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Kinetic Analysis of the Dual Phospholipid Model for Phospholipase A₂ Action*

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A kinetic scheme is proposed for the action of cobra venom phospholipase A_2 on mixed micelles of phospholipid and the nonionic detergent Triton X-100, based on the "dual phospholipid model."

$$E + S \xleftarrow{k_1} ES = ES + S \xleftarrow{k_2} ESS \xrightarrow{k_3} ESS + Prod$$

The water-soluble enzyme binds initially to a phospholipid molecule in the micelle interface. This is followed by binding to additional phospholipid in the interface and then catalytic hydrolysis. A kinetic equation was derived for this process and tested under three experimental conditions: (i) the mole fraction of substrate held constant and the bulk substrate concentration varied; (ii) the bulk substrate concentration held constant and the Triton X-100 concentration varied (surface concentration of substrate varied); and (iii) the Triton X-100 concentration held constant and the bulk substrate concentration varied. The substrates used were chiral dithiol ester analogs of phosphatidylcholine (thio-PC) and phosphatidylethanolamine (thio-PE), and the reactions were followed by reaction of the liberated thiol with a colorimetric thiol reagent. The initial binding $(K_s = k_1/k_{-1})$ was apparently similar for thio-PC and thio-PE (between 0.1 and 0.2 mM) as were the apparent Michaelis constants $(K_m = (k_{-2} + k_3)/k_2)$ (about 0.1 mol fraction). The V_{max} values for thio-PC and thio-PE were 440 and 89 µmol min⁻¹ mg⁻¹, respectively. The preference of cobra venom phospholipase A₂ for PC over PE in Triton X-100 mixed micelles appears to be an effect on k_3 (catalytic rate) rather than an effect on the apparent binding of phospholipid in either step of the reaction.

Phospholipase A_2 , like most other lipolytic enzymes, acts preferentially on substrates which are aggregated at a lipidwater interface (1, 2). The presence of this interface makes kinetic analysis of the enzymatic reaction more complicated than those reactions with simple water-soluble substrates. The Triton X-100¹/phosphatidylcholine mixed micelle system allows kinetic analysis of the enzyme reaction under conditions in which the surface concentration of the substrate can be varied independently of the bulk concentration. An earlier kinetic analysis of phospholipase A_2 from cobra venom (*Naja naja naja*) assumed initial nonspecific interaction of the enzyme with the interface followed by binding of the phospholipid substrate in its active site (3). More recently a "dual phospholipid model" was suggested in which the enzyme binds a phospholipid molecule in the first step rather than nonspecifically to the interface, and then the interfacial enzyme binds a second phospholipid in the interface (4, 5). Several detailed models for this process have been formulated, some of which involve a conformational change upon binding to the first phospholipid and a possible role for a dimeric enzyme (2).

The earlier kinetic study on cobra venom phospholipase A₂ was based on a pH-stat assay which is limited in sensitivity and subject to sizable experimental error, particularly at low initial rates. A more sensitive assay was developed by Aarsman et al. (6) based on the use of thiol ester substrate analogs. This reaction can be followed spectrophotometrically by reaction of the liberated thiol with a colorimetric thiol reagent. Volwerk et al. (7) reported on the use of a dithiol ester analog of phosphatidylcholine for a phospholipase A2 assay. This substrate, however, was racemic and not suitable for detailed kinetic analysis since the enzymatically inactive enantiomer would act as a competitive inhibitor and thus complicate the analysis. The availability of a *chiral* thio- PC^2 substrate (8) and the greater sensitivity of the thiol assay prompted a more detailed evaluation of the kinetics of cobra venom phospholipase A₂ in Triton X-100/thio-PC mixed micelles. The kinetic analysis for the dual phospholipid model is derived herein, and the kinetic parameters for cobra venom phospholipase A₂ action on both thio-PC and thio-PE in mixed Triton X-100 micelles are obtained.

