Phospholipase A2 Engineering: Structural and Functional Roles of Highly Conserved Active Site Residues Tyrosine-52 and Tyrosine-73

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ABSTRACT: Site-directed mutagenesis was used to probe the structural and functional roles of two highly conserved residues, Tyr-52 and Tyr-73, in interfacial catalysis by bovine pancreatic phospholipase A2 (PLA2, overproduced in Escherichia coli). According to crystal structures, the side chains of these two active site residues form H-bonds with the carboxylate of the catalytic residue Asp-99. Replacement of either or both Tyr residues by Phe resulted in only very small changes in catalytic rates, which suggests that the hydrogen bonds are not essential for catalysis by PLA2. Substitution of either Tyr residue by nonaromatic amino acids resulted in substantial decreases in the apparent $k_{cat}$ toward 1,2-dioctanoyl-sn-glycero-3-phosphocholine (DC$_8$PC) micelles and the $v_0$ (turnover number at maximal substrate concentration, i.e., mole fraction = 1) toward 1,2-dimyristoyl-sn-glycero-3-phosphothanolamine (DC$_{14}$PM) vesicles in scooting mode kinetics [Berg, O. G., Yu, B.-Z., Rogers, J., & Jain, M. K. (1991) Biochemistry 30, 7283–7297]. The Y52V mutant was further analyzed in detail by scooting mode kinetics: the E → E* equilibrium was examined by fluorescence; the dissociation constants of $E^*$, $E^*$P, and $E^*$1 ($K_{M}^*$, $K_{P}^*$, and $K_{I}^*$, respectively) in the presence of Ca$^{2+}$ were measured by protection of histidine-48 modification and by difference UV spectroscopy; the Michaelis constant $K_{M}^*$ was calculated from initial rates of hydrolysis in the absence and presence of competitive inhibitors; and the turnover number under saturating conditions ($k_{cat}$, which is a theoretical value since the enzyme may not be saturated at the interface) was calculated from the $v_0$ and $K_{M}^*$ values. The results indicated little perturbation in the interfacial binding step (E to $E^*$) but ca. 10-fold increases in $K_{P}^*$, $K_{I}^*$, $K_{M}^*$, and a <10-fold decrease in $k_{cat}$. Such changes in the function of Y52V are not due to global conformational changes since the proton NMR properties of Y52V closely resemble those of wild-type PLA2; instead, it is likely to be caused by perturbed enzyme-substrate interactions at the active site. Tyr-73 appears to play an important structural role. The conformational stability of all Tyr-73 mutants decreased by 4–5 kcal/mol relative to that of the wild-type PLA2. The proton NMR properties of Y73A suggested significant conformational changes and substantially increased conformational flexibility. These detailed structural and functional analyses represent a major advancement in the structure-function study of an enzyme involved in interfacial catalysis.

Phospholipase A2 (PLA2) catalyzes the hydrolysis of the 2-acyl ester bond of 3-sn-phosphoglycerides. Central to the activity of this enzyme are the absolutely conserved Asp-99 and His-48, termed the catalytic dyad in the fashion of serine proteases. Hydrogen-bonded to Asp-99 are the highly conserved Tyr-52 and Tyr-73. With the exception of bee venom PLA2 (which has only one analogous tyrosine; Scott et al., 1990), the "catalytic network" (Brunie et al., 1985) formed by these four residues is invariant among all secretory phospholipases A2 (van den Bergh et al., 1987; Verheij et al., 1981; Maraganore et al., 1987) and occupies virtually identical positions in all crystal structures (Renetseder et al., 1985; Achari et al., 1987). Additionally, it remains intact in the crystal structures of PLA2 complexes with an sn-2 amide analogue (Thunnissen et al., 1990) and with a transition-state analogue inhibitor (White et al., 1990; Scott et al., 1990). As depicted in Figures 1 and 2, the catalytic network is connected via a water molecule to the N-terminal region located on the surface of the enzyme, which has been suggested to be part of the "interfacial recognition site" on the basis of spectroscopic studies and the use of chemically modified or semisynthetic enzymes (Volwerk & de Haas, 1982; van Dam-Mieras et al., 1975; van Scharrenburg et al., 1982; Dijkstra et al., 1984; Volwerk & de Haas, 1982). Speculation regarding the roles of the two tyrosines in PLA2 function has persisted (Brunie et al., 1985; Renetseder et al., 1985) ever since these structural

