Physicochemical Studies on the Interaction of Pancreatic Phospholipase A_2 with a Micellar Substrate Analogue†

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ABSTRACT: Porcine pancreatic phospholipase A_2 interacts with micelles of the substrate analogue *n*-octadecylphosphocholine to form a specific complex over considerably wide concentration ranges of both lipid and protein. UV absorption difference spectroscopy measurements indicate that the ratio of lipid to protein molecules in the complex is ~50. This number is confirmed by using other techniques to study the composition of the complex, namely, ultracentrifugation experiments and light scattering. The latter techniques furthermore demonstrate that the lipid–protein complex consists of 100 lipid and 2 enzyme molecules. Thus, the number of lipid molecules in the free micelle (200) is halved when the complex with phospholipase is being formed. The consequences of the results are discussed in relation to a theoretical model of the lipid–protein interaction.

Phospholipase A_2 (EC 3.1.1.4) binding to monomolecular and micellar lipid substrate analogues has been extensively studied in order to obtain a better understanding of lipid–protein interaction in general and of the mode of action of the enzyme in particular (Verger & de Haas, 1976). As confirmed by recent 1.7-Å resolution X-ray analysis (Dijkstra, 1980), pancreatic phospholipase A_2 contains a region, distinct from its active site, that preferentially interacts with organized lipid–water interfaces (Verger et al., 1973). This region, called the interface recognition site (IRS),[1] includes several hydrophobic amino acid residues, among which is the only tryptophan in the protein, and also involves the N-terminal amino group Ala[1] (Slotboom & de Haas, 1975). van Dam-Mieras et al. (1975) used the presence of Trp^3 in the IRS to study the interaction of porcine pancreatic phospholipase A_2 with the micellar interface, formed by the enzymatically nondegradable substrate analogue *n*-hexadecylphosphocholine ($C_{16}PN$), by fluorescence and UV absorption difference spectroscopy. Fluorescence has the drawback that as a result of this interaction the Trp^3-emission maximum shifts to shorter wavelengths. Reliable quantitative data can only be obtained when the fluorescence signals of free and bound enzyme species are separated.

More quantitative information about the composition of the complex formed by phospholipase A_2 and $C_{16}PN$ was supplied by de Araujo et al. (1979). By use of isothermal microcalorimetry, light scattering, and equilibrium gel filtration, they showed the formation of a stable complex consisting of 2 enzyme molecules and about 80 lipid monomers. In this paper, the "molecular weight" of the complex was only determined by equilibrium gel filtration. Because of differences in density and probably in hydration between proteins and lipid micelles, it is not certain that the molecular weight of the complex, thus determined, is correct.

Up to now, only one substrate analogue was used to study the lipid–protein interaction. In the present study, another substrate analogue is chosen, i.e., *n*-octadecylphosphocholine ($C_{18}PN$), having a 10 times lower cmc. The aim of this paper is to accurately define binding parameters. In the following paper in this issue (Donné-Op den Kelder et al., 1981), these parameters are used to obtain detailed information on the lipid–protein interaction by using several micellar substrate analogues. Although with isothermal calorimetry the thermodynamic parameters can be obtained directly, UV absorption difference spectroscopy is used to save time and material. From the spectroscopic data, obtained by UV absorption difference spectroscopy, the lipid to protein (L/P) molar ratio and the dissociation constant of the complex formed by phospholipase A_2 and $C_{18}PN$ are determined. Binding of aggregated lipids to protein involves a complication, which is often ignored; i.e., the number of lipid monomers in the aggregate that interacts with the protein is not known beforehand. Thus, a dissociation constant obtained from lipid titrations has no physical significance when its value is expressed in lipid monomer concentration. Such experiments need rigorous curve fitting analyses to derive valuable data of the binding parameters (de Araujo et al., 1979). In this respect, it is often more advantageous to titrate the lipid with the enzyme. In the present paper, both protein and lipid titrations are described for the system porcine pancreatic phospholipase A_2–$C_{18}PN$, and it is checked whether or not the L/P molar ratio in the complex is constant over the concentration range. Light scattering and equilibrium gel filtration experiments were done to check the results from UV spectroscopy and to determine the lipid–protein complex composition. Finally the equilibrium sedimentation technique was used to determine the number of protein molecules in the lipid–protein complex when the enzyme is saturated with lipid.

