Phospholipase A_2 Complexes with Gadolinium(III) and Interaction of the Enzyme–Metal Ion Complex with Monomeric and Micellar Alkylphosphorylcholines. Water Proton Nuclear Magnetic Relaxation Studies†

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ABSTRACT: Gadolinium(III) binds competitively with calcium(II) to porcine pancreatic phospholipase A_2 (EC 3.1.1.4) and its zymogen. The enzyme–Gd^{3+} complex exhibits 4% of the hydrolytic activity of the corresponding Ca^{2+} complex toward a dispersion of dioctanoyllecithin. Dissociation constants for the Gd^{3+} complex of enzyme and proenzyme were evaluated from water proton relaxation rate (PRR) titrations. At pH 5.8, the dissociation constants for the Gd^{3+} complexes of enzyme and zymogen are 0.50 and 0.18 mM, respectively. Dissociation constants for the complexes of enzyme with Ca^{2+}, Eu^{3+}, and Tb^{3+} were evaluated in PRR titrations by competition of these cations with Gd^{3+} binding. PRR enhancement factors for the Gd^{3+} complexes of enzyme and proenzyme are 16.4 and 5.8, respectively, at 22 °C and 24.3 MHz. Binding of a homologous series of *n*-alkylphosphorylcholines to the enzyme–Gd^{3+} complex was investigated through the influence of monomeric and micellar forms of these amphiphiles on the PRR enhancement factor for the enzyme-bound Gd^{3+}. Separate monomer and micelle binding regions were observed in titrations using *n*-alkylphosphorylcholines with critical micelle concentrations ranging from 15 μM to 13 mM. In every case, the enhancement factors for the enzyme–Gd^{3+} complexes were significantly greater than that for the ternary complex, enzyme–Gd^{3+}–monomer. Moreover, a synergism was observed in the binding of Gd^{3+} and micelles to the enzyme. The magnitudes of the PRR enhancement factors for the enzyme–Gd^{3+} complexes with micelles of *n*-alkylphosphorylcholines indicate that the bound Gd^{3+} is freely accessible to the bulk solvent. These results suggest a model for the enzyme–micelle complex in which the active site is spatially removed from the enzyme–micelle interface.

Phospholipase A_2 (EC 3.1.1.4) catalyzes the specific hydrolysis of the fatty acid ester bond at the 2 position of 1,2-diacyl-*sn*-glycero-3-phosphorylcholines (deHaas et al., 1971). Pancreatic phospholipase A_2 is secreted as a zymogen, prophospholipase A_2, which can be converted to phospholipase A_2 by tryptic cleavage of a heptapeptide from the N terminus. Both phospholipase A_2 and prophospholipase A_2 catalyze hydrolysis of monomeric short-chain lecithins[1] at comparable rates. However, there is a large enhancement of the V_{max} for phospholipase A_2 catalyzed hydrolysis of short-chain lecithins present in a micellar aggregate which is not observed for prophospholipase A_2 (Pieterson et al., 1974a). Binding studies show that porcine pancreatic phospholipase A_2 forms a complex with micelles of lecithin, but no enzyme–micelle complex is detected for the zymogen (Pieterson et al., 1974a). The functionally important interactions between phospholipase A_2 and micelles make this an attractive system for investigations of enzyme–lipid interactions at a molecular level.

Ca^{2+}, the only metal ion heretofore shown to activate porcine pancreatic phospholipase A_2, does not possess spectroscopic properties which are useful to probe its environment within the enzyme. The magnetic and spectral properties of trivalent ions of the lanthanide series have been used to investigate the metal ion binding sites of enzymes and proteins (Smolka et al., 1971; Reuben, 1971; Valentine and Cottam, 1973; Gomez et al., 1974; Epstein, et al., 1974). The present paper reports activation of phospholipase A_2 by Gd^{3+} and nuclear magnetic resonance studies of the binding of Gd^{3+} to both the enzyme and proenzyme. In addition, a series of *n*-alkylphosphorylcholines covering a broad range of cmc's are used to investigate the interaction of the enzyme–Gd^{3+} complex with monomeric and micellar amphiphiles.

Experimental Procedure

Materials. Porcine pancreatic phospholipase A_2 and prophospholipase A_2 were prepared according to the method of Nieuwenhuizen et al. (1974) and both proteins were homogeneous as judged by gel electrophoresis. Protein concentrations were determined from the absorbance at 280 nm using $\epsilon_{1\,cm}^{1\%} = 13.0$ and 12.5 for the enzyme and proenzyme, respectively (Pieterson, 1973). Assays of enzyme activity, using a dispersion of dioctanoyllecithin (7 mg/ml) as substrate, were conducted using the pH-stat assay of deHaas et al. (1971). The conditions for the assay were 0.1 M NaCl, 4 mM sodium acetate (pH 6.0), 40 °C.

Lanthanide compounds were obtained from Alfa Inorganics. Standard solutions of $Gd(NO_3)_3$ and $Eu(NO_3)_3$ were made from the corresponding oxides and $Tb(NO_3)_3$ from the chloride salt. *n*-Decylphosphorylcholine ($C_{10}PN$), *n*-dodecyl-

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[1] Abbreviations used are: cmc, critical micelle concentration; PRR, proton relaxation rate; lecithin, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine; $C_{10}PN$, *n*-decylphosphorylcholine; $C_{12}PN$, *n*-dodecylphosphorylcholine; and $C_{16}PN$, *n*-hexadecylphosphorylcholine; Mes, 4-morpholinoethanesulfonic acid.

phosphorylcholine ($C_{12}PN$), and *n*-hexadecylphosphorylcholine ($C_{16}PN$) were prepared as described previously (van Dam-Mieras et al., 1975). Solutions were buffered at pH 5.8 with 3.5 mM sodium cacodylate acidified with HCl or Mes. Sodium cacodylate and Mes were purchased from Sigma Chemical Co., and all other materials were of reagent grade.