1 Abbreviations: CD, circular dichroism; CMC, critical micelle concentration; COSY, correlated spectroscopy; 1D, one-dimensional; 2D, two-dimensional; DC$_8$PC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DC$_{14}$PC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DC$_8$PM, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DC$_{14}$PM, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DC$_8$P, 1,2-dioleoyl-sn-glycero-3-phosphophytyl; DC$_{14}$P, 1,2-dioleoyl-sn-glycero-3-phosphophytyl; edeoy-LPC, 1-hexadecylpropanoic acid; DTPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DTPM, 1,2-dioleoyl-sn-glycero-3-phosphophytyl; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; PLA2, phospholipase A2; Tris, 2-amino-2-((hydroxymethyl)-1,3-propanediol; WT, wild-type.
The results show that the H-bonds between Y52/Y73 and the carbonylate of Asp-99 are not essential to catalysis, but the aromatic side chains of both are required for optimal activity. Tyr-73 has been shown to play important structural roles whereas Tyr-52 has been shown to play significant functional roles. A preliminary account of part of this result has been published (Dupereur et al., 1990). Related results for porcine PL2a have also been reported to Kuipers et al. (1990). These two earlier papers reported only conventional micellar and monomeric kinetic data, but the results are generally consistent between porcine and bovine PL2a.

MATERIALS AND METHODS

Materials and Routine Procedures. Oligonucleotides were obtained from the Biochemical Instrument Center at the Ohio State University. The following lipids used in this study were prepared and described as previously: DTPM (Jain et al., 1986); DC-PC (Jain & Gelb, 1989); HDNS (Jain & Vas, 1987); MJ33 (Jain et al., 1991d). All other phospholipid substrates were purchased from Avanti Polar Lipids (Birmingham, AL). Ultrapure guanidino hydrochloride was purchased from ICN Biochemicals. Other chemicals and biochemicals were of the highest quality available commercially. CD spectra were recorded on a JASCO J-500C spectropolarimeter using a thermostatted quartz microcell and processed using DP-500/AT system (version 1.29) software.

Construction and Purification of Mutant Enzymes. Site-directed mutants were constructed using an Amersham mutation kit according to the manual provided by the manufacturer. The position 52 mutant enzymes were constructed from the oligonucleotides 5'GC TGG TCT CTT GCA ATT ATG 3' (Y52K) and 5'GC TGG TCT AGA A/G/A/C/A 3' (G52R). Confirmed mutations with the second oligonucleotide were AAQ (Y52F) and AAC (Y52V).

Recombinant PL2a was isolated from the Escherichia coli expression host, BL21 (DE3)[pLYS], carrying the pTO-prop2 plasmid (Deng et al., 1990). All PL2a enzymes were purified as described elsewhere (Nieuwhuizen et al., 1991) with the following modifications. Following lysing and refolding, the pH of the protein solution was lowered to 4.8 with glacial acetic acid and most of the material was removed by centrifugation. The supernatant was dialyzed overnight against distilled water at 4°C and subsequently loaded onto a small Sepharose-4B column. This column was bound to the 9S form and removed many impurities prior to trypsin digestion. Activation and anion-exchange chromatography were conducted as previously described.

Kinetic Analysis. Activities toward micellar and monomeric PC substrates were conducted at 45°C on a Radiometer RIKS titration system as previously described (Nieuwhuizen et al., 1991). The apparent Vmax and Kms were determined from Eadie-Hofstee plots (Atkins & Nimmel, 1975) by v vs [S] by linear regression analysis. The apparent Km were calculated from Vmax on the basis of a molecular weight of 13,500. The specificity activity toward monomeric DC-PC was assayed at 5 mM. The negatively charged head group of DC-PC was deoxycholate (at 15 mM) were prepared and assayed as described previously (Nieuwhuizen et al., 1991). Kinetic analysis of PL2 in the micelles were carried out as described previously using a pH-stat method under first-order (Jain et al., 1986; Jain & Gelb, 1989; Berg et al., 1991) or zero-order conditions (Berg et al., 1991a; Jain et al., 1991c).

The data reported for DC-PC are the initial rates with 1 mM substrate at 23°C. Since the substrate concentration dependence of the rate of hydrolysis of this substrate does not exhibit anomalous behavior at the CMC, presumably because proteinase PL2a forms premicellar aggregates with small CMC, the data were analyzed using the same method as that used for the full bound to the interface in this system (Jain & Rogers, 1989).

