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Characterization of Phospholipase A_2 (PLA_2) from Taiwan Cobra: Isoenzymes and Their Site-Directed Mutants[1]

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Extracellular and secretory phospholipase A_2 (PLA_2), a class of phospholipid digesting enzyme, is widely distributed in animal venoms of reptiles and insects. Two cDNAs encoding PLA_2 isoenzymes from Taiwan Cobra (*Naja naja atra*) were cloned into *p*QE-30 plasmid vector and expressed in *Escherichia coli*. The recombinant products were subjected to refolding using sulfonation under reduction/oxidation conditions with glutathione and enterokinase removal of His-tag, resulting in the active recombinant PLA_2 with the same molecular masses of native enzymes as determined by mass spectrometry. The recombinant PLA_2 was also shown by circular dichroism to possess a secondary structure similar to native PLA_2. The enzymatic activity of the major isoenzyme ($PLA_{2\text{-}1}$) is higher than the other minor isoenzyme ($PLA_{2\text{-}2}$), which shows two amino acid difference from $PLA_{2\text{-}1}$. Site-directed mutagenesis was used to probe the structure/function relationship of two highly conserved residues among all reported PLA_2, *i.e.*, His-47 and Asp-93. Replacement of His-47 residue by either Ala or Arg resulted in the complete loss of activity. Similarly, the mutant Asp-93 → Asn (D93N) also retained little activity. These results suggest that both His-47 and Asp-93 are essential for the catalytic activity of PLA_2. Computer graphic study, based on homology modelling, highlights the differences between native PLA_2 isoenzymes and their site-directed mutants, which may account for the differences in the observed biological activity. © 1998 Academic Press

The enzyme phospholipase A_2 (PLA_2; EC 3.1.1.4, phosphatidate 2-acylhydrolase) is widely distributed among various species in the animal kingdom, notably in the pancreatic tissues of mammals and animal venoms of reptiles and insects. It can catalyze efficiently and specifically the hydrolysis of the 2-acyl ester bond of 1,2-diacyl-3-sn-phosphoglycerides (1). Snake PLA_2 are a group of polypeptides ranging about 120-130 amino acids in length, each chain is invariably cross-linked by 7 disulfide bonds (2). In addition to its enzymatic activity for the cleavage of ester bonds in phosphoglycerides, venom PLA_2 has been shown to possess diverse pharmacological effects such as neurotoxic, myonecrotic, cardiotoxic, hemolytic and anticoagulant actions (3,4).

Currently more than 50 primary sequences of PLA_2 from various tissues of different animal families have been solved by conventional protein sequencing coupled with cDNA cloning and sequencing (5,6). The protracted and important issue is generally concerned with the elucidation of the evolutionary and/or catalytic mechanisms for the generation of multiple PLA_2 isoforms in the same or closely-related species with widely different activities (7). It is imperative to characterize and compare various isoforms of these toxins with diverse enzyme activity with regard to their primary structures in order to gain some insight into the molecular basis underlying the process of sequence variation among these proteins.

On the other hand, detailed and comparative studies of the catalytic mechanism(s) of various PLA_2 require the use of pure and homogeneous forms of this enzyme. In spite of the relative ease in the isolation of individual forms of PLA_2 by conventional protein techniques, the characterization and purification of PLA_2 from the recombinant DNA technology remains the method of choice in the generation of site-directed mutants for catalytic and mechanistic studies of PLA_2. In order to determine the specific role(s) of various catalytically relevant residues in PLA_2, we have employed the protein engineering approach based on site-directed mutants derived from expressed proteins of cloned PLA_2 cDNAs reported previously (8,9).

[1] The sequence data of cDNA for the precursors of two native phospholipase A_2 (*Naja naja atra*) isoenzymes, $PLA_{2\text{-}1}$ and $PLA_{2\text{-}2}$, have been deposited in the EMBL Data Library under Accession Numbers X73225 and X77755, respectively.

