, the side chain of this residue is not critically important for the conformational stability.

(2) ^1H NMR Properties. The structural assessment of mutant enzymes was first carried out by one-dimensional proton NMR. The aromatic region of the NMR spectra for Ca^{2+}-free mutants (D49N, D49E, and D49A) and wild type is shown on the left side in Figure 3. Striking similarity between WT and mutant enzymes can be observed. Further structural evaluation of mutants was conducted by using two-dimensional NOESY spectra. Partial NOESY spectra for WT, D49N, and D49E are shown in Figure 4. Qualitatively, the similarity of the mutant to WT follows the order WT ≥ D49N > D49E ≥ D49A on the basis of 1D and 2D NOESY spectra.

Partial resonance assignment of the spin systems for each mutant was accomplished by analyzing its NOESY and COSY (not shown) spectra along with previously published resonance assignments for WT and mutants (Fisher et al., 1989; Dupureur et al., 1992a,c; Li & Tsai, 1993). Table 2 summarizes the partial assignment data for WT, D49N, D49E, and D49A. The designation of spin systems listed in this table is based on that described by Fisher et al. (1989). All the spin systems identified for WT can be found for the mutant enzymes. The resonances which differ by >0.10 ppm between WT and mutants are underlined in Table 2. For D49N and D49E, there is no resonance that differs from the corresponding resonance in WT by >0.10 ppm among all 35 identified spin systems. Only one resonance (Xb, Tyr-28) differs from those of the WT by more than 0.10 ppm for D49A. Examination of the crystal structure reveals that the side chain of Tyr-28 is located in close vicinity of the substitution position. In fact, the carbonyl oxygen of this residue is directly involved in Ca^{2+} binding by providing one of the ligands. Additionally, most of the NOEs between aromatic and aliphatic residues (L1, L2, IG, A1, and M) can be observed for all the mutant enzymes. The preservation of the NOE cross-peaks provides strong evidence that the structural integrity of mutant enzymes is maintained. Overall, structural analysis of mutant enzymes by high-resolution 2D ^1H NMR experiment reveals that, in the absence of Ca^{2+}, there is no global structural perturbation upon substitution at position-49 by Asn, Glu, and Ala, which is consistent with the data from the conformational stability study.

Calcium Ion Binding Properties. (1) NMR Analysis. One-dimensional proton NMR spectra of WT, D49N, D49E, and D49A in the presence of Ca^{2+} are also shown in Figure 3. While the differences between WT PLA2 with and without Ca^{2+} are clearly visible especially in the aromatic region near 6.4 ppm, the spectra of mutants with and without Ca^{2+} are practically identical. This result indicates weak or no Ca^{2+} binding for these mutant enzymes. Further analysis of 2D NOESY NMR spectra of WT and mutant enzymes both with and without Ca^{2+} confirms this conclusion. The 2D NOESY spectra of mutant enzymes with and without Ca^{2+} are almost identical (data not shown). The quantitative binding affinity of Ca^{2+} to the mutants was then determined by UV difference spectroscopy.

(2) UV Difference Spectroscopy. It has been shown that Ca^{2+} binding to PLA2 causes local conformational changes which can be evidenced by UV difference spectra with a maximal absorption at 242 nm (Pieterson et al., 1974; Tsai et al., 1985). By monitoring the change in intensity of this peak (ΔA) on the change of calcium concentration ($[Ca^{2+}]$), a hyperbolic dependence between these two parameters can be established for enzymes with specific binding of Ca^{2+}. At straight line is yielded when the double-reciprocal plot of ΔA *vs* $[Ca^{2+}]$ is performed, and ΔA_{max} is obtained by extrapolating $[Ca^{2+}]$ to infinity. The binding constant was determined by applying the method of Fletcher et al. (1970) with the equation:

$$\gamma/[\text{ligand}] = n/K_{d,Ca} - \gamma/K_{d,Ca} \tag{2}$$

where γ is the degree of saturation which, in our experiment, is the ratio of protein bound with Ca^{2+} ($[\text{protein}]_b$) to the total amount of protein ($[\text{protein}]_t$) that in turn equals $\Delta A/\Delta A_{max}$ (Pieterson et al., 1974), [ligand] is the ligand concentration which is same as $[Ca^{2+}]$ in our case, n is the number of binding sites on the enzyme, and $K_{d,Ca}$ is the average dissociation constant. Rearrangement of eq 2 gives

$$\gamma = n - (\gamma/[\text{ligand}])K_{d,Ca} \tag{3}$$

A plot of γ/[ligand] *vs* γ gives the values of n (from the intercept) and $K_{d,Ca}$ (from the slope) directly.

