Hydrolysis of Micellar Diheptanoylphosphatidylcholine Catalyzed by Bovine Pancreatic Phospholipase A$_2$: Kinetic Characterization of Group I and II Enzymes

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Initial velocity data for the hydrolysis of micellar 1,2-diheptanoyl-sn-glycero-3-phosphatidylcholine (diCPC) catalyzed by bovine pancreatic PLA$_2$ (Group I) were analyzed using the Michaelis-Menten equation. The $K_v$ value for the micellar substrate was found to be independent of Ca$^{2+}$ concentration, as was the $K_v$ value for the monodispersed substrate. The pH dependence curve of $K_v$ in the presence of saturating amounts of Ca$^{2+}$ showed three transitions reflecting large $pK$ shifts of two ionizable groups from 5.0 to 5.45 and from 9.5 to 10.25, whereas the $K_v$ value for the monodispersed substrate was independent of pH [Fujii et al. (1991) J. Biochem. 110, 1008-1015]. The pH dependence curve of $k_{cat}$ showed three transitions, indicating the participation of three ionizable groups with $pK$ values of 5.45, 8.4, and 10.25. Deprotonation of the first group and protonation of the third group were found to be essential for catalysis. The respective groups were assigned as the catalytic group His 48, the N-terminal α-amino group, and invariant Tyr 52. The present results as well as those for another Group I PLA$_2$ (Naja naja atra) are very different from those for Group II PLA$_2$ (Agkistrodon halys blomhoffii and Trimeresurus flavoviridis), which showed Ca$^{2+}$-dependent substrate binding and no participation of the α-amino group in catalysis [Teshima et al. (1989) J. Biochem. 106, 518-527; Nishimura et al. (1992) J. Biochem. 111, 210-218]. Although the catalytic efficiency of the bovine enzyme is very low as compared with those of snake enzymes, the present results confirmed that this enzyme shows properties characteristic of Group I enzymes as described above, indicating that no special catalytic mechanism needs to be assumed for the bovine pancreatic enzyme.

Phospholipases A$_2$ (PLA$_2$s) [EC 3.1.1.4] catalyze the hydrolysis of the acyl-ester bonds at the sn-2 position of 1,2-diacyl-sn-3-phosphoglycerides and require binding of Ca$^{2+}$ to the enzymes for catalysis. They are classified into two groups, I and II, according to differences in the primary structure (1). However, their macroscopic tertiary structures, including those of the active site, are believed to be very similar to each other. Both types of PLA$_2$s show much higher enzymatic activity on micellar substrates than on monodispersed substrates (2); when the substrate forms micelles, not only the binding constants of the substrates but also the catalytic center activities of the enzymes increase markedly.

The Ca$^{2+}$ concentration and pH dependences of the kinetic parameters for hydrolysis of monodispersed and micellar substrates by Group I and II PLA$_2$s from snake venoms (Naja naja atra, Agkistrodon halys blomhoffii, and Trimeresurus flavoviridis) have been compared in our previous papers (3-8). The results indicated the existence of differences between the two enzyme types in the Ca$^{2+}$ dependence of substrate binding and the participation of the α-amino group in catalysis, reflecting some differences in the catalytic mechanism. These snake venom PLA$_2$s exhibit generally higher turnover numbers and have higher affinities for phospholipid molecules aggregated in micelles than do the mammalian pancreatic PLA$_2$s. It is therefore of great interest to compare the kinetic properties of pancreatic PLA$_2$s in detail with those of snake venom PLA$_2$s. In the previous study (9), we examined the Ca$^{2+}$ and pH dependence of kinetic parameters for the hydrolysis of monodispersed substrate by bovine pancreatic PLA$_2$ and found that the pancreatic enzyme was kinetically similar to snake venom enzyme of Group I, but not to Group II enzymes.

In the present study, we examined the Ca$^{2+}$ and pH dependence of kinetic parameters for the hydrolysis of a micellar substrate, 1,2-diheptanoyl-sn-glycero-3-phosphorylcholine (diCPC), and confirmed the difference in the catalytic mechanism between Group I and II PLA$_2$s.