Experimental Section

Materials and Methods. In all experiments, glass-distilled water was used. Standard buffer solutions contained 50 mM sodium acetate, 100 mM sodium chloride, and 25 mM calcium chloride at pH (or pH*) 6.0, unless stated otherwise.

Porcine pancreatic prophospholipase was isolated and converted into active enzyme as described by Nieuwenhuizen et al. (1974). Protein concentrations were determined from the absorbance at 280 nm, using an $E^{1\%}_{1cm}$ of 12.5. This value was

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[1] Abbreviations used: IRS, interface recognition site; $C_{16}PN$, *n*-hexadecylphosphocholine; $C_{18}PN$, *n*-octadecylphosphocholine; DMPC, dimyristoylphosphatidylcholine; cmc, critical micelle concentration; L/P, lipid to protein ratio; pH*, uncorrected pH meter reading in $^2H_2O/H_2O$ mixtures.

0006-2960/81/0420-4068$01.25/0

determined by ultracentrifugation experiments.

The partial specific volume of the protein, $\bar{v}_p = 0.707$ cm^3/g, was measured in an Anton Paar Model DMA 02C precision densimeter at 25 °C with monomeric enzyme concentrations below 3 mg/mL. The different protein solutions were dialyzed for 48 h against standard buffer. The partial specific volume was in good agreement with the partial specific volume $\bar{v}_p$ = 0.70 cm^3/g, calculated from the amino acid composition (Cohn & Edsall, 1943).

The substrate analogue C_{18}PN was prepared and purified as previously described (van Dam-Mieras et al., 1975). C_{18}PN concentrations were determined by weight or by measuring the lipid phosphorus as described by Chen et al. (1956).

The partial specific volume of the lipid at concentrations above cmc was measured in the Anton Paar densimeter at different solvent densities, realized by varying the $^2H_2O/H_2O$ ratio, and proved to be $\bar{v}_L = 0.981 \pm 0.004$ cm^3/g. The cmc of C_{18}PN was determined by the Wilhelmy plate method to be 1–2 μM in standard buffer at 25 °C (Davies & Rideal, 1961).

Sedimentation Equilibrium Studies. Equilibrium experiments were done with a Beckman Model E analytical ultracentrifuge equipped with electronic speed control, using interference optics. Samples (100-μL solutions of the lipid–protein complex) were equilibrated at least 48 h against buffer at 25 °C and placed in one side of a 12-mm double sector cell with an Al-filled epon centerpiece. Dialysis buffer was placed in the other sector. The equilibrium studies were carried out at 32 000–40 000 rpm at 25 °C. Photographs of the interference pattern were taken after 24 and 30 h. The photographic plates were measured on a Nikon Model 6C T_2 profile-projector.

The concentrations of enzyme and lipid in the equilibrium experiments were determined with the interference optics after Babul & Stellwagen (1969) by using a capillary-type synthetic boundary centerpiece. The sedimentation equilibrium experiments for the complex formation between phospholipase A_2 and C_{18}PN were done by the method of Reynolds & Tanford (1976). With this method, it is possible to determine the molecular weight of the protein part in an enzyme–detergent complex by masking the detergent part at a solvent density $\rho = 1/\bar{v}_L$ ($\bar{v}_L$ is the partial specific volume of the detergent), using different $^2H_2O/H_2O$ mixtures. In eq 1,

$$M(1 - \phi'\rho) = M[(1 - \bar{v}_p\rho) + \delta_L(1 - \bar{v}_L\rho)] \quad (1)$$

derived by Casassa & Eisenberg (1964), where M is the molecular weight of the protein (excluding bound lipid), ϕ' is the apparent partial specific volume of the protein bound to lipid, v_p is the true partial specific volume of the protein, and δ_L is the binding ratio (W_L/W_p). The second term in the right-hand part vanishes at this density, and ϕ' equals $\bar{v}_p$.

