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Phospholipids Chiral at Phosphorus. Use of Chiral Thiophosphatidylcholine To Study the Metal-Binding Properties of Bee Venom Phospholipase A_2†

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ABSTRACT: It has been shown recently by ^{31}P nuclear magnetic resonance (NMR) that phospholipase A_2 (PL A_2) from bee venom shows a high degree of stereoselectivity toward the "isomer B" of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine (DPPsC) [Bruzik, K., Jiang, R.-T., & Tsai, M.-D. (1983) *Biochemistry 22*, 2478–2486]. We now report a quantitative kinetic study of PL A_2 using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and (R_P)-, (S_P)-, and (R_P + S_P)-DPPsC by a spectrophotometric assay. The substrates were mixed with Triton X-100 to form mixed micelles, and steady-state kinetic theories were applied. The enzyme was activated by Ca^{2+}, which induced a conformational change of the enzyme, as shown by UV difference spectra. The apparent dissociation constant of Ca^{2+}/PL A_2 is 2.5 mM. In the presence of Ca^{2+}, large substrate specificity and stereospecificity in V_{max} (in μmol min^{-1} mg^{-1}) were observed: DPPC, 1850; (R_P)-DPPsC, 76; (R_P + S_P)-DPPsC, 64; (S_P)-DPPsC, 0.044. On the other hand, relatively small variation in K_m was observed, which suggests that the interfacial interaction is relatively nonspecific among the substrates studied. (S_P)-DPPsC and Cd^{2+} were shown as competitive inhibitors for the hydrolysis of DPPC by Ca^{2+}/PL A_2. Binding of Cd^{2+} with apo-PL A_2 was also demonstrated by UV difference spectra, with a dissociation constant of 0.59 mM. Activation of apo-PL A_2 by Cd^{2+} was unequivocally demonstrated for (S_P)-DPPsC by use of ^{31}P NMR. The V_{max} values of Cd^{2+}/PL A_2 were DPPC/(R_P)-DPPsC/(S_P)-DPPsC = 17.6/0.069/0.0044 μmol min^{-1} mg^{-1}. Thus, substitution of Ca^{2+} by Cd^{2+} caused the ratio of maximum velocity of R_P and S_P isomers, V_R/V_S, to decrease by a factor of 110, which is a large change though not enough to cause a complete reversal of stereospecificity. Comparison with the literature data on the metal ion dependence of stereospecificity of various enzymes suggests that the high degree of stereospecificity of Ca^{2+}/PL A_2 and the large change in V_R/V_S support a direct coordination between Ca^{2+} and the *pro-S* oxygen of DPPC in the active site of PL A_2. The effect of chirality on the interfacial interaction between PL A_2 and phospholipid micelles is also discussed.

Phospholipase A_2 (PL A_2)[1] (EC 3.1.1.4) catalyzes hydrolysis of the fatty acid ester at the *sn*-2 position of phospholipids (Van Deenen & de Haas, 1964). The mechanism of the enzyme has been investigated extensively [for recent reviews, see Verheij et al. (1981), Volwerk & de Haas (1982), and Slotboom et al. (1982)]. In a popular catalytic mechanism of pancreatic PL A_2 proposed by Verheij et al. (1980), Ca^{2+} was suggested to interact with P—O^- and C=O directly. However, there has been little evidence for such substrate–cofactor coordination, and the interaction of the enzyme with the substrate at the active site or on the membrane surface remains unclear.

Recently it has been shown that PL A_2 from several different sources catalyze stereospecific hydrolysis of the "isomer B" of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine (DPPsC) (Bruzik et al., 1982, 1983) and the corresponding DPPsE (Orr et al., 1982; Jiang et al., 1984). The configuration at phosphorus for the isomer B of DPPsC and DPPsE was later determined as R_P (Jiang et al., 1984). Such results are intriguing because the C–O bond cleaved in the reaction catalyzed by PL A_2 is five bonds away from the phosphorus center, and the enzyme is relatively nonspecific to the structure of the phosphate head group (phosphatidylcholine, phosphatidylethanolamine, and phosphatidic acid are all substrates of PL A_2).

However, the stereochemical result reported previously was only qualitative. No quantitative kinetic data of chiral thiophospholipids have yet been reported, presumably due to the fact that kinetic study of lipolytic enzymes is often complicated by interfacial interactions, by the phase property of substrates, and by the heterogeneous nature of the reaction mixture. Thus, it remains unanswered whether the observed stereospecificity is caused by a difference in interfacial interactions as a result of different phase properties or by a difference in the binding of substrates to the active site of the enzyme. In either case, what is the mechanistic significance of such a high stereospecificity?

In this paper we report kinetic results on highly purified bee venom PL A_2 (Shipolini et al., 1971), by use of R_P and S_P isomers of DPPsC (Figure 1) of very high diastereomeric purity (>97%). To enhance our understanding of the mech-

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[1] Abbreviations: ATPαS, adenosine 5′-(1-thiotriphosphate); ATPβS, adenosine 5′-(2-thiotriphosphate); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPsC, 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine; EDTA, ethylenediaminetetraacetate; K_a, activation constant; K_m, Michaelis constant; Mops, 3-(*N*-morpholino)propanesulfonic acid; MPPsC, 1-palmitoyl-*sn*-glycero-3-thiophosphocholine; NMR, nuclear magnetic resonance; PL A_2, phospholipase A_2; PRPP synthetase, phosphoribosylpyrophosphate synthetase; TF_1ATPase, adenosinetriphosphatase from thermophilic bacterium PS3; TLC, thin-layer chromatography; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; V_{max}, maximal velocity.

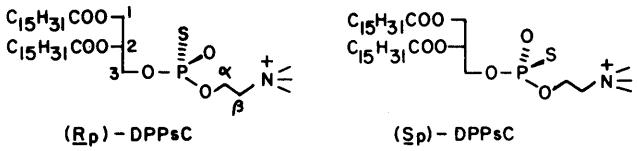

FIGURE 1: Structure and configuration of chiral thiophosphatidylcholine.

anistic significance of the kinetic results, we further investigated the Ca^{2+} binding property of the enzyme, as well as the effect of Cd^{2+} on the kinetic properties of thiophospholipids. The results are discussed in terms of the enzyme–substrate interfacial interaction and the Ca^{2+}–substrate interaction in the active site of PL A_2.

Materials and Methods

Materials. DPPsC isomers were prepared as described previously (Bruzik et al., 1982, 1983). The diastereomeric purity of (R_P)- and (S_P)-DPPsC was 97% and >98%, respectively, as determined by ^{31}P NMR in CH_3OD (Bruzik et al., 1983) at 121.5 MHz, with high resolution and high signal/noise ratios. DPPC, Triton X-100, Mops and EDTA (tetrasodium form) were obtained from Sigma. *p*-Nitrophenol (99%) was purchased from Aldrich. $CaCl_2$ (reagent grade) was obtained from Allied Chemical. Ultrapure $Ca(NO_3)_2$, $Cd(NO_3)_2$, $CdCl_2$, and NaOH were purchased from Alfa. Chelex-100 was purchased from Bio-Rad. All other chemicals used were of reagent grade or highest purity available commercially.