MATERIALS AND METHODS

Preparation of Enzyme—Bovine pancreatic PLA$_2$ (iso-electric point, pI 6.4) was prepared by a method similar to those of Nieuwenhuizen et al. (10) and Dutilh et al. (11), as described previously (9). The lyophilized final prepara-

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tion was dissolved in 0.2 M NaCl solution containing 1 mM HCl and then subjected to Sephadex G-75 chromatography. The enzyme eluted with 0.2 M NaCl solution was stored at 4°C. The enzyme concentration was determined spectrophotometrically using the absorbivity $A_280 = 12.3$ or the molar absorption coefficient $\varepsilon = 1.70 \times 10^4 \text{M}^{-1} \cdot \text{cm}^{-1}$ at 278 nm (11, 12).

Synthesis of Substrate—diCPC was synthesized by acylation of $\alpha$-glycerophosphorylcholine with n-enanthic acid anhydride (Tokyo Kasei Kogyo) according to the method of Robles and van den Berg (13). The starting material for synthesis of the substrate, hen egg-yolk lecithin, was obtained from QP, and purified according to the reported method (14). A methanolic solution of 1 M tetra-n-butylammonium hydroxide was from Aldrich. The product was purified on a column of Silicagel 60 (Merck, mesh 230-400) with a mixture of chloroform, methanol, and water [65 : 25 : 2 (v/v)] and then on a column of UniSil (Clarkson, 100-200 mesh) by stepwise elution with mixtures of chloroform and methanol. The final product was dried over P2O5 in a desiccator and stored at —20°C. The purity was checked on a Silicagel 60 plate (Merck) using a mixture of chloroform, methanol, and water [65 : 25 : 4 (v/v)].

Kinetics of the Hydrolysis of diCPC—Enzymatic hydrolysis of the substrate was followed at 25°C and ionic strength 0.2 by a pH-stat assay method using a system consisting of a Radiometer PHM 82 standard pH meter, a TTT 80 titrator, and an ABU 80 autoburette, as described previously (6). Substrate solution (1.0 ml) containing a given concentration of CaCl2 (Merck) was transferred to a cell, and the pH of the solution was adjusted to a desired value by adding a small volume of 30 mM NaOH solution. To this solution, 5-25 μl of the enzyme stock solution was added and the released n-enanthic acid was titrated with 30 mM NaOH under a nitrogen stream so as to keep the pH at the initial value. The concentration of 30 mM NaOH used for titrating the released fatty acid was corrected by titration of a standard solution of sulfamic acid (Nacalai Tesque). At pH values below 6.5, the observed titration curves were apparent values, since the carboxyl group of the released fatty acids would not have been dissociated completely. The data were therefore corrected using the degree of dissociation of the fatty acid, $\alpha = [1/(1 + 10^{P_K - pH})]$. The $P_K$ value of n-enanthic acid was determined to be 4.85 by acid-base titration in the presence of 3 mM diCPC and 10 mM Ca2+ at 25°C and ionic strength 0.2.

RESULTS

Hydrolysis of diCPC Catalyzed by Bovine PLA2—Figure 1A shows the initial velocity, $v$, of the enzymatic hydrolysis of diCPC, plotted as a function of molar concentration of the substrate, $C_s$, at 25°C, pH 7.5, and ionic strength 0.2 in the presence of 20 mM Ca2+, which can saturate more than 96% of the enzyme. The figure also shows the corresponding data for diCPC (9).

The $v$ values at substrate concentrations above the critical micelle concentration (cmc) [≈ 1.58 mM (6)] were much higher than those below the cmc. Similar observations have been reported for other PLA2s such as the porcine pancreatic (15), N. naja atra (3), A. halyi blomhoffii (4), and T. flavoviridis (5) enzymes.