Proton Relaxation Measurements. The longitudinal proton relaxation time, T_1, of water protons was obtained with a pulsed magnetic resonance spectrometer using the 180°-τ-90° pulse sequence of Carr and Purcell (1954). The spectrometer was operated at frequencies of 8.13, 15.0, 24.3, 40.0, and 60.0 MHz and all titrations were performed at 24.3 MHz and 22 ± 1 °C. The T_1's of the protons of $C_{10}PN$ were determined by pulsed Fourier transform techniques using the McDonald-Leigh sequence (1973) on a Varian HR-220 instrument.

Analysis of the Data. Binding of Gd^{3+} to phospholipase A_2 and prophospholipase A_2 enhances the effect of the paramagnetic ion on the longitudinal nuclear spin relaxation rate of water protons. The enhancement[2] is described by eq 1 (Mildvan and Cohn, 1970)

$$\epsilon^* = \sum_i \frac{[M_i]}{[M]_T} \epsilon_i = \frac{[Gd^{3+}]_F}{[Gd^{3+}]_T} + \frac{[E\text{-}Gd^{3+}]}{[Gd^{3+}]_T} \epsilon_b \qquad (1)$$

where ϵ^* is the observed enhancement, ϵ_b is the characteristic enhancement for the binary, enzyme-Gd^{3+} complex, and the subscripts T and F refer to the total and free concentrations of Gd^{3+}, respectively. The enhancement for free Gd^{3+} is defined as unity. The dissociation constant, K_D, and the characteristic enhancement for the enzyme-Gd^{3+} complex, ϵ_b, were obtained from titrations of solutions of constant Gd^{3+} concentration with enzyme by a least-squares fit of the data to eq 1.

Displacement of Gd^{3+} from the enzyme-Gd^{3+} complex by another metal ion changes the observed enhancement for the solution. If the competing ion is diamagnetic (e.g., Ca^{2+}), titration of a solution of Gd^{3+} and enzyme with a second metal ion can also be fitted directly to eq 1 to obtain the dissociation constant for the second metal ion. Because the electron spin relaxation times for other paramagnetic lanthanide ions are short, their influence on the nuclear spin relaxation time of water protons is much less than that of Gd^{3+}, and the relaxation effect is not enhanced upon binding of the ions to the enzyme. However, at a concentration of Tb^{3+} in the order of 1 mM, there are small contributions to the observed relaxation rate of water protons, from free Tb^{3+} and from the Tb^{3+} complex with the enzyme, which must be considered in the analysis of the competition experiments of Tb^{3+} with Gd^{3+}. The paramagnetic contribution to the relaxation rate of water protons, $1/T_{1p}$, in solution of enzyme, Gd^{3+}, and Tb^{3+} is given by

$$\frac{1}{T_{1p}} = \sum_i [M]_i R_i = [Gd^{3+}]_F R_{Gd^{3+}} + [Tb^{3+}]_F R_{Tb^{3+}} + [E\text{-}Gd^{3+}] R_{E\text{-}Gd^{3+}} + [E\text{-}Tb^{3+}] R_{E\text{-}Tb^{3+}} \qquad (2)$$

where R_i's are the respective molar relaxivities evaluated in separate experiments. The dissociation constants for Tb^{3+} and Eu^{3+} complexes with the enzyme were obtained by a least-squares fit of the data to eq 2.

Coupled equilibria involving the competition of two metal ions for the same binding site on the enzyme were solved numerically using a computer routine provided by Dr. M. Pring (University of Pennsylvania).

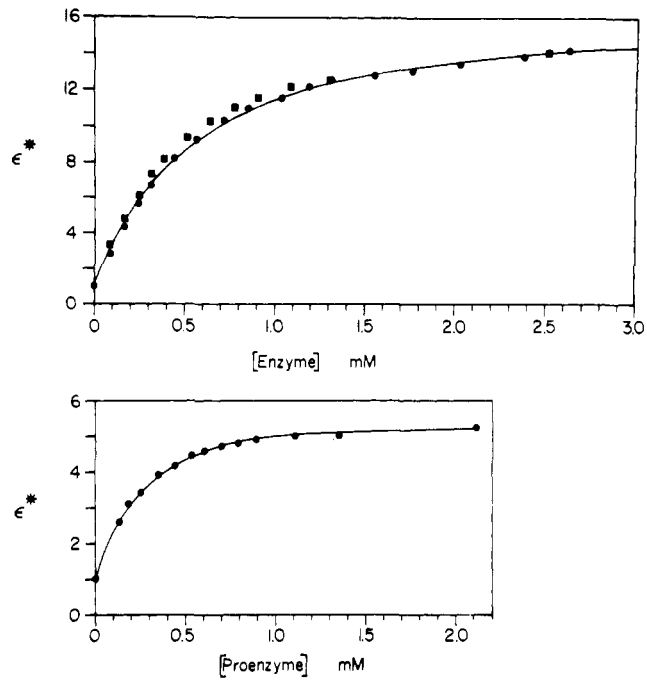


FIGURE 1: PRR titration of Gd^{3+} binding to phospholipase A_2 and prophospholipase A_2. Circles represent the experimental data and the solid lines represent the titration curve computed for K_D = 0.50 mM and ϵ_b = 16.4 for phospholipase A_2 and K_D = 0.18 and ϵ_b = 5.8 for prophospholipase A_2. 3.5 mM sodium cacodylate, pH 5.8, [Gd^{3+}] = 50 μM, 22 °C, and 24.3 MHz. The standard deviation calculated from the individual data points was 0.1 and 0.2 for the enzyme and proenzyme, respectively. The squares represent the titration of Gd^{3+} binding to phospholipase A_2 performed in 3.5 mM Mes, pH 5.8, [Gd^{3+}] = 50 μM, 22 °C, 24.3 MHz.