EXPERIMENTAL PROCEDURES

Materials—Phospholipase A_2 was purified to homogeneity from N. naja naja (cobra) venom as described by Deems and Dennis (9) and modified by Darke et al. (10). Thio-PC was synthesized by the same procedure as described by Hendrickson et al. (8) for the synthesis of the bisheptanoyl homolog. The starting material was D-mannitol which was successively converted to 1 - trityl - sn - glycerol; 2, 3 - bis(tosyl)-1-trityl-sn-glycerol; 3-trityl-1,2-dideoxy-1,2-(thiocarbonyldithio)-sn-glycerol; 3-trityl-1,2-dideoxy-1,2-(dithio)-sn-glycerol; 3trityl-1,2-bis(decanoylthio)-1,2-dideoxy-sn-glycerol; 1,2-bis(decanoylthio)-1,2-dideoxy-sn-glycerol; and finally to 1,2-bis(decanoylthio)-1,2-dideoxy-sn-glycerol-3-phosphocholine. The two thio-PC homologs had identical IR spectra and R_F values on silica gel TLC.

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¹Triton X-100 is a polydisperse preparation of p-(1,1,3,3-tetramethylbutyl)phenoxypolyoxyethylene glycols containing an average of 9.5 oxyethylene units/molecule.

² The abbreviations used are: thio-PC, 1,2-bis(decanoylthio)-1,2-dideoxy-*sn*-glycerol-3-phosphorylcholine; thio-PE, 1,2-bis(decanoyl-thio)-1,2-dideoxy-*sn*-glycerol-3-phosphorylethanolamine; diapalmitoyl-PC, 1,2-bis(palmitoyl)-*sn*-glycerol-3-phosphorylcholine; CMC, critical micelle concentration.

Thio-PE was prepared from thio-PC by phospholipase D-catalyzed exchange of ethanolamine for choline (11) and purified by preparative high pressure liquid chromatography. 4,4'-Dithiobispyridine was purchased from Aldrich, and Triton X-100 was obtained from Rohm and Haas Co., Philadelphia, PA.

Preparation of Mixed Micelles—A measured amount of phospholipid in chloroform solution was dried under a stream of nitrogen and then in vacuo. Triton X-100 in buffer was added, and the mixture was warmed to about 40 °C and vortexed several times until a clear solution was obtained.

Enzyme Assay—The standard buffer contained 0.1 M KCl, 25 mM Tris chloride, pH 8.5, and 10 mM CaCl₂. Micellar substrate in buffer (0.3 ml) was added to a microcuvette (2 × 10 mm), followed by 5 μ l of 4,4'-dithiobispyridine (50 mM in ethanol). The cuvette was placed in the cell compartment of a Perkin-Elmer Model 552 spectrophotometer thermostated at 30 °C and balanced against a blank containing 4,4'-dithiobispyridine in buffer. The absorbance at 324 nm was recorded until a stable base-line was obtained. Phospholipase A₂ (10 μ l containing 50–125 ng of protein) was added and the absorbance was recorded for several minutes. Activity was calculated using an extinction coefficient of 19,800 M⁻¹ cm⁻¹ (12). Enzyme activity was linear with respect to the amount of enzyme within the range used in these assays. The average error for triplicate determinations of kinetic points was ±5%.

Computer Curve Fitting—Data were fitted by nonlinear regression analyses using the programs in KINFIT, a kinetics program written by Knack and Röhm (13). These programs include fits to polynomials, the Michaelis-Menten equation, and the Hill equation.