Figure 1B shows the Lineweaver-Burk plot of the data for bovine pancreatic PLA2 with diCPC at substrate concentrations below the cmc. The apparent Michaelis constant, $K_m^\text{monodisp}$ and apparent maximum velocity, $V_{\text{max}}^\text{monodisp}$, were thus determined by using a nonlinear regression analysis to be 5.13 ± 0.20 x 10⁻³ M and 20.0 ± 0.3 pmol/min/mg, respectively. Each error is expressed as the standard error. Using the molecular weight of the enzyme, 13,795, the catalytic center activity (turnover number), $k_{\text{cat}}$, was calculated to be 2.76 ± 0.04 x 10⁶ min⁻¹. The velocity data at substrate concentrations above the cmc were also analyzed by using the equation (16):

$$v = \frac{(cmc) \cdot \frac{V_{\text{max}}^\text{monodisp}}{K_m^\text{monodisp} + C_s} \cdot \frac{C_s - (cmc)}{K_m^\text{monodisp} + C_s}}{1 + \frac{(cmc) \cdot \frac{V_{\text{max}}^\text{monodisp}}{K_m^\text{monodisp} + C_s} \cdot \frac{C_s}{K_m^\text{monodisp} + C_s}}{1 + \frac{(cmc) \cdot \frac{V_{\text{max}}^\text{monodisp}}{K_m^\text{monodisp} + C_s} \cdot \frac{C_s}{K_m^\text{monodisp} + C_s}}},$$

where $K_m^\text{monodisp}$ and $V_{\text{max}}^\text{monodisp}$ are the apparent Michaelis constant and apparent maximum velocity for the micellar substrate, respectively. This equation can be rewritten as:

$$v_{\text{mic}}' = \frac{C_m}{K_m^\text{mic}} - \frac{C_m}{K_m^\text{mic} + C_m},$$

where $v_{\text{mic}}'$ is the velocity minus the corresponding value at the cmc: $v_{\text{mic}}' = v - v_{\text{cmc}}$, and $C_m$ is the molar concentration of the substrate in the micellar phase: $C_m = C - (cmc)$. The value of $K_m^\text{mic}$ is expressed as $K_m^\text{mic} = [1/(1 - f) \cdot K_m^\text{monodisp}]$, where $f$ is the degree of enzyme saturation with monodispersed substrate at the cmc: $f = [(cmc)/(K_m^\text{monodisp})]/(1 + (cmc)/K_m^\text{monodisp})$. The $f$ value was calculated by using the kinetic parameter for the monodispersed state of the substrate. The value of $V_{\text{max}}^\text{mic}$ is expressed as $V_{\text{max}}^\text{mic} = V_{\text{max}}^\text{monodisp} - v_{\text{cmc}}$.

Figure 1C shows the plot of the kinetic data above the cmc.
**Bovine Pancreatic Phospholipase A**

Fig. 2. Lineweaver-Burk plots of the kinetic data for the hydrolysis of micellar diC_{16}PC catalyzed by bovine PLA, at 25°C, pH 7.5, and ionic strength 0.2 in the presence of various fixed concentrations of Ca\(^{2+}\); □, 0.8 mM; ▲, 1.1 mM; △, 2.2 mM; ●, 5.0 mM; ○, 20 mM Ca\(^{2+}\).

Fig. 3. Analysis of the Ca\(^{2+}\)-dependence of the kinetic parameters for the hydrolysis of micellar diC_{16}PC catalyzed by bovine PLA. The reciprocal of the apparent maximum velocity, 1/V_{max,app} (○), and the apparent parameter, k_{cat,app}/V_{max,app} (●), were plotted as a function of 1/Ca\(^{2+}\) according to Eqs. 4 and 3, respectively.

cmc according to Eq. 2, from which the values of K_{m,app} and V_{max,app} were determined to be 1.48±0.16×10^{-3} M and 179.8±9.7 μmol/min/mg, respectively. Using the determined values of K_{m,app}=5.13×10^{-3} M and v_{max}=5.56 μmol/min/mg, the values of K_{m,app} and V_{max,app} were then calculated to be 1.12±0.13×10^{-3} M and 185.4±9.7 μmol/min/mg, respectively. From the molecular weight, 13,795, the catalytic center activity for the micellar substrate, k_{cat,app}, was calculated to be 2.6±0.1×10^{5} min^{-1}.