Dissociation Constants. (a) Protection Methods. The equilibrium constant for the association of lipid for light scattering (calcium, inhibitors, products, and the other analogue DTPM) bound to the active site of PL2a at the interface or in the aqueous phase was determined calorimetrically. A stock solution of alkylation of His-48 by 2-bromo-4-nitrocatecholophosphate as described elsewhere (Jain et al., 1991a). Briefly, PL2a (30 mM) was dissolved in 0.1 M sodium phosphate buffer, pH 7.3, in the presence of 2 mM dodecyl-PP, 0.8 mM 2-bromo-4-nitrocatecholophosphate, and the appropriate ligands. At various time intervals, aliquots were taken, diluted 1:100 with EDTA (pH 7.3), and the residual enzyme was diluted into an appropriate assay mixture (Jain et al., 1991c; Nieuwhuizen et al., 1974; Radvanyi et al., 1989). The nonlinear regression profile of the residual PLA2 activity as a function of time provided the rate constant for inactivation. The equilibrium dissociation constant under a given set of conditions was calculated from the Scatchard-Unter equation described elsewhere (Jain et al., 1991a).

(b) Spectroscopic Methods. Binding of PL2a to DTPM vesicles was studied by monitoring the resonance energy transfer from the tryptophan residue (Trp-3) on the PL2a (excitation at 285 nm) to the dansyl probe on the HDNS present in the vesicles or by direct excitation at 347 nm) monitored the fluorescence emission of the HDNS without energy transfer (Jain & Vas, 1987). All measurements were carried out on an SLM 4800S spectrofluorometer. Solutions contained vesicles of DTPM (0.2 mg) and HDNS (4 μg) in 1.5 mL of 10 mM Tris-HCl, 0.5 mM CaCl2, pH 8.0. The fluorescence of the solution was monitored following addition of appropriate amounts of the enzyme. Emission was monitored at 495 nm and the slit width was 4 nm for both emission and excitation.

The absorbance of PL2 in the 230-340-nm region was monitored by a UV-visible spectrophotometer (Hewlett-Packard Model 8425) equipped with a diode array detector. The standard software package for this spectrophotometer included automated quantitation and concentration algorithms that were necessary for obtaining the difference spectra. The resolution of the spectra was 2 nm, and therefore the peaks in the difference spectra appear sharper than those reported by Hilte et al. (1983). Under certain conditions, we noticed minor contributions from scattering by micelles. In such cases, the experiments were designed such that these contributions could be subtracted. Typically, the PL2a concentration was 35 mM in 20 mM Tris-HCl, 0.5 mM CaCl2. The first addition was usually the neutral detergent (deoxycholate-LPC), and then appropriate amounts of ligands were added in small volumes such that the overall dilution was less that 5%. The spectra were corrected for such dilution before substractions were made for obtaining the difference spectra.

GdnHCl-Induced Denaturation and Circular Dichroism Spectroscopy. Enzyme samples for GdnHCl denaturation were prepared by dissolving the lyophilized enzyme into 10 mM sodium borate, 0.1 mM EDTA, pH 8.0, to a concentration of approximately 3 mg/mL, as determined spectrophotometrically (Völker & de Haas, 1982). A stock solution of GdnHCl near 8.5 M was prepared in the same buffer and adjusted to the refractive index (Nondon, 1972). Both solutions, along with the buffer, were used to prepare 15-20 different solutions at various concentrations of GdnHCl. All solutions were made in 20-mM Tris buffer near 0.05 mM/μL. Samples were incubated at 30°C for at least 10 min prior to measurement. Typically five scans were collected at a signal averaged to produce a CD spectrum from 250 to 200 nm. For each sample, a background spectrum of a solution of the appropriate concentration of GdnHCl in borate was collected and subtracted from the sample spectrum. The resulting observed ellipticity at 222 nm was recorded as a measure of the degree of structure present in the enzyme at each concentration of GdnHCl (Greenfield & Fasman, 1969).

NM R Analysis. The enzyme sample was dissolved in D2O (99.9 atom % D, MSTD Isotopes), and salts were added with stock solutions of NaCl, KCl, both in D2O. The solution was kept at room temperature for 1 h to allow for deuteron exchange and then lyophilized. After the exchange process was repeated, the sample was dissolved in 0.4-0.5 mM of D2O (99.99 atom % D, Cambridge Isotope) and the pH (uncorrected pH from pH meter reading) was adjusted to 4.0-4.1 with DCl and Na2OD stock solutions (MSTD Isotopes). The final NMR samples contained 1.0 mM enzyme, 50 mM CaCl2, and 300 mM NaCl. Proton NMR spectra were recorded on a Bruker AM 500 spectrometer at 37 °C, unless otherwise specified. Chemical shift references are referenced to internal sodium 3-trimethylsilyl[1,2,3,3-d3]propionate (TSPM).