Light-Scattering Measurements. The buffer was filtered under pressure through Millipore filters (25-nm pores). The solution of protein and/or lipid was directly put into the light-scattering cuvettes. These cuvettes (floating in saturated cesium chloride solution) were centrifuged for 1 h at 20 000 rpm (45000g, SW25.1) and 25 °C in a Beckman L5-50 B preparative ultracentrifuge in order to remove dust. For the light-scattering measurements, a FICA 50 photometer was used.

The L/P molar ratio in the complex was determined by the method described by de Araujo et al. (1979). The molecular weights of micelles and complex were determined according to Doty & Edsall (1951) by using eq 2, which describes light scattering of particles much smaller than the wavelength of the used light, where K is a constant, depending on the

$$\frac{Kc}{R_{90} - R_{90}^\circ} = \frac{1}{M} + 2Bc \quad (2)$$

wavelength of the incident light (in this study, λ = 546 nm) and the refractive index increment, c is the concentration of the particle, R_{90} is the Rayleigh ratio at the scattering angle of 90°, R_{90}° is the Rayleigh ratio at 90° of the pure solvent, M is the anhydrous mass-average molecular weight of the particles, and B is the second virial coefficient, correcting for deviation from the ideal case due to pair interaction. From the diffusion coefficient, determined by quasi-elastic light scattering (Berne & Pecora, 1976) with an argon laser (λ = 514 nm), it was possible to calculate the hydrodynamic radius of the hydrated C_{18}PN micelle in solution $r_h = 37 \pm 2$ Å.

Refractive Index Increment Measurements. The refractive index increments were measured with a Rayleigh interferometer (Aus Jena, DDR) and with the interference optics of the analytical ultracentrifuge at λ = 546 nm and 25 °C. For C_{18}PN and phospholipase A_2, the values 0.141 ± 0.002 and 0.189 ± 0.006 mL/g, respectively, were found.

UV Absorption Difference Spectroscopy. Difference spectra were recorded by using an Aminco Model DW 2-a spectrophotometer equipped with a MIDAN data analyzer (containing 4 KB of RAM storage capacity). The data analyzer was used to store the reference difference spectrum. The wavelength range was 75 nm with 10 data points per nm resolution. Spectra were recorded by direct automatic subtraction of the stored spectrum from the actually measured one at 0.2 nm/s wavelength sweep by using noise suppression (slow response).

A set of quartz tandem cuvettes was used (2 × 1 cm light path), containing, e.g., for lipid titrations, standard buffer and a buffered enzyme solution in the left and right compartments of either cuvette, respectively. After storage of the reference difference spectrum, equal volumes of a lipid solution (10 μL or more) were added to the enzyme in cuvette A and to buffer in cuvette B, while the same volume of buffer was added to the enzyme in cuvette B. The mixtures were magnetically stirred for ~1 min or longer. The solutions were not stirred during recording of the actual difference spectrum. In some cases, recordings were repeated to check for the stability of the measured signal. The observed difference signal (Q_{obsd}) was routinely taken from the value of the absorption maximum at 292 nm relative to the base line (see, e.g., Figure 3). Binding parameters were obtained as previously described by de Araujo et al. (1979). Calculations were performed on a desk top computer (Apple II, 48 KB of RAM storage capacity). A program was written in BASIC for nonlinear regression analysis based on the method of Fletcher & Powell (1963), involving a combination of the steepest descent and Gauss–Newton regression methods (program available on request). Data input consists of the values for total enzyme concentrations (E_T), total lipid (monomer) concentrations (L_T), and the corresponding observed signal, Q_{obsd}. The unweighted data are essentially fitted to eq 3 (see also Appendix) where K_d rep-

$$K_d = \frac{(E_T - X)[(L_T/N) - X]}{X} \quad (3)$$

resents the dissociation constant of the lipid–protein complex, N the number of lipid molecules per enzyme molecule in the complex, and X the concentration of that complex. Equation 1, in fact a quadratic function in X, is solved for X (yielding only one physically possible root) by using initial guessed values for K_d and N. A calculated signal, Q_{calcd}, is then obtained from the relation

