Deletion of D-Helix in Bovine Pancreatic Phospholipase A2

Shigenobu Kimura, Toshiaki Tanaka, Ichio Shimada,*
Yasuhiro Shiratori, Setsuko Nakagawa,† Haruki Nakamura,
Fuyuhiko Inagaki* and Yoshimi Ota

Protein Engineering Research Institute, Suita, Osaka 565, Japan
*The Tokyo Metropolitan Institute of Medical Science,
Bunkyo-ku, Tokyo 113, Japan
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D-Helix-deleted bovine pancreatic phospholipase A2 was designed using molecular mechanic calculations, and synthesized. Effects of the deletion on the enzymatic activity and on the structure were investigated. The enzymatic activity of the mutant protein was about 40% of that of the native one for a micellar substrate of 1,2-dioctanoylphosphatidylcholine. Although the Michaelis constant of the mutant enzyme was not changed, the catalytic constant was decreased. The dissociation constant of calcium ion was also changed. 1H NMR study revealed a slight conformational change around the active site of the mutant enzyme in addition to changes around the mutated region. The effect on the activity of the mutation seems to be due to the conformational changes around the active site.

Recently the technique of site-directed mutagenesis has been used widely as a tool to elucidate the structure and function of protein molecules. Along with many kinds of single site mutations, it is also an useful approach to mutate and delete the whole of the secondary structure such as α-helix or β-sheet.

Phospholipase A2 (PLA2) (EC 3.1.1.4) is an ubiquitous enzyme which specifically hydrolyzes the ester bond at the C2 position of 1,2-diacylphosphoglycerides. PLA2s from the mammalian pancreas and platelets have homologous primary structures to those of snake venom PLA2s, and calcium ion is required for enzymatic activity.1–3) Since these enzymes have higher activity on aggregated substrates, it is considered that the enzymes recognize the lipid–water interface.1) Three-dimensional structures of some PLA2s have been analyzed.4–8) Bovine pancreatic PLA2 is a monomeric enzyme which has five α-helices (A to E) and one antiparallel β-strand.4,5) However, snake venom PLA2s lack the amino acid residues corresponding to the region of the fourth short α-helix (D-helix) in pancreatic PLA2s, while in all pancreatic PLA2s D-helix regions are conserved.1) The role of the D-helix is still obscure, although some speculations have been brought up.9)

We thus attempted to delete the D-helix of bovine PLA2 to obtain information about the role of D-helix. In this article, the design and production of D-helix-deleted bovine PLA2 mutant protein (ΔD-PLA2), its enzymatic activity and the results of 1H NMR analysis of the mutant protein in solution are described.

Materials and Methods

Model building of mutant protein. The model structure of Δ-PLA2 was constructed using molecular mechanics calculations and computer graphics on a DEC VAX 8600 computer and an Evans and Sutherland PS 300 system. The programs CHARMM and HYDRA were used for the energy minimization and the graphics display, respectively.

The X-ray crystallographic coordinates of bovine PLA2,
refined to 1.7 Å resolution were used as the starting coordinates. Energy minimization was applied to the initial model that lacked six residues, to idealize the peptide bond geometry in the regions where the polypeptide chain bond was to be annealed. Energy was minimized further in a 20 Å sphere of bulk water using a box of water, obtained from a Monte Carlo simulation, to refine the structure.

Production of ΔD-PLA2. ΔD-PLA2 was produced essentially as previously described. The DNA fragment between the Hind III and Asu II sites of the synthesized PLA2 gene was substituted for a newly synthesized one which encoded the designed D-helix-deleted amino acid sequence. The mutant PLA2 gene was inserted into the E. coli-yeast shuttle vector pAT405, and pro-ΔD-PLA2 was produced using the yeast secretion system.

Purification procedure. Native bovine pro-PLA2 was purified by the procedure of Dutilh. Pro-ΔD-PLA2 was purified as follows. After the pH of the culture fluid had been adjusted to 4.0 with sodium hydroxide, it was put on a SP-Sephadex C-25 (Pharmacia Fine Chemicals) column equilibrated with 50 mM sodium acetate (pH 4.0) and eluted with a linear gradient of 0 to 0.5 M sodium chloride. The main peak fraction eluted between 0.25 and 0.3 M sodium chloride was collected and desalted using Sephadex G-25 (fine grade, Pharmacia Fine Chemicals) in 0.1 M sodium chloride and eluted with a linear gradient of 0 to 0.3 M sodium chloride. The main peak was eluted at the start of the gradient. It was collected, desalted, and lyophilized as a fraction of purified pro-ΔD-PLA2.

Both pro-PLA2S were activated with L-[1-tosylamido-2-phenyl]ethyl chloromethyl ketone-treated trypsin (Sigma Chemical Co.) (1/500 w/w amount of pro-enzyme) on ice for 90 min. The peptides were separated using Sephadex G-25 (fine grade, Pharmacia Fine Chemicals) in 0.1 M acetic acid, and lyophilized. The product was rechromatographed on a DE52 (Whatman Biosystems Ltd.) column equilibrated with 5 mM Tris–HCl buffer (pH 8.0) and eluted with a linear gradient of 0 to 0.3 M sodium chloride.