Results

Kinetic Assays. Gd^{3+} activation of phospholipase A_2 catalyzed hydrolysis of a dioctanoyllecithin dispersion was measured with the pH-stat assay (cf. Experimental Procedure). The rate reported was corrected for the blank rate of hydrolysis of dioctanoyllecithin which was negligible in the absence of added Gd^{3+}. The maximum velocity for phospholipase in the presence of Gd^{3+} was 30 IU and the activator constant for Gd^{3+} was 25 μM. The corresponding maximum velocity and activator constant for phospholipase in the presence of Ca^{2+} under these conditions are 750 IU and 550 μM, respectively.

Gd^{3+} Binding to Phospholipase A_2 and Prophospholipase A_2. Titrations of Gd^{3+} with enzyme and proenzyme are shown in Figure 1. The solid lines were computed using the dissociation constant and characteristic enhancement factor, ϵ_b, for the enzyme or proenzyme obtained from a least-squares fit of the data to eq 1 for a single binding site model (Pieterson et al., 1974b). K_D's for the enzyme-Gd^{3+} and proenzyme-Gd^{3+} complexes of 0.50 and 0.18 mM, respectively, were obtained from analysis of the data in Figure 1. Characteristic enhancement factors, ϵ_b, were 16.4 and 5.8, respectively, for the enzyme and proenzyme complexes. Reuben has shown that the cacodylate anion binds to Gd^{3+}, and in order to estimate the possible influence of the buffer anion on the results, the enzyme titration was performed in 3.5 mM Mes/NaOH, pH 5.8 (Figure 1). A dissociation constant and enhancement factor

[2] The enhancement is defined as:

$$\epsilon^* = \frac{1/T_1^* - 1/T_{1o}}{1/T_1 - 1/T_{1o}} = \frac{1/T_{1p}^*}{1/T_{1p}}$$

where the subscript o denotes a measurement in the absence of Gd^{3+} and the asterisk denotes a measurement in the presence of a complexing agent (e.g., an enzyme, amphiphile, or both) (Mildvan and Cohn, 1970).

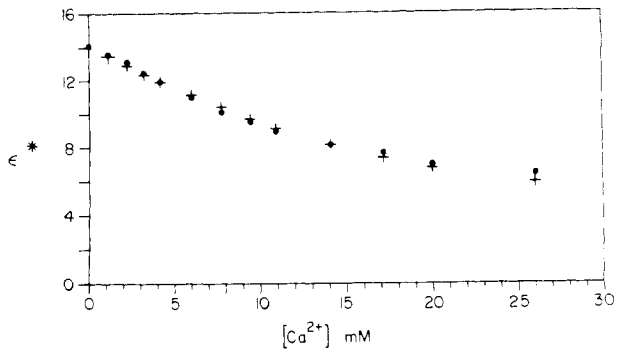


FIGURE 2: PRR titration of Ca^{2+} competition with Gd^{3+} binding to phospholipase A_2. Circles represent the experimental data and the crosses the theoretically determined values calculated using $K_{D\,Gd^{3+}} = 0.50$ mM, $\epsilon_b = 16.4$ and $K_{D\,Ca^{2+}} = 3.6$ mM. 3.5 mM sodium cacodylate, pH 5.8, $[enzyme]_{initial} = 2.67$ mM, $[Gd^{3+}]_{initial} = 50\ \mu M$, 22 °C, 24.3 MHz.

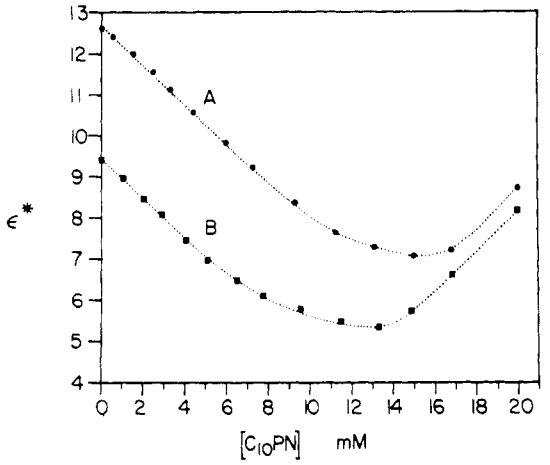


FIGURE 3: PRR titration of Gd^{3+} binding to alkylphosphorylcholines. $[Gd^{3+}] = 50\ \mu M$ for titrations of $C_{10}PN$ and $C_{16}PN$ and 86 μM for $C_{12}PN$. 3.5 mM sodium cacodylate, pH 5.8, 22 °C, 24.3 MHz. The dotted lines are sketched through the experimental points.

of 0.45 and 16.3 mM were obtained from the data in the Mes buffer. The apparent dissociation constant for the Mes-Gd^{3+} complex at pH 5.8 is >200 mM (Reed and Kayne, unpublished results). The similarity of K_D's and ϵ_b's for the enzyme-Gd^{3+} complex in the Mes and cacodylate buffers shows that there is no appreciable effect of the buffer on the results. Values of the observed enhancement, ϵ^*, for solutions of Gd^{3+} and enzyme or proenzyme were constant over a period of at least 5 h and precipitation of the enzyme-Gd^{3+} or proenzyme-Gd^{3+} complex could not be detected.

Competitive Binding Experiments. Competition between Ca^{2+} and Gd^{3+} for the metal ion binding site on the enzyme is illustrated in Figure 2 where ϵ^* for a solution of Gd^{3+} and enzyme is plotted vs. the concentration of Ca^{2+}. The crosses in Figure 2 represent computed values of ϵ^* using values of ϵ_b and K_D for the enzyme-Gd^{3+} complex determined in a previous titration and the best fit value for the dissociation constant of the enzyme-Ca^{2+} complex. The dissociation constant for the proenzyme-Ca^{2+} complex was obtained by the same method.

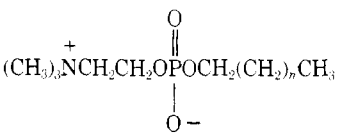
Dissociation constants for the enzyme-Eu^{3+} and enzyme-Tb^{3+} complexes were evaluated from a least-square fit of the data from competition experiments to eq 2. The dissociation constants are included in Table I.