$$Q_{calcd} = CX \quad (4)$$

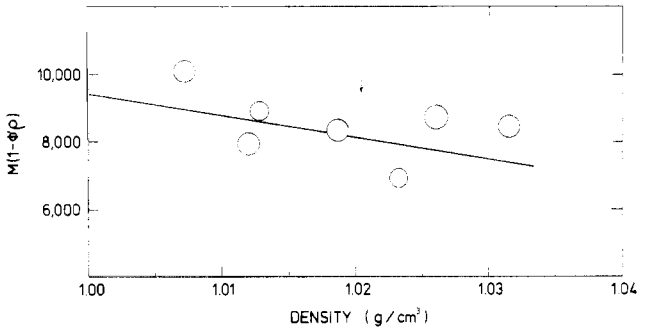


FIGURE 1: Plot of $M(1 - \phi'\rho)$ for the complex formed between phospholipase A_2 and *n*-octadecylphosphocholine complex vs. solvent density (ρ) at 25 °C. M is the molecular weight of the protein part of the complex, and ϕ is the apparent partial specific volume of the protein in the complex. All solutions contained 50 mM sodium acetate, pH 6.0, 100 mM sodium chloride, 30 mM calcium chloride, 0.5 mg/mL lipid–protein mixture (L/P molar ratio of 160/1), and varying ^2H_2O percentages.

Table I: Molecular Weight (M_r) of Phospholipase A_2, C_{18}PN Micelles, and the Complex Formed between Phospholipase A_2 and C_{18}PN Micelles at 25 °C[a]

exptl method	phospholipase A_2 ($M_r \times 10^{-4}$)	C_{18}PN micelles ($M_r \times 10^{-4}$)	complex ($M_r \times 10^{-4}$)
analytical	1.35 ± 0.06[b]		3.0 ± 0.2 (protein part) ~5 (lipid part)
ultracentrifuge light scattering	1.5 ± 0.1	8.3 ± 0.3	7.9 ± 0.5
equilibrium gel filtration	1.4 ± 0.1	13 ± 1[c]	9.2 ± 0.5[c]

[a] Values are presented as the mean ± SD. [b] From de Haas et al. (1968). [c] These values are hydrated molecular weights.

where C is the molar UV difference extinction coefficient. Its guessed value is given as input to the regression program.[2] The program searches for the minimum of the summed squared difference $[\sum^n(Q_{obsd} - Q_{calcd})^2]$ over all n data points. When a minimum is found, the parameters K_d, N, and C are given as output, together with their variances and covariances from which standard deviations and correlation coefficients are calculated.

Results

Determination of the Composition of the Lipid–Protein Complex by Sedimentation Equilibrium Studies. The sedimentation equilibrium experiments to determine the composition of the complex formed by phospholipase A_2 from pig pancreas and C_{18}PN were done at 25 °C at different solvent densities by varying the $^2H_2O/H_2O$ ratio. At each density at the slowest possible speed, the overall ln c vs. r^2 plot, where c is the protein concentration at the radial position r, showed no significant upward curvature, and therefore the value for $M(1 - \phi'\rho)$ was calculated by using the least squares of the entire plot. All measurements were done at a L/P molar ratio of 160/1 where the enzyme was at least 90% saturated with lipid (see below). The apparent molecular weights as a function of the density are shown in Figure 1. The arrow indicates the point where $\rho = 1/\bar{v}_L$. The corresponding molecular weight for the protein part of the complex is (3.0 ± 0.2) × 10^4 (Table I). From the molecular weight of phospholipase A_2, being 1.4 × 10^4, it can be concluded that there are two enzyme molecules in the complex.

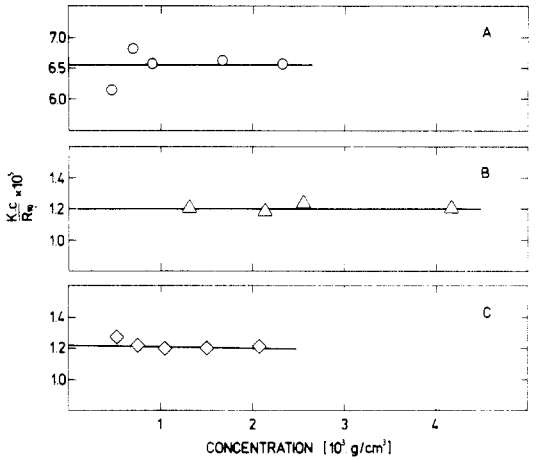


FIGURE 2: Plots of the scattered intensity at 90° as a function of the concentrations of phospholipase A_2 from pig pancreas in (A) (○), *n*-octadecylphosphocholine in (B) (△), and *n*-octadecylphosphocholine–phospholipase A_2 complex in a lipid to protein molar ratio of 54:1 in (C) (◇). Here, the concentration c is the sum of the weights of lipid and protein in the above-mentioned molar ratio. All measurements were done at pH 6.0, the temperature was 25 °C, and the used wavelength was 546 nm.