Wild-type PLA2 shows a typical hyperbolic profile on a plot of ΔA vs $[Ca^{2+}]$ (Figure 5). Further treatment of this these gives $n = 1.0$ and $K_{d,Ca} = 2.0$ mM. This n value is consistent with the fact that bovine pancreatic PLA2 has only one calcium binding site (Dijkstra et al., 1981). The value

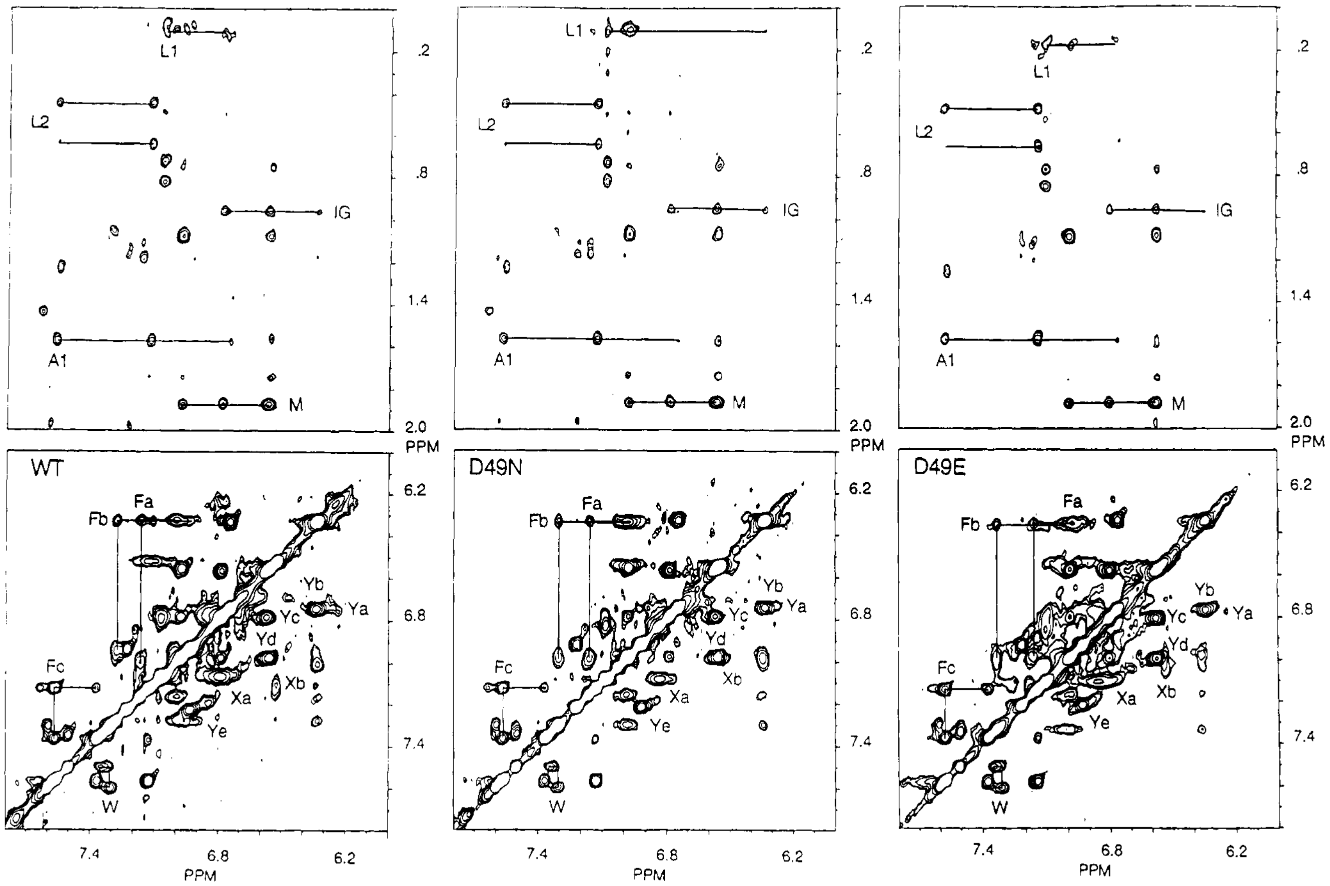

FIGURE 4: Phase-sensitive NOESY spectra (showing the aromatic−aromatic and aromatic−aliphatic regions) of WT, D49N, and D49E in D_2O, at 37 °C, at 500 MHz in the absence of Ca^{2+}. Sample conditions, data acquisition, and data processing parameters were described under Materials and Methods. The aromatic and some of the aliphatic spin systems (F, Y, W, L, M, A, and IG designating Phe, Tyr, Trp, Leu, Met, Ala, and Ile, respectively) have been described previously (Fisher et al., 1989; Dupureur et al., 1992a,c; Li & Tsai, 1993).