RESULTS

Catalytic Rates. The activities of the mutant enzymes were measured at 37°C in buffer containing DC-PC micelles, DC-PC monomers, DC-PC micelles, and DC-PC/PC deoxycholate micelles. The results are listed in Table 1, and the significance of each assay is briefly ex-
Table I: Summary of Kinetic Data for WT and All Mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>DCPC Miecle</th>
<th>DCPC Monomer</th>
<th>DCPC Micelle</th>
<th>DCPC-Me Micelle</th>
<th>DCPC-PG Micelle</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>330</td>
<td>675</td>
<td>1.4</td>
<td>1.2</td>
<td>930</td>
</tr>
<tr>
<td>Y23F</td>
<td>330</td>
<td>265</td>
<td>1.4</td>
<td>0.9</td>
<td>382</td>
</tr>
<tr>
<td>Y73F</td>
<td>560</td>
<td>580</td>
<td>2.0</td>
<td>0.4</td>
<td>1700</td>
</tr>
<tr>
<td>Y13F/Y73F</td>
<td>380</td>
<td>265</td>
<td>2.8</td>
<td>0.14</td>
<td>2350</td>
</tr>
<tr>
<td>Y13V</td>
<td>11</td>
<td>5.1</td>
<td>0.7</td>
<td>0.07</td>
<td>90</td>
</tr>
<tr>
<td>Y23K</td>
<td>40</td>
<td>5.1</td>
<td>1.7</td>
<td>0.9</td>
<td>670</td>
</tr>
<tr>
<td>Y73A</td>
<td>40</td>
<td>4.0</td>
<td>1.8</td>
<td>0.7</td>
<td>330</td>
</tr>
<tr>
<td>Y73K</td>
<td>30</td>
<td>4.0</td>
<td>1.5</td>
<td>0.5</td>
<td>130</td>
</tr>
</tbody>
</table>

*Specific activity (pU) at 5 mM substrates. *Specific activity (pU) at 1 nM substrates. *Mixed micelles with 3 equivalents of sodium deoxycholate.

Plained as follows. The data reported for DCPC monomers, DCPC micelles, and DCPC-PG/deoxycholate micelles are all specific activities at 1 nM enzyme (per enzyme molecule at a fixed substrate concentration) and will not be seriously interpreted. In the DCPC micelle assay, KD and Kd were derived with the assumption that classical Michalis–Menten kinetics are obeyed. Since the assumption may not be valid as explained in the Discussion, KD and Kd should be considered as apparent values (Kd,app) and KD,app respectively. The DCPC-PG system is the slowest mode assay developed by Jain and co-workers (Jain & Berg, 1989; Jain et al., 1991), which permits measurements of kinetic constants exclusively at the interface, without being complicated by the E to E* equilibrium shown in Figure 3. The v0 values listed in Table I are defined by equation (1), i.e., V0 = kcat*Enzyme/[(Km + E)].

This equation is analogous to the Michaelis–Menten equation, except that the substrate concentration X0 (therefore also Kd*), the Michaelis constant in the soaking mode is represented by molar fractions. The initial v0 is actually the turnover number at the concentration "seen" by the enzyme bound to the interface. It can be considered as the turnover number at the maximal rate concentration since the actual molar fraction cannot exceed 1 (X0 = 1 for DCPC-PG vehicles), but it is not necessarily a saturating concentration for the formation of the E*5 complex. The results from all the assay methods indicate that substitution of Tyr-52 and/or Tyr-73 by phenylalanine resulted in only small changes in the activity, which suggests that the hydrogen bonding between these two tyrosine residues and the carbonyl of Asp-99 is not essential to catalysis. However, replacement of Thr-52 or Tyr-73 by nonaromatic amino acids, including some which can function as H-bond donors, resulted in substantial decreases in the Kd,app toward DCPC micelles.

Figure 4: CD spectra of WT PL2A in the native form (curve a) and the fully denatured form (curve b). The CD spectra were run for 10 mM sodium borate, 0.1 mM EDTA, 0.05 mg/ml PL2A, pH 8.0. (b) in the presence of 8.5 M GdnHCl.

(120-750-fold) and smaller changes in the v0 toward DCPC-PG vesicles (<30-fold). The possible reasons for the quantitative variations in the different assay systems will be discussed later. However, the results clearly suggest that the aromatic rings of Tyr-52 and Tyr-73 are important in the function and/or structure of PL2A.

Structural Properties. The lack of functional roles for the phenolic hydroxyls in the hydrophobic regions of Tyr-52 and Tyr-73 raises two structural questions: (i) whether the roles of the phenolic hydroxyls have been replaced by water molecules or other residues at the active site; and (ii) whether the two phenolic hydroxyls contribute to conformational stability. The high-resolution X-ray structure of the Y23F/Y73F double mutants does not reveal any additional water around the two residues (C. Sekharadู, B. Ramakrishnan, B. Huang, R.-T. Jiang, C. M. Dupereur, M. Sundaralingam, and M.-D. Tsai, unpublished results). The second question was addressed by determining the free energy of denaturation. For nonaromatic mutants, it is important to determine if the decreased activity is caused by changes in the local conformation of the enzyme before the perturbation in the kinetic data can be interpreted. This problem was addressed by 1D and 2D proton NMR analysis.