The solid curve in Fig. 1A is the most probable theoretical one drawn using the determined parameters.

Effect of Ca\(^{2+}\) on the Enzymatic Hydrolysis of Micellar diC_{16}PC—Figure 2 shows the Lineweaver-Burk-plots of the data for the hydrolysis of micellar diC_{16}PC by bovine PLA at 25°C, pH 7.5, and ionic strength 0.2 in the presence of various concentrations of Ca\(^{2+}\). The f value was less than one and was unchanged by Ca\(^{2+}\) concentration since the value of k_{cat,app} was independent of Ca\(^{2+}\) concentration (9), and the v_{max} value was much smaller than the V_{max,app} value, as shown before (Fig. 1). We could therefore assume K_{m,app}=K_{m,app} and V_{max,app}=V_{max,app}. The plots were therefore based on Eq. 2.

As the Ca\(^{2+}\) concentration increased, the V_{max,app} value also increased, whereas the K_{m,app} value remained unchanged. The same observations were reported previously for the hydrolysis of monodispersed diC_{16}PC by the same enzyme (9). A putative interaction scheme for the hydrolysis can be expressed as

$$EM + S\text{ micelle} \rightarrow EM + S_{app} \rightarrow EM + S_{app} + P,$$

where E, M, P, and S represent the enzyme, Ca\(^{2+}\), product, and substrate micellar unit of N monomer molecules which can interact with one enzyme molecule. ES\(_{N}\) and N in ES\(_{N}\) represent the enzyme-micellar substrate complex and the number of micellar substrate molecules bound to one enzyme molecule, respectively. K_{EM} and K_{EM} are the respective dissociation constants of Ca\(^{2+}\) (M) from the

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The two curves were very similar in shape to each other and had three transitions: below pH 6, between pH 8 and 9.5, and above pH 9.5. However, all the transitions for the micellar substrate were significantly shifted to the alkaline side as compared with those for the monodispersed substrate. The two large transitions, one below pH 6 and the other above pH 9.5, which seem to have slopes of +1 and -1, respectively, are not due to protein denaturation, as judged from the pH dependence of the far ultra-violet CD spectrum below 250 nm (9). These results indicate that the deprotonated and protonated states of corresponding ionizable groups are critical for catalysis. Both of the pH-dependence curves also showed the minor participation of an additional ionizable group with a pK value around 8.6, indicating that the ionization state of this group also influences the catalysis.

Previously, we analyzed the pH dependence data for monodispersed substrate and determined the pK values of the three participating ionizable groups, $\mathrm{pK}_{1}^\text{M} = 5.0$, $\mathrm{pK}_{3}^\text{M} = 8.4$, and $\mathrm{pK}_{4}^\text{M} = 9.5$ (9). The respective ionizable groups were tentatively assigned as the catalytic group His 48, N-terminal $\alpha$-NH$_2$ group, and Tyr 52, which is located in close proximity to His 48. Therefore, the three corresponding transitions for the micellar substrate are due to the deprotonation of these ionizable groups.

Two transitions in the pH-dependence data of the logarithm of $1/K_{M}^\text{mic}$ shown for the micellar substrate (Fig. 4) are therefore thought to correspond to the pK shifts of His 48 and Tyr 52, respectively. No significant perturbation of the $\alpha$-NH$_2$ group is thought to occur. The data were analyzed on the basis of the interaction scheme shown in Fig. 6, where $EM(1,1), EM(1,0), EM(0,1)$, and $EM(0,0)$ represent the microscopic forms of the enzyme-Ca$^{2+}$ complex, and $EMS_{w}(1,1), EMS_{w}(1,0), EMS_{w}(0,1)$, and $EMS_{w}(0,0)$ represent those of the enzyme-Ca$^{2+}$-substrate complex. The first and second numerals in parentheses indicate the ionization states of the two ionizable groups in question: 1 and 0 indicate their protonated and deprotonated forms, respectively. The logarithm of $1/K_{M}^\text{mic}$, observed at a given pH value, may therefore be expressed by