Enzyme. PL A_2 was purified from lyophilized whole venom of *Apis mellifera* (honey bee) (Sigma, grade IV) according to the procedure of Shipolini et al. (1971). The final preparation showed a single band in polyacrylamide gel electrophoresis. The protein concentrations were determined by the procedure of Lowry et al. (1951) by using bovine serum albumin as a standard. Purified PL A_2 was routinely quantitated by UV absorption maximum at 278 nm ($E_{278}^{0.1\%} = 1.54$). The molecular weight was first reported as 19 000 per monomer (Shipolini et al., 1971) and later revised to 15 000 based on its amino acid composition (Shipolini et al., 1974a). Like PL A_2 from several other sources (Slotboom et al., 1982), the bee venom PL A_2 also exists as a dimer (Shipolini et al., 1971). In this paper we treat the enzyme as a monomer, with M_r 15 000. For long-term storage, the purified enzyme was dialyzed against Tris–citrate buffer (pH 8.25), lyophilized, and kept at −20 °C.

To prepare the apoenzyme, we first passed doubly distilled, demineralized water through a Chelex-100 column (1.5 × 10 cm) and used the Chelex-treated water for all buffers. The lyophilized PL A_2 (10 mg) was dissolved in 2 mL of 10 mM EDTA, incubated at room temperature for 30 min, and dialyzed against 2 L of 0.2 mM Mops buffer (pH 7.2) containing 1 mM EDTA overnight. This was followed by 2 days of dialysis against the same buffer without EDTA, with four changes of 1 L of Chelex-treated Mops buffer. The EDTA-free, Ca^{2+}-free PL A_2 was further passed through a small column of Chelex-100 (0.5 × 5 cm) preequilibrated with the Mops buffer. The apoenzyme obtained was then adjusted to pH 7.2, filtered through glass wool, diluted with an appropriate amount of buffer, and used for metal ion binding and kinetic studies. The apoenzyme used for the NMR experiment in Figure 8 was obtained by the same procedure, except that the Mops buffer used was 10 mM.

Methods of Kinetic Analysis. The procedures used were modified from the spectrophotometric assay of Kupferberg et al. (1981), which monitored the evolution of fatty acid by a *p*-nitrophenol indicator. For determination of the K_m and V_{max} of DPPC and DPPsC isomers with Ca^{2+}/PL A_2, we prepared a stock buffer containing 0.2 mM Mops·Na, 0.1 mM *p*-nitrophenol, 5 mM $CaCl_2$, 0.1 mM EDTA, and 0.1% (w/w) Triton X-100 (1.5 mM), pH 7.23 (measured at 23 °C). The micelle solutions were prepared fresh daily by sonicating 10 μmol of DPPC or DPPsC in 10 mL of the stock buffer for 10 min in a 50 °C water bath. After being cooled to room temperature, the micelle solution was adjusted back to pH 7.23, diluted to various concentrations with the stock buffer, and aged under argon at 37 °C for 2.5 h. The enzyme was first dialyzed against 0.2 mM buffer (pH 7.2) containing 5 mM $CaCl_2$, then diluted with the proper amount of the stock buffer, and preincubated in ice–water. The cuvette (1 cm^2 × 3 cm) was capped with a rubber septum, flushed with dry argon through needles, and incubated at 37 °C in the cell holder. To start the reaction, 2 mL of substrate solution was transferred to the cuvette by a syringe, and absorbance at 400 nm was recorded for several minutes to ensure a flat base line (improper sonication or exposure to CO_2 would cause drastic instability of the base line). A fixed amount of PL A_2 solution (usually <20 μL) was then added to the cuvette by a Hamilton syringe, followed by quick mixing by use of a micro magnetic stirring bar. The decrease in the absorbance at 400 nm was then recorded, usually with the full scale set at 0.05 OD. On the basis of calibration by back-titration of reaction mixtures with standardized KOH, a decrease of 0.01 in absorbance corresponds to 4.24×10^{-3} μmol of DPPC hydrolyzed by PL A_2. For a 1 mM DPPC solution, a decrease of 0.01 in A_{400} corresponds to hydrolysis of 0.21% of the substrate. Thus, the initial velocity v was measured within the first 1% of the reaction in most cases. For the hydrolysis of (S_P)-DPPsC, a fast reaction was observed in the first 1–2 min due to a trace amount of the R_P isomer followed by a slow reaction from which the v was measured. The K_m and V_{max} were calculated from Lineweaver–Burk plots.

The K_m and V_{max} of DPPC and (R_P)-DPPsC for Cd^{2+}/PL A_2 were measured by the same procedure except that the apoenzyme was used, and the stock buffer contained 5 mM $CdCl_2$ in place of 5 mM $CaCl_2$.

For the activation study of apo-PL A_2 by Ca^{2+}, the $CaCl_2$ was omitted in the stock buffer and added, with varying concentration, to the substrate solution (2 mM DPPC) before the 2.5-h aging.

For the inhibition study of Cd^{2+}, the DPPC solution (1 mM) was prepared in the stock buffer without Ca^{2+}. A Cd^{2+} stock solution was prepared by dissolving $CdCl_2$ (5 mM) in the stock buffer without Ca^{2+}. The reaction mixtures were prepared by mixing 1 mL of the DPPC solution, 0–0.8 mL of the Cd^{2+} stock solution, 1.0–0.2 mL of diluting buffer (the stock buffer without Ca^{2+}), and the appropriate amount (2.5–20 μL) of 0.1 M $CaCl_2$ (pH 7.2), to a final volume of 2.0 mL. After the mixtures were aged for 2.5 h, the reaction was started by mixing with PL A_2 as described above.

For the inhibition study of (S_P)-DPPsC, the DPPC micelles (1 mM) and DPPsC micelles (1 mM) were prepared in the stock buffer and aged separately. Appropriate amounts of substrate, inhibitor, and the stock buffer were mixed just before the reaction. Each line in Figure 5 was measured in a day, and the DPPC and DPPsC micelles were prepared fresh daily. In order to minimize errors due to micelle preparation, the data were normalized by fitting the data with [DPPsC] = 0 to the Lineweaver–Burk plot of DPPC (Figure 2a).

In all experiments involving Cd^{2+}, the Mops buffer and substrates were pretreated with Chelex-100 before use.

Demonstration of Cd^{2+} Activation of PL A_2 by ^{31}P NMR. (S_P)-DPPsC (55 mg) was dissolved in 7.5 mL of 10% Triton X-100 in a 1:1 mixture of H_2O/D_2O by stirring in a 50 °C water bath. The solution was then passed through a column of Chelex-100 (0.5 × 5 cm) preequilibrated with 10% Triton X-100. Three 2.0-mL fractions (I–III) were collected, and each was mixed with 0.2 mL of 0.5 M Mops buffer, pH 7.2. Forty-four microliters of 0.5 M $Ca(NO_3)_2$ was added to fraction I, the same amount of $Cd(NO_3)_2$ to fraction II, and the same volume of water to fraction III. Each fraction was adjusted to pH 7.2. Six NMR samples were prepared consecutively at 1-h intervals: sample Ia was a mixture of 0.9 mL of fraction I and 1.34 mg of apo-PL A_2 in 1.0 mL of 10 mM Mops, pH 7.2 (final concentrations were 4.1 mM DPPsC, 4.2% Triton X-100, 26.4 mM Mops, and 4.6 mM Ca^{2+}); sample Ib was the same as Ia without the enzyme; sample IIa was a mixture of 0.9 mL of II and 1.0 mL of the enzyme solution; sample IIb was the same as IIa without the enzyme; sample IIIa was a mixture of 0.9 mL of III and 1.0 mL of the enzyme solution; sample IIIb was the same as IIIa without the enzyme. The samples were incubated at 37 °C, and the reactions were monitored by ^{31}P NMR at 37 °C (Figure 8). The qualitative results have been reproduced twice by use of TLC [see Bruzik et al. (1983) for TLC conditions].