Measurement of calcium binding. PLA2 activity was measured in various calcium concentrations from 0 to 50 mM. The enzymatic activities were in proportion to the amounts of active enzymes that bound calcium ions (data not shown). The ratio of these velocities to maximum velocity is therefore thought to correspond to the saturation concentration of the protein for calcium ions. The data obtained was treated to calculate the dissociation constant (Kd) in accordance with the method of Fletcher et al. for the Scatchard model of ligand–protein interaction.

$^1$H NMR analysis. NMR spectra were recorded on a JEOL JNM GX-500 $^1$H 500 MHz NMR spectrometer. NMR samples of native PLA2 and ΔD-PLA2 were dissolved in D$_2$O at a concentration of 1.0 mM. The pH of the sample solution was adjusted to pH 5.0 by the addition of 1 M DCl or NaOD. All two dimensional spectra (DQF-COSY, HOHAHA, and NOESY) were recorded in a pure phase mode at 28°C. HOHAHA and NOESY spectra were recorded with a mixing time of 55 msec and 150 msec, respectively. 512 blocks of 2 K data points were accumulated with 64-128 scans. The digital resolution of all 2D spectra was usually 5-6 Hz/pixel in both dimensions by zero-filling in the t$_1$ dimension. The Gauss function was used for apodization. The relaxation delay was 1.2 sec.

Results

Design and model building of mutant protein

To delete the D-helix from bovine PLA2, a new amino acid sequence was designed comparing the primary structures of snake venom PLA2S, which lack D-helix regions and have higher enzymatic activities than bovine PLA2.

In bovine PLA2, the D-helix is formed by the peptide fragment from Leu58 to Asp66. Cys61 in this helix forms a disulfide bond with Cys91 on E-helix. If Cys61 was deleted, a free –SH group would remain and this might cause a disulfide bond interchange reaction in the folding process. To delete the n-
helix and conserve this disulfide linkage, nine amino acid residues from Asp59 to Asn67 were replaced by three amino acid residues containing a Cys, Gly-Cys-Tyr (Fig. 1). This sequence often appeared in this region of elapid snake PLA₂.⁵,¹²,²¹ A molecular model of the tertiary structure of this designed mutant protein was constructed (Fig. 2). Comparing with the three-dimensional structure of the native bovine PLA₂, no large structural change is expected except in the region around the deleted D-helix. The distance between alpha carbon atoms of Cys60 and Cys85 was 6.3 Å, and the dihedral angle of \( \text{C}_{\beta}\text{S}_{\beta}\text{S}_{\beta}\text{C}_{\beta} \) was 76°. The standard values of the distance and the angle are about 6 Å and 90°, respectively.²² This suggests that the formation of a new disulfide bond is possible.

Production and purification of mutant protein

According to the protocols described in Materials and Methods, a synthetic gene coding pro-ΔD-PLA₂ was introduced into yeast cells and expressed. The amount of secreted pro-ΔD-PLA₂ and the cultural pattern was almost the same as for the production of the native enzyme.¹² From 91 of culture fluid, 22 mg of pro-ΔD-PLA₂ was purified by SP-Sephadex C-25 and DE52 chromatographies. The pro-ΔD-PLA₂ obtained was activated by specific digestion. The purity and the activation was confirmed by SDS-PAGE and reversed phase HPLC (data not shown).
The mutation of the purified protein was confirmed by peptide mapping and amino acid sequence analysis after pyridylethlylation. A shifted peak in the map of ΔD-PLA2 was collected and its N-terminal amino acid sequence was found to be Lys-Leu-Gly-pyridylethylated Cys-Tyr-Pro-Tyr-Asn-Asn-Asn-(data not shown). This sequence corresponds to the designed one, and it was confirmed that the primary structure of the mutant protein is as expected one.

To confirm the formation of a disulfide bond by the newly introduced cysteine residue, sulfhydryl groups were measured. Only 0.03 mol of free -SH groups was detected in one mol of ΔD-PLA2 under denaturing conditions using DTNB. This indicates that almost all cysteine residues form disulfide linkages in the ΔD-PLA2 molecule, and the newly introduced cysteine residue forms a disulfide bond.

**Kinetic parameters of ΔD-PLA2 and native PLA2**

The kinetic parameters were measured by the hydrolysis of diC8-PC above the critical micelle concentration. From the Lineweaver-Burk plots of the native and mutant enzymes a Michaelis constant (Km) and catalytic constant (kcat) were calculated (Table I). Both ΔD-PLA2 and native PLA2 have the same Km, although the kcat of ΔD-PLA2 was about 40% of the native one. This suggests that the mutation did not change the affinity for the micellar substrate, but decreased the ability to hydrolyze. The dissociation constants of ΔD-PLA2 and the native enzyme for calcium ions were 1.1 mM and 4.2 mM respectively, by Scatchard