$$\log \left( \frac{1}{K_{M}^\text{mic}} \right) = \log \left( \frac{[\mathrm{H}^{+}]^{2}}{K_{EM}^\text{mic} [\mathrm{EM}]_{\text{mic}}^{\text{PP}}} + \frac{[\mathrm{H}^{+}]^{2}}{K_{EM}^{\text{mic}*} [EM^{*}]_{\text{mic}}^{\text{PP}}} + 1 \right) + \log \left( \frac{1}{K_{M}^\text{mic}} \right),$$

where $K_{EM}^\text{mic}$ and $K_{EM}^{\text{mic}*}$ are the macroscopic dissociation constants of protons from the two ionizable groups of enzyme-Ca$^{2+}$ complex, and $K_{EM}^{\text{mic}}$ and $K_{EM}^{\text{mic}*}$ are the corresponding constants for the enzyme-Ca$^{2+}$-substrate complex. $1/K_{M}^\text{mic}$ is the limiting value of $1/K_{M}^\text{mic}$ when the two ionizable groups in question are completely deprotonated.

The solid curve for the micellar substrate is the most probable theoretical one, drawn according to Eq. 5 using the determined parameters of $pK_{1}^\text{M} = 5.00$ and $pK_{3}^\text{M} = 9.50$ (9), and the assumed values of $pK_{1}^{\text{mic}} = 5.45$, $pK_{3}^{\text{mic}} = 10.25$, and $1/K_{M}^{\text{mic}} = 138$. The pH-dependence data of the logarithm of $k_{\text{cat}}$ were analyzed similarly on the basis of the reaction scheme shown in Fig. 7, where $EMS_{s}(1,1,1), EMS_{s}(1,1,0), EMS_{s}(1,0,1), EMS_{s}(0,1,0), EMS_{s}(0,1,1), EMS_{s}(0,0,1), EMS_{s}(0,0,0)$ represent the microscopic forms...
of enzyme-Ca\textsuperscript{++}-substrate complex. The first, second, and third numerals in parentheses represent the ionization states of the three ionizable groups: 1 and 0 indicate their protonated and deprotonated forms, respectively. Among these molecular complexes, only two species having deprotonated His 48 and protonated Tyr 52, EMS\textsubscript{N}(0,1,1) and EMS\textsubscript{N}(0,0,1), were assumed to produce the product with rate constants of \(k_{cat.1}\) and \(k_{cat.2}\), respectively.

The logarithm of \(k_{cat}^{mic}\) observed at a given pH value may thus be expressed by

\[
\log k_{cat}^{mic} = \log \left( A \left[ H^+ \right]^2 + B \left[ H^+ \right] \right) / \left( K_{EMS_{N}}^{K_{EMS_{N}}^{K_{EMS_{N}}} + [H^+]^2 + K_{EMS_{N}}^{K_{EMS_{N}}} + [H^+] + 1} \right),
\]

where \(K_{EMS_{N}}^{K_{EMS_{N}}^{K_{EMS_{N}}}}, K_{EMS_{N}}^{K_{EMS_{N}}^{K_{EMS_{N}}}}, \) and \(K_{EMS_{N}}^{K_{EMS_{N}}^{K_{EMS_{N}}}+1}\) are the macroscopic dissociation constants of protons from His 48, the \(\alpha\)-amino group, and Tyr 52 of the enzyme-Ca\textsuperscript{++}-substrate complex. \(A\) and \(B\) are the respective constants expressed by

\[
A = k_{cat.1}^{K_{EMS_{N}}^{K_{EMS_{N}}^{K_{EMS_{N}}}+1}}
\]

and

\[
B = k_{cat.2}^{K_{EMS_{N}}^{K_{EMS_{N}}^{K_{EMS_{N}}}+1}}
\]

where \(k_{cat.1}^{K_{EMS_{N}}^{K_{EMS_{N}}^{K_{EMS_{N}}}+1}}\) and \(k_{cat.2}^{K_{EMS_{N}}^{K_{EMS_{N}}^{K_{EMS_{N}}}+1}}\) are the microscopic dissociation constants of protons from the \(\alpha\)-amino group of EMS\textsubscript{N}(0,1,1) and from Tyr 52 of EMS\textsubscript{N}(0,0,1), respectively.

The solid curve for the micellar substrate in Fig. 5 is the most probable theoretical one constructed according to Eq. 6, using the determined values of \(k_{cat.1}^{K_{EMS_{N}}^{K_{EMS_{N}}^{K_{EMS_{N}}}+1}} = 5.45, k_{cat.2}^{K_{EMS_{N}}^{K_{EMS_{N}}^{K_{EMS_{N}}}+1}} = 8.40, \) and \(K_{EMS_{N}}^{K_{EMS_{N}}^{K_{EMS_{N}}}+1} = 10.25, \) and the assumed values of \(A = 1.10 \times 10^{23} \text{M}^{-1} \cdot \text{min}^{-1}\) and \(B = 3.63 \times 10^{23} \text{M}^{-1} \cdot \text{min}^{-1}\).

**DISCUSSION**

**Difference in the Ca\textsuperscript{++} Dependence of Substrate Binding between Group I and II PLA\textsubscript{2}s**—Previously, we studied the pH dependence of the binding constant of Ca\textsuperscript{++} to various PLA\textsubscript{2}s (17–22). The results indicated that the binding mode of Ca\textsuperscript{++} which had been proposed on the basis of crystallographic data for the porcine and bovine pancreatic enzymes (23, 24) is common to both enzyme types. However, our present results suggest that the mode of Ca\textsuperscript{++}-binding to the enzyme-substrate complex differs significantly between Group I and II PLA\textsubscript{2}s.

As can be seen from Figs. 2 and 3, the binding of micellar diC\textsubscript{7}PC to bovine PLA\textsubscript{2} is independent of Ca\textsuperscript{++} binding. This was also the case for the binding of monodispersed diC\textsubscript{6}PC (9). Similar observations have also been reported for other Group I enzymes from porcine pancreas (25, 26) and N. flavoviridis venom (6, 7). In these cases, there might be no significant direct interaction between the bound Ca\textsuperscript{++} ion and the substrate molecule.

On the other hand, the Ca\textsuperscript{++} binding to Group II enzymes from A. halya blohoffii and T. flavooirdia venom enhanced the binding constants of both monodispersed and micellar substrates by a factor of more than 10, indicating that the substrate binding enhanced the binding constant of Ca\textsuperscript{++} by the same factor (6, 7). This suggests the presence of direct interaction between the bound Ca\textsuperscript{++} ion and the substrate molecule. Similar observations have also been noted for the PLA\textsubscript{2} from Crotalus adamanteus venom (27).

According to the catalytic mechanism proposed by Verheij et al. (28), an intermediate complex should be stabilized by coordination of the bound Ca\textsuperscript{++} ion with the phosphoryl group and the carboxyl group at the sn-2 position of the bound substrate molecule. Our results seem compatible with this hypothesis for Group II PLA\textsubscript{2}s, but not for Group I PLA\textsubscript{2}s.

Recently, Thunmänn et al. performed an X-ray crystallographic study on a complex of a porcine PLA\textsubscript{2} mutant with a substrate analog, (R)-2-dodecanoyl-amino-1-hexanolphosphoglycol in which an acylaminolinkage had been introduced instead of an ester bond at the sn-2 position (29). More recently, Dekker et al. studied the interaction of this analog with porcine PLA\textsubscript{2} in solution by the NMR method (30). The results suggested the presence of direct interaction between the bound Ca\textsuperscript{++} ion with the phosphoryl group and the carboxyl group of the amide bond at the sn-2 position of the analog molecule bound to Group I enzyme.