UV Difference Spectra. For Ca^{2+} binding studies, 1 mL of apoenzyme solution (containing 0.94 mg of PL A_2; 63 μM) in 10 mM Mops buffer, pH 7.2, was placed in sample and reference cells. The spectrum, scanned from 340 to 230 nm, was stored in the microprocessor and zeroed. Varying amounts of $CaCl_2$ solution (0.5 M, pH 7.2) were added to the sample cell and the corresponding amounts of water to the reference cell. A spectrum was recorded after each addition.

For Cd^{2+} binding studies, a smaller concentration (11 μM) of apo-PL A_2 was used in order to avoid light scattering caused by trace amounts of precipitate upon addition of varying amounts of 0.2 M $CdCl_2$. The pH of 0.2 M $CdCl_2$ was not adjusted, but the pH of the sample cell was not changed upon addition of up to 5 mM $CdCl_2$. The base line was set to zero at 340 nm for each curve.

Instrumental Methods. ^{31}P NMR was performed on a Bruker WM-300 (for determination of diastereomeric purity) and WP-200 (for monitoring DPPsC hydrolysis). Under the conditions used in Figures 8 and 9, we found that the integrals were approximately 1:1 for an equimolar mixture of DPPsC/MPPsC, justifying the use of integrals to measure rates of hydrolysis. Chemical shifts were referenced to external 85% H_3PO_4, with a plus sign indicating a downfield shift. Spectrophotometric assays were performed on a Uvikon 820 UV–vis spectrophotometer. Sonication was carried out in a Brownwill Biosonik with a microtip.

Results

Kinetic Analysis. Full discussion of the advantages and disadvantages of various assay methods and kinetic models for phospholipase A_2 can be found in Verheij et al. (1981), Verger (1980), Slotboom et al. (1982), and Volwerk & de Haas (1982). Although short-chain micellar substrates seem to show normal steady-state kinetics (Bonsen et al., 1972), there is yet no unified kinetic theory for long-chain phospholipid substrates. Because our specific purpose was to compare DPPC and DPPsC isomers quantitatively under identical conditions, we chose a combined and compromised procedure, as described under Materials and Methods, which gave apparently normal Michaelis curves and allowed determination of apparent K_m and V_{max}. The spectrophotometric detection by use of *p*-nitrophenol as an indicator (Kupferberg et al., 1981) was used because of its high sensitivity that allowed accurate measurements of linear initial velocity (<1% of reaction) without being complicated by product inhibition. The mixed micelles of phospholipids with the nonionic detergent Triton X-100 were used because of the advantages in providing an isotropic solution of long-chain phospholipids (Dennis, 1973a,b, 1974a,b; Dennis & Owens, 1973) and in activating bee venom PL A_2 (Upreti & Jain, 1978). The importance of Triton in this study is shown in Figure 2. Curve a shows a linear Lineweaver–Burk plot for the hydrolysis of DPPC under the conditions described under Methods of Kinetic Analysis, in which the total concentration of Triton was maintained at 1.5 mM. Curve b was obtained by a similar procedure, except that the ratio of [Triton]/[DPPC], rather than the total Triton concentration, was kept constant. Curve c was obtained by use of "small unilamellar vesicles" obtained from ultrasonication of DPPC in the absence of detergents (Fendler, 1982). Since only curve a is linear, the conditions of curve a were used for kinetic studies in this work.

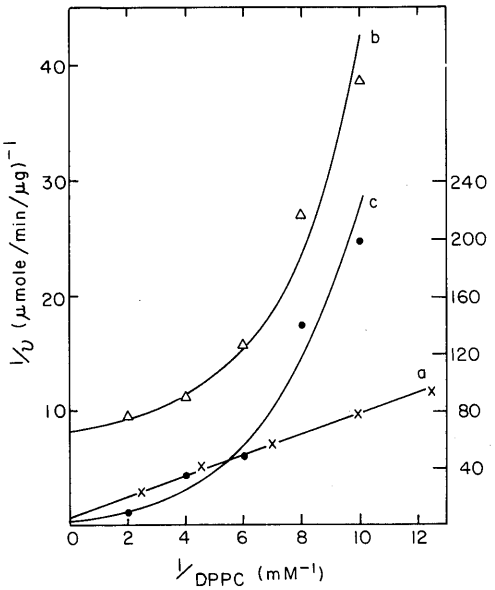

FIGURE 2: Comparison of Lineweaver–Burk plots for the hydrolysis of DPPC by phospholipase A_2 under different conditions. (a) Total Triton X-100 was kept at 1.5 mM while the DPPC concentration varied, as described under Methods of Kinetic Analysis. (b) The ratio [Triton]/[DPPC] was kept at 4 while the total [Triton] varied with [DPPC]. The procedure was the same as for (a), except that DPPC (1 mM) was sonicated in 4 mM Triton X-100, and the dilution buffer contained no detergents. (c) Use of small unilamellar vesicles without detergents. The scale of the *y* axis on the left-hand side is for (a) (×) and (b) (△), whereas that on the right-hand side is for (c) (●).

Another potential problem is that phase properties of DPPC and DPPsC isomers may have different temperature dependence (i.e., different gel–liquid–crystalline transition temperatures; Jiang et al., 1984). The phase property of substrates may have a large effect on the activity of PL A_2. If this is the case, comparison of DPPC and DPPsC isomers should be made at the same phase, rather than at the same temperature. The use of mixed micelles seemed to have eliminated this problem. The Arrhenius plot of the velocity of DPPC hydrolysis is approximately linear in the range 30–45 °C, showing no evidence of complication by possible phase changes (Kensil & Dennis, 1981). Comparison of kinetic properties at a constant temperature (37 °C) is therefore justified.

Ca^{2+} Activation and Binding. Like PL A_2 from many different sources, the bee venom PL A_2 also shows a high specificity for Ca^{2+} as an activator (Shipolini et al., 1971). However, the stoichiometry of Ca^{2+} binding and the disso-

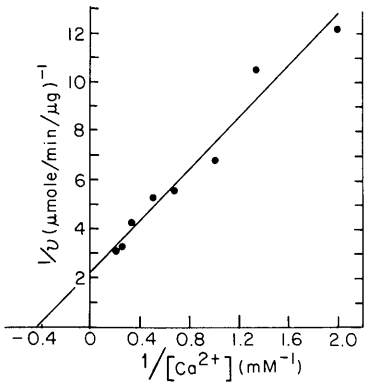

FIGURE 3: Plot of $1/v$ vs. $1/[Ca^{2+}]$ for the activation of apo-PL A_2 by Ca^{2+}. The initial velocity v was measured for the hydrolysis of DPPC (2 mM) as described under Methods of Kinetic Analysis.