<table>
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<tr>
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<th>ΔD-PLA2</th>
<th>Native PLA2</th>
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<tr>
<td>Specific activity (μmol/min·mg)</td>
<td>1.2 x 10^3</td>
<td>2.9 x 10^3</td>
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<td>Km (mM)</td>
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<td>1.2 x 10^3</td>
</tr>
<tr>
<td>Kd (mM)</td>
<td>1.1</td>
<td>4.2</td>
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**Table 1. Enzymatic Activities and Dissociation Constants of Calcium**

**Fig. 3.** 1H NMR Spectra of (a) ΔD-PLA2 and (b) Native PLA2.

In the spectrum of ΔD-PLA2, the signals of slowly exchangeable amide protons were observed in the lowfield region.
Fig. 4. The Residues Whose Proton Signals were Appreciably Shifted (Red) and Shifted a Little (Yellow) in AD-PLA$_2$ are Shown on the Backbone Structure of Native PLA$_2$ (Blue).

Normal stereo vision was used.

analysis. This indicates that AD-PLA$_2$ binds calcium more tightly than the native enzyme.

$^1$H NMR spectra

To obtain information on the induced structural change in AD-PLA$_2$, $^1$H NMR analysis was done. Figure 3 shows $^1$H NMR spectra of native PLA$_2$ and AD-PLA$_2$ in D$_2$O solution. In spite of the deletion of D-helix in AD-PLA$_2$, the spectra were similar, showing that an appreciable conformational change does not occur. For more structural information, the DQF-COSY and HOHAHA spectra of AD-PLA$_2$ were analyzed and aromatic proton resonances were assigned to the specific types of amino acid residues. The aromatic proton resonances of AD-PLA$_2$ were subsequently assigned to the specific residues compared with those of native PLA$_2$ (manuscript in preparation). On the basis of these assignments, the chemical shifts of the aromatic proton resonances of AD-PLA$_2$ and native PLA$_2$ were compared. Some of the aromatic proton resonances, Phe5, Phe106, Tyr52, Tyr69, Tyr73, and His48, showed small but appreciable chemical shift changes. On the contrary, other aromatic proton resonances, Trp3, Phe22, Phe94, Tyr28, Tyr75, Tyr111 and His115, showed only minor chemical shift changes. (The numbering of the residue corresponds to that of native PLA$_2$.) These results suggest that the induced conformational change by the deletion of D-helix is not global but localized to the specific region of the enzyme (Fig. 4).

Discussion

AD-PLA$_2$ had about 40% of the enzymatic activity of the natural enzyme for micellar diC$_8$-PC. Although the enzymatic activity was not increased by the deletion of D-helix, it indicates that, like snake venom PLA$_2$, bovine PLA$_2$ can have an enzymatic activity without the D-helix region. The kinetic parameter $Km$ of AD-PLA$_2$ was not changed from that for the native one (Table I). It indicates that the affinity for micellar substrates
does not change. Dijkstra et al. identified 21 residues that were possibly involved in the interaction with micellar substrates from the three-dimensional structure of bovine PLA₂. Among these residues, Val65 and Asn67, located around the C-terminal of the d-helix, were deleted. The 1H NMR chemical shift of Tyr69 changed in the mutant protein. From these results, it is concluded that d-helix region, at least associated with these three residues, is not essential for recognition of the lipid-water interface.

The decrease of enzymatic activity is explained as the result of the decrease of hydrolytic activity, because only the $k_{cat}$ of ΔD-PLA₂ was changed (Table I). Among 1H NMR signals observed in the aromatic region, chemical shifts of His48, Tyr52, Tyr73, Phe5, and Phe106 changed. It means that environments of these residues changed. His48 is a catalytic residue, and other residues except Tyr73 are positioned in the bottom or around the active site. The binding affinity of ΔD-PLA₂ for calcium ion is also increased relative to the native enzyme (Table I). It is known that the calcium binding site is near the catalytic residues. These results suggest that, as well as causing conformational changes in the mutated region, the d-helix deletion causes slight changes in conformation around the active site including the calcium binding site to decrease the catalytic activity.

Tyr73 is located on the junction of d-helix and β-sheet and it also forms hydrogen bond network with His48 and Tyr52 through Asp99 in native PLA₂. Tyr73 is considered to be important for the catalytic activity of PLA₂ through this hydrogen bond network (Teshima et al. submitted, Ikeda personal communication). So Tyr73 of ΔD-PLA₂ also seems to be related to the hydrolytic activity change, although it is not positioned near the catalytic site. To elucidate the mechanism of this enzymatic activity change it is necessary to obtain more detailed structural information of ΔD-PLA₂ in solution. Detailed 1H NMR analysis of ΔD-PLA₂ is now in progress (manuscript in preparation).

An article on d-helix-deleted pancreatic porcine PLA₂ was published during the preparation of this manuscript. In this case, no structural changes of the active site residues were detected by X-ray crystallographic studies, although some structural change in this region should have occurred because the calcium binding activity and the catalytic activity were changed. It seems that NMR analysis is effective in detecting a slight structural change in solution. It may be possible to detect some structural changes in the porcine mutant enzyme, as well as in the case of bovine mutant enzyme.

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