Table 2: Chemical Shifts of the Aromatic and Some of the Aliphatic Residues of WT and D49 Mutants in the Absence of Ca^{2+} [a]

spin system	possible assignments	WT ($-Ca^{2+}$)		WT ($+Ca^{2+}$)[b]		D49N		D49E		D49A	
Fa	(F5)	6.33	7.00	6.28	7.00	6.34	6.98	6.36	6.99	6.37	6.96
		7.17		7.15		7.16		7.17		7.16	
Fb	F106	6.33	6.95	6.43	6.88	6.34	6.98	6.37	7.00	6.37	7.03
		7.27		7.26		7.30		7.34		7.30	
Fc	F94	7.13	7.37	7.10	7.35	7.12	7.37	7.15	7.38	7.14	7.38
		7.57		7.55		7.57		7.58		7.58	
Ya	Y111	6.24	6.76	6.18	6.72	6.27	6.74	6.24	6.78	6.25	6.78
Yb	Y52	6.34	6.75	6.34	6.74	6.33	6.75	6.34	6.77	6.32	6.77
Yc	Y73	6.57	6.79	6.55	6.78	6.57	6.79	6.58	6.81	6.58	6.81
Yd	Y75	6.56	6.98	6.52	6.95	6.56	6.98	6.58	7.00	6.58	7.00
Ye	Y69	6.94	7.23	6.92	7.20	6.92	7.22	6.94	7.22	6.93	7.23
W	W3	7.31	7.35	7.30	7.34	7.30	7.37	7.31	7.35	7.32	7.35
		7.50	7.60	7.48	7.58	7.50	7.60	7.51	7.61	7.51	7.61
Xa	(F22)	6.79	7.07	6.75	7.04	6.83	7.08	6.87	7.11	6.88	7.11
Xb	(Y28)	6.52	7.11	6.49	7.15	6.55	7.04	6.54	7.06	6.56	6.88
L1	(L41)	0.12		0.07		0.11		0.17		0.17	
L2	L58	0.45	0.65	0.44	0.63	0.46	0.65	0.49	0.67	0.49	0.67
IG	I95	0.96		0.94		0.96		0.96		0.96	
A1	(A55)	1.58		1.56		1.57		1.59		1.57	
M	(M8)	1.87		1.86		1.87		1.89		1.89	

[a] The underlined are resonances which differ by >0.10 ppm between WT (no Ca^{2+}) and mutant enzyme. Parentheses indicate tentative assignments. The designation of spin systems is based on that described by Fisher et al. (1989). [b] Sample with Ca^{2+}.

of $K_{d,Ca}$ is comparable to those of PLA2s from porcine pancreas and bee venom which are 2.8 mM (Pieterson et al., 1974) and 2.5 mM (Tsai et al., 1985), respectively. As shown in Figure 5, substitution of Asp-49 with Asn resulted in complete loss of the Ca^{2+} binding ability. This result suggests that both carboxyl group oxygen atoms of the aspartate residue and/or the negative charge are critical for calcium binding. Replacements of Asp-49 with Ala, Gln, and Lys produce similar results to that of D49N; little affinity toward Ca^{2+} can be detected even at 150 mM Ca^{2+}. However, unlike the previous report that D49E *porcine* PLA2 does not bind Ca^{2+} ($K_{d,Ca} > 100$ mM) (van den Bergh et al.,

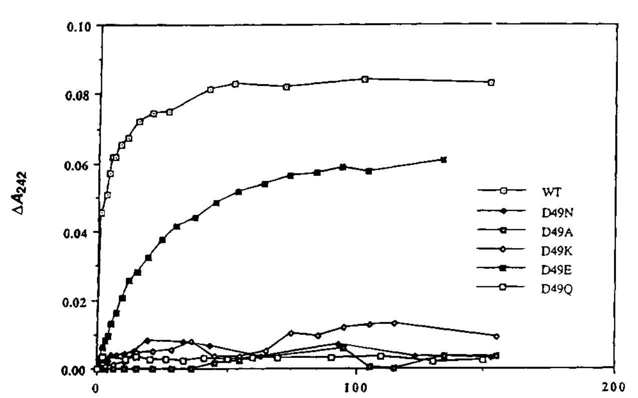

FIGURE 5: Binding profile of Ca^{2+} to WT, D49A, D49N, D49E, D49Q, and D49K. The measurements were carried out in a buffer containing 50 mM Tris, 100 mM NaCl, and various concentrations (0–150 mM) of $CaCl_2$, at pH 8.0 and 20 °C. The final concentration of the enzyme was 0.7 mg/mL, and the UV difference spectra were recorded at 242 nm.

Table 3: Summary of Kinetic Data for WT and D49 Mutants

enzyme	sp act. [μmol/(mg·min)][a]	$k_{cat,app}$ (s^{-1})	$K_{m,app}$ (mM)	v_0[c] (s^{-1})
WT	3.5×10^3	675	1.4	330
D49N	6.5	2.0	3.0	0.4
D49E[b]	0.08	na[d]	na	<0.001
D49Q[b]	0.006	na	na	<0.001
D49K[b]	0.03	na	na	0.005
D49A[b]	0.18	na	na	0.011

[a] Specific activities were measured at 6 mM DC_8PC. The error limits are estimated to be ±5% for $k_{cat,app}$, ±10% for specific activity, and ±15% for $K_{m,app}$ for WT PLA2. [b] The specific activity is too low to do further kinetic study on pH-stat. The background rate for the specific activity is ca. 0.003 μmol/(mg·min). [c] The background rate of v_0 is ca. 0.001 s^{-1}. [d] na, data not available.

1988), our data indicate that D49E *bovine* PLA2 does possess an affinity toward calcium ion. The dependence of ΔA on [Ca^{2+}] for D49E shows a typical hyperbolic curve (Figure 5). Calculation from this curve gave $n = 0.9$ and $K_{d,Ca} = 23$ mM.