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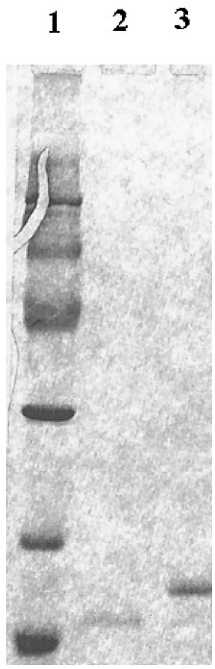

FIG. 1. SDS-PAGE of recombinant PLA_2. Lane 1, low molecule weight markers in kDa: phosphorylase b (94), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (30), soybean trypsin inhibitor (20), and lysozyme (14). Lane 2, refolded recombinant PLA_2 after enterokinase cleavage. Lane 3, recombinant PLA_2 before enterokinase cleavage. Note that the mobility of PLA_2 is slower than that of lysozyme (14 kDa) probably due to insufficient binding of SDS detergent to PLA_2. $PLA_{2\text{-}1}$ and $PLA_{2\text{-}2}$ show similar results.

MATERIALS AND METHODS

Preparation of recombinant PLA_2. For induction of PLA_2 gene expression, *E. coli* M15[*p*REP4] cells containing plasmid *p*QE-30/$PLA_{2\text{-}1}$ and *p*QE-30/$PLA_{2\text{-}2}$ (8,9) were grown at 37°C in LB-broth containing 100 μg/ml ampicillin, 25 μg/ml kanamycin until A_{600} reaches 0.7-0.9. The cultures were induced by 2 mM IPTG for 3 h. The cells were harvested by centrifugation at 4,000 × g for 10 min. The recombinant PLA_2 was then purified by Ni-NTA resin as previously described (8,9). Purified PLA_2 was sulfonated with 2-nitro-5-(sulfothio)-benzoate (NTSB) in 8 M urea solution containing 0.3 M Na_2SO_3, pH 8.0. The sulfonated protein was precipitated by dialysis against 0.25% acetic acid and solubilized in 50 mM sodium borate (pH 8.4) containing 5 mM EDTA, 8 M urea, 4 mM reduced/2 mM oxidized glutathione. Refolding was performed by a 4-fold dilution with the same buffer without urea. The resulting solution was incubated at room temperature for 24 h. The protein solution was then dialyzed against distilled water at 4°C overnight. The protein solution was centrifuged to remove insoluble materials and lyophilized. The $(Asp)_4$-Lys plus His-affinity tag was removed by enterokinase cleavage. The recombinant PLA_2 was further purified by HPLC on a reversed-phase C_{18} column, equilibrated with 0.1% TFA and eluted with a linear gradient of 35-70% acetonitrile for 35 min. The eluate was monitored at 235 nm.

PLA_2 activity assay. PLA_2 activity was measured by the pH-stat titration method. The assay was conducted at 37°C with a Metrohm 718 STAT Titrino titration system (Metrohm, Switzerland). The reaction mixture consisted of a 2 ml solution containing 6 mM dipalmitoyl-phosphotidylcholine (Sigma), 6 mM sodium deoxycholate, 0.1 mM EDTA, 100 mM NaCl, and 2.5 mM $CaCl_2$. PLA_2 was added to start the reaction. The liberated fatty acid was titrated with 4 mM NaOH in order to maintain a constant pH of 7.5 within 6 min.

SDS–PAGE analysis. The purity and molecular mass of PLA_2 were analyzed by SDS-gel electrophoresis (SDS-PAGE) according to Laemmli (10).

Circular dichroism. CD spectra of recombinant PLA_2 was obtained on a Jasco J-720 spectropolarimeter at a concentration of 1 mg/ml in H_2O with a cell pathlength of 0.1 mm. The CD spectra were measured in a wavelength range of 260 to 200 nm. CD spectra was then recorded by signal averaging of five scans.

Molecular mass analysis. PLA_2 was dissolved in 50% acetonitrile containing 0.1% acetic acid to make a final concentration of 10 μM. The sample was then analyzed in an API 100 mass spectrometer (PE SCIEX) at an infusion rate of 5 μl/min. The spectra were analyzed with a software (BioToolBox) from the manufacturer.