Gd^{3+} Binding to n-Alkylphosphorylcholines. *n*-Alkylphosphorylcholines (I) in monomeric form bind to phospholipase A_2 in a 1:1 complex and induce changes in the ultraviolet spectrum of the enzyme identical with those produced by D-lecithins which are competitive inhibitors (van Dam-Mieras et al., 1975). *n*-Alkylphosphorylcholines are not hydrolyzed by the enzyme and are useful for the investigation of the interaction of phospholipase A_2 with monomeric and micellar amphiphiles.

TABLE I: Dissociation Constants for Metal Ion Complexes with Phospholipase A_2[a]

Metal Ion	K_D enzyme (mM)	K_D proenzyme (mM)
Gd^{3+}	0.50	0.18
Gd^{3+}	0.45[b]	—
Ca^{2+}	3.6 (2.5)[c]	2.2 (2.5)[c]
Tb^{3+}	0.08	—
Eu^{3+}	0.07	—

[a] Dissociation constants were obtained from PRR titrations at 24.3 MHz and 22 °C in 3.5 mM sodium cacodylate buffer, pH 5.8. Uncertainty in the dissociation constants is estimated to be 15%. [b] Titration performed in 3.5 mM Mes, pH 5.8. [c] Values for the dissociation constants for Ca^{2+} from the enzyme and proenzyme in parentheses are from Pieterson et al. (1974b) and were measured in 50 mM sodium acetate at pH 6.0.



I

A complete description of the distribution of Gd^{3+} in solutions of enzyme and *n*-alkylphosphorylcholine requires a knowledge of the affinity of the *n*-alkylphosphorylcholine for Gd^{3+}. The dissociation constant for the Gd^{3+}-$C_{10}PN$ complex was determined by measurements of the T_1 of the protons on the 1-carbon of the alkyl chain of $C_{10}PN$ as a function of the Gd^{3+} concentration. An analysis of the data indicates a weak dissociation constant for the Gd^{3+}-$C_{10}PN$ complex of approximately 1 M. Since the same liganding group is present in $C_{10}PN$ as in $C_{12}PN$ and $C_{16}PN$, it is reasonable to assume a similar value for the dissociation constants of the latter two compounds.

Figure 3 shows ϵ^* for titrations of Gd^{3+} vs. the concentration of $C_{10}PN$, $C_{12}PN$, and $C_{16}PN$. For $C_{10}PN$ and $C_{12}PN$, there is an initial deenhancement (i.e., $\epsilon^* < 1$) which is within the experimental error of the T_1 measurement (~3% of the T_1 value). An increase in ϵ^* occurs when the total concentration of lipid exceeds the cmc, which reflects interactions between Gd^{3+} and the aggregated amphiphiles. The cmc for $C_{12}PN$ measured by van Dam-Mieras et al. (1975) is 1.1 mM, which corresponds approximately to the concentration of $C_{12}PN$ where ϵ^* increases (1.3 mM). Similarly, the cmc of $C_{10}PN$ was inferred to be 13 mM, i.e., the concentration where ϵ^* increases. The monotonic increase in ϵ^* upon addition of $C_{16}PN$ to solutions of Gd^{3+} is consistent with a low cmc for $C_{16}PN$. Approximate dissociation constants for the Gd^{3+}-micelle complexes, based on the concentration of micellar amphiphile which gives a half-maximum change in ϵ^*, are 8, 4, and 2 mM for $C_{10}PN$, $C_{12}PN$, and $C_{16}PN$, respectively. These constants should be viewed phenomenologically, since the number of amphiphiles comprising the micellar binding site for Gd^{3+} is not established. These data show that the affinities of Gd^{3+} for both monomeric and micellar *n*-alkylphosphorylcholines are

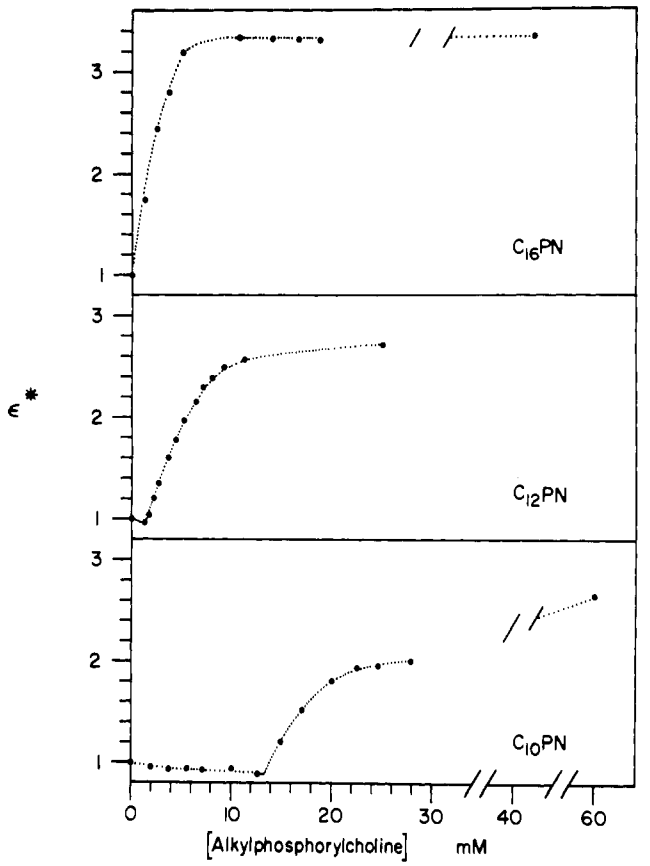


FIGURE 4: PRR titration of $C_{10}PN$ binding to phospholipase A_2 in the presence of Gd^{3+}. Curve A (●) [enzyme] = 1.33 mM, $[Gd^{3+}]$ = 43 μM; curve B (●) [enzyme] = 0.53 mM, $[Gd^{3+}]$ = 43 μM. 3.5 mM sodium cacodylate, pH 5.8, 22 °C, 24.3 MHz. The dotted lines are sketched through experimental points.