RESULTS

Kinetic Equation—The proposed kinetic scheme involves binding of the water-soluble enzyme to phospholipid in the micelle surface. This is followed by binding to additional phospholipid in the interface and then catalytic hydrolysis. This is summarized in Equation 1.

$$E + S \xrightarrow{k_1} ES \qquad ES + S \xrightarrow{k_2} ESS \xrightarrow{k_3} ESS + Prod \quad (1)$$

Triton X-100 present in the micelle is assumed to be an inert diluter with negligible affinity for the enzyme. Thus, E and the first S should be expressed in bulk terms and will be denoted E_B and S_B , whereas ES, ESS, product, and the second S should be expressed in surface concentration terms. The following symbols are used in the kinetic derivation: E_0 (mole/ volume), total enzyme; $T_0 = T_t - T_{mon}$ (mole/volume), total Triton X-100 in the micelle where T_t (mole/volume) is the total concentration of Triton X-100 utilized and T_{mon} (mole/ volume) is the monomer concentration present which is assumed to equal its CMC of 0.24 mM (14); S_0 (mole/volume), total phospholipid substrate (bulk concentration); X_s (mole fraction, unitless), total phospholipid substrate (surface concentration); $[S_B]$ (mole/volume), free phospholipid substrate (bulk concentration); [S] (mole fraction, unitless), free phospholipid substrate (surface concentration); $[E_B]$ (mole/volume), free enzyme (bulk concentration); [ES] and [ESS] (mole fraction, unitless), intermediates (surface concentration); [Prod] (mole fraction, unitless), product (surface concentration); $K_s = k_{-1}/k_1$ (mole/volume); $K_m = (k_{-2} + k_3)/k_2$ (mole fraction, unitless), apparent Michaelis constant (surface concentration); $L_0 = S_0 + T_0$ (mole/volume), total interface in bulk concentration units (bulk concentration) assuming equal surface areas for Triton and phospholipid molecules (3); v ((mole/volume)time⁻¹), velocity (bulk concentration); and $V_{\text{max}} = k_3 E_0$ ((mole/volume)time⁻¹), maximal velocity.

Equations for velocity and steady state conditions are shown in Equations 2-4. L_0 is used to convert from surface units (mole fraction, unitless) to bulk units (mole/volume) and vice versa.

$$v = L_0 d[\operatorname{Prod}]/dt = L_0 k_3 [ESS]$$
⁽²⁾

$$L_0 d[ES]/dt = k_1 [E_B] [S_B] + (k_{-2} + k_3) L_0 [ESS]$$

$$-k_{-1}L_0[ES] - k_2L_0[ES][S] = 0$$

$$d[ESS]/dt = k_2[ES][S] - (k_{-2} + k_3)[ESS] = 0$$
(4)

The total enzyme, E_0 , is given by Equation 5.

$$E_0 = [E_B] + [ES]L_0 + [ESS]L_0$$
(5)

Equations 3-5 can be combined to give an expression for [ESS].

$$[ESS] = E_0[S][S_B]/L_0(K_sK_m + K_m[S_B] + [S][S_B])$$
(6)

Substituting Equation 6 into Equation 2 gives the velocity at steady state in terms of the total initial bulk concentration of substrate S_0 , where $S_0 = [S_B]$, and the total initial surface concentration of substrate, X_s , where $X_s = [S]$.

$$v = \frac{V_{\max}X_{s}S_{0}}{K_{m}K_{s} + K_{m}S_{0} + X_{s}S_{0}}$$
(7)

Kinetic Approaches—The simplest system in which to test the kinetic formulation for the dual phospholipid model would be a system with monomeric substrates. Unfortunately, such an approach is not feasible since the apparent K_m for the enzymatic reaction as determined for monomeric systems appears to occur near or above the CMC for these substrates (regardless of chain length) where the substrate becomes micellar (1). Decreasing the chain length results in an increased CMC, but also an increased apparent K_m . This necessitates the use of a micellar system. Triton X-100 has proven useful in such systems since the detergent is uncharged, has a low CMC, and allows the surface concentration of the substrate to be varied independently of the bulk concentration. In a micellar system, two types of binding can take place: (i) initial binding of the enzyme to the micelle surface, which is dependent on the bulk concentration of substrate; and (ii) secondary binding of enzyme within the interface, which is dependent on the surface concentration of substrate.