From the slope, which is equal to $-d[M(1 - \phi'\rho)]/d\rho = -M(\bar{v}_p + \delta_L\bar{v}_L)$, the binding ratio δ_L and thus the number of lipid monomers in the complex can be obtained. This yields a value of about 100 lipid monomers per 2 protein molecules in the complex. Because the density is varied over a small range, the uncertainty in the slope and therefore in the number of lipid monomers in the complex is rather large (about 50%).

Determination of the Composition of the Lipid–Protein Complex by Light-Scattering Measurements. In all experiments, there was no angle dependence of the scattering intensity at angles between 30 and 150°. The dissymmetry ($Z_{45°}/Z_{135°}$) was always smaller than 1.05. The Rayleigh ratio at 90° was linear with phospholipase A_2 and C_{18}PN concentrations, as can be seen from Figure 2. The slopes of the lines, being zero, indicate that these solutions are more or less ideal. The intercepts yielded a molecular weight of (1.5 ± 0.1) × 10^4 for phospholipase A_2 and of (8.3 ± 0.3) × 10^4 for the anhydrous C_{18}PN micelle (see Table I). This latter value gives a number of 192 ± 7 monomers per micelle.

When the complex formation between enzyme and lipid was studied by varying the lipid concentration at a constant enzyme concentration, there was always a break in the curve at a L/P molar ratio of 54 ± 5 to 1. Samples with different concentrations at this molar ratio were prepared. The Rayleigh ratio at 90° and the refractive index increment were measured. Both were linear with concentration (defined in this case as the sum of lipid and enzyme concentrations in g/mL). This means that over the whole concentration range the same type of complex is formed. When the reduced intensity $[Kc/(R_{90} - R_{90}°)]$ was plotted as a function of the concentration, as shown in Figure 2, the molecular weight of the complex could be determined from the intercept. This yielded a value of (7.9 ± 0.5) × 10^4 (Table I). The molecular weight for a complex consisting of 1 enzyme molecule and 54 lipid monomers is 3.8 × 10^4. From this, one can conclude that there are 2 enzyme molecules and about 100 lipid monomers in the complex. This result is in good agreement with the complex composition found in the sedimentation equilibrium study.

Estimation of the Molecular Weight of the Lipid–Protein Complex by Equilibrium Gel Filtration. For estimation of

[2] Good estimates for C are obtained by adding small amounts of enzyme to a large excess of lipid. Under those conditions the measured signal is linear with enzyme concentration. The value for C is then found from the slope of the straight line.

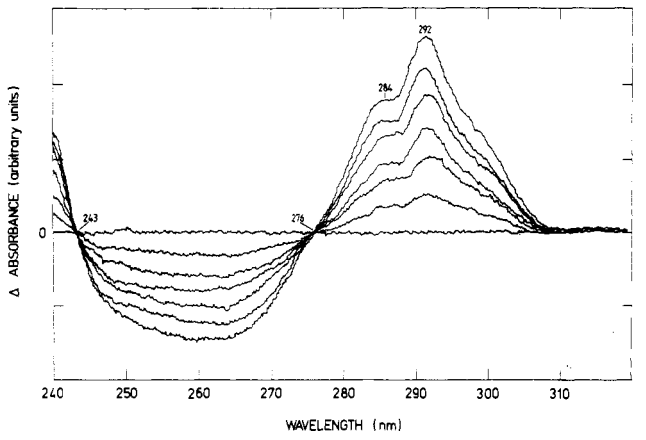


FIGURE 3: Ultraviolet absorption difference spectra produced by the interaction of phospholipase A_2 with increasing amounts of *n*-octadecylphosphocholine at 25 °C and pH 4.0. Absorbances are in arbitrary units.