(a) Conformational Stability. Since this is the first systematic study of this type for bovine PL2A and its mutant enzymes, we experimented with a number of denaturing and observation methods in an effort to select the most sensitive approach. Chemical denaturation provides the most complete and reversible data for unfolding proteins (Pace & Marshall, 1980), and GdnHCl is among the strongest of reversible chemical denaturants (Pace & Marshall, 1980) and is especially suited for application to the rugged PL2A. Circular dichroism spectroscopy is quite sensitive to changes in the secondary structure of proteins, particularly those with high helical content like PL2A (Banci et al., 1989). Elution pressure and m values, along with the midpoint of the denaturation curve (D), are listed in Table II. Substitution of Tyr-52 or Tyr-73 by Ala lowered the D of GdnHCl by 4-5 kcal/mol, suggesting that Tyr-73 contributes significantly to the conformational stability of PL2A. The changes for the Tyr-52 mutants, particularly Y73A, are much less pronounced.

Figure 5: Denaturation curves. Conditions are as described in Figure 4. Fractions eluted with 1-6 (A-B)/(F(A)-F(6)), where F(6) and F(6) are the observed ellipticities at 222 nm for native and denatured states, respectively, and F(0) is the observed ellipticity at a given GdnHCl concentration.

Figure 6: One-dimensional proton NMR spectra of WT (A), Y23F (B), Y73F (C), and Y73A (D). Sample conditions are described in Materials and Methods.

Figure 7: The phase-sensitive NOESY spectrum of WT, with a 200-ms mixing time, at 37 °C. The identified spin systems are labeled as follows: 1) of the 7 Tyr's (7Yr), 2) of the 4 phenylalanines (F7-F20), and the single tryptophan (W). Two of the unidentified spin systems are labeled as Xa and Xb. The NOE spectra of WT, Y23F, and Y73F by 4-5 kcal/mol, suggesting that Tyr-73 contributes significantly to the conformational stability of PL2A. The changes for the Tyr-52 mutants, particularly Y73A, are much less pronounced.

Figure 8: CD spectra of WT PL2A in the native form (curve a) and the fully denatured form (curve b). The CD spectra were run for 10 mM sodium borate, 0.1 mM EDTA, 0.05 mg/ml PL2A, pH 8.0. (b) in the presence of 8.5 M GdnHCl.
The phase-sensitive NOESY spectrum of Y73A with a 200-ms mixing time at 37 °C shows interresidue NOEs to the tyrosine residues of Y72A and Y69 which are also identified in Y73A. Features led to the assignment of Tyr-52 to Yb in the tenative assignment of Tyr-69 to Ye since Tyr-69 is the tyrosine residues closer to Tyr-52 according to the crystal structure shown in Figure 2. Although the proton NMR resonances of porcine PLA2 have been completely assigned (Dekker et al., 1991a), only some spin systems have been identified for bovine PLA2 (Fishel et al., 1989). Our assignments on the basis of the differences between WT and Y52V agree with theirs. For WT and Y52V, the chemical shifts of the aromatic rings have been identified from the COSY spectra (not shown) and NOESY spectra and are listed in Table III. Only five resonances (underlined in Table II) in the NOE spectrum of WT and Y52V, and the largest difference is only 0.09 ppm. Thus it can be concluded that the conformations of WT and Y52V are little perturbed. On the other hand, the conformations of Y73A appears to be substantially perturbed judging from the two-dimensional NOESY and COSY spectra, and it was not possible to identify all of the corresponding spin systems for Y73A. The NMR properties of Y73S are very nearly the same (Dupureur et al., 1992). These results are consistent with the observation that Y52V is least perturbed and Y73A and Y73S are greatly perturbed in conformational stability, as described in the previous section.

The lack of intermediate NOEs in the NOESY spectrum of Y73A led us to suspect whether this mutant retains the integrity of the PLA2 structure. The answer is positive since the 2D NOESY spectrum of Y73A (not shown) is almost identical to that of WT and the mutant still retains substantial activity as shown in Table I. It is also unlikely that Y73A consists of mixture of active and denatured proteins since the N values determined from the same amount of Y73A and WT are the same within experimental errors (see a later section). The lack of NOE, therefore, reflects flexibility in conformation, which again agrees with the large decrease in the \( \Delta G_{\text{HDD}}^{20} \) of this mutant.