Protein concentration determination. The protein concentrations of native and recombinant PLA_2 were determined by bicinchoninic acid (BCA)-Protein Assay Reagent kit (Pierce) using bovine serum albumin as standard.

Site-directed mutagenesis. Site-directed mutants were constructed from the following oligonucleotides:

5′GGTGCTGCCAGGTT**GC**TGACAACTGCT 3′ (H47A),
5′GGTGCTGCCAGGTTC**G**TGACAACTGCT 3′ (H47R), and
5′GTCTGTGATTGT**A**ACCGCTTGGCAGCC 3′ (D93N).

Substitutions were made using overlap extension PCR mutagenesis (11). The mutants were selected and confirmed by DNA sequencing.

Computer modelling. The atomic coordinates for the published PLA_2 structure were obtained from the Protein Data Bank (entry 1POA). Substitution of mutated residues and subsequent energy minimization calculations were carried out on a Silicon Graphics Iris 4D/35 and Iris Indigo Elan 4000 workstation using the macromolecular computer modelling system "Insight II" from Biosym (version 2.2). Plots of the active site residues were produced by employing the program MOLSCRIPT and Raster-3D.

RESULTS AND DISCUSSION

On the basis of structural characteristics (4,12), PLA_2 can be divided into two groups: (A) Group I comprising PLA_2 obtained from mammalian pancreas and venoms of two snake families, *i.e. Elapidae* and *Hydrophiidae*, and (B) Group II containing PLA_2 found in venoms of *Crotalidae* and *Viperidae* snakes. We have established the cDNA library and solved two primary structures of PLA_2 isoforms from Taiwan cobra (*Naja naja atra*) belonging to *Elapidae* family in order to allow a defined structure-function comparison and

TABLE 1

Properties of Recombinant Phospholipase A_2 Isoenzymes

	Amino acid residue	Relative activity (%)	Molecular mass
Native PLA_2		100	13256, 13220
$PLA_{2\text{-}1}$	49-N, 64-F	113	13256
$PLA_{2\text{-}2}$	49-H, 64-S	80	13220

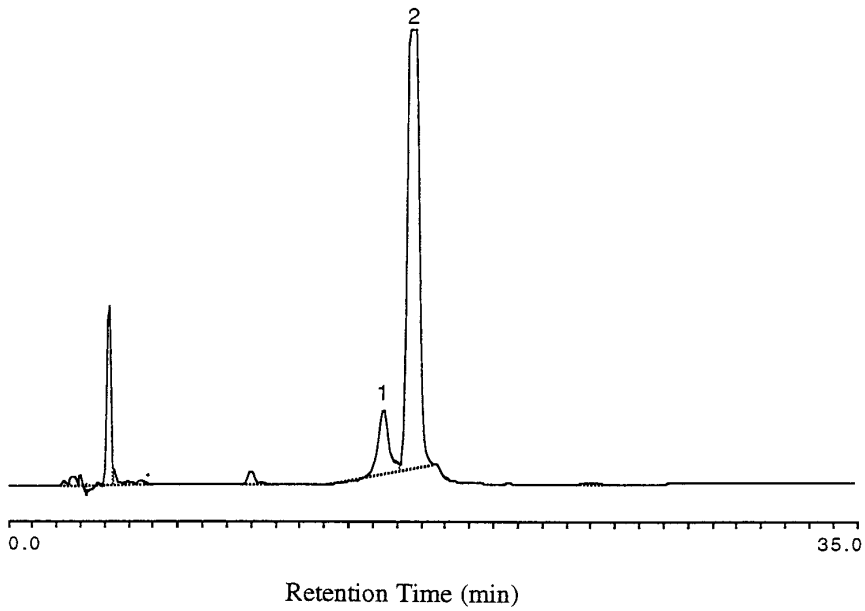

FIG. 2. Purification of the recombinant PLA_{2-1} by reversed-phase HPLC. The enterokinase-treated PLA_{2-1} was applied on a reversed-phase C_{18} column and eluted with a linear gradient of 35–70% acetonitrile for 35 min. The flow rate was 1.0 ml/min. The eluate was monitored at 235 nm. Peak 1 indicates the refolded PLA_{2-1} with His-affinity tag. Peak 2 indicates the refolded PLA_{2-1} whose His-affinity tag has been removed by enterokinase.