The difference in the Ca\textsuperscript{++} dependence of the bindings of true substrates between Group I and II PLA\textsubscript{2}s could therefore be interpreted in terms of the existence of two kinds of enzyme-substrate complexes: formation of one complex is Ca\textsuperscript{++}-independent (Complex I) and that of the other complex is dependent on Ca\textsuperscript{++} (Complex II). The former complex might be nonproductive and the latter productive. Complex II may involve such direct interactions between the bound Ca\textsuperscript{++} ion and the substrate molecule, as suggested above for the enzyme-substrate analog complex. Complexes I and II might thus be predominant in the Michaelis complexes of Groups I and II PLA\textsubscript{2}s, respectively.

**Difference between Group I and II Enzymes in Participation of the \(\alpha\)-Amino Group in Catalysis**—As can be seen from Figs. 4 and 6, the pK values for both His 48 (pK 5.0) and Tyr 52 (pK 9.5) of the enzyme-Ca\textsuperscript{++} complex shifted significantly to the alkaline side upon binding of the micellar substrate, whereas no such phenomenon was observed upon binding of the monodispersed substrate (9). The same result has been shown qualitatively for porcine PLA\textsubscript{2} (25). Similar observations have also been reported.
for another Group I PLA₂ from the venom of *N. naja atra* (7) and also for Group II PLA₆ from the venom of *A. halys blomhoffii* (8) and *T. flavoviridis* (7).

The pK shifts of His 48 and Tyr 52 upon binding of micellar substrates are therefore a property common to both types of enzyme and are thought to be very important for elucidating the catalytic mechanism of PLA₂.

As can be seen from Fig. 5, the catalytic group His 48 (pK 5.45), the N-terminal α-amino group (pK 8.4), and Tyr 52 (pK 10.25) of the enzyme-Ca²⁺-substrate complex participate in catalysis. Deprotonation of His 48 and protonation of Tyr 52 are critical for the catalysis. Importance of the ionization state of the α-amino group is also indicated. This was also the case for the monodispersed substrate (9).

Similar observations have also been made for another Group I PLA₂ from *N. naja atra* venom (7). On the other hand, Group II PLA₆ such as the enzymes from *A. halys blomhoffii* (8) and *T. flavoviridis* venom (7) showed no significant participation of the α-amino group. This difference was compatible with the X-ray crystallographic finding that the α-amino group of bovine PLA₂ (Group I) was incorporated via one water molecule into the hydrogen bonding network of the active site (24), whereas this was not the case for *Crotalus atrox* PLA₂ (Group II) (31).

In our previous studies on snake venom enzymes, the mixed micelle of a long-chain phosphoglyceride, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (dC₁₂PC) with Triton X-100 was used as a micellar substrate. In that case, the effects of Triton X-100 on the kinetic results were thought to be important in discussing the differences in the catalytic mechanisms for monodispersed and micellar substrates. In the present study, we therefore used a simple substrate. The same result as for another Group I PLA₂ (diC₇PC) as a substrate to study the Ca²⁺ and pH dependence of the catalytic parameters for hydrolysis of micellar substrate. The same result as for another Group I PLA₂ from the venom of *N. naja atra* was obtained for the bovine enzyme. This result indicated that no fundamental effect of Triton X-100 on the kinetic properties needs to be assumed.

Generally, mammalian pancreatic PLA₂₈ have lower *kₐ₉* value and higher *Kₐ₉* value than those of snake venom enzymes. In spite of the difference in the enzymatic activity between pancreatic and snake venom enzymes, bovine pancreatic PLA₂ was found to show similar kinetic properties to those of snake venom Group I PLA₂. It is very interesting that the kinetic properties have been conserved during the evolution of Group I PLA₂. There might be some important implications of the difference of kinetic properties between Group I and II enzymes.

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