Equation 7 is in the form of a rate equation for an ordered bisubstrate reaction (15). The determination of kinetic parameters for such a reaction involves variation of the concentration of one substrate at a fixed concentration of the other. There are three variables involved in experiments with Triton X-100/phospholipid mixed micelles: S_0 , X_s , and T_0 . If one is fixed and the other two varied, three types of experiments can be performed.

In Case I, the surface concentration of substrate, X_s , is fixed and the bulk concentrations of substrate, S_0 , and of Triton, T_0 , are varied. This is accomplished experimentally by increasing the concentrations of phospholipid and Triton proportionally. A plot of v versus S_0 should give a hyperbolic saturation curve with the maximal velocity equal to $V_{max}/(K_m/X_s + 1)$ and the value of S_0 at one-half maximal velocity equal to $K_s/(X_s/K_m + 1)$. This experiment allows determination of the initial binding of enzyme to the interface, K_s , where the secondary binding in the interface is fixed. A linear transformation of Equation 7 in terms of the variables v and S_0 is given in Equation 8.

$$\frac{1}{v} = \frac{K_m K_s}{V_{\max} X_s} \frac{1}{S_0} + \frac{(K_m / X_s + 1)}{V_{\max}}$$
(8)

A plot of 1/v versus $1/S_0$ should give a straight line which intersects the 1/v axis at $(K_m/X_s + 1)/V_{max}$ and the $1/S_0$ axis at $-(X_s/K_m + 1)/K_s$.

In Case II, the bulk concentration of substrate, S_0 , is fixed but the surface concentration of substrate, X_s , and the bulk concentration of Triton, T_0 , are varied. This is accomplished experimentally by just increasing the concentration of Triton.

(3)

A plot of v versus X_s should give a hyperbolic saturation curve with the maximal velocity equal to V_{max} and the value of X_s at one-half maximal velocity equal to $K_m(K_s/S_0 + 1)$. This experiment allows determination of the apparent interfacial Michaelis constant, K_m (mole fraction units), where the initial binding to the interface is fixed. A linear transformation of Equation 7 in terms of the variables v and X_s is given in Equation 9.

$$\frac{1}{v} = \frac{K_m(K_s/S_0 + 1)}{V_{\max}} \frac{1}{X_s} + \frac{1}{V_{\max}}$$
(9)

A plot of 1/v versus $1/X_s$ should give a straight line which intersects the 1/v axis at $1/V_{max}$ and the $1/X_s$ axis at $-1/K_m(K_s/S_0 + 1)$.

In Case III, the bulk concentration of Triton X-100, T_0 , is fixed and the bulk concentration of substrate, S_0 , and its surface concentration, X_s , are varied. This is accomplished experimentally by just increasing the phospholipid concentration. The kinetic expression for this case can be obtained directly from Equation 7 (where $X_s = S_0/L_0$ and $L_0 = S_0 + T_0$) and is given in Equation 10.

$$v = \frac{V_{\text{max}}S_0^2}{K_m K_s T_0 + (T_0 + K_s) K_m S_0 + (K_m + 1)S_0^2}$$
(10)

A plot of v versus S_0 should be sigmoidal. The double reciprocal form of Equation 10 is given in Equation 11.

$$\frac{1}{v} = \frac{K_m K_s T_0}{V_{\max}} \left(\frac{1}{S_0}\right)^2 + \frac{(T_0 + K_s) K_m}{V_{\max}} \left(\frac{1}{S_0}\right) + \left(\frac{K_m + 1}{V_{\max}}\right)$$
(11)

This equation is in the form of a second order polynomial, $Y = aX^2 + bX + c$. Data can be fit to this equation by nonlinear regression analysis using the polynomial fit in the computer program KINFIT. A plot of 1/v versus $1/S_0$ should curve upward and fit this polynomial.

Thio-PC Substrate—Kinetic data for the phospholipase A_2 catalyzed hydrolysis of thio-PC were obtained in the three cases defined above. When the mole fraction of thio-PC was kept constant and the bulk concentration of substrate varied (Case I), saturation curves were obtained (Figs. 1 and 2). These points fit Equation 8, and double reciprocal plots of the points gave reasonable straight lines. From the maximal velocity, a value for K_m was calculated using the V_{max} obtained in Case II. This value of K_m was used to calculate K_s from the value of S_0 at one-half maximal velocity. The results are shown in Table I.

For Case II, the bulk concentration of thio-PC was kept constant and the amount of Triton X-100 varied. A plot of v



FIG. 1. Activity of phospholipase A_2 toward Triton X-100/ thio-PC mixed micelles as a function of total thio-PC (Case I). Constant $X_s = 0.0196$; standard assay conditions. Solid lines, computer fits to Equations 7 and 8.



FIG. 2. Activity of phospholipase A_2 toward Triton X-100/ thio-PC mixed micelles as a function of total thio-PC (Case I). Constant $X_s = 0.0385$; standard assay conditions. Solid lines, computer fits to Equations 7 and 8.

versus X_s appeared more sigmoidal than hyperbolic (Fig. 3). From a computer fit to the Hill equation

$$v = V_{\max} X_s^n / (K'^n + X_s^n); K' = K_m (K_s / S_0 + 1)$$

values of V_{max} and K_m were calculated using a value of 0.1 mM for K_s (Table I).

When the concentration of thio-PC was varied at constant Triton X-100 concentration (Case III), plots of v versus S_0 (Fig. 4) were sigmoidal. Plots of 1/v versus $1/S_0$ fit nicely to second order polynomials (Fig. 5) as given by Equation 11.

Thio-PE Substrate—Kinetic data for the hydrolysis of thio-PE by phospholipase A_2 were obtained in experiments similar to those described above for thio-PC. For Case I, a plot of vversus S_0 at constant X_s (Fig. 6) was used to calculate K_s . For Case II, a plot of v versus X_s at constant S_0 (Fig. 7) was used to calculate K_m . This plot could be fit reasonably well to a hyperbolic curve. These kinetic parameters are also given in Table I. For Case III, a plot of v versus S_0 at constant T_0 (Fig. 8) appeared sigmoidal and fit Equation 10, through a computer fit to the polynomial as given by Equation 11. Kinetics for the enzyme reactions with thio-PE and thio-PC was initially linear and eventually decreased as the percentage of hydrolysis increased. Lag phases were not observed with either substrate.

DISCUSSION

The use of thio-PC and thio-PE as substrates for phospholipase A_2 allows precise determination of reaction rates by spectrophotometric detection of the thiol products using 4,4'dithiobispyridine as a thiol reagent. Plots of velocity as a function of substrate concentration at constant Triton X-100 concentration show sigmoidal character. This is a strong indication that the reaction involves at least two types of substrate binding, consistent with the dual phospholipid model suggested earlier (4, 5). A kinetic expression (Equation 7) was derived for the model involving the binding of two substrate molecules to the enzyme.

Kinetic experiments at constant surface concentration of substrate allowed calculation of the dissociation constant, K_s , for initial binding of enzyme to the interface. Any deviation of the lowest points from the theoretical curves in Figs. 1 and 2 may result from the fact that in this region the concentration of bulk Triton X-100 approaches its CMC of 0.24 mM (14), so the assumption that the concentration of monomers is equal to the CMC may not be perfect. The calculation of surface concentration of substrate should really utilize the concentration of micellar Triton X-100, T_0 , where $T_0 = T_t$ –

TABLE I Kinetic parameters for phospholipase A_2 -catalyzed hydrolysis of thio-PC and thio-PE

Substrate	Case ^a	So	Χ,	K,	K _m	Vmax
		mM	mol fraction	тM	mol fraction	µmol min ⁻¹ mg ⁻¹
Thio-PC	I	0.02-0.34	0.0385	0.18 ± 0.03		
	Ī	0.005-0.23	0.0196	0.12 ± 0.03		
	ĪI ^b	0.476	0.01 - 0.2		0.11 ± 0.01	440 ± 20
Thio-PE	Ī	0.02-0.39	0.0385	0.19 ± 0.01		
	Ī	0.02-0.39	0.0196	0.18 ± 0.03		
	ĪI	0.476	0.02 - 0.11		0.10 ± 0.01	89 ± 8

^a Experiments were carried out in triplicate. Errors represent the standard deviation of a computer fit of the data by nonlinear regression analysis.

^b The plot of v versus X, was sigmoidal and fit the Hill equation with a Hill coefficient of $n = 1.40 \pm 0.05$.



FIG. 3. Activity of phospholipase A₂ toward Triton X-100/ thio-PC mixed micelles as a function of mole fraction thio-PC (Case II). Constant $S_0 = 0.476$ mM; standard assay conditions. Solid lines, computer fits to Equation 9.



FIG. 4. Activity of phospholipase A_2 toward Triton X-100/ thio-PC mixed micelles as a function of thio-PC concentration (Case III). Constant Triton X-100 concentration: 0.952 mM (Δ), 4.76 mM (\bigcirc) and 9.52 mM ($\textcircled{\bullet}$). Solid lines, computer fits to Equation 11. Standard assay conditions.

[Triton X-100 monomers] (16). Since it would be very difficult to measure the monomer concentration of detergent under the conditions of the experiments, this calculation is not feasible and it assumed that the concentration of Triton X-100 monomers is equal to its CMC. However, as the total



FIG. 5. Double reciprocal plot of Fig. 4. Symbols are the same as described in the legend to Fig. 4. Solid lines, computer fits to polynomial $1/v = a(1/S_0)^2 + b(1/S_0) + c$. Constants: Δ , a = 5.47 (± 0.38) × 10⁻⁶, b = 8.02 (±7.5) × 10⁻⁶, and c = 3.02 (±0.25) × 10⁻³; \bigcirc , a = 7.17 (±0.06) × 10⁻⁴, b = -2.06 (±5.0) × 10⁻⁵, and c = 3.29 (±0.08) × 10⁻³; \bigoplus , a = 2.43 (±0.07) × 10⁻³, b = 3.28 (±4.69) × 10⁻⁴, and c = 3.48 (±0.57) × 10⁻³.



FIG. 6. Activity of phospholipase A_2 toward Triton X-100/ thio-PE mixed micelles as a function of total thio-PE (Case I). Constant $X_s = 0.0196$; standard assay conditions. Solid lines, computer fits to Equations 7 and 8.

concentration of Triton X-100 approaches its CMC, the error introduced by this assumption becomes significant. Additional experiments at constant surface concentrations greater



FIG. 7. Activity of phospholipase A_2 toward Triton X-100/ thio-PE mixed micelles as a function of mole fraction thio-PE (Case II). Constant $S_0 = 0.476$ mM; standard assay conditions. Solid lines, computer fits to Equation 9.



FIG. 8. Activity of phospholipase A₂ toward Triton X-100/ thio-PE mixed micelles as a function of thio-PE concentration. Constant Triton-100 concentration was 9.52 mM. Solid lines, computer fit to Equation 11. A double reciprocal plot of Fig. 7 was fit by computer to the polynomial $1/v = a(1/S_0)^2 + b(1/S_0) + c$ with the constants a = 1.32 (+0.08) × 10⁻³, b = 1.91 (+0.009) × 10⁻², and c =2.07 (+1.78) × 10⁻³. Standard assay conditions.

than $X_s = 0.0385$ would allow slope and intercept replots and determination of K_m and V_{max} . Unfortunately, above $X_s =$ 0.0385, the total concentration of Triton X-100 in these experiments approaches and falls below its CMC where T_{mon} and thus T_0 cannot be reasonably estimated based on the CMC of pure Triton.

Experiments at constant bulk concentration of substrate allowed calculation of the apparent surface Michaelis constant (K_m) and V_{max} . For thio-PC, the curve in Fig. 3 was not hyperbolic, but appeared somewhat sigmoidal. In order to determine the maximal velocity and S_0 value at one-half

maximal velocity, these data were fitted to a Hill equation. The deviation from hyperbolic shape observed with thio-PC could be due to a number of causes. The assumption of equal surface areas for Triton X-100 and thio-PC (17, 18) is implicit in the kinetic treatment and allows the use of L_0 and mole fraction units, but this may not be completely valid and the surface areas may actually change as the surface concentration of substrate changes. Curvature in the analogous plots in the earlier kinetic analysis (Fig. 5 of Ref. 3) was explained in this way. Of course, Triton X-100 may have some small inhibition which is not negligible under certain conditions and this could contribute to the data. Alternatively, there may be secondary binding of the enzyme with more than one phospholipid within the interface in a cooperative manner or with other enzyme molecules. Experiments with thio-PE differed from thio-PC in that within the range of experimental conditions tested, the data could be fitted better to hyperbolic curves. This may indicate a basic difference in the secondary interactions of thio-PC and thio-PE with the enzyme.

By the nature of the system, binding of enzyme to the substrate involves two ordered steps: initial binding to the interface and secondary binding within the interface. Because different units are involved in each binding, the relative affinities at these two steps cannot be compared. If the secondary binding is tighter than the initial binding, this would imply a conformational change of the enzyme upon initial binding. Unfortunately, the experiments reported herein do not allow such a distinction. Furthermore, secondary binding of more than one substrate molecule within the interface might actually occur and would go unrecognized because the expected fits of experiments in Cases II and III would not be significantly different from the secondary binding of only one phospholipid.

 K_s values for thio-PC and thio-PE are similar (between 0.1 and 0.2 mM) as are the values of K_m (about 0.1 mol fraction). The V_{max} values for thio-PC and thio-PE, however, are quite different (440 and 89 μ mol min⁻¹ mg⁻¹, respectively). The above kinetic analysis was applied to the earlier kinetic data for the natural phospholipid substrate, dipalmitoyl-PC (3). The earlier data were obtained at 40 °C, while data reported here were obtained at 30 °C. Although these earlier data were not very precise due to the inaccuracy of the pH-stat assay method, values for K_s ranged from 0.36 to 1.5 mM and K_m was about 0.9 mol fraction with a large error. Comparing these values with the kinetic parameters for thio-PC, it appears that the first binding step with thio-PC is of tighter affinity by perhaps as much as an order of magnitude. This difference was also observed by Volwerk *et al.* (7) for apparent K_m values for pancreatic phospholipase A2-catalyzed hydrolysis of monomeric thio-PC homologs. This could be attributed to increased hydrophobicity of the sulfur atom. The $O \rightarrow S$ substitution also lowers the CMC of thio-PC by about an order of magnitude (8). The value of V_{max} for dipalmitoyl-PC was 4 $\times 10^3 \,\mu$ mol min⁻¹ mg⁻¹ (3). This is somewhat greater than that for thio-PC, even when the difference in temperature is taken into consideration.

The dual phospholipid model for phospholipase action, upon which this kinetic analysis is based, involves an initial bimolecular interaction of a water-soluble enzyme with a phospholipid molecule in the interface, followed by interaction with additional phospholipid in the interface and then catalysis. The kinetic data presented here do not differentiate which of the two binding steps involves the catalytic site, nor do they differentiate between a monomeric or dimeric active enzyme. The initial binding of enzyme to a phospholipid molecule in the interface may serve to anchor the enzyme to the interface where it then interacts with substrate. Interaction with additional phospholipid in the interface may induce a favorable conformational change in the enzyme allowing efficient catalysis. These are possible factors which may contribute to the marked preference of phospholipase A_2 for interfacial rather than monomeric substrate. These data do show that the preference of cobra venom phospholipase A_2 for Triton X-100/PC mixed micelles over Triton X-100/PE mixed micelles (19) is an effect on k_3 (catalytic rate) rather than the apparent binding of phospholipid in either step of the reaction.

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