Equilibrium Dissociation Constants of WT and Y52V. Since Y52V showed the largest decrease in turnover numbers and very small perturbations in structure, it was chosen for detailed analysis of kinetic and equilibrium constants. The purpose is to pinpoint the specific step(s) which differ between Y52V and WT. Most of the measurements have been performed according to the recent reports for porcine pancreatic PLA2 (Berg et al., 1991; Jain et al., 1991a). Since our system is bovine pancreatic PLA2, it was necessary to use the constants for both WT and Y52V. The specific experiments are explained as follows.

(a) The E to E' Step. As shown in Figure 10, the relative fluorescence of HDNS in DTPM vesicles increases with the amount of WT or Y52V. As shown elsewhere for porcine pancreatic PLA2 (Jain & Vaz, 1987), such an increase in the fluorescence intensity is due to the resonance energy transfer from the tryptophan donor on the protein (Trp-3) to the dansyl acceptor localized at the bilayer interface; the change is not observed when the protein is added to a suspension of HDNS alone. Thus, the equilibration among the conformational states of the enzyme, the maximal change is observed when the lipid to enzyme mole ratio approaches 40 for both Y52V and WT. Since the initial rising phase is linear and steep in both cases, it is not possible to compute the equilibrium dissociation constant for the protein bound to the interface (Jain et al., 1992). However, in the case of porcine PLA2, other experiments (Jain et al., 1982, 1986a; Jain & Vaz, 1987) suggested that the apparent dissociation constant is \( < 1 \mu M \) when such binding curves are observed, i.e., virtually all molecules of both Y52V and WT were located at the interface under the conditions used for the kinetic measurements with DTPM vesicles. This conclusion was further confirmed by the \( N_p \) values determined under catalytic conditions described in a later section.

(b) Interfacial Binding of Ligands to \( \text{E}^* \) by the Protection Method. The equilibrium dissociation constants for the binding of a variety of ligands to PLA2 bound to the interface of deoxy-LPC are summarized in Table IV. As described by Jain et al. (1991a), these parameters were obtained from the halffat times for the hydrolysis of HDNS-48 at the catalytic site of PLA2 at the interface of the neutral detergent, deoxy-LPC, in the presence and in the absence of the ligand. The half-times of inactivation for the hydrolysis of WT and Y52V in the aqueous phase (without Ca\(^{2+}\)) were approximately the same (first row in Table IV), which suggested that the accessibility of the His-48 at the catalytic site was not significantly altered in this mutant. The second and third rows in Table IV indicate that the mutants behave similarly to WT in the affinity for calcium in the aqueous phase (E-Ca) and in the interface of deoxy-LPC micelles (E-Ca). The next two rows show that
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Table IV: Equilibrium Dissociation Constants of WT and Mutant Enzymes* complexes parameter unit WT Y52V Y52F Y73S E inactive halftime min 1.5 1.3 0.4 0.7 E-Ca⁺-mM 1.8 0.31 0.34 0.13 0.7 E-Ca⁺-DPC 1.2 0.1 0.12 0.5 0.33 E-deoxy-LPC mole fraction 0.28 0.01 0.15 0.2 0.1 E-deoxy-LPC · E-DTPM mole fraction 0.05 0.02 0.09 0.04 0.02 E-Ca⁺-DPC · E-DTPM mole fraction 0.01 0.03 0.02 0.04 0.02 E-Ca⁺-DPC · E-Ca⁺-DTPM mole fraction 0.04 0.05 0.04 0.02 0.01 E-Ca⁺-DTPM · E-DTPM mole fraction 0.03 0.04 0.02 0.02 0.01 * Determined by spectrophotometric characterization.

Figure 1: UV difference spectra for WT (0.035 mM) obtained by adding (a) 1.9 mM deoxy-LPC, (b) 1.9 mM deoxy-LPC and 0.06 mM M33J, and (c) 0.2 mM CaCl₂. The buffer contained 50 mM Tris-chloride and 1 mM CaCl₂, pH 8.0.

The affinities for deoxy-LPC in the absence of calcium (E* · deoxy-LPC) and in the presence of calcium (E* · Ca⁺-deoxy-LPC) are weak for both WT and the mutants, suggesting that a good degree of distinction between both WT and the mutants. These results justify the use of this technique to compare the relative binding affinities of WT and mutants to substrate analogues and inhibitors.

The data in rows 7 and 8 show that the dissociation constants for the substrate analogue DTPM and the products of hydrolysis of DCIPC are higher by an order of magnitude for Y52S than for WT and Y52F. These results suggest that both the E* to E* equivalence and the E* to E* equilibrium shift toward E* for a factor of 10.

The affinity toward M33J is different between WT and Y52S, as shown by the concentration dependence curves in Figure 12. In Figure 12, the intensities at 292 nm in the difference spectra are plotted against the concentration of M33J in the absence (A) and presence (B) of deoxy-LPC.

Figure 12: Intensity at 292 nm in the difference spectra for WT and Y52V, as a function of the concentration of M33J in the absence (A) and the presence (B) of 1 mM deoxy-LPC. Notice that the concentration is millimolar in panel A but in micromolar in panel B. The smooth lines in panel B were the theoretical curves for a rectangular hyperbola from which the values of Kₐ* were obtained. Other conditions are as in the legend of Figure 1.

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The affinity toward M33J is different between WT and Y52S, as shown by the concentration dependence curves in Figure 12. In Figure 12, the intensities at 292 nm in the difference spectra are plotted against the concentration of M33J in the absence (A) and presence (B) of deoxy-LPC. Notice that the concentration is millimolar in panel A but in micromolar in panel B. The smooth lines in panel B were the theoretical curves for a rectangular hyperbola from which the values of Kₐ* were obtained. Other conditions are as in the legend of Figure 1.

Discussion

The preceding sections present rigorous analyses of the structural and functional roles of the highly conserved active site residues Tyr-52 and Tyr-73. The results indicate that the non-equivalence of Tyr-52 with valine (and other nonaromatic residues) lowers the affinities of the enzyme toward substrates, products, and inhibitors by ca. 10-fold and also causes a decrease in kₐ by ca. 5-10-fold. Tyr-73 clearly plays a structural role, whereas whether it also plays a functional role similar to that of Tyr-52 is uncertain. The broader implications of our results are discussed in the following sections.

Binding of the Enzyme to the Substrate. The mutation of residues 52 and 73 did not noticeably influence the E* to E* equivalence as monitored by the kinetic and equilibrium dissociation constant experiments. The fact that the E* to E* transition, but not the E* to E* transition, was perturbed in Y52S provides strong support to the previous suggestion (Ramaz & Jain, 1991; Berg et al., 1991; Jain et al., 1991a,b) that these two steps are completely independent. The results in Figure 11 clearly show that the changes in kₐ are caused by the E* form but not the E* form. These results should have a far-reaching impact on the field, since these two forms were not distinguished in most spectroscopic (Kuipers et al., 1991; Desser et al., 1991b) and calorimetric (Biltonen et al., 1991) studies. Since the alkylphosphocholine often used in these studies for converting E* to E* actually also binds to the active site (Jain et al., 1991), the E* species in these reports is a mixture of E* and E*.

Comparison between Micellar and Scooting Mode Assay Systems. In agreement with previous reports, we find that the micellar assay system is able to detect roughly 10-fold more activity than the scooting assay system. This is consistent with the notion that the scooting assay system is much more sensitive to the influence of sodium ions than the micellar assay system. The scooting assay system allows a more accurate determination of the kinetic parameters of the enzyme under physiological conditions.

The micellar assay system shows a clear advantage in terms of speed and simplicity, while the scooting assay system offers a more accurate determination of the kinetic parameters of the enzyme under physiological conditions.

Table V: Catalysts Parameters for the Hydrolysis of DCIPC by WT and Y52V parameter unit WT Y52V

The data for Y52F are similar to those of WT. The data for Y73S are not interpreted because of the structural perturbations in this mutant (Dupueur et al., 1992).

The values for WT, Y52V, and Y73S are 30, 2.5, and 7.5 s⁻¹, respectively.

* Determined by spectrophotometric characterization.

Figure 1: UV difference spectra for WT (0.035 mM) obtained by adding (a) 1.9 mM deoxy-LPC, (b) 1.9 mM deoxy-LPC and 0.06 mM M33J, and (c) 0.2 mM CaCl₂. The buffer contained 50 mM Tris-chloride and 1 mM CaCl₂, pH 8.0.

The affinities for deoxy-LPC in the absence of calcium (E* · deoxy-LPC) and in the presence of calcium (E* · Ca⁺-deoxy-LPC) are weak for both WT and the mutants, suggesting that a good degree of distinction between both WT and the mutants. These results justify the use of this technique to compare the relative binding affinities of WT and mutants to substrate analogues and inhibitors.

The data in rows 7 and 8 show that the dissociation constants for the substrate analogue DTPM and the products of hydrolysis of DCIPC are higher by an order of magnitude for Y52S than for WT and Y52F. These results suggest that both the E* to E* equivalence and the E* to E* equilibrium shift toward E* for a factor of 10.

The affinity toward M33J is different between WT and Y52S, as shown by the concentration dependence curves in Figure 12. In Figure 12, the intensities at 292 nm in the difference spectra are plotted against the concentration of M33J in the absence (A) and presence (B) of deoxy-LPC. In panel A, both WT and Y52V showed linear increases which were unrelated to the concentration of M33J in the absence (A) and presence (B) of deoxy-LPC. In panel B, the smooth lines in panel B were the theoretical curves for a rectangular hyperbola from which the values of Kₐ* were obtained. Other conditions are as in the legend of Figure 1.

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What factors might contribute to the larger "dynamic range" in the kcat values? There are two possibilities here: what kcat values and kcat,act values actually measure, and why the decreases in the kcat values are so large for DCPC-RC. Regarding the first possibility, the presence of noncovalent interactions of the enzyme to micelles (similar to the E* step in the scooting mode). The relatively small perturbation in the kcat values of micelles in the E* step in the scooting mode. However, even if the kcat values were very different, they should not be responsible for the difference in the kcat values mentioned when all enzyme molecules are bound to micelles. The kcat values of micelles at can be constantly compared with the ν values of the scooting mode. Therefore, micelles associated with micelles, the active site may not be saturated (the actual mol fraction of substrate cannot exceed 1). In reality, kcat values could be further limited by the need to accommodate the contributions of the absolutely conserved phenolic hydroxyls Tyr-52 and Tyr-73 to this network to critical activity toward agarose in the absence of sugar. This is not the case. However, kinetic results alone do not suggest any alternative structure-function relationships for these phenolic hydroxyls. Such networks in other enzymes has also been responsible for contributing to stability (Gruetter, 1987). This prompted us to pursue the possible contributions of these residues (through the hydrogen bonding network) to conformational stability. Interestingly, removal of the Tyr-73 phenolic hydroxyl by mutating to Phe, Ala, Ser, or Lys lowered the ΔG° in 4-5 kcal/mol. These changes are not significant for every direct interaction of Tyr-73 with other tyrosine residues, although the decrease in activity. Our knowledge of the largest among active site mutants. The contribution of the hydroxy of Tyr-52 appears to be less critical.

Close inspection of the crystal structure of bovine pancreatic PL2A (PDB reference 1CTB) on active site and substrate, it is found that Tyr-52, Tyr-73, and Asp-99 are located in the C-helix, the &-wing, and the E-helix, respectively (see Figure 2). The C-helix has the active site (Salter et al., 1990). However, our data analysis suggests that the conformation of Y52S is little perturbed. For Tyr-73 removal, the tyrosyl hydroxyl group is more likely to undergo a remarkable effect on ΔG°. Interestingly, further removal of the aromatic group leads to a decrease in the binding of the tyrosine residues to different functional and structural properties of the mutant enzymes in detail and have gained considerable insight into the subtle structural interactions for these tyrosines we must await further exploration of the active site before these interrelations can be fully understood.

After submission of this paper, we observed that the conformational flexibility in Y73A also occurred in Y73S and D99N and perceived that such a behavior is characteristic of (a) the active site catalytic domain to the "miniglobule" (b) aromatic residues and has been shown to be connected by hydrogen bonding. Indeed, the function of such structure-function relationships for residues participating in hydrogen bonding would be significantly diminished (Salter et al., 1990; Gruetter et al., 1987). There are also a number of examples in which aromatic residues serve to connect secondary structure elements (Nobold et al, 1972; Morre & Kretsinger, 1975). PL2A structure appears to utilize an elegant combination of both stabilizing features.

Roles of Tyrosine and Cysteine in the Conformational Stability of PL2A. The conformational stability of the porcine enzyme is reported to be near 7 kcal/mol (Pickering et al., 1991). Our results for the highly homologous bovine enzyme are in agreement with this result. The conformational stability of PL2A among the most stable of enzymes (Pace, 1990). This is consistent with the many references in the literature to the remarkable stabilities of this enzyme under many otherwise dehydrating conditions.

However, the contributions of the aromatic rings of Tyr-52 and Tyr-73 to the conformational stability of PL2A appear to be in contrast to reports of this type for other enzymes. Experiments on the model tripeptide Leu-Arg-Lys have shown that Tyr-52 and Tyr-73 each participates in edge-to-face, T-shaped, aromatic-aromatic interactions with Tyr-69 and Tyr-73. Regardless of the extent of affinity and the aromatic-aromatic side chains near the active site (including Phe-5, -22, -106 and Tyr-52, -69, -73, -75), provides a unique hydrophobic, aromatic environment that somehow augments catalysis.
Mechanism of Phospholipase A₂


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