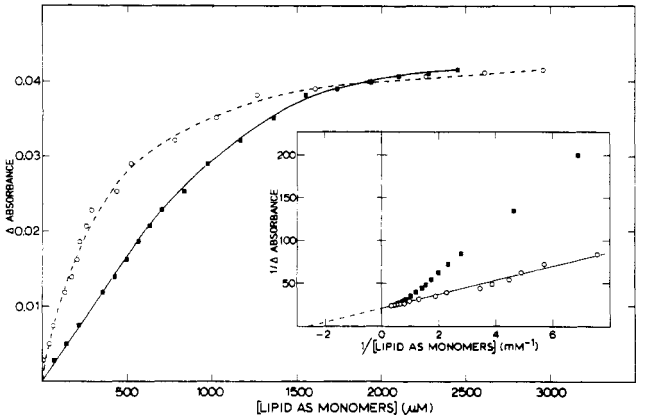


FIGURE 4: Direct plot of the ultraviolet absorption difference spectroscopy signal at 292 nm relative to the *n*-octadecylphosphocholine concentration expressed as monomers. The difference signal at 292 nm relative to total lipid concentration (■) is shown, and the solid curve through these points represents the result of the computer fit. In addition, the observed signal is plotted as a function of free lipid (○). The broken curve gives the calculated difference signal relative to free lipid. (Inset) Double-reciprocal plot of the observed difference signal at 292 nm as a function of total lipid (■) and free lipid (○), respectively. The concentration of phospholipase A_2 is 27.4 μM. All measurements are done at 25 °C and pH 4.0.

the molecular weight of the lipid–protein complex by a third method, equilibrium gel filtration experiments were done. A column (0.9 × 60 cm) was packed with Sephadex G-100 superfine, equilibrated at 25 °C in standard buffer, containing monomeric C_{18}PN (0.9 μM) and phospholipase A_2 (10.9 μM). The column was used in an upward flow direction. An amount of lipid (3 mmol) was passed through the column. The enzyme activity and the lipid phosphorus peaks overlapped completely, and a constant L/P molar ratio of 48/1 was calculated. The molecular weight of the complex could be estimated by calibration of the column with the Boehringer protein Combithek size II unit and appeared to be (9.2 ± 0.3) × 10^4 (see Table I). This value represents the hydrated molecular weight.

On the same column, now packed with Sephadex G-100, calibrated, and flowing in an upward direction, the C_{18}PN micelles alone eluted at a volume which pointed to a molecular weight of 1.3 × 10^5. This value also represents a hydrated molecular weight.

UV Absorption Difference Spectroscopy. Figure 3 shows a typical set of UV absorption difference spectra obtained for porcine phospholipase A_2 titrated with the substrate analogue C_{18}PN. Well-defined isosbestic points can be observed at 243 and 276 nm, respectively. The difference spectrum mainly

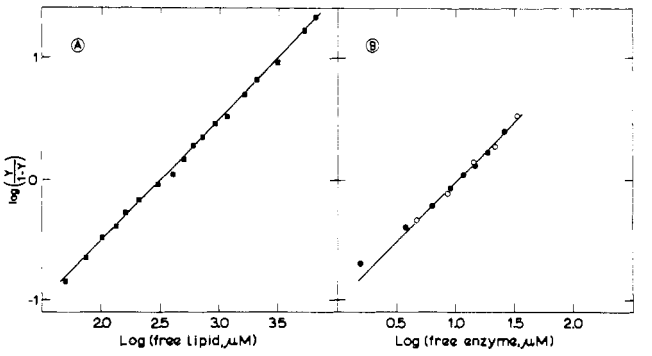


FIGURE 5: Hill plots of the interaction between phospholipase A_2 and *n*-octadecylphosphocholine, relating $Y/(1 - Y)$ to free titrant, where Y is the fractional saturation of the titrated compound with the titrant. (A) The result of a lipid titration at pH 4.0 is shown. The phospholipase A_2 concentration is 27.4 μM, and the temperature is 25 °C. (B) An enzyme titration at pH 6.0 is shown at two different lipid concentrations, 1.02 mM (●) and 0.62 mM (○). The temperature is 25 °C.