Mg^{2+} was used to probe the possibility of a change of metal specificity for the D49E mutant. Although D49E does show slightly higher affinity toward Mg^{2+} than WT (not shown), the nonspecific nature of the binding prevents us from obtaining the dissociation constant and making any meaningful interpretation.

Catalytic Properties. (1) Micellar Substrate. The kinetic parameters $k_{cat,app}$ and $K_{m,app}$ were determined as described previously for DC_8PC micelles (Noel et al., 1991). The values are considered as apparent since the assays using micelles do not separate the steps of E to E* (enzyme bound to the interface) and E* to E*S (enzyme·substrate complex at the interface). According to our recent studies, however, the relative $k_{cat,app}$ values of DC_8PC micelles accurately reflect the relative k_{cat} values in the scooting mode assay system for a series of PLA2 mutants (Dupureur et al., 1992a,c). This is even more likely to be the case for D49 mutants for which the chemical step will be rate-limiting and the E to E* step has been shown to be unperturbed in D49 mutants (see results from Equilibrium Dissociation Constants). Due to low activity, only the specific activity at a fixed DC_8PC concentration was obtained for D49A, D49E, D49Q, and D49K. The results from these kinetic studies are listed in Table 3.

The activity of D49N listed in Table 3 should be considered only as an upper limit since it is likely to arise from WT impurity as a result of nonenzymatic deamidation of the mutant at position 49 (see next section) (Wright, 1991). The dramatic decrease in the specific activity (4.3×10^4-fold) of D49E, which still retains Ca^{2+} binding capacity though with 12-fold lower affinity, suggests that a correct geometry at the Ca^{2+} binding site is required for the catalysis by this enzyme. The significant decreases in specific activity for D49A, D49Q, and D49K ranging from 1.9×10^4- to 5.8×10^5-fold are likely to be caused by the inability of these mutants to bind Ca^{2+}.

Further kinetic study on D49E reveals that Ca^{2+} is still required for the activity of D49E. In the absence of Ca^{2+}, there is no detectable activity for D49E. Upon addition of Ca^{2+} (25 mM), the activity of D49E recovers completely. However, no activity of D49E can be observed in the presence of 50 mM Mg^{2+}. This result indicates that although there is an apparent increase in the affinity toward Mg^{2+} for D49E as judged by UV difference spectral measurement, Mg^{2+} cannot be used to substitute Ca^{2+} for the catalysis by D49E. Kinetic measurements on D49A and D49K also indicate that Ca^{2+} is critical for the residual activities of these two mutants.

(2) Activities on $DC_{14}PM$ Vesicles by the Scooting Mode Assay System. The initial velocities of hydrolysis of DC_{14}-PM vesicles (v_0) for all the mutants along with that of the wild-type enzyme are also listed in Table 3. The hydrolytic rates for D49A, D49E, D49Q, and D49K are all less than 0.02 s^{-1}, compared to a value of 330 s^{-1} observed for the WT enzyme. In agreement with the micellar kinetic studies, the v_0 of D49N, although decreasing dramatically in comparison with that of the WT (750-fold), is considerably higher than those of the other mutants. Detailed scooting mode kinetic studies described in the following sections suggest that the activity of D49N is mainly caused by WT impurity:

(a) Under the first-order conditions with 0.5 mM Ca^{2+} and 0.15 mg/mL $DC_{14}PM$, a first-order curve was seen with an initial burst of activity. The k_i value is the same as with the WT (about 0.2 min^{-1}), whereas the amplitude per enzyme (N_s) is about 0.025% of what is seen with WT (this experiment was conducted with 50 times the usual enzyme concentration in order to obtain an accurate N_s value). This suggests that there is only 0.025% of active enzyme in D49N. On the basis of the v_0 value with $DC_{14}PM$, it is 0.1%, and on the basis of the $k_{cat,app}$ with DC_8PC, it is 0.03%.

(b) Calcium is required for activity. The K_{Ca}*($DC_{14}PM$) for the hydrolysis of $DC_{14}PM$ under v_0 conditions is 0.13 mM compared to 0.12 mM observed for WT. In other words, the kinetic Ca^{2+} affinity of the active portion of D49N is the same as that of the WT enzyme.

(c) The rates of inactivation with *p*-nitrophenacyl bromide in the E, E*, and E*Ca states of D49N are similar to those of WT (Dupureur et al., 1992a). This is also evident in the values of $K_{Ca}^* = 0.4$ mM and $K_I^* = 0.009$ mM for the D49N preparation because these values are virtually the same as those obtained for WT.

Equilibrium Dissociation Constants. The results from the previous sections have established that the D49 mutants showed very low catalytic and calcium binding activities. The low catalytic activity is apparently related to the low calcium binding affinity. However, it remains to be established whether the low calcium affinity also affects binding

Table 4: Summary of Fluorescence Study on E to E* for WT and D49 Mutants[a]

enzyme	λ_{max} (nm)	F_{rel} E	F_{rel} E* on DTPM
WT	351	1.00	1.28
D49A	344	2.00	2.25
D49N	350	1.40	1.95
D49E	351	1.17	1.68
D49K	347	1.96	2.74

[a] F_{rel} is the intensity at 333 nm compared to that of the E form for the WT under identical conditions.

of the enzyme to the interface (i.e., the E to E* step), and ligand binding at the interface (i.e., the E* to E*L step). We therefore performed binding experiments using the spectroscopic protocols developed elsewhere (Jain et al., 1982, 1986b, 1991a; Jain & Maliwal, 1985). The specific experiments carried out with D49A are outlined below:

(1) Binding to the Zwitterionic Interface. The E to E* binding studies were carried out either by resonance energy transfer to dansyl-GPE (Jain & Vaz, 1987) or by monitoring the emission intensity for tryptophan (Jain et al., 1982, 1986b). It has been established previously that binding of PLA2 to micelles of deoxy-LPC, which is a zwitterionic neutral diluent, causes an increase in the fluorescence emission intensity of Trp-3. Since deoxy-LPC has very low affinity for the catalytic active site of the enzyme, the bound enzyme is predominantly in the E* state (Jain & Gelb, 1991). The increase in the emission intensity of D49A shows a hyperbolic dependence on the bulk concentration of the neutral diluent. Curve-fitting yielded an apparent dissociation constant $K_d = 0.85$ mM and a 12% increase in the fluorescence emission intensity at 335 nm. These values are appreciably different from those observed with WT: $K_d =$ 7 mM and an increase in fluorescence emission intensity of 60%. The specific reason for the differences is unclear. However, the data indicate that D49A is fully capable of binding to the surface of zwitterionic micelles.

(2) Binding to the Anionic Interface. The fluorescence properties of D49 mutants in the E and E* forms at the anionic DTPM vesicles were also significantly different from those of WT. As summarized in Table 4, the fluorescence intensity (twice that of WT) and the emission maximum (344 nm versus 351 nm for WT) for D49A in the aqueous phase (E form) suggest that Trp-3 in this mutant is in a somewhat more hydrophobic environment. The corresponding values for other D49 mutants (Table 4) also suggest that the environment of Trp-3 depends significantly on the amino acid residue at position 49. This effect is also reflected in the relative intensity of these mutants on binding to DTPM vesicles (the E* form, last column in Table 4). It may be noted here that although D49N contains a minor amount of WT impurity, it should influence only the catalytic behavior but not the spectroscopic behavior.

Despite changes in the fluorescence intensity and the emission maximum, the binding affinity to the anionic surface does not change in the D49 mutants. From the binding isotherms [results not shown; however, see Jain et al. (1986b)], it was estimated that the D49 mutants bind to about 30 DTPM molecules with an apparent K_d estimated to be <10 μM. These results are similar to those reported earlier for PLA2s from porcine pancreas (Jain et al., 1991a; Yu et al., 1993) and bovine pancreas (Dupureur et al., 1992a) in the presence of Ca^{2+}.

Overall, the results indicate that the D49 mutants are fully capable of binding to the interfaces of zwitterionic micelles and anionic vesicles. However, the changes in the spectroscopic properties are surprising because Trp-3 is located in the interfacial recognition region which is not a part of the catalytic region. We do not understand these results yet, but they do indicate that a perturbation in the calcium binding region has a significant effect on the microenvironment of the N-terminal region of PLA2. As discussed elsewhere (Jain & Maliwal, 1993), such a behavior would be predicted if the orientation of the N-terminus in these mutants was different enough to alter the relationship to the neighboring quenching groups including the bound water molecules.

(3) The E to E*L Step.* With the WT enzyme, titration of E* at the deoxy-LPC interface with an active site-directed ligand caused a further change in the fluorescence emission as well as a change in the absorbance in the 292 nm region (Dupureur et al., 1992a). These changes were not observed with the D49A mutant when MJ33 or MG14 was used as an active site-directed ligand. These results suggest that calcium binding is required for the binding of a substrate analog to the active site of the enzyme. The same conclusion about the obligatory requirement of calcium for the sequential binding of the active site-directed ligands was arrived at independently from the kinetic and equilibrium binding measurements carried out by monitoring the protection of His-48 from alkylation (Yu et al., 1993).

Binding of an active site-directed ligand to PLA2 also finds its expression in the value of K_d^I, i.e., the apparent affinity of the enzyme to the interface of a neutral diluent in the presence of an active site-directed ligand (Jain et al., 1993). For bovine WT PLA2, the value of K_d (7 mM) is about 25-fold larger than the value of K_d^I (0.28 mM), which does not seem to depend on the structure of the inhibitor. On the other hand, although the K_d value for the D49A mutant was about 0.8 mM, the apparent affinity for the interface was unaffected in the presence of an active site-directed inhibitor; i.e., for these D49 mutants, $K_d = K_d^I$. Such a behavior would be expected if EI or E*I species are not formed with D49 mutants.

Use of D49A To Study the Binding of PLA2 to the Substrate Interface. Binding of WT and catalytically active enzymes to the substrate interfaces cannot be monitored directly because the substrate is hydrolyzed. Such studies in the past were done with the unnatural enantiomer or the ether analogs of phospholipids (Jain et al., 1982, 1986b, 1991a), or with alkylated enzymes (Volwerk et al., 1974; Jain et al., 1991b), which leave open the possibilities about the role of the ester groups in the binding of the enzyme to the interface. With catalytically inactive D49 mutants, which do not bind a substrate molecule to the catalytic site as established in the previous section, it is possible to monitor the binding of the enzyme to the interface of ester phospholipids. The result showed that binding of D49A to vesicles of zwitterionic DOPC or POPC could not be detected by the fluorescence change of Trp-3. This finding agrees with the previous report that WT PLA2 binds poorly to the ether analog of $DC_{14}PC$ in the presence or absence of Ca^{2+} unless anionic additives are present (Jain et al., 1982, 1986b).

On the other hand, a significant increase in fluorescence intensity was observed on the binding of D49A and other catalytically inactive mutants to the vesicles of anionic DC_{14}-PM. Such spectroscopic changes were qualitatively similar to those obtained with WT enzyme and the corresponding ether analog DTPM. Collectively, these results confirm that the binding of PLA2 to the interface does not depend upon the ester or ether functions of phospholipids. Surprisingly, however, the quantitative differences between these systems suggest that the presence of calcium at the active site does influence the spectroscopic property of Trp-3. The possible origin of these spectroscopic effects is being investigated.

DISCUSSION

The preceding sections present rigorous analyses of the structural and functional roles of the highly conserved residue Asp-49 using multifaceted approaches. Our results suggest that Asp-49 does not play a critical role in either conformational stability or global structural integrity of the enzyme. However, the carboxyl group of Asp-49 is critically important for the Ca^{2+} binding ability of the enzyme, and calcium binding is essential for catalysis. The mutants were then used to evaluate the requirement of calcium binding in various binding steps using neutral and zwitterionic micelles and vesicles. The results indicate that the mutants behave similarly to WT in the E to E* step, but are unable to bind substrate analogs at the active site. The broader implications of our results are discussed in the following sections.

Roles of Asp-49 in Conformational Stability and Global Structural Integrity of PLA2. Conformational stabilities of PLA2 have been reported to be around 7 and 9.5 kcal/mol for porcine and bovine enzymes (Pickersqill et al., 1991; Dupureur et al., 1992a), respectively. Both of these values put PLA2 among the most stable of enzymes (Pace, 1990). Although the unusual abundance of disulfide bonds (7 disulfide bonds for a protein of 123 amino acids in the case of bovine enzyme) may contribute significantly to the stability of PLA2, certain residues other than cysteine are shown to be of great importance in conformational stability (Dupureur et al., 1992a,b; Li & Tsai, 1993).

Results from this study show that Asp-49 does not play an important role in the conformational stability of PLA2 since the conformational stabilities of the mutants are close to that of WT PLA2. The CD spectra of all mutants share striking similarity to that of the WT (not shown). Although the one-dimensional ^1H NMR spectra show small but notable differences between mutant and WT, thorough examination of the 2D NOESY and COSY spectra unveils the overall similarity among WT and mutants. For D49A in the absence of Ca^{2+}, only one resonance from Tyr-28 differs from those of WT by >0.10 ppm. This is not unexpected considering that Tyr-28 is located in the immediate region of the substitution and it actually provides one of the ligands for the metal binding. Equally convincing is the identification of most of the NOE cross-peaks between aromatic and aliphatic spin system for the mutant enzymes.

Although conformational stability and structure are two independent properties, they seem to always agree with each other in evaluating the perturbation in the mutants of PLA2 as shown by the results from this work and previous reports (Dupureur et al., 1992a,b,c; Li & Tsai, 1993).

Calcium Binding Site of Bovine Pancreatic PLA2. The calcium binding site of PLA2 is different from those of many receptor proteins. Instead of having several negatively charged side chains along with several polar side chains to form a cagelike Ca^{2+} binding site such as the one in D-galactose and D-glucose receptors (Falke et al., 1991), the Ca^{2+} binding site of PLA2 has only one negatively charged side chain (Asp-49). The rest of the ligands needed for Ca^{2+} binding are provided by three backbone carbonyl oxygens and two water molecules. How does PLA2 attain its Ca^{2+}-specific affinity with such a simple machinery? What is the exact role of each participant? Answers to these questions will no doubt shed light on the apprehension of the metal binding specificity of proteins.

The results from this study clearly show the importance of the side chain carboxylate of Asp-49 for the Ca^{2+} binding ability of the enzyme. Replacement of Asp-49 with the isosteric amide group resulted in complete loss of Ca^{2+} binding capability, suggesting that both oxygen atoms of the carboxyl group and/or the negative charge are required for binding of Ca^{2+} to this enzyme. Substitution of Asp with Glu retains the ability of Ca^{2+} binding although a 12-fold increase in the dissociation constant is observed. Isosteric replacement of Glu with Gln at position 49 again knocks out the Ca^{2+} binding ability.

The result of D49E raises an interesting question as to how it retains some of the Ca^{2+} binding ability and how its catalytic activity compares with those mutants that have no Ca^{2+} binding affinity. It has been suggested that similar positioning of the carboxyl group with different side chain lengths can be achieved through local backbone and side chain readjustment (Judice et al., 1993). A similar readjustment could lead to the ability of D49E to bind Ca^{2+}, though with a 12-fold lower affinity. The bound Ca^{2+} in D49E, however, is insufficient to support catalysis. It should also be noted that an earlier report for porcine pancreatic PLA2 suggested no binding ability of D49E toward Ca^{2+} ($K_{d,Ca}$ > 100 mM) (van den Bergh et al., 1988). The reason for the disagreement between the studies of the two highly homologous PLA2s from porcine and bovine pancreas is unclear.

Roles of Asp-49 in Interfacial Catalysis. The involvement of Asp-49 and calcium binding in the catalysis by PLA2 has long been suggested by other studies (Fleer et al., 1981; van den Bergh et al., 1988). However, the use of D49 mutants coupled with structural analysis, scooting mode kinetic assays, and UV and fluorescent binding studies described in this report has allowed detailed dissection of the roles of Asp-49 and calcium binding in the interfacial catalysis by bovine pancreatic PLA2.

As demonstrated by the scooting mode kinetic analysis, the activity of D49N reported in Table 3 is likely to arise mainly from WT impurity through nonenzymatic deamidation of asparagine. It has been well documented that the deamidation of asparagine could be enhanced by high ionic strength, and Asn is located between two polar residues (Wright, 1991). The purification of PLA2 does involve a high ionic strength refolding procedure (Noel et al., 1991), and Asn-49 is indeed located between two polar residues, His-48 and Asn-50. The deamidation most likely occurs in the denatured state since the activity of D49N samples does not increase with time. In the native state, the deamidation process in most proteins becomes much slower because of rigidity of the structure and less accessibility of water (Wright, 1991).

The results of fluorescence studies indicate that all mutants are fully capable of binding to zwitterionic micelles (deoxy-LPC) and anionic vesicles (DTPM and $DC_{14}PM$), but they have little affinity for the surface of zwitterionic vesicles (DOPC and POPC). These results are similar to the behavior of WT, except that previous binding studies could only be performed with substrate analogs (Jain et al., 1982, 1986b, 1991a). The availability of inactive mutants such as D49A has permitted direct binding study for binding to the surface of substrates such as $DC_{14}PM$, DOPC, and POPC.

The residue Asp-49, presumably through its central role in calcium binding, is found to be crucial in the E* to E*L step. Fluorescence binding studies suggest that D49A PLA2 is unable to bind the active site-directed inhibitor MJ33 or MG14. Thus, binding of Ca^{2+} is important for both ground state and transition state binding of substrates, but is not required for the E to E* step. The energetic contribution of bound Ca^{2+} along with the side chain of Asp-49 to the catalysis can be estimated at ~6 kcal/mol on the basis of v_0 values of WT and D49A. It is important to note that the Ca^{2+} is still required for the residual activity of the mutants, even though the Ca^{2+} binding affinity has decreased or become undetectable. Thus, Ca^{2+} is required for the catalytic activity of WT PLA2, and the side chain of Asp-49 is required for proper orientation of the bound Ca^{2+} ion.

The results from our mutagenesis study also suggest that lysine cannot replace calcium to stabilize the transition state, since the activity of D49K is only 10^{-5} relative to that of WT. In fact, the activity of D49K also requires the presence of Ca^{2+}. This finding agrees with a related study with PLA2 from a different source (van den Bergh et al., 1988), and disagrees with the suggestions by Heinrikson and co-workers (Maraganore et al., 1984; Maraganore & Heinrikson, 1986) that Asp-49 is not essential for catalysis and that the ϵ-amino group of Lys-49 can substitute for Ca^{2+} in supporting the catalysis by PLA2.

REFERENCES

Atkins, G. L., & Nimmo, I. A. (1975) *Biochem. J. 149*, 775–779.

Berg, O. G., Yu, B.-Z., Rogers, J., & Jain, M. K. (1991) *Biochemistry 30*, 7283–7297.

Bodenhausen, G., Kogler, H., & Ernst, R. R. (1984) *J. Magn. Reson. 58*, 370–388.

Davidson, F. F., & Dennis, E. A. (1990) *J. Mol. Evol. 31*, 228–238.

Deng, T., Noel, J. P., & Tsai, M.-D. (1990) *Gene 93*, 229–234.

Dijkstra, B. W., Drenth, J., & Kalk, K. H. (1981) *Nature 289*, 604–606.

Dupureur, C. M., Yu, B.-Z., Jain, M. K., Noel, J. P., Deng, T., Li, Y., Byeon, I.-J. L., & Tsai, M.-D. (1992a) *Biochemistry 31*, 6402–6413.

Dupureur, C. M., Li, Y., & Tsai, M.-D. (1992b) *J. Am. Chem. Soc. 114*, 2748–2749.

Dupureur, C. M., Yu, B.-Z., Mamone, J. A., Jain, M. K., & Tsai, M.-D. (1992c) *Biochemistry 31*, 10576–10583.

Falke, J. J., Snyder, E. E., Thatcher, K. C., & Voertler, C. S. (1991) *Biochemistry 30*, 8690–8697.

Fisher, J., Primrose, W. U., Roberts, G. C. K., Dekker, N., Boelens, R., Kaptein, R., & Slotboom, A. J. (1989) *Biochemistry 28*, 5939–5946.

Fleer, E. A. M., Verheij, H. M., & de Haas, G. H. (1981) *Eur. J. Biochem. 113*, 283–288.

Fletcher, J. E., Spector, A. A., & Ashbrook, J. D. (1970) *Biochemistry 9*, 4580–4586.

Jain, M. K., & Maliwal, B. P. (1985) *Biochim. Biophys. Acta 814*, 134–140.

Jain, M. K., & Vaz, W. L. C. (1987) *Biochim. Biophys. Acta 906*, 1–8.

Jain, M. K., & Gelb, M. H. (1991) *Methods Enzymol. 197*, 112–125.

Jain, M. K., & Maliwal, B. P. (1993) *Biochemistry 32*, 11838–11846.

Jain, M. K., Egmond, M. R., Verheij, H. M., Apitz-Castro, R. J., Dijkman, R., & de Haas, G. H. (1982) *Biochim. Biophys. Acta 688*, 341–348.

Jain, M. K., Rogers, J., Jahagirdar, D. V., Marecek, J. F., & Ramirez, F. (1986a) *Biochim. Biophys. Acta 860*, 435–447.

Jain, M. K., Maliwal, B. P., de Haas, G. H., & Slotboom, A. J. (1986b) *Biochim. Biophys. Acta 860*, 448–461.

Jain, M. K., Yu, B.-Z., Rogers, J., Ranadive, G. N., & Berg, O. G. (1991a) *Biochemistry 30*, 7306–7317.

Jain, M. K., Ranadive, G. N., Yu, B.-Z., & Verheij, H. M. (1991b) *Biochemistry 30*, 7330–7340.

Jain, M. K., Tao, W., Rogers, J., Arenson, C., Eibl, H., & Yu, B.-Z. (1991c) *Biochemistry 30*, 10256–10268.

Jain, M. K., Yu, B.-Z., & Berg, O. G. (1993) *Biochemistry 32*, 11319–11329.

Judice, J. K., Gamble, T. R., Murphy, E. C., De Vos, A. M., & Schultz, P. G. (1993) *Science 261*, 1578–1581.

Kumar, V. A., Sekharaudu, Y. C., Dupureur, C. M., Zhu, H., Tsai, M.-D., & Sundaralingam, M. (1994) *Protein Sci.* (in press).

Li, Y., & Tsai, M.-D. (1993) *J. Am. Chem. Soc. 115*, 8523–8526.

Maraganore, J. M., & Heinrikson, R. L. (1986) *J. Biol. Chem. 261*, 4797–4804.

Maraganore, J. M., Merutka, G., Cho, W., Welches, W., Kezdy, F. J., & Heinrikson, R. L. (1984) *J. Biol. Chem. 259*, 13839–13843.

Marion, D., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967–974.

Noel, J. P., Bingman, C., Deng, T., Dupureur, C. M., Hamilton, K. J., Jiang, R.-T., Kwak, J.-G., Sekharudu, C., Sundaralingam, M., & Tsai, M.-D. (1991) *Biochemistry 30*, 11801–11811.

Nozaki, Y. (1972) *Methods Enzymol. 26*, 43–50.

Pace, C. N. (1986) *Methods Enzymol. 131*, 266–279.

Pace, C. N. (1990) *Trends Biochem. Sci. 15*, 1–4.

Pickergill, R. W., Sumner, I. G., Collins, M. E., Warwicker, J., Perry, B., Bhat, K. M., & Goodenough, P. W. (1991) *FEBS Lett. 281*, 219–222.

Pieterson, W. A., Volwerk, J. J., & de Haas, G. H. (1974) *Biochemistry 13*, 1439–1445.

Rance, M., Sorensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479–485.

Rosario-Jansen, T., Pownall, H. J., Noel, J. P., & Tsai, M.-D. (1987) *Phosphorus Sulfur 30*, 601–604.

Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H., & Sigler, P. B. (1990) *Science 250*, 1541–1546.

Tsai, T.-C., Hart, J., Jiang, R.-T., Bruzik, K., & Tsai, M.-D. (1985) *Biochemistry 24*, 3180–3188.

van den Bergh, C. J., Bekkers, A. C. A. P. A., Verheij, H. M., & de Haas, G. H. (1988) *Eur. J. Biochem. 182*, 307–313.

Volwerk, J. J., Pieterson, W. A., & de Haas, G. H. (1974) *Biochemistry 13*, 1446–1454.

Wright, H. T. (1991) *Crit. Rev. Biochem. Mol. Biol. 26*, 1–52.

Yu, B.-Z., Berg, O. G., & Jain, M. K. (1993) *Biochemistry 32*, 6485–6492.