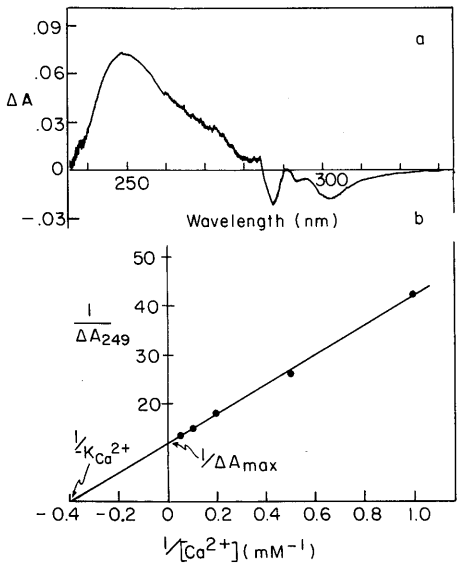

FIGURE 4: (a) UV difference spectrum of bee venom PL A_2 induced by $CaCl_2$. Both sample and reference cells contained 63 μM enzyme in 10 mM Mops buffer, pH 7.2. The sample cell also contained 20 mM $CaCl_2$. (b) Double-reciprocal plot $1/\Delta A_{249}$ vs. $1/[Ca^{2+}]$, obtained from the above experiment with varying concentration of Ca^{2+}.

ciation constant (K_d) have not been determined for this particular enzyme. Because the main theme of this paper is the dependence of stereospecificity on metal ions, it is necessary to study the Ca^{2+} binding properties of the enzyme, such that saturating concentration of Ca^{2+} can be used and that quantitative comparison can be made between Ca^{2+}/PL A_2 and Cd^{2+}/PL A_2.

Figure 3 shows the effect of Ca^{2+} on the rate of hydrolysis of DPPC catalyzed by PL A_2. The activation constant K_a = 2.3 mM can be obtained from the approximately linear double-reciprocal plot.

The activation of PL A_2 by Ca^{2+} was shown to be caused by a Ca^{2+}-induced conformational change. Figure 4a shows the Ca^{2+}-induced UV difference spectrum of PL A_2, with a large peak at 249 nm and two small negative peaks at 287.6 and 302 nm. The peaks at 287.6 and 302 nm can be attributed to perturbations of aromatic side chains. Such a conformational change has also been observed in pancreatic PL A_2 (Pieterson et al., 1974a), but the UV difference spectrum is somewhat different (large peak at 242 nm and small peak at 280–290 nm, both positive), presumably due to different amino acid sequences of the two enzymes (Verheij et al., 1981; Shipolini et al., 1974a,b).

The dependence of ΔA_{249} on Ca^{2+} concentration showed a hyperbolic curve. The double-reciprocal plot of ΔA_{249} vs. $[Ca^{2+}]$ gave a straight line (Figure 4b), from which the apparent dissociation constant $K_{Ca^{2+}}$ = 2.5 mM was obtained, in good agreement with the activation constant (2.3 mM) obtained from Figure 3. The maximal absorbance (ΔA_{max}) at 249 nm calculated from the y intercept is 0.085, and the molar extinction coefficient thus obtained is 1350 cm^{-1} M^{-1}.

Table I: Summary of Kinetic Data of Bee Venom PL A_2

metal	substrate	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
Ca^{2+}	DPPC	1.67	1850
Ca^{2+}	(R_P)-DPPsC	0.85	76
Ca^{2+}	(S_P)-DPPsC	0.30	0.044
Ca^{2+}	(R_P + S_P)-DPPsC	2.1 (1.05)[a]	64
Ca^{2+}	DPPC + (S_P)-DPPsC	2.4 (1.2)[b]	1430
Cd^{2+}	DPPC	6.4	17.6
Cd^{2+}	(R_P)-DPPsC	0.24	0.069
Cd^{2+}	(S_P)-DPPsC		0.0044

[a] Number in parenthesis is the K_m of (R_P)-DPPsC only. [b] Number in parentheses is the K_m of DPPC only.

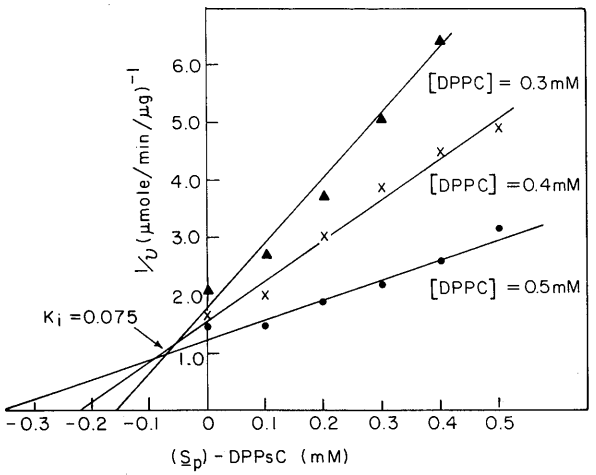

FIGURE 5: Dixon plot for competitive inhibition of PL A_2 catalyzed DPPC hydrolysis by (S_P)-DPPsC. The concentration of Ca^{2+} was 5 mM.

Kinetic Properties of DPPC and DPPsC Isomers. The K_m and V_{max} values derived from the Lineweaver–Burk plots of the hydrolysis of (R_P)-, (S_P)-, and (R_P + S_P)-DPPsC catalyzed by bee venom PL A_2 are summarized in Table I. The corresponding values for DPPC are obtained from Figure 2a. The V_{max} of (R_P)-DPPsC is $^1/_{24}$ of that of DPPC, whereas the V_{max} of (S_P)-DPPsC is only $^1/_{1727}$ of that of (R_P)-DPPsC.

The data in Table I suggest a few interesting mechanistic features. (a) The stereospecificity of PL A_2 toward (R_P)- and (R_P)-DPPsC is unexpectedly high. (b) The low activity of (S_P)-DPPsC is not due to poor affinity of the enzyme for the substrate, because it has a lower K_m than the R_P isomer. (c) In the mixed micelles (R_P + S_P)-DPPsC, the S_P isomer does not seem to have an appreciable effect on either K_m or V_{max} of the R_P isomer.

To further understand the possible inhibitory effect of (S_P)-DPPsC, we measured the K_m and V_{max} of a 1:1 mixture of DPPC/(S_P)-DPPsC micelles. Again, (S_P)-DPPsC had no significant effect on the hydrolysis of DPPC, as shown in Table I. On the other hand, if DPPC micelles and (S_P)-DPPsC micelles were prepared separately and mixed together just before the reaction, (S_P)-DPPsC functioned as a competitive inhibitor with a low K_i (0.075 mM), as shown by the Dixon plot in Figure 5.

Inhibitory and Binding Properties of Cd^{2+}. Recently Jaffe & Cohn (1979) have shown that a "reversal of stereospecificity" upon substitution of Mg^{2+} or Ca^{2+} (which

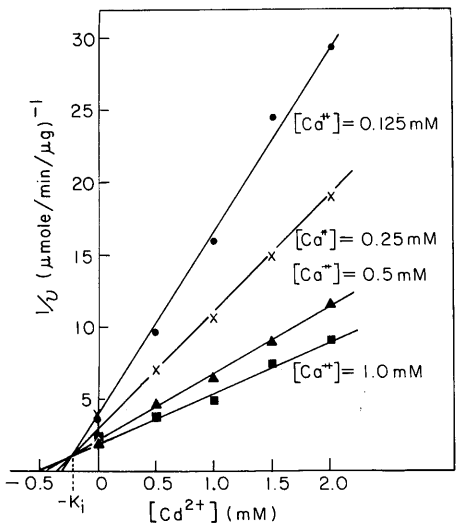

FIGURE 6: Dixon plot for competitive inhibition of PL A_2 catalyzed DPPC hydrolysis by Cd^{2+}. The concentration of DPPC micelles was 1 mM.

prefers oxygen over sulfur) by Cd^{2+} (which prefers sulfur over oxygen) can be viewed as direct evidence for the coordination between the divalent metal ion and the sulfur-substituted phosphate group. We therefore investigated the effect of Cd^{2+} on the stereospecificity of PL A_2 toward DPPsC isomers.