Table II: Nonlinear Regression Analysis of Binding Data Obtained from Addition of C_{18}PN Micelles to 27.4 μM Phospholipase A_2 at pH 4.0 and 25 °C in the Absence of Ca^{2+}

dissociation constant of the complex	$K_d = 4.2 \pm 0.4$ μM
UV difference extinction coefficient	$C = 1944 \pm 24$ M^{-1} cm^{-1}
lipid to enzyme molar ratio in complex	$N = 48 \pm 0.5$
correlation coefficients (r)	r_{K_d} (with C) = 0.95 r_{K_d} (with N) = 0.78 r_C (with N) = 0.56
minimum function value	$\Sigma^n(Q_{calcd} - Q_{obsd})^2 = 1.61 \times 10^{-6}$ $(\Delta OD_{292nm})^2$

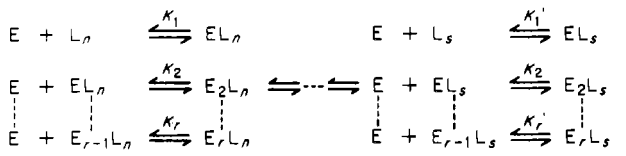
arises from perturbation of Trp^3 on binding of enzyme to the lipid micelles, i.e., a broad minimum at 260 nm and maximum absorption at 292 nm (Donovan, 1969). Furthermore, perturbation of one or more tyrosine residues is indicated by the shoulder at 284 nm.

In Figure 4, a direct plot of the observed signal at 292 nm is shown relative to the total lipid concentration expressed as lipid monomers. The solid curve represents the result of the computer fit. In addition, the observed signal is plotted as a function of the concentration of free lipid expressed as monomers. This plot gives a hyperbolic relationship, unlike the plot vs. total lipid, as is also indicated by the inset in Figure 4 showing double-reciprocal plots of signal vs. either total lipid or free lipid. The result of the lipid titration was also fitted to a Hill plot, relating log $[Y/(1 - Y)]$ to log (free lipid), where Y represents fractional saturation of the titrated enzyme with C_{18}PN (Figure 5A). The Hill plot of a typical protein titration under similar conditions as the lipid titration is shown in Figure 5B. Here Y represents the fractional saturation of the lipid with enzyme. The Hill constants were 1.0 and 0.95, found for the lipid and protein titrations, respectively. The computer output for the lipid titration is summarized in Table II.

Discussion

In order to better understand the interaction of lipolytic enzymes at lipid–water interfaces, we studied the binding parameters and the composition of the complex formed by pig pancreatic phospholipase A_2 and the substrate analogue *n*-octadecylphosphocholine. In Figure 4, where the UV absorption difference signal is plotted relative to total and free lipid, the necessity of nonlinear regression analysis in lipid titrations is clearly shown. Only after computer fitting does the curve in the direct plot become a hyperbolic saturation type

Scheme I



(or, in the double-reciprocal plot, a straight line). In the previous study on this lipid–protein interaction (van Dam-Mieras et al., 1975), uncorrected total lipid concentrations were used. Consequently, the apparent binding constants were too high.

From the computer fitted results of lipid and enzyme titrations it can be concluded that phospholipase A_2 has a high affinity for a micellar binding site, consisting of approximately 48 $C_{18}PN$ monomers (Table II). Light-scattering experiments gave a L/P molar ratio in the complex of 54/1, which is in good agreement with the UV results. The molecular weight 79 000 (Table I) points to a complex of 2 enzyme molecules and about 100 lipid monomers.