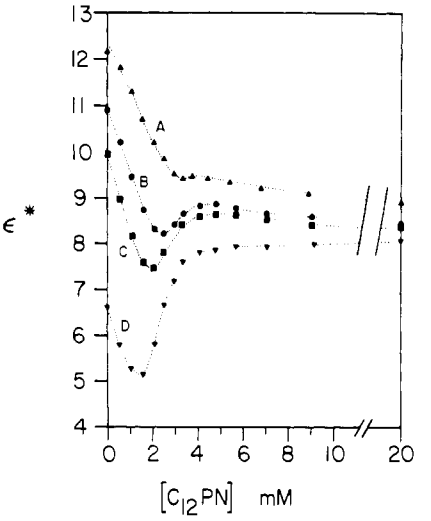


FIGURE 5: PRR titration of $C_{12}PN$ binding to phospholipase A_2 in the presence of Gd^{3+}. $[Gd^{3+}]$ = 43 μM for all titrations. Curve A (▲) [enzyme] = 1.31 mM; curve B (●) [enzyme] = 0.79 mM; curve C (■) [enzyme] = 0.53 mM; curve D (▼) [enzyme] = 0.26 mM. 3.5 mM sodium cacodylate, pH 5.8, 22 °C, 24.3 MHz. The dotted lines are sketched through the experimental points.

substantially weaker than the affinity of Gd^{3+} for the enzyme.

Interaction of $C_{10}PN$ with Phospholipase A_2–Gd^{3+}. Titrations of phospholipase A_2 and Gd^{3+} with $C_{10}PN$, shown in Figure 4, exhibit two phases. The initial phase of the titration curves occurs below the cmc for $C_{10}PN$, and the decrease in ϵ^* in this region is attributed to the formation of a ternary complex, enzyme–Gd^{3+}–monomer, with an enhancement factor, ϵ_t, which is less than ϵ_b. Equilibria pertinent to the first phase of the titration curves are:

$$\text{enzyme–}Gd^{3+}\text{–monomer} \overset{K_3}{\rightleftharpoons} \text{enzyme–}Gd^{3+} + \text{monomer} \quad (3)$$

$$\text{enzyme–monomer} \overset{K_s}{\rightleftharpoons} \text{enzyme} + \text{monomer} \quad (4)$$

An approximate dissociation constant, K_3, of 5 mM was obtained by fitting the initial phases of the titration curves according to the method described by Reed et al. (1970). In the curve fitting, values for the dissociation constant and enhancement of the enzyme–Gd^{3+} complex were taken from the experiments described, and a range of K_s values from 1 to 3 mM was used (van Dam-Mieras et al., 1975).

The transition between the two phases of the titration curves (i.e., the concentration of $C_{10}PN$ where ϵ^* is at a minimum) occurs at concentrations of $C_{10}PN$ greater than the cmc measured in the absence of enzyme (13 mM). Furthermore, the transition shifts to higher concentrations of $C_{10}PN$ with increases in the concentration of enzyme.

The second phase of the titration curves is attributed to the formation of complexes between the enzyme–Gd^{3+} species and micelles of $C_{10}PN$. Since both the enzyme–Gd^{3+} and enzyme–Gd^{3+}–monomer complexes are present at the transition point, it is not possible to attribute the increase in ϵ^* to the micelle complex of either species separately, and the following equilibria are applicable:

$$\text{enzyme–}Gd^{3+}\text{–micelle} \rightleftharpoons \text{enzyme–}Gd^{3+} + \text{micelle} \quad (5)$$

$$\text{enzyme–}Gd^{3+}\text{–monomer–micelle} \rightleftharpoons \text{enzyme–}Gd^{3+}\text{–monomer} + \text{micelle} \quad (6)$$

The characteristic enhancements for either the enzyme–Gd^{3+}–micelle complex or the enzyme–Gd^{3+}–monomer–micelle complex or both must be greater than that of the ternary complex in order for ϵ^* to increase in the second phase of the titration curves.

Interaction of $C_{12}PN$ with Phospholipase A_2–Gd^{3+}. Titrations of enzyme and Gd^{3+} with $C_{12}PN$, shown in Figure 6, also exhibit more than one phase. All of the titration curves show an initial phase characterized by a decrease in ϵ^*, which is attributed to the formation of a ternary enzyme–Gd^{3+}–monomer complex (eq 4). Comparison of the first phase of the titration curves for $C_{12}PN$ with $C_{10}PN$ indicates that the dissociation constant of $C_{12}PN$ from the ternary complex is substantially lower than that for $C_{10}PN$.

When the concentration of $C_{12}PN$ exceeds the cmc, ϵ^* increases in a second phase which is attributed to the binding of the enzyme–Gd^{3+}–monomer complex or enzyme–Gd^{3+} complex or both to $C_{12}PN$ micelles (cf. eq 5 and 6). For a fixed concentration of Gd^{3+}, the transition between the two phases occurs at progressively higher concentrations of $C_{12}PN$ for higher concentrations of enzyme. The concentration of enzyme also has a pronounced effect on the shape of the titration curves. At higher concentrations of enzyme (Figure 5, curves A, B, and C) both local minima and maxima in ϵ^* are observed. In contrast, at lower concentrations of enzyme (curve D of Figure 5), the titration curve at concentrations of $C_{12}PN$ greater than the cmc appears to follow a simple binding isotherm. The local maxima at ~4 mM $C_{12}PN$ (curves A, B, and C of Figure 5) are tentatively ascribed to the presence of a significant amount of the enzyme–Gd^{3+}–micelle complex

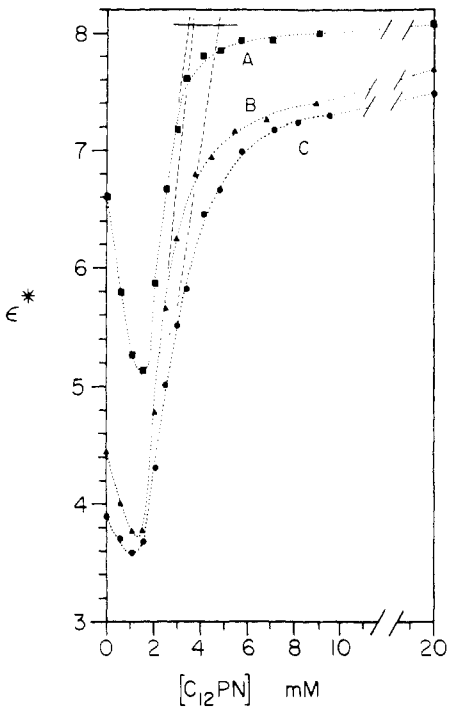


FIGURE 6: Effect of [Gd^{3+}] on the binding of C_{12}PN to phospholipase A_2. Curve A (■) [enzyme] = 0.26 mM, [Gd^{3+}] = 43 μM; curve B (▲) [enzyme] = 0.15 mM, [Gd^{3+}] = 43 μM; curve C (●) [enzyme] = 0.15 mM, [Gd^{3+}] = 86 μM. 3.5 mM sodium cacodylate, pH 5.8, 22 °C, 24.3 MHz. The dotted lines are sketched through the experimental points.

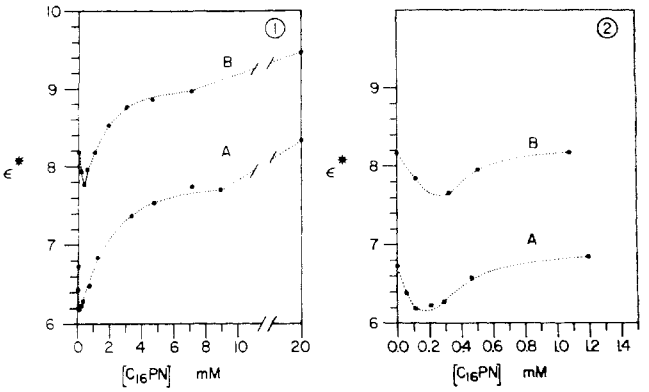


FIGURE 7: PRR titration of C_{16}PN binding to phospholipase A_2 in the presence of Gd^{3+}. Curve A [enzyme] = 0.25 mM, [Gd^{3+}] = 43 μM; curve B [enzyme] = 0.50 mM, [Gd^{3+}] = 43 μM. 3.5 mM sodium cacodylate, pH 5.8, 22 °C, 24.3 MHz. Expansion of the initial portions of the curves is shown on the right. The dotted lines are sketched through the experimental points.

without a monomer bound at the active site (cf. eq 5). The absence of a local maximum in the titration curve for the lowest concentration of enzyme (Figure 5, curve D) suggests that the ratio [enzyme-Gd^{3+}-monomer]:[enzyme-Gd^{3+}] at the transition point is greater for the titration represented by curve D of Figure 5 than in the other titrations. It follows from the preceding arguments that the decrease in ϵ^* (Figure 5, curves A, B, and C) at concentrations of C_{12}PN greater than that where the local maximum occurs could reflect the equilibrium:

$$\text{E-Gd}^{3+}\text{-micelle} \rightleftharpoons \text{E-Gd}^{3+}\text{-monomer-micelle} \quad (7)$$

However, since the concentration of C_{12}PN already exceeds the cmc in this region, an increase in the concentration of free monomer cannot be the driving force for this equilibrium.

The limiting enhancement (at 20 mM C_{12}PN) for the titration represented by curve D of Figure 5 is substantially greater than ϵ^* in the absence of C_{12}PN. On the other hand, the limiting enhancements (at 20 mM C_{12}PN) in curves A, B, and C of Figure 5 are less than ϵ^* in the absence of C_{12}PN, indicating that the characteristic enhancement for the enzyme-Gd^{3+}-monomer-micelle complex is less than ϵ_b. The higher ϵ^* at 20 mM C_{12}PN for curve D of Figure 5 indicates that C_{12}PN micelles synergize the binding of Gd^{3+} to the enzyme. Moreover, synergism is also indicated by the fact that ϵ^*'s at 20 mM C_{12}PN for all four titration curves lie within one unit, whereas in the absence of C_{12}PN there is a wide variation in the fraction of Gd^{3+} bound to the enzyme. Synergism in binding of Ca^{2+} and micelles to the enzyme also has been observed (Pieterson, 1973). At high concentrations of C_{12}PN there is a possibility for effective competition between micelles and enzyme for Gd^{3+}. However, it is unlikely that such a competition is important, because in the presence of micelles the affinity of the enzyme for Gd^{3+} is enhanced ~20-fold.

In the second phase of the titration, shown in Figure 5, curve D, ϵ^* increases abruptly to a limiting value of ~8.1. A linear extrapolation of the initial region of this phase to the limiting enhancement of 8.1 gives an equivalence point at 3.4 mM C_{12}PN. In order to use this equivalence point to estimate the stoichiometry of micellar amphiphile to enzyme, it is necessary to assess the influence of the Gd^{3+}-free enzyme on the position of the equivalence point. The titrations shown in Figure 6, curves A and B, indicate that lowering the total concentration of enzyme from 0.26 mM to 0.15 mM has little effect on the position of the equivalence point. However, doubling the concentration of Gd^{3+} shifts the position of the extrapolated equivalence point to ~4.8 mM C_{12}PN (curve D, Figure 6). At these levels of enzyme, $[Gd^{3+}]_T$ is the primary determinant of the position of the extrapolated equivalence point. With the approximation that at the equivalence point [enzyme-Gd^{3+}-micelle] + [enzyme-Gd^{3+}-monomer-micelle] $\simeq$ $[Gd^{3+}]_T$, the minimum number of C_{12}PN monomers which comprise the micelle, m, is obtained from the concentration of C_{12}PN at the equivalence point, $[C_{12}PN]_{ep}$, with eq 8.

$$m \simeq ([C_{12}PN]_{ep} - [\text{cmc}])/[Gd^{3+}]_T \quad (8)$$

The equivalence points of the titrations in curves A, B, and C of Figure 6 give m values of 46, 48, and 39, respectively.

Interaction of C_{16}PN with Phospholipase A_2-Gd^{3+}. Titration curves of enzyme and Gd^{3+} with C_{16}PN are shown in Figure 7. The shape of the curves is similar to those of titrations of enzyme and Gd^{3+} with C_{10}PN and C_{12}PN. In contrast to C_{10}PN and C_{12}PN, where transition points are not more than a factor of two greater than the cmc, measured in the absence of enzyme, the transition points between the two phases of the titration curves with C_{16}PN occur at concentrations in excess of ten times the cmc.

The decrease in ϵ^* in the first phase reflects an increasing saturation of the active site with monomer, which implies that the concentration of free monomer increases throughout the first phase. Since the concentration of free monomer is limited by the cmc, 15 μM, a dissociation constant for the enzyme-monomer and enzyme-Gd^{3+}-monomer complexes of ~75 μM would be required to suppress the concentration of free monomer to a value of less than the cmc throughout the first phase. This interpretation neglects the possibility that the enzyme may extract monomers directly from the micelle. If extraction occurs to a significant extent in the first phase of the titration, then the estimated dissociation constant quoted above represents a lower limit.

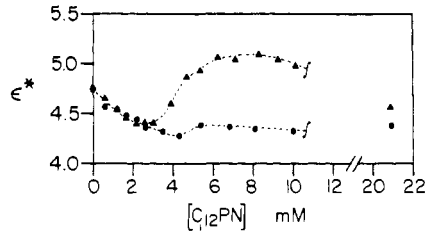


FIGURE 8: PRR titration of $C_{12}PN$ binding to prophospholipase A_2 in the presence of Gd^{3+}. Curve A (●) [proenzyme] = 0.50 mM, $[Gd^{3+}]$ = 43 μM; curve B (▲) [proenzyme] = 0.50 mM, [enzyme] = 0.029 mM, $[Gd^{3+}]$ = 43 μM. 3.5 mM sodium cacodylate, pH 5.8, 22 °C, 24.3 MHz. The dotted lines are sketched through the experimental points.

The observation of a first phase which occurs over a concentration range which extends far above the cmc supports the concept that the binding of monomer to the enzyme competes with monomer aggregation. Competition between monomer binding to the enzyme and aggregation is further exemplified by the continuous shift of the transition point to higher concentrations of amphiphile as the enzyme concentration is increased. The steep decrease in ϵ^* in the first phase of the titration for $C_{16}PN$ indicates that the affinity of the enzyme-Gd^{3+} complex for $C_{16}PN$ is substantially greater than that for $C_{12}PN$ or $C_{10}PN$. Comparison of the second phase of the titrations for $C_{16}PN$ with those for $C_{12}PN$ shows that the binding of enzyme-Gd^{3+} or enzyme-Gd^{3+}-monomer to the micelles is weaker in the case of $C_{16}PN$ than for $C_{12}PN$. As observed for $C_{12}PN$ micelles, a synergistic effect of $C_{16}PN$ micelles on Gd^{3+} binding to the enzyme is also indicated for $C_{16}PN$ micelles by the higher value of ϵ^* at the end of the titration curves than in the absence of $C_{16}PN$. Titrations (not shown) at higher concentrations of enzyme show that ϵ for the enzyme micelle complexes is less than ϵ_b.

Interaction of Prophospholipase A_2-Gd^{3+} with $C_{12}PN$. Equilibrium gel filtration studies (Pieterson et al., 1974a) demonstrate the existence of an enzyme-micelle complex, but no evidence for a complex between the proenzyme and micelles has been found. The titration of the proenzyme-Gd^{3+} complex with $C_{12}PN$ (Figure 8, curve A) resembles the first phase (e.g., monomer binding region) of the corresponding titrations with the enzyme (Figure 6). However, there is a slight increase in ϵ^* at concentrations of $C_{12}PN$ above the cmc. Since there is a possibility of a trace contamination of the proenzyme sample with enzyme, the slight increase in ϵ^* in the titration of the proenzyme sample could be attributed to complexes between the micelle and contaminating enzyme. Indeed, addition of enzyme to the proenzyme sample at a ratio of 1:17 amplified the increase in ϵ^* significantly (Figure 8, curve B). From the magnitude of the effect produced by a concentration of enzyme of 6% the concentration of proenzyme, a less than 1% contamination of the proenzyme sample with enzyme could account for the slight "bump" in the titration of the proenzyme sample with $C_{12}PN$. The synergistic effect of micelles on the binding of Gd^{3+} to the enzyme can account for the ability of a low concentration of enzyme to compete effectively with proenzyme for Gd^{3+} at concentrations of $C_{12}PN$ greater than the cmc. Because an approximately 1% trace of enzyme in the proenzyme sample is probable, it is unlikely that the "bump" in the titration curve at concentrations of $C_{12}PN$ greater than the cmc reflects a proenzyme-micelle interaction.

Interpretation of the Enhancement Factors. The magnitude of the enhancement factor for a complex of a paramagnetic metal ion depends on the correlation time, τ_c, for modulation of the dipolar coupling of the unpaired electrons of the metal ion and the protons of coordinated water molecules, and on the number of first coordination sphere water molecules which exchange freely with the bulk solvent (cf. Mildvan and Cohn, 1970). In favorable cases, the correlation time can be evaluated from the dependence of the PRR on the nuclear resonance frequency. The frequency dependence of the PRR (not shown) for solutions in which the enzyme-Gd^{3+}, enzyme-Gd^{3+}-$C_{12}PN$(monomer), or enzyme-Gd^{3+}-$C_{12}PN$(monomer)-micelle complexes are, respectively, the predominant paramagnetic species in each case shows a frequency-dependent correlation time[3] (cf. Reuben and Cohn, 1970). While the frequency dependence of the correlation time makes it difficult to evaluate the magnitude of the correlation time at a given frequency, this observation does show that differences in the sizes of the enzyme-Gd^{3+} and enzyme-Gd^{3+}-micelle complexes are not solely responsible for the differences in the enhancement factors for these species.[4]