shed some light on the biological significance of these stable PLA_2 in snake venoms. Upon the extension of our previous work on the structural analysis of two major acidic PLA_2, *i.e.* PLA_{2-1} and PLA_{2-2} by PCR technique (8,9), we have further employed site-directed mutagenesis on these cloned cDNAs to aid in the study of structure/function relationship of these PLA_2 isoforms.

Preparation of site-specific mutants of PLA_2. We have previously generated cDNA clones by using PCR amplification of total venom cDNA mixtures. With these clones we have further prepared several mutant enzymes based on site-specific mutagenesis. The first few mutants were based on the active-site residues, the conserved His-48 and Asp-99 (based on the numbering system of pancreatic PLA_2), termed the catalytic dyad among all members of Group-I PLA_2 (2-4) and supposedly to be central to the activity of this enzyme. Our focus of this work is to use site-directed mutagenesis to evaluate the contribution of His-47 and Asp-93 (corresponding to His-48 and Asp-99 of pancreatic PLA_2) to the structure/function of Taiwan cobra PLA_2 overexpressed in *E. coli* system. By overcoming the complicated refolding process, we have achieved the active recombinant PLA_2 enzymes using refolding conditions of sulfonation and reduction/oxidation with a glutathione redox couple and subsequent enterokinase removal of His-tag.

Based on the cDNAs cloned in our previous studies (8, 9), PLA_2 attached with six-histidine tag and the N-terminal extension pentapeptide $(Asp)_4$-Lys was expressed in *E. coli,* which could be recognized by enterokinase and specifically cleaved at the C-terminal side of the lysine residue (15). The expressed PLA_2, appeared to be exclusively present in the inclusion body of *E. coli.* It can only be partially purified by Ni-nitrilotriacetic acid (Ni-NTA) affinity column. It was not surprising that the expressed PLA_2 might not show the full enzymatic activity when compared with native PLA_2. The presence of seven pairs of disulfide bonds in PLA_2 posed one difficult task for the expression of functional protein. After refolding the expressed protein was then subjected to enterokinase removal of His-affinity tag and $(Asp)_4$-Lys pentapeptide (Fig. 1). To our satisfaction the expressed PLA_{2-1} and PLA_{2-2} isoforms showed comparable activity when compared with the native enzyme.

Characterization and structural analysis of the expressed recombinant PLA_2. In order to probe the structural basis for the activity of PLA_2 mutants, the refolded PLA_2 was purified by reversed-phase HPLC on a C_{18} column (Fig. 2). From the viewpoint of site-specific mutagenesis it is important to ensure that no inadvertent base changes were introduced during the mutagenesis procedure. Therefore the most straightforward way to check the identities of wild-type and mutant enzymes is to determine their molecular masses by current mass spectrometry with high precision. The molecular masses of recombinant PLA_2 were analyzed, which were in complete agreement with the calculated masses of 13256 and 13220 for PLA_{2-1} and PLA_{2-2} respectively (Fig. 3). Molecular masses of all other mutant enzymes were also found to be in accord

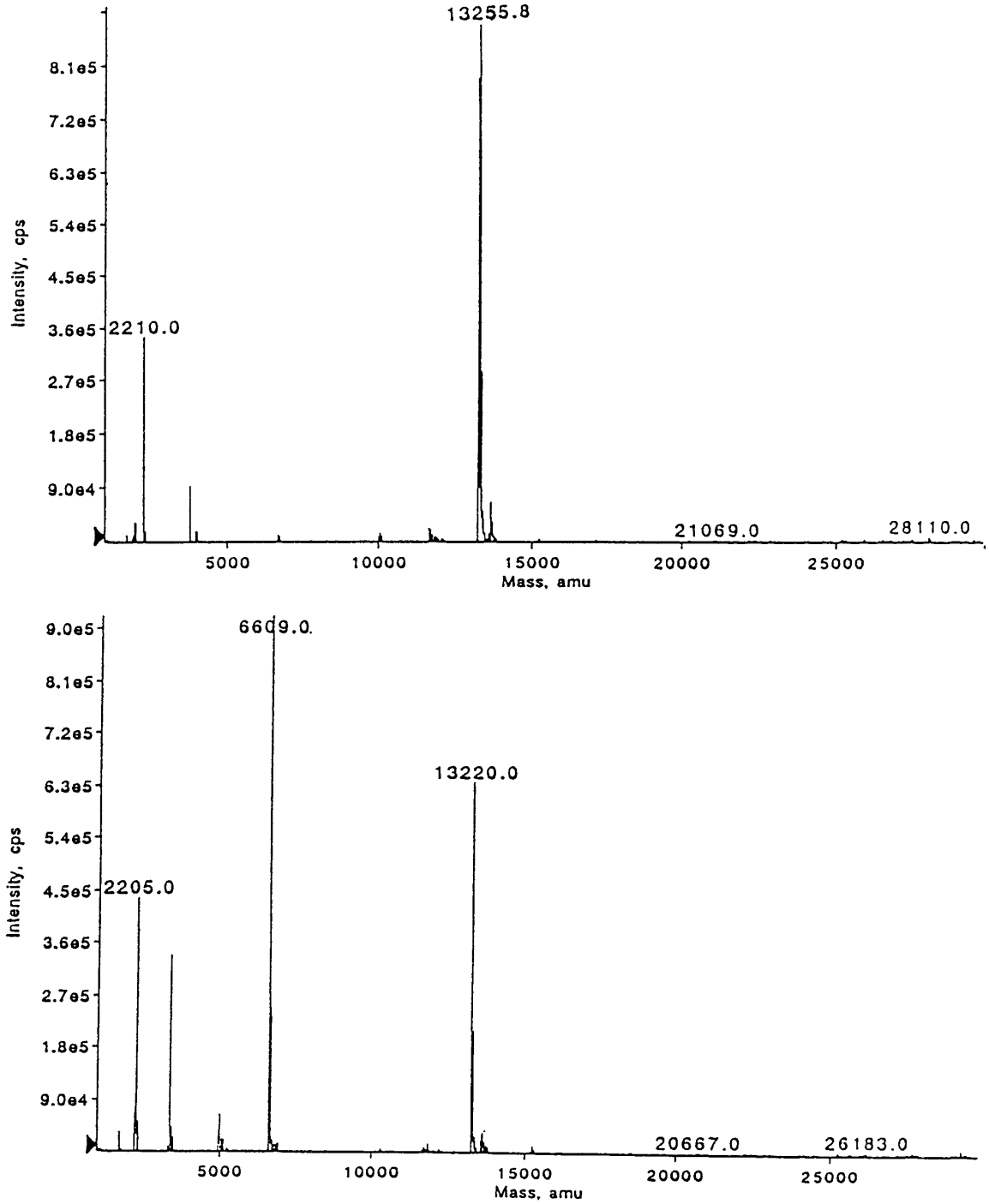

FIG. 3. Mass spectra of recombinant $PLA_{2\text{-}1}$ and $PLA_{2\text{-}2}$ determined by electrospray ionization mass spectrometry. The refolded and lyophilized $PLA_{2\text{-}1}$ and $PLA_{2\text{-}2}$ were dissolved in 50% acetonitrile containing 0.1% acetic acid to make a final concentration of 10 μM. The sample was then analyzed in an API 100 mass spectrometer (PE SCIEX) at an infusion rate of 5 μl/min. The spectra were analyzed with software (BioToolBox) from the manufacturer.

with the expected values. As shown in Fig. 4, the CD spectrum of the recombinant wild-type $PLA_{2\text{-}1}$ was also similar to native PLA_2 isolated and purified from crude venom (16), reflecting the prominent α-helical secondary structure present in both recombinant and native PLA_2 and attesting to the validity of our refolding conditions.

To further characterize the recombinant PLA_2, the enzymatic activity was measured using the pH stat technique. Recombinant $PLA_{2\text{-}2}$, which is the minor compo-

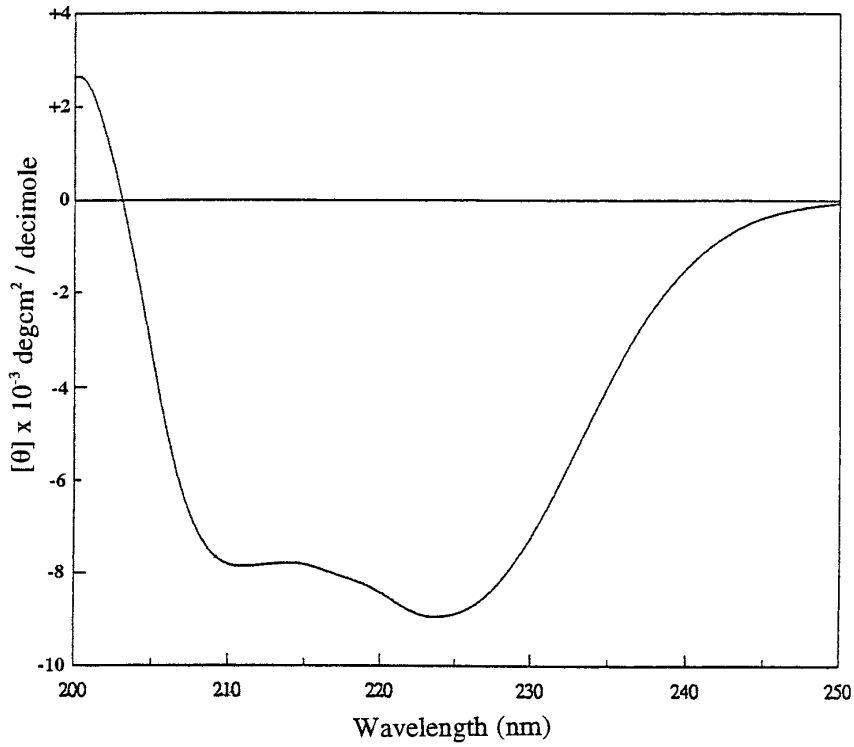

FIG. 4. CD spectrum of the recombinant $PLA_{2\text{-}1}$. CD spectra were measured on a Jasco J-720 spectropolarimeter at a concentration of 1 mg/ml in H_2O with a cell pathlength of 0.1 mm. CD spectra were generally recorded and smoothed by signal averaging of five scans. The final spectrum shows the typical α-helical secondary structure with two minima at 209 and 223 nm.

nent with two amino acid residues differences from the major $PLA_{2\text{-}1}$, shows a lower activity. The alterations in conformation around the vicinity of active site could be a possible cause to account for the difference in activity. The activity of $PLA_{2\text{-}1}$ was a little higher than native PLA_2 (Table 1). The result is quite reasonable when considering the relative contribution of $PLA_{2\text{-}1}$ and $PLA_{2\text{-}2}$ to the native PLA_2. However when L-α-phosphatidylcholine from egg yolk (Sigma) was used as a substrate, the activity of $PLA_{2\text{-}1}$ was about 3/4 that of native PLA_2 (data not shown). There is probably some difference in substrate specificity between $PLA_{2\text{-}1}$ and $PLA_{2\text{-}2}$.

Characterization of the expressed PLA_2 mutants. Having developed the ability to express and refold the two isoforms of PLA_2 with activity, we then used site-directed mutagenesis to evaluate the structural and functional roles of the catalytic dyad (Asp-93 and His-47) commonly present in the Group-I PLA_2. The His-47 was changed to Ala and Arg, and the Asp-93 to Asn. The resulting mutants are named H47A, H47R, and D93N, respectively. The activities of all mutant enzymes decrease significantly in comparison with that of the wild-type enzyme (Tables 2 and 3). Among them, H47A and H47R mutants show no enzymatic activity, indicating the essential role of His-47 in PLA_2 catalytic activity. Change of the His residue might alter the H-bonding network connecting the active residues as exemplified in the catalytic mechanism of most serine proteases and secretory PLA_2 (17). It is noteworthy that mutant D93N retained little enzymatic activity, in contrast to that of the homologous porcine pancreatic PLA_2

TABLE 2

Activities of Recombinant $PLA_{2\text{-}1}$ and Its Mutants

	Amino acid residue	Relative activity (%)
$PLA_{2\text{-}1}$	47-H, 93-D	100.0%
$PLA_{2\text{-}1}$ (H47A)	47-**A**, 93-D	No activity
$PLA_{2\text{-}1}$ (H47R)	47-**R**, 93-D	No activity
$PLA_{2\text{-}1}$ (D93N)	47-H, 93-**N**	14.1%

TABLE 3

Activities of Recombinant $PLA_{2\text{-}2}$ and Its Mutants

	Amino acid residue	Relative activity (%)
$PLA_{2\text{-}2}$	47-H, 93-D	100.0%
$PLA_{2\text{-}2}$ (H47A)	47-**A**, 93-D	No activity
$PLA_{2\text{-}2}$ (H47R)	47-**R**, 93-D	No activity
$PLA_{2\text{-}2}$ (D93N)	47-H, 93-**N**	1.5%

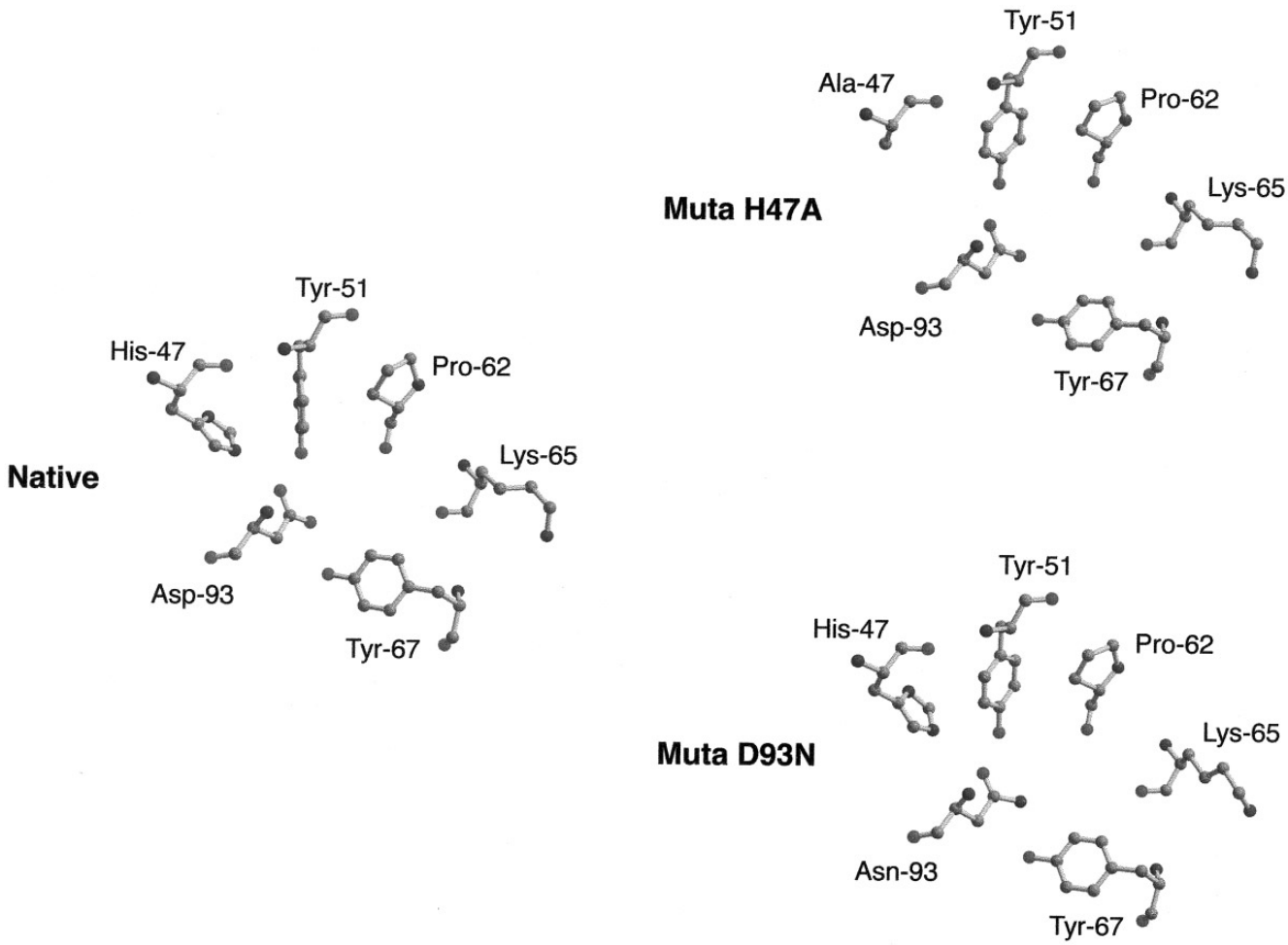

FIG. 5. Computer graphic stereoview of the active sites within the catalytic network for native PLA_{2-1} and its two site-specific mutants H47A and D93N. Note that the aromatic ring of Tyr-51 of native enzyme has been twisted to a great degree in both mutants.

mutant (D99N) which showed little loss in enzyme activity (18). In pancreatic PLA_2, the catalytic triad is composed of Asp, His, and a water molecule and there is a delicate arrangement with other residues such as N-terminal residue plus Tyr-52 and Tyr-73 to form an extensive H-bonding network to effect the efficient phospholipid hydrolysis (19,20). For venom PLA_2 this H-bonding network may be affected much more than that of pancreatic PLA_2 by Asp-93 → Asn mutation resulting in an almost inactive enzyme such as that of PLA_{2-2} (D93N) (Table 3). In this respect, venom PLA_2 is similar to trypsin of serine proteases, whose catalytic triad is composed of Asp, His, and Ser. Replacing the charged Asp residue by a neutral Asn, there was a dramatic loss of catalytic activity by a factor of 104 folds due to some drastic conformational change in the active site (21). The present work corroborates that the mutation of Asp-93 residue to Asn could possibly cause conformational changes of the surrounding residues in the active catalytic center as envisioned and depicted in Fig. 5. It is conceivable that perturbation in hydrogen-bonding network by site-specific mutation substantially effects the connection between the catalytic site and the interfacial binding site, which involves binding of the enzyme to the lipid-water interface. Therefore Asp-93 may be involved in the stabilization of the imidazolium intermediate of His-47 and the orientation of His-47 relative to Tyr-51 plus Asp-93 and Tyr-67, the four most conserved and essential residues involved in PLA_2 catalysis. With the exception of bee venom PLA_2 (22), the catalytic network formed by these four residues is invariant among all secretory PLA_2, including Group I snake enzymes in this study (23).

CONCLUSION

The conformational stability of PLA_2 is very high when compared with most enzymes, giving credence to the claim of being among the most stable of enzymes in nature (24). In addition to enzymatic activity, snake

PLA_2 show diverse pharmacological effects. Such an extensive functional diversity within this group of structurally similar proteins has aroused great interests regarding the question of the relationship between their structure and function, especially in light of the recent finding that this enzyme may be involved in the metabolism of arachidonic acid and eicosanoids (25). Since we have developed a highly efficient system for the expression and refolding of two major PLA_2 isoforms with full activity in this study based on our previous cDNA sequencing and cloning of cobra PLA_2 (8,9), it would be of importance to do detailed structural and functional analyses on some site-specific mutant enzymes in order to gain some insight into the structural variations between different members of Group-I PLA_2 such as those from snake and pancreas sources and to unravel the intriguing evolutionary and developmental process leading to generation of multiple isoenzymes in animal kingdom.

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