An independent technique like equilibrium sedimentation was used to determine the molecular weight of the protein part in the complex. These measurements fully confirmed the light-scattering experiments. Equilibrium gel filtration yielded a hydrated molecular weight of 92 000 (Table I) for the lipid–protein complex. From the length of the extended $C_{18}PN$ molecule (30 Å) and the hydrodynamic radius (r_h = 37 ± 2 Å), the number of water molecules per lipid monomer could be estimated. This gave a value of 12 ± 5 H_2O molecules per lipid monomer. For other lipids like *n*-hexadecylphosphocholine (de Araujo et al., 1979; Kaatze et al., 1980), *n*-dodecylphosphocholine (Lauterwein et al., 1979), and lysolecithins (Pottel et al., 1978), similar hydration values were found. A complex of 2 enzyme molecules and 100 hydrated $C_{18}PN$ monomers has a molecular weight of 93 000, which is in complete agreement with the measured molecular weight. The calculated hydrated molecular weight for a $C_{18}PN$ micelle of 200 monomers is the same as found on the calibrated column. This gives another confirmation for the composition of the complex.

When the results of the phospholipase A_2–$C_{18}PN$ interaction are compared with the earlier obtained values for the $C_{16}PN$-micellar interface (de Araujo et al., 1979), it is striking that in both cases the micelles have fallen apart into two particles, each containing two binding sites for the enzyme phospholipase. The affinity of the enzyme for the micellar binding site is larger for longer chain lipids, while more lipid chains constitute one binding site.

The results from the binding studies gave Hill constants close to 1 for either protein or lipid titrations (see Figure 5). It should be noted that free lipid concentrations are calculated from total lipid added minus lipid bound to the protein, using a constant lipid to protein molar ratio of 50:1 (see also Appendix). From this can be concluded that (1) the two binding sites for the enzyme mentioned above are independent and (2) the number of lipid molecules in the lipid protein complex does not appear to change over the investigated titration ranges. Thus, no evidence is found for cooperative lipid binding processes. Second, the same type of complex is predominantly formed over lipid to protein molar ratios ranging from 10:1 to 1000:1.

The phenomenon of getting smaller particles upon interaction of enzyme on organized lipid–water interfaces has been described for other lipid–protein systems as well. Lauterwein et al. (1979) investigated the protein–lipid interaction in melittin-containing *n*-dodecylphosphocholine micelles and found a complex formation of one melittin molecule and approximately 40 detergent molecules. Jonas et al. (1980) studied the interaction of human and bovine A-I apolipoproteins with sonicated dimyristoylphosphatidylcholine (DMPC) vesicles at different L/P molar ratios. Two types of stable complexes were formed, vesicles with a maximum of 3–4 bound apo-A-I's and unique complexes with a composition of 300 DMPC molecules and 3 protein molecules. The same paper referred to Aune et al. (1977) where a similar mode of interaction between DMPC vesicles and apolipoprotein C-III is described. Fung et al. (1979) showed the "solubilization" of various large phospholipid aggregates by adding D-lactate dehydrogenase from *Escherichia coli* by electron microscopy and by ^{31}P and ^1H NMR.

The result that over a large concentration range one type of complex is formed containing two phospholipase A_2 molecules should not be interpreted to indicate that only an enzyme dimer is functionally active in catalysis. There is still no indication that in lipid-free solutions porcine pancreatic enzyme tends to dimerize. In equilibrium gel filtration and in light-scattering experiments (Table I), a molecular weight of about 15 000 was found, which is close to the molecular weight of a single phospholipase A_2 molecule (M_r 13 800, calculated from amino acid sequence). In sedimentation velocity experiments, only at enzyme concentrations above 3 mg/mL was a (small) increase in the sedimentation constants seen.

The number 50 lipid monomers per enzyme molecule is close to the lipid to protein ratios in various studies on reconstituted membrane proteins in pure lipid/water systems, which emphasizes the concept of "boundary lipids" or "lipid annulus". This "annulus" consists of 30–40 lipid molecules immobilized by 1 protein molecule surrounding the hydrophobic region of the protein. In the case of phospholipase A_2, the 50 lipid monomers cannot be accommodated in annulus form around the interface recognition site, because this site is too small. It is surprising that still the same number of lipid molecules constitutes a binding site for the protein molecule. Possibly the numbers found for lipid to protein ratios in all studies on lipid–protein interactions emphasize the properties of the lipid more than those of the protein. Unpublished ^1H NMR T_2 studies suggest that in the case of phospholipase A_2 the overall environment is hardly changed for the bulk of the alkyl chains upon complex formation.

Acknowledgments

This paper is dedicated to Professor A. Rossi Fanelli in honