Phospholipase A2 Engineering. The Structural and Functional Roles of Aromaticity and Hydrophobicity in the Conserved Phenylalanine-22 and Phenylalanine-106 Aromatic Sandwich

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ABSTRACT: The highly conserved phenylalanine-22 and phenylalanine-106, arranged as an aromatic sandwich, form part of an invariant hydrophobic wall that shields the active site of bovine pancreatic phospholipase A2 (PLA2) from bulk solvent [Dijkstra, B. W., Drenth, J., & Kalk, K. H. (1981) Nature 289, 604-606]. The residues have also been suggested to interact with the sn-2 acyl chain of bound phospholipid substrate [White, S. P., Scott, D. L., Otwinowski, Z., Gelb, M. H., & Sigler, P. B. (1990) Science 250, 1560-1563]. We now report the importance of these two residues in the structure and function of PLA2 in terms of aromaticity (changing to ile) and hydrophobicity (changing to Ala) and hydrophilic (changing to Tyr) character of these residues. The structural properties of the mutants were analyzed by proton NMR and by guanidine hydrochloride-induced denaturation. The functional properties were determined by measuring kinetic parameters toward various substrates in the forms of monomers, micelles, and vesicles, and by measuring equilibrium dissociation constants at the interface. The results show that (i) The conformational stability of each mutant was as good as that of wild-type PLA2; none of the mutants was significantly perturbed structurally as judged from detailed 1H NMR analysis. These results suggest that neither the Phe-22/ Phe-106 face-to-face pair nor the Phe-22/Tyr-111 edge-to-face pair plays a significant structural role. (ii) Mutations to Ile at either position 22 or position 106 resulted in only minor perturbations in activity. This suggests that the aromaticity is not important to the function of these two residues. (iii) Substitution at either of these two positions with Ala resulted in 10-200-fold decreases in catalytic activities, suggesting that the hydrophobic bulk of both residues is important for catalysis, possibly by interacting with the sn-2 acyl chain. (iv) Replacement of Phe-106 by Tyr resulted in decreased activity; the same substitution at position 22 produced an enzyme with wild-type activity. The differential behavior of the two mutants can be rationalized by the orientation of the side chains: Phe-22 points outward facing solvent, whereas Phe-106 points into the active site. (v) Detailed analysis of all the mutants using scooting mode kinetics and equilibrium measurements suggested that the perturbation in the function of F22A, F106A, and F106Y lies primarily in kcat; binding of the enzyme to the interface and binding of ligands to the enzyme at the interface are less perturbed.

The structure of phospholipase A2 (PLA2) is characterized by a large number of disulfide bonds (seven in a molecular weight of 14000 for pancreatic PLA2), an extensive hydrogen-bonding network at the active site, and a few aromatic-aromatic pairs surrounding the active site. In this study, we extend our structure-function studies of PLA2 with an exploration of the conserved aromatic pair composed of Phe-22 and Phe-106. These residues first gained attention with the report of the crystal structure of the bovine enzyme (Dijkstra et al., 1991a,b), where it was noted that the core of the active site was shielded by a highly invariant "hydrophobic wall" composed of residues Ile-9, Ala-102, Ala-103, Phe-22, and Phe-106 (Figure 1). As shown in Figure 1, the hydrophobic interactions further extend to Phe-5, Leu-41, and Tyr-111. Two of the residues involved in this hydrophobic network, Phe-5 and Ile-9, have been suggested to be part of a "hydrophobic channel" critical to binding substrates in interfacial catalysis by PLA2 (White et al., 1990).

The potential structural roles of the Phe-22/Phe-106 aromatic pair are interesting. There is growing evidence that many conserved aromatic residues participate in a variety of interactions that can contribute to enzyme structure and stability (Burley & Petsko, 1985, 1988; Serrano et al., 1991). The near-parallel geometry of Phe-22 and Phe-106 is uncommon and is expected to contribute very little stabilization...
energy (Singh & Thornton, 1985; Burley & Petkova, 1985). However, to date no experimental evidence has been reported which supports this prediction. Furthermore, Phe-22 is further involved in an edge-to-face interaction with Tyr-111, which can contribute 1.5 kcal/mole of stabilization (Burley & Petkova, 1988; Serrano et al., 1991). To assess the possible structural roles of Phe-22 and Phe-106, we evaluated a number of PLAsA mutants at these positions by measuring their conformational stability and performing 1D and 2D 1H NMR analyses.

Information regarding possible functional roles for Phe-22 and Phe-106 developed a few years ago when we coupled cryo-electron microscopy to biochemical studies to propose one of the first enzyme–substrate binding models for PLAsA (Rosario-Janes et al., 1987). We further speculated that the cryo-electron microscopy (Cryo-EM) studies were supported by the observed conformational changes in the enzyme.

The Cryo-EM studies revealed the proximity of Phe-22 and Phe-106 to the active site of the enzyme, and the possibility that these residues may play a role in the mechanism of catalysis. The Cryo-EM studies also provided insights into the structural features of the enzyme, including the location of the active site and the orientation of the substrate.

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 as described previously: DTPM (Jain et al., 1986a); DC,DM (Jain & Gelli, 1991); HDNS (Jain & Vaz, 1987); M333 (Jain et al., 1996b). All other phospholipid substrates were purchased from Avanti Polar Lipids (Birmingham, AL). Ultrapure guanidinium hydrochloride was purchased from ICN Biomedicals. 99.96% amino-9% D,O was obtained from Aldrich; TMSP and 2.0% D,O were purchased from MSD Isotopes. DCl was obtained from Cambridge Isotopes. Other chemicals and biochemicals were of the highest quality available.

The pH-stat enzyme assay reactions were performed on a Radiometer TR2S Titrator System. CD spectra were recorded on a JASCO J-500C spectropolarimeter using a thermostatted quartz microcell and processed using a D-500/AT system (version 1.29) software. Proton NMR spectra were recorded on a Bruker AM 500 spectrometer coupled to an Aspect 3000 processing unit.

Construction and Purification of Mutant Enzymes. Site-directed mutants were constructed with an Amersham or USB mutagenesis kit according to the manufacturer's instructions. The position 22 mutants were constructed from oligonucleotides in which the underscored bases in 5' CAT AAT TGT AAA TAT CAA 3' were replaced with AAT, AAT, and AGC for F22Y, F22I, and F22A, respectively. The oligonucleotides used for the construction of the position 106 mutants were 5' TTT TGA AAT ACA AAT 3' (F106Y), 5' TTT TGA AAT ACA AAT 3' (F106Y), and 5' TTT TGA AAT ACA AAT 3' (F106A). Recombinant PL2 was isolated from the E. coli strain expressing the plasmid pET-21a (DE3) or pET-32a, harboring the pGEM-2Z plasmid (Deng et al., 1990). All PL2 enzymes were purified as described elsewhere (Noel et al., 1991; Depurkar et al., 1992b).

Conformational Stability. The conformational stability of each enzyme, represented by the free energy of unfolding, ΔG, was determined by inducing the reversible denaturation of the enzyme with either 5 M guanidinium hydrochloride (Gdn-HCl) or a combination of 8 M urea. Following the loss of secondary structure by circular dichroism (CD) spectroscopy and previously described (Depurkar et al., 1992b). Measurements were taken on enzyme solutions at 0.1 mg/ml, 10 mM sodium borate, and 0.1 mM EDTA, pH 8.0, at 30 °C (Ca2+ was omitted since it further stabilizes the enzyme to the extent that a complete denaturation curve cannot be obtained within the solubility limit of Gdn-HCl).

Table 1: Free Energies of Denaturation Induced by Gdn-HCl

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ΔG (kcal/mol)</th>
<th>ΔH (kcal/M)</th>
<th>ΔS (cal/M·K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT PL2A</td>
<td>9.5</td>
<td>6.9</td>
<td>1.47</td>
</tr>
<tr>
<td>F22Y</td>
<td>11.0 (+1.5)</td>
<td>7.0</td>
<td>1.58</td>
</tr>
<tr>
<td>F22I</td>
<td>12.2 (+2.2)</td>
<td>7.0</td>
<td>1.73</td>
</tr>
<tr>
<td>F22A</td>
<td>10.0 (+0.5)</td>
<td>6.9</td>
<td>1.46</td>
</tr>
<tr>
<td>F106Y</td>
<td>11.4 (+1.1)</td>
<td>6.9</td>
<td>1.65</td>
</tr>
<tr>
<td>A68A</td>
<td>8.6 (+0.9)</td>
<td>6.9</td>
<td>1.47</td>
</tr>
</tbody>
</table>

* The error limit is estimated to be 0.5 kcal/mol.

Kcat and Km were determined from Eadie–Hofstee plots (Acton & Numino, 1975) by the use of linear regression analysis. The Kcat was calculated from Vmax and [S]0 at each substrate concentration. The Kcat was determined at 0.5 M substrate. Specific activities toward DCl were determined at 0.5 M substrate in the presence of 50 mM DCl (10 °C). These conditions, enzyme concentrations of the preincubated complex, and pH 8.0 were determined under the following conditions: reaction enzymes were formed from preincubated complexes with this substrate (Jain & Vaz, 1987). Kinetic analysis of PL2A was carried on a modified DC/PM reaction vehicle was carried out at 22 °C and pH 8.0 as described previously using the pH-stat method under first-order (Jain et al., 1986a; Jain & Gelli, 1991; Berg et al., 1991a, 1991b).

Dissociation Constants. (A) Protection Conditions. The equilibrium dissociation constants for the dissociation of ligands (calcium, inhibitors, products, and the ether substrate analog DTPM) to the active site of PL2A at the interface or in the aqueous phase were determined by monitoring the rate of dissociation of the free ligands by p-nitrophenylphosphoryl thioester (PLA) (Jain et al., 1991a). Briefly, PLA (20 mM) was incubated at 22 °C in 50 mM PIPES buffer, pH 7.2, with 5 μM DCl/PM vesicles and monitored for 1 h using a stopped-flow spectrophotometer (Model 810, Applied Photophysics, England). The rate of dissociation of the PLA was determined using an appropriate assay mixture (Jain et al., 1991a, 1991b; Niewoehnrich et al., 1974; Radzun, 1989). The nonlinear regression of the plot of the residual PLA activity as a function of time provided the rate constant for inactivation. The equilibrium dissociation constant under a set of conditions was calculated from the Scrutton–Uitter 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 PL2A (excitation at 283 nm) to the danyl probe HDNS present in the vesicles or by direct (excitation at 347 nm) monitoring the fluorescence emission of HDNS without energy transfer (Jain & Vaz, 1987). All measurements were carried out on an SLM 4800S spectrofluorimeter. Solutions contained vesicles of DTPM (0.2 mg) and HDNS (4 μg) in 1.5 ml of 10 mM Tris-HCl and 0.5 mM CaCl2, pH 8.0. The fluorescence of the solution was monitored following increasing amounts of increasing amounts of vesicles of DTPM monitored at 495 nm, and the slit width was 4 nm for emission and excitation. Under certain conditions, in the absence of reducing conditions, it was also possible to obtain values of effective equilibrium constants from measurements of the change in the absorbance at 292 nm.
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**Table I:** Chemical Shifts of the Aromatic Spin Systems for WT and Mutants

<table>
<thead>
<tr>
<th>spin system</th>
<th>assignments</th>
<th>F22A</th>
<th>F106I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fx</td>
<td>6.28</td>
<td>7.03</td>
<td>7.15</td>
</tr>
<tr>
<td>Yh</td>
<td>7.30</td>
<td>7.37</td>
<td>7.45</td>
</tr>
<tr>
<td>Yz</td>
<td>6.35</td>
<td>6.74</td>
<td>7.07</td>
</tr>
<tr>
<td>Yg</td>
<td>7.35</td>
<td>7.30</td>
<td>7.30</td>
</tr>
<tr>
<td>Wz</td>
<td>7.30</td>
<td>7.45</td>
<td>7.48</td>
</tr>
<tr>
<td>Xa</td>
<td>6.72</td>
<td>6.95</td>
<td>6.95</td>
</tr>
<tr>
<td>Xg</td>
<td>6.52</td>
<td>6.95</td>
<td>6.95</td>
</tr>
<tr>
<td>Xh</td>
<td>7.35</td>
<td>7.30</td>
<td>7.30</td>
</tr>
</tbody>
</table>

*The underlined are resonances which differ by >0.1 ppm between WT and mutated. Parentheses indicate tentative assignments.*

**Partial Resonance Assignments:** The chemical shifts of the aromatic spin systems and a few aliphatic residues for WT, F22A, and F106I are summarized in Table I. Three sources of information were utilized in our limited partial assignments: the partial assignments reported previously for WT bovine PL-A2 (Fisher et al., 1989), the spectra of various mutants of PL-A2 in our laboratory (Dasgupta et al. 1992b) and unpublished results, and distance relationships revealed by the crystal structure. Since the last criterion depends on the accuracy of the crystallographic data and the crystal structures are all, in any case, our assignments should be considered tentative until complete assignments can be obtained and solution structures calculated. The assignments and conformational calculation have been performed for the PL-A2 from porcine pancreas (Decker et al., 1991b), but unfortunately the spectral properties of the bovine and the porcine homologues are different enough that the assignments of one cannot be applied directly to the other.

**Summary of spin systems:** The designation of spin systems in Table II is based on those described by Fisher et al. (1989). Fb disappears in F106A and F106I, confirming the assignment of this spin system to the side chain Xa. The other spin systems are tentatively assigned to F22A. Fh, which persisted through all the mutants and was perturbed in the aliphatic mutants, is tentatively assigned to F5. Our NOESY spectrum of WT displays NOEs near 0 ppm; these have been named L1 and L2 and assigned to Leu-41 and Ile-9 by Fisher et al. (1989). Since these residues are located near F22 and F106 in the surrounding aromatic/aliphatic NOEs are affected by the aromatic-to-aliphatic mutations, as shown by the shifts of these aromatic peaks in the spectra of the mutants.

**Four additional aliphatic cross peaks assigned by Fisher and co-workers were located in our chemical spectrum and labeled according to their system: L2, assigned to Leu-58, and IG, assigned to Ile-95, are independently confirmed by our COSY data (not shown). M, assigned to Met-8, and A1, assigned to Ala-55, are identifiable in our spectra and are tentatively confirmed in the crystal structure, those aliphatics are all close to aromatic that are distant from the mutation site (Pro-94, Tyr-73, and Tyr-52) and provide additional information which fit with the class of conformational changes due to Pro-22 and Phe-106 substitutions.

**1H NMR Properties: Semiquantitative Comparison:** The ratios which differ between WT and the mutated residues with >0.1 ppm are underlined in Table II. There are only two such resonances in F106I: those for F5 and Y111. As shown in Figure 2, these residues are in the proximity to position 106. For F22A, there are five residues with >0.1 ppm changes in chemical shifts: F5, F106, Y111, Y52, and 19. The first three are in direct proximity to the mutated residue, and the other two are perturbed only <0.2 ppm. Thus, in all of the aliphatic mutants characterized, the changes in chemical shifts are confined to residues in the immediate environment of the mutation. The same is true for NOE cross peaks: most of the missing aromatic–aromatic NOEs in the mutants are due to interactions with proximal perturbations; the NOEs related to the active site residues Y52, Y69, and Y73 and the more distant residues W3, Y75, F94, M8, A55, L58, and D59 remain virtually undisturbed in most cases. Thus, NMR results are consistent with the structural study results that these residues appear to make a few global structural contributions.

In summary, the results of structural analysis suggest that changing the Cys-22 residue to Pro, Ile, or Ala causes little perturbation in conformation or in conformational stability. The Ile mutants are considerably more stable than WT. In addition to the perturbations in the structural roles of aromatic pairs (see Discussion), these results permit us to interpret kinetic data directly.

**Catalytic Properties:** Table III summarizes the activities of all mutants PL-A2 toward six substrates: DC-Pc, DC-PC, and DC-Pm micelles; DC-Pm vesicles, and DC-PC and DC-PC monomers. Mutations at either position resulted in only a minor reduction in activity. This suggests that the aliphatic residue is not important to the function of these two residues. Substitution at either of the two positions with Ala caused a large decrease in activity. This result suggests that the hydrophobic bulk of both residues is important for catalysis. Substitution with Tyr at position 106 results in decreased activity. This result suggests that a possible functional role of the Phe residues is to maintain the aromatic-to-aliphatic mutations, as shown by the shifts of these aromatic peaks in the spectra of the mutants.
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Table IV: Equilibrium Dissociation Constants of WT and Mutant Enzymes<sup>1</sup>

<table>
<thead>
<tr>
<th>pH</th>
<th>WT</th>
<th>F22A</th>
<th>F60E</th>
<th>F60D</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>1.5</td>
<td>1.3</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td>5.8</td>
<td>0.12</td>
<td>0.13</td>
<td>0.14</td>
<td>0.12</td>
</tr>
<tr>
<td>6.6</td>
<td>0.08</td>
<td>0.09</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td>7.4</td>
<td>0.02</td>
<td>0.03</td>
<td>0.04</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<sup>1</sup> Determined by the protection method. The dissociation constant is for dissociation of the last complex shown in the table. The error limits of the parameters vary depending on how they are derived. However, the accuracy is within ±30% even in the worst circumstance. Units in mol·L<sup>-1</sup>.

**Table V: Equilibrium Dissociation Constants Measured by the Fluorescence Method<sup>2</sup>**

<table>
<thead>
<tr>
<th>pH</th>
<th>WT F22A</th>
<th>F60E</th>
<th>F60D</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>0.02</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>5.8</td>
<td>0.02</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>6.6</td>
<td>0.02</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>7.4</td>
<td>0.02</td>
<td>0.03</td>
<td>0.07</td>
</tr>
</tbody>
</table>

<sup>2</sup> The error limits are generally ±10%. Units in mol·L<sup>-1</sup>.

**Table VI: Catalytic Parameters for the Hydrolysis of DC-P<sub>34</sub>M by WT and Mutants**

<table>
<thead>
<tr>
<th>pH</th>
<th>WT F22A</th>
<th>F60E</th>
<th>F60D</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>5.8</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>6.6</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>7.4</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
</tbody>
</table>

**Discussion**

We have undertaken a multifaceted study which probes the structure-function relationship of the phospholipase A<sub>2</sub> using a polypeptide substrate in which the hydrolysis of residue 22 would point toward solvent and as such would likely have little impact on the character of the active site. A very similar situation was the case with the polypeptide substrate which we used in the present study. In both cases, the polypeptide substrate is more or less conserved among natural variants. To our knowledge, there are no other known natural substitutions of F22 to Lys and Phe, and the question of why this position is so critically important and how the hydrolysis of residue 22 is so important is one of the most intriguing questions in the field of PKA.

**Figure 4** shows the relative fluorescence intensity at 333 nm of WT (squares), F22A (circles), F22D (diamonds), and F60E (triangles) at pH 5.0. The fluorescence emission at 333 nm of WT is shown by the solid line. The relative fluorescence of the WT is taken as 1.0 for purposes of comparison. The fluorescence of the mutants is reduced in comparison to the WT, and this reduction is similar for all mutants at pH 5.0. The data indicate that F60E is only marginally perturbed in both structure and function and are consistent with this natural variant.
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renburg et al., 1985) is consistent with this hypothesis. When the hydrophobicity is not optimal as in F22A, F106A, and F106Y, catalysis is perturbed. Indeed, the behavior of the semisynthetic mutant F5Y (van Scharrenburg et al., 1982), in which the new tyrosyl side chain points in the same direction as Tyr-106, is similar to that of F106Y.

While our data are sufficient to pinpoint $k_{cat}$ as the primary parameter of perturbation in the interfacial catalysis by F22 and F106 mutants, the magnitudes of the overall effects are not sufficient for further dissection. The data in Tables IV and V show that the dissociation constants for substrate, product, and inhibitor are also perturbed, but to a smaller extent. On the other hand, the $K_m^*$ values of the mutants decrease relative to WT. Considering the relatively large range of errors for lipid substrates (generally ±30%), the data should not be overinterpreted. It is difficult to attribute a factor of 10 in overall activity to microscopic rate constants even in a well-established and well-defined assay system.

Conclusion. Recent major developments in structural studies (White et al., 1990; Thunnissen et al., 1990; Dekker et al., 1991) and kinetic analysis (Jain & Berg, 1989; Berg et al., 1991) of PLA2 have provided a basis for integrated structure–function studies of interfacial catalysis such as the work described in this paper. The results of structural analysis indicate that disrupting the Phe-22/Phe-106 aromatic face-to-face pair or the Phe-22/Tyr-111 edge-to-face pair does not lead to notable perturbations in the conformation or conformational stability of PLA2. The results of functional studies suggest that bulky hydrophobic side chains at positions 22 and 106 are required for optimal catalytic activity, possibly by aligning the 2-acyl chain as suggested by structural analysis and/or by maintaining hydrophobicity at the active site.

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REFERENCES