The maximum enhancement for a complex occurs when τ_c = (nuclear Larmor frequency)$^{-1}$ and when a minimum number of water molecules are displaced upon complexation. Assuming a hydration number of nine for H_2O-Gd^{3+}, at 24.3 MHz, a maximum enhancement of ~35 (i.e., ~4.4/bound water) is calculated if eight water ligands remain in the first coordination sphere.[5] Reuben (1975) has shown that the water proton relaxation rate for solutions of the H_2O-Gd^{3+} complex is not exchange limited. Hence, a minimum number of ~4 freely exchanging water ligands in the first coordination sphere of the Gd^{3+} in the binary, enzyme-Gd^{3+} complex is obtained from the ratio of ϵ_b to the maximum enhancement per water molecule. Similarly, the enzyme-Gd^{3+}-monomer-micelle complex must have at least two rapidly exchanging water molecules.

Discussion

The enhanced catalytic activity of phospholipase A_2 from porcine pancreas (deHaas et al., 1971) and from the venom of *Crotalus adamanteus* (Wells, 1972) toward aggregated substrates has stimulated interest in the structure of the enzyme-micelle complex. Several explanations of the enhanced activity involve either a favorable organization of the substrate in the aggregated phase (Wells, 1972) or regulation of the active site by binding of the micelle to the enzyme (Verger et al., 1973; Pieterson et al., 1974a). Detailed structural studies of the complexes of the enzyme with monomeric and aggregated amphiphiles should provide a basis for resolution of these proposals. Substitution of Gd^{3+} for Ca^{2+} in complexes with phospholipase A_2 permits studies of the interaction of the enzyme with amphiphilic molecules by paramagnetic probe techniques.

PRR studies show that binding of monomeric *n*-alkylphosphorylcholines to the enzyme competes effectively with the aggregation equilibrium and increases the apparent cmc of the amphiphile. Hence, the concentration of monomer bound to the enzyme is not limited by the cmc of the amphi-

[3] The paramagnetic contribution to the relaxation rate of water protons is less at 8.13 MHz than at 15 and 24.3 MHz which shows the correlation time varies with the magnetic field strength. The electron spin relaxation time of Gd^{3+}, τ_s, is the only contribution to the correlation time which can be magnetic field dependent.

[4] The contribution of τ_s to the correlation time for each complex shows that the rotational correlation times of the complexes are not the sole determinant of the enhancement factors.

[5] A maximum value of 35 for the enhancement is obtained from the ratio of the correlation function for $\tau_c \simeq 8 \times 10^{-11}$ s for aquo-Gd^{3+} and for $\tau_{c_{max}} \simeq 1/\omega_I$ with eight-ninths of the hydration sphere remaining (ω_I is the nuclear Larmor frequency).

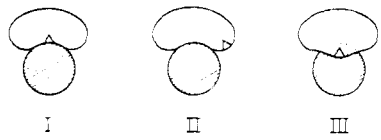


FIGURE 9: Hypothetical models for the phospholipase A_2-micelle complex. The triangle represents the metal ion binding site and the shaded area represents the micelle.

phile measured in the absence of enzyme. Rather, the magnitude of the dissociation constant of the enzyme-monomer complex relative to the cmc will determine the degree of saturation of the enzyme with monomer. The complex titration curves with $C_{12}PN$ also indicate that binding of the enzyme to the micelle does not simultaneously saturate the active site with the corresponding monomer. These results imply that binding of monomer to the active site and binding of micelles to the enzyme are independent events which may occur at distinct sites on the enzyme. However, the two events could be coupled indirectly by monomer- and micelle-induced changes in the structure of the enzyme. Conformational changes in the enzyme upon binding of monomeric and micellar *n*-alkylphosphorylcholines have been observed by ultraviolet difference spectroscopy (van Dam-Mieras et al., 1975).

The synergism evident in binding of Gd^{3+} and micelles to the enzyme shows that structural effects are transmitted between the binding sites for metal ion and micelle. Although a quantitative estimate of this synergism has not been obtained from the equilibrium measurements with *n*-alkylphosphorylcholines, the activator constant of Gd^{3+} for phospholipase A_2 catalyzed hydrolysis of dioctanoyllecithin is 20-fold lower than the dissociation constant of the enzyme-Gd^{3+} complex. Micelle-induced structural changes at the metal ion binding site on the enzyme are also indicated by a significant increase in the PRR enhancement on binding of the micelle to the enzyme-Gd^{3+}-monomer complexes. However, this latter effect may be due, in part, to the increased rotational correlation time of the enzyme-micelle complex.

The enhancement factor of greater than eight (at 24.3 MHz) for the enzyme-Gd^{3+}-monomer-micelle complex shows that the binding site of the metal ion is situated such that at least two water molecules in the first coordination sphere of the Gd^{3+} exchange freely with the bulk solvent (i.e., a residence time of water on Gd^{3+} in the order of 1 μs). The facile accessibility of the coordination sphere of Gd^{3+} to the bulk solvent must be considered in any model for the enzyme-micelle complex. For example, three possible models for the location of the Gd^{3+} binding site in the enzyme-micelle complex are given in Figure 9. Model II is most easily accommodated by the PRR data since the location of the metal ion is remote from contacts between the enzyme and the micelle. On the other hand, models I and III are less compatible with the PRR data since each model requires that a pathway for access of solvent to the metal ion exists through the enzyme or in the area of contact between the enzyme and the micelle.

The models presented above consider only the spatial relationship of the metal ion binding site to the micelle binding site and are not intended to specify the nature of the enzyme-micelle interaction. The presence of a paramagnetic probe at the site of the activating cation in complexes of porcine pancreatic phospholipase A_2 should permit further elucidation of the structure of complexes of the enzyme with monomeric and aggregated amphiphiles.

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