In most of the reported studies on metal ion dependence of stereospecificity for diastereomers of ATPαS and ATPβS (Cohn, 1982; Eckstein, 1983), the enzymes do not show absolute specificity for metal ions, and it is usually possible to substitute Mg^{2+} with Zn^{2+}, Cd^{2+}, or others. PL A_2 is known to have very high specificity for Ca^{2+}. Although Cd^{2+} and Ca^{2+} have similar ionic radii (0.97 and 0.99 Å, respectively), Cd^{2+} cannot be substituted for Ca^{2+} as an activator of PL A_2 under normal conditions. In this section we show that Cd^{2+} can indeed compete with Ca^{2+} for binding with PL A_2; in the next section we demonstrate the activation of PL A_2 by Cd^{2+} using the less reactive isomer (S_P)-DPPsC.

The Dixon plot in Figure 6 shows that Cd^{2+} is a competitive inhibitor (against Ca^{2+}) of PL A_2, with K_i = 0.25 mM. Binding of Cd^{2+} to PL A_2 also induces a conformational change, as shown in Figure 7a. The spectrum differs from Figure 4a in that at 287 nm there is a small positive, instead of negative, peak, and the large positive peak at 249 nm is now shifted to 235 nm. These differences suggest that the Cd^{2+}·PL A_2 interaction is somewhat different from that of Ca^{2+}·PL A_2. The double-reciprocal plot ΔA_{235} vs. [Cd^{2+}] gives a linear curve (Figure 7b), from which $K_{Cd^{2+}}$ = 0.58 mM can be obtained.

The above results suggest that Cd^{2+} competes with Ca^{2+} for the same binding site, with a smaller dissociation constant. Although the effect of Cd^{2+} is inhibitory, and the UV difference spectra are somewhat different, we have shown that Cd^{2+} can also function as an activator of PL A_2, as described in the following section.

Activation of Apo-PL A_2 by Cd^{2+}. Before we can compare the stereospecificity between Ca^{2+}/PL A_2 and Cd^{2+}/PL A_2, it is necessary to show that Cd^{2+} can actually activate apo-PL A_2, other than function as a competitive inhibitor of Ca^{2+}/PL A_2. A problem encountered was that the apo-PL A_2 still retained 1–2% of residual activity, which might be higher than the true activity of Cd^{2+}/apo-PL A_2. Addition of a trace amount of EDTA completely eliminated the residual activity of apo-PL A_2, and addition of excess Cd^{2+} reactivated the enzyme. However, such a result does not unequivocally show activation of PL A_2 by Cd^{2+} because EDTA has a higher

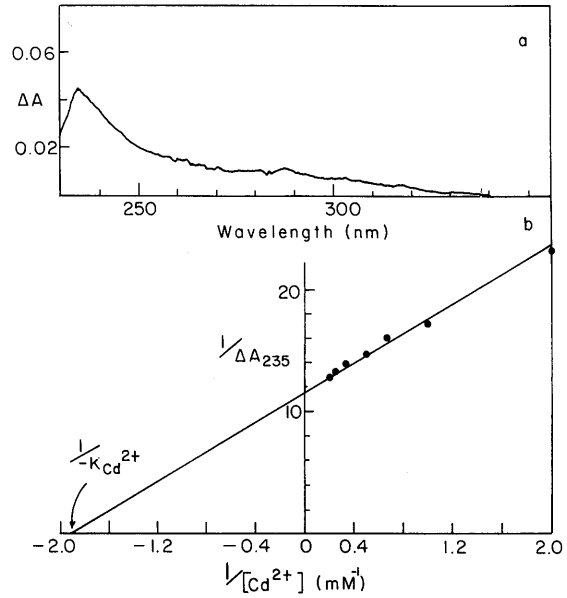

FIGURE 7: (a) UV difference spectrum of bee venom PL A_2 induced by $CdCl_2$. Both sample and reference cells contained 11 μM enzyme in 10 mM Mops buffer, pH 7.2. The sample cell also contained 0.5 mM $CdCl_2$. (b) Double-reciprocal plot $1/\Delta A_{235}$ vs. $1/[Cd^{2+}]$ obtained from the above experiment with varying concentration of Cd^{2+}.

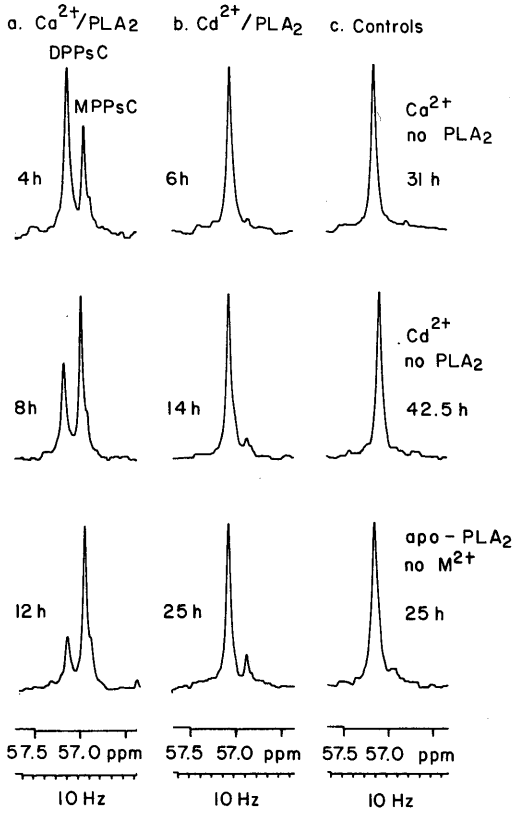

FIGURE 8: ^{31}P NMR spectra (at 81.0 MHz) showing hydrolysis of (S_P)-DPPsC by Ca^{2+}/PL A_2 (a) and by Cd^{2+}/PL A_2 (b), as well as three control experiments with Ca^{2+} only, Cd^{2+} only, and apo-PL A_2 only (c). The assignments of DPPsC and MPPsC are based on Bruzik et al. (1983). Sample conditions: 4.1 mM DPPsC, 4.2% Triton X-100, 4.6 mM M^{2+}, 1.34 mg of apo-PL A_2, and 26.4 mM Mops, pH 7.2. Spectral parameters: spectral width 1000 Hz, acquisition time 2.05 s, acquisition delay 1 s, pulse width 60°, broad band ^1H decoupling, 2000 transients, 37 °C, 2 Hz line broadening.

affinity for Cd^{2+}, and addition of excess Cd^{2+} could have released the trace amount of contaminating Ca^{2+}.

Use of the least active isomer (S_P)-DPPsC allowed unequivocal demonstration of activation of apo-PL A_2 by Cd^{2+},

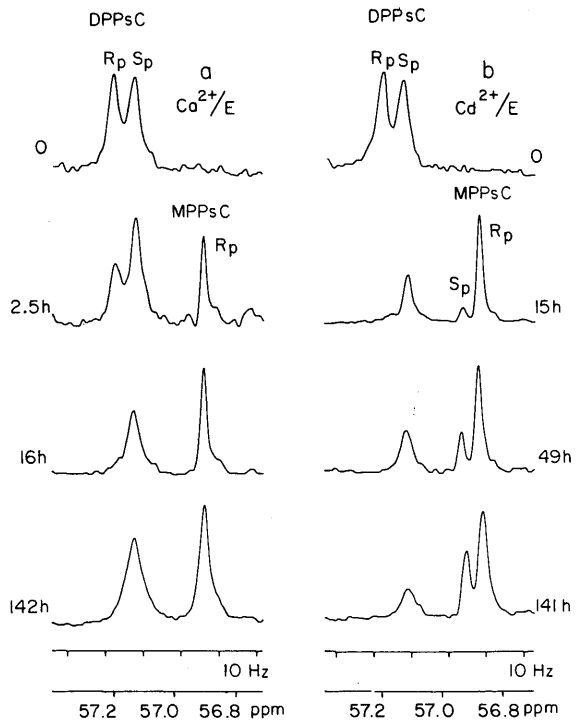

FIGURE 9: ^{31}P NMR spectra (at 81.0 MHz) showing hydrolysis of (R_P + S_P)-DPPsC by Ca^{2+}/PL A_2 (a) and by Cd^{2+}/PL A_2 (b). Sample conditions: 10 mM DPPsC, 5% Triton X-100, 35% D_2O, 0.25 mM EDTA, 2.5 mM $M(NO_3)_2$, 50 mM Mops, pH 7.2, and 3.8 μg of apo-PL A_2 (a) and 350 μg of apo-PL A_2 (b). The PL A_2 used in this particular experiment was obtained from Sigma. Spectral parameters: spectral width 1000 Hz, acquisition time 4.1 s, pulse width 45°, broad band ^1H decoupling, 37 °C, Gaussian multiplication (LB −1, GB 0.05).

as well as determination of the relative activity of Ca^{2+}/PL A_2 and Cd^{2+}/PL A_2 for the hydrolysis of (S_P)-DPPsC, by use of ^{31}P NMR. Figure 8 shows the time course of hydrolysis of (S_P)-DPPsC (4.1 mM) by Ca^{2+}/PL A_2 (a) and Cd^{2+}/PL A_2 (b), as well as control experiments (c) in the absence of PL A_2 or metal ions. In the absence of PL A_2, neither Ca^{2+} nor Cd^{2+} catalyzed hydrolysis of (S_P)-DPPsC substantially. At 25 h, the hydrolysis was ca. 18% for Cd^{2+}/PL A_2, but only barely detectable (<5%) for apo-PL A_2, which clearly showed the activation of apo-PL A_2 by Cd^{2+}. The hydrolysis of Ca^{2+}/PL A_2 was 30% at 4 h, approximately 10 times faster than that of Cd^{2+}/PL A_2. It is not necessary to subtract the rate of apo-PL A_2 from the rate of Cd^{2+}/PL A_2 because the residual activity of apo-PL A_2 should have been mostly suppressed in the presence of 4.6 mM Cd^{2+} due to the low K_i of Cd^{2+}.

Effect of Cd^{2+} on the Stereospecificity of PL A_2. The K_m and V_{max} of DPPC and (R_P)-DPPsC for Cd^{2+}/PL A_2 were measured spectrophotometrically as described for Ca^{2+}/PL A_2 and are listed in Table I. The V_{max} of (S_P)-DPPsC was too low to be measured spectrophotometrically. Since the ^{31}P NMR result (at saturating substrate concentrations) gave a relative velocity of 10/1 for Ca^{2+}/Cd^{2+}, the V_{max} of (S_P)-DPPsC for Cd^{2+}/PL A_2 can be assigned as 0.0044 μmol min^{-1} mg^{-1}, i.e., $^1/_{10}$ of that for Ca^{2+}/PL A_2.

According to the data in Table I, the ratio V_R/V_S is 1727 for Ca^{2+}/PL A_2 and 15.7 for Cd^{2+}/PL A_2. Thus, substitution of Ca^{2+} by Cd^{2+} causes a decrease in the V_R/V_S ratio by a factor of 110, which is a large change though not large enough for a complete reversal of stereospecificity ($V_R/V_S < 1$). The qualitative relationship in the metal ion dependence of stereospecificity is best summarized by another set of ^{31}P NMR experiments by use of (R_P + S_P)-DPPsC. As shown in Figure 9, the R_P isomer was hydrolyzed faster than the S_P isomer by both Ca^{2+}/PL A_2 (Figure 9a) and Cd^{2+}/PL A_2 (Figure 9b), confirming $V_R/V_S > 1$ in both cases. By use of 92 times of PL A_2 in the Cd^{2+} sample as in the Ca^{2+} sample, it was shown in Figure 9 that (R_P)-DPPsC was hydrolyzed faster in (a) than in (b), whereas (S_P)-DPPsC was hydrolyzed faster in (b) than in (a). This confirmed the result (from Table I) that, for (R_P)-DPPsC, $V_{Ca^{2+}}/V_{Cd^{2+}}$ = 1100 (>92) and, for (S_P)-DPPsC, $V_{Ca^{2+}}/V_{Cd^{2+}}$ = 10 (<92). The "partial reversal of stereospecificity" is also obvious from this figure: in (a), hydrolysis of (R_P)-DPPsC was complete at 16 h, but no hydrolysis of (S_P)-DPPsC occurred up to 142 h; in (b), hydrolysis of the S_P isomer occurred before hydrolysis of the R_P isomer was complete (at 15 h).

DISCUSSION

Specificity of Phospholipase A_2. The catalysis of PL A_2 is characterized by three types of specificity: (1) substrate specificity, which requires a phosphate ester adjacent to a fatty acid ester (Bonsen et al., 1972); (2) surface specificity, which requires aggregated substrates with a suitable chain length for optimal activity (Pieterson et al., 1974; de Haas et al., 1971); (3) stereospecificity, which requires L conformation at the C_2 of lecithin (Bonsen et al., 1972). Thus, pancreatic PL A_2 hydrolyzes L-dioctanoyllecithin with an optimal activity, whereas L-didecanoyllecithin is not hydrolyzed at all (de Haas et al., 1971), as a result of surface specificity. When the surface quality is made equal, the stereospecificity can be demonstrated: in the equimolar mixtures of 3-*sn*-dioctanoyllecithin plus 1-*sn*-didecanoyllecithin and 3-*sn*-didecanoyllecithin plus 1-*sn*-dioctanoyllecithin, only the 3-*sn*-lecithin is hydrolyzed in both cases, *at the same rate* (Verger et al., 1973).

The difference between kinetic properties of DPPC and DPPsC can be caused by either the surface specificity or the substrate specificity, or both, of bee venom PL A_2. The difference between the R_P and S_P isomers of DPPsC can be caused by either the stereospecificity or the surface specificity, or both. Although the difference in the surface quality between DPPC and DPPsC isomers is expected to be minimal since they all bear the same acyl chains and choline group, our previous study showed that there are small, yet distinct, differences in the physical properties of DPPC and DPPsC isomers in unilamellar (Tsai et al., 1984) as well as multilamellar phases (Tsai et al., 1983). Are these differences responsible for the different kinetic properties of DPPC and DPPsC isomers as substrates of PL A_2?

The question is difficult to answer quantitatively, as there is still no unified model for the mysterious "interfacial interaction" between lipids and lipolytic enzymes (Verger, 1980). However, the apparent K_m values in Table I seem to suggest that the less active isomers bind better (lower K_m). Whether the observed K_m is a reflection of the interfacial interaction or the binding of substrate to the active site is discussed in a later section. In any case, the relative invariance in K_m values suggests that the 24-fold difference in the V_{max} of DPPC and (R_P)-DPPsC for Ca^{2+}/PL A_2 is mainly caused by substrate specificity instead of surface specificity. Such a difference is not unusual in enzyme–nucleotide interactions (Cohn, 1982; Eckstein, 1983), but it is intriguing for PL A_2.

Stereospecificity of PL A_2. Again, the apparent K_m values suggest that the 1727-fold difference in the V_{max} of (R_P)- and (S_P)-DPPsC is mainly caused by stereospecificity (at phosphorus) rather than by the surface specificity of PL A_2.

Such a high degree of stereospecificity, induced simply by

Table II: Summary of Results on the Metal Ion Dependence of Stereospecificity

enzyme	substrate	V_R/V_S Mg^{2+}	Ca^{2+}	Zn^{2+}	Cd^{2+}	Mg/Cd	Ca/Cd	reference
acetate kinase	ATPαS	1/4.6	–	–	1/52.1	11.4		Romaniuk & Eckstein (1981)
	[ATPβS]	>1667	–	–	1/1.1	[>1834]		
arginine kinase	[ATPαS]	16.8	–	–	1/10	[168]		Cohn et al. (1982)
	[ATPβS]	24.2	–	–	1/137	[3343]		
glucokinase	ATPαS	1/104	–	–	1/2.6	1/40		Darby & Trayer (1983)
	[ATPβS]	14.9	–	–	1/6.3[a]	[93]		
3-P-glycerate kinase	[ATPαS]	1/235	–	1.02	–	–		Jaffe et al. (1982)
	ATPβS	1/1000	–	1/3000	–	–		
hexokinase (yeast)	ATPαS	1/20.4	–	–	1/23	1.1	–	Jaffe & Cohn (1979)
	[ATPβS]	590	39	1.6	1/36.7	[21650]	[1431]	
hexokinase (mammalian)	ATPαS	1/30.5	–	–	1/10[a]	1/3		Darby & Trayer (1983)
	[ATPβS]	19.8	–	–	1/2.5	[50]		
myokinase (yeast)	ATPαS	1/8.9	–	–	1/9.7	1.1		Tomasselli & Noda (1983)
	[ATPβS]	8.9	–	–	1/28.3	[252]		
myosin ATPase	ATPαS	3.1	–	–	1/1.9	5.9		Connolly & Eckstein (1981)
	[ATPβS]	1/2777	–	–	4.3	[1/11940]		
Met-tRNA synthetase	[ATPβS]	<1/21000	–	20	–	–		Smith & Cohn (1982)
Phe-tRNA synthetase	[ATPβS]	2.7	–	–	1/50	[135]		Connolly et al. (1980)
PRPP synthetase	[ATPαS]	1/2500	–	–	1.2	[1/3000]		Gibson & Switzer (1980)
pyridoxal kinase	ATPαS	1.2	–	–	1/1.2	1.4		Churchich & Wu (1982)
	[ATPβS]	1/30	–	1/7	5.0	[1/150]		
RNA polymerase	[ATPβS]	1/1.1	–	–	large	–		Armstrong et al. (1979)
TF_1ATPase	ATPαS	1/753	–	–	1/22	1/34		Senter et al. (1983)
	[ATPβS]	≤1/575	–	–	2	[<1/1150]		
phospholipase A_2	DPPsC		1727	–	15.7		110	this work

[a] Initial velocity instead of V_{max} was reported in these cases.

substituting an oxygen atom with a sulfur atom at a position five bonds away from the center of reaction, provides significant information regarding the interaction between phospholipids and PL A_2. Since the choline side chain is not a required feature of substrates, the phosphate group is the only site available for an ionic interaction with some charged residue or cofactor of the enzyme. Such an ionic interaction must be specific to one of the two diastereotopic oxygen atoms of the phosphate and responsible for the high stereospecificity toward the R_P isomer of DPPsC. Because Ca^{2+} is required for catalysis, it is most likely to be involved in the stereospecific binding with the phosphate group. This is further supported by the "metal ion dependence of stereospecificity" discussed in the following section.

Metal Ion Dependence of Stereospecificity. A number of nucleotide-related enzymes show a "reversal of stereospecificity" upon substitution of Mg^{2+} by Cd^{2+} (Jaffe & Cohn, 1979; Cohn, 1982; Eckstein, 1983), presumably caused by the preference of Mg^{2+} to oxygen ligands and Cd^{2+} to sulfur ligands. In the reaction catalyzed by hexokinase, $(V_R/V_S)_{Mg^{2+}} = 590$ and $(V_R/V_S)_{Cd^{2+}} = 0.027$ were obtained with ATPβS and interpreted as evidence for direct coordination between Mg^{2+} and the *pro-S* oxygen of β-phosphate. Such a metal ion dependence, however, was not observed with ATPαS for the same enzyme ($V_R/V_S = 20.4$ and 23 for Mg^{2+} and Cd^{2+}, respectively). The latter can be interpreted by two different ways: either the metal ion does not bind to the P_α of ATP or the geometric constraint is very high near the α-phosphate, such that Cd^{2+} is forced to bind to the oxygen even though it prefers the sulfur.

Hexokinase/ATPβS is probably an ideal case in which there is little steric or electronic constraint on the β-phosphate–metal binding site, such that the stereospecificity of binding is dictated predominantly by the oxygen vs. sulfur ligand preference of M^{2+}. Table II summarizes the ratios V_R/V_S of PL A_2 along with those published before 1983 (the reports by use of initial velocity rather than V_{max} are not included). The cases in brackets show a reversal in stereospecificity going from Mg^{2+} to Cd^{2+} (or Zn^{2+}) and have been used as evidence for direct coordination between M^{2+} and the phosphate.

In nonideal cases the ligand preference of metal ions is obscured, to a varying degree, by steric or electronic constraint at the coordination site. Under this condition the ratio $(V_R/V_S)_{Mg^{2+}}/(V_R/V_S)_{Cd^{2+}}$, defined as the "Mg/Cd" ratio, should be considered in interpreting the data. In all cases where a reversal of stereospecificity occurred, the Mg/Cd ratio is always >50 or $<^1/_{150}$. In all cases where a reversal of stereospecificity was not observed, the Mg/Cd ratio is close to 1 (from $^1/_3$ to 11.4), except the cases of glucokinase/ATPαS (Mg/Cd = $^1/_{40}$) (Darby & Trayer, 1983) and TF_1ATPase/ATPαS (Mg/Cd = $^1/_{34}$) (Senter et al., 1983). Although the authors of the former concluded that the α-P is not involved in coordinating the metal on the enzyme, the authors of the latter expressed some reservation in their interpretation of the unusually small Mg/Cd ratio.

The Ca/Cd ratio of PL A_2/DPPsC is 110, which should correspond to an even larger Mg/Cd ratio since the O/S preference of Mg^{2+} is larger than that of Ca^{2+} (Jaffe & Cohn, 1979; Pecoraro et al., 1984). In the case of hexokinase/ATPβS, Mg/Cd = 15 (Ca/Cd). Thus, the Mg/Cd ratio of PL A_2/DPPsC could be as high as ca. 1650, which strongly supports direct $Ca^{2+}\cdots{}^{-}O—P{=}O$ coordination, even though the stereospecificity is not fully reversed by substituting Ca^{2+} with Cd^{2+}. Recently Pecoraro et al. (1984) reported a detailed analysis of various factors that may influence the observed metal ion dependence of stereospecificity. Such a quantitative analysis is not yet possible for PL A_2 because we do not have complete K_m values, and the K_m values may reflect more of the interfacial interaction than binding of the substrate to the active site, as discussed in the following section.

Implication of the Results of Inhibition Studies. Study of inhibition kinetics has been useful in understanding the mechanism of many enzymes. In the case of PL A_2, any incorporation of inhibitor may change the quality of interface and complicate kinetic analysis. Among the few known competitive inhibitors of PL A_2 (porcine pancreas) are 1-*sn*-phosphatidylcholines and the 2-amide analogue of 3-*sn*-phosphatidylcholines (Bonsen et al., 1972).

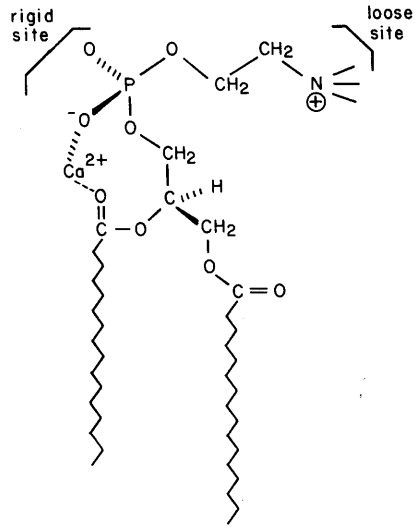

FIGURE 10: Structure showing binding of Ca^{2+} with the *pro-S* oxygen and possibly with the 2-carbonyl oxygen of phospholipid substrate at the active site of PL A_2.

Our inhibition study has allowed us to differentiate the "surface interaction" (step 1) and the "active site binding" (step 2) (Deems et al., 1975):

$$E{\cdot}Ca^{2+} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}Ca^{2+}{\cdots}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}Ca^{2+}{\cdot}S \xrightarrow{\text{step 3}} E{\cdot}Ca^{2+}{\cdot}P$$

(step 1 over k_1, k_{-1}; step 2 over k_2, k_{-2})

In the experiment by use of mixed micelles prepared from 1:1 mixture of DPPC/(S_P)-DPPsC, there is only one type of micelles, and the inhibitor does not compete with the substrate until step 2. Since only small differences between the kinetic properties of DPPC and (S_P)-DPPsC and between that of (R_P)-DPPsC and (S_P)-DPPsC were observed, the inhibitor does not seem to compete well at step 2. On the other hand, when DPPC micelles and (S_P)-DPPsC micelles were prepared separately and mixed just before the reaction, (S_P)-DPPsC was a competitive inhibitor. This suggests that the inhibition effect occurs mainly at step 1, the surface interaction.

Thus, (S_P)-DPPsC should have larger intrinsic K_m at step 2 relative to the R_P isomer. The observed K_m and K_i of (S_P)-DPPsC are both small, which suggests that the observed K_m is mainly a reflection of the first binding step, the interfacial interaction.

The competitive inhibition by Cd^{2+} suggests that it binds to the same site of PL A_2 as Ca^{2+} does, but the UV-difference spectrum suggests that $E{\cdot}Cd^{2+}$ may have a somewhat different conformation from $E{\cdot}Ca^{2+}$. Such a conformational difference could be due to different ligation properties of Cd^{2+} in the active site of the enzyme and could be the main cause of the low activity of the PL $A_2{\cdot}Cd^{2+}$ complex. However, the PL $A_2{\cdot}Cd^{2+}$ complex still retains the capability of substrate binding and catalysis, as shown by our kinetic data.

Catalytic Role of Ca^{2+} in PL A_2. The role of Ca^{2+} in the catalysis of PL A_2 has not been well understood. Our results strongly suggest that Ca^{2+} is involved in two functions. The first is to maintain the proper conformation of the enzyme. Cd^{2+} also binds to the enzyme with high affinity but does not keep the enzyme in the most appropriate conformation for catalysis. The second function of Ca^{2+} is to bind the *pro-S* oxygen of the phosphate group of the substrate and orient the substrate in the proper conformation, as shown in Figure 10. According to the mechanism proposed by Verheij et al. (1980), another function of Ca^{2+} is probably to polarize the 2-C=O group of the substrate and facilitate the nucleophilic attack by H_2O at the carbonyl carbon. This third function is yet to be demonstrated experimentally.

Although the PL A_2 from bee venom has a quite different amino acid sequence than PL A_2 from other sources (Shipolini et al., 1974a,b; Slotboom et al., 1982), the mechanistic features described in this work are most likely applicable to PL A_2 from other sources because Ca^{2+} is commonly required by PL A_2 and the enzyme from four different sources (bee venom, porcine pancreas, *Naja naja* venom, and *Crotalus adamanteus* venom) all show a high stereoselectivity toward the R_P isomer of DPPsC (Bruzik et al., 1983).

For pancreatic PL A_2, the residues of the enzyme interacting with Ca^{2+} have been well characterized (Slotboom et al., 1982). The Ca^{2+}-induced difference spectrum (small peaks at 280–290 nm; large peak at 242 nm) was attributed to the perturbation of tyrosine and histidine residues by Ca^{2+} (Pieterson et al., 1974a). In addition, a second, loose Ca^{2+} binding site has been characterized (Slotboom et al., 1978; Donné-Op den Kelder et al., 1983; Drakenberg et al., 1984). The interaction of Ca^{2+} with the bee venom PL A_2 has not been characterized. However, the Ca^{2+}- and Cd^{2+}-induced peak at 287 nm is consistent with the perturbation of an aromatic residue, most likely tyrosine.

Conclusion. The kinetic properties of bee venom PL A_2 have been investigated by use of DPPC and DPPsC isomers. The differences between DPPC and DPPsC are attributed to the substrate specificity, whereas the differences between DPPsC isomers are attributed to the stereospecificity of the enzyme. The surface specificity does not play an important role in present cases. The binding studies of Ca^{2+} and Cd^{2+} show conformational changes of PL A_2 induced by the metal ions. The Cd^{2+} ion inhibits the reaction competitively, but its function as an activator has also been demonstrated by use of (S_P)-DPPsC. Substitution of Ca^{2+} by Cd^{2+} causes the ratio of the maximal velocity, V_R/V_S, to decrease by a factor of 110, which supports direct binding of Ca^{2+} with the *pro-S* oxygen of the phosphate group of DPPC in the active site of PL A_2. The implication of the results on the interfacial interaction and on the catalytic roles of Ca^{2+} in the catalysis of PL A_2 is also discussed.

ACKNOWLEDGMENTS

We are indebted to Dr. C. Steginsky of this laboratory for useful discussion and to Dr. W. W. Cleland for providing us a manuscript (Pecoraro et al., 1984) prior to publication.

Registry No. PL A_2, 9001-84-7; DPPC, 63-89-8; (R_P)-DPPsC, 82482-77-7; (S_P)-DPPsC, 82482-78-8; Ca, 7440-70-2; Cd, 7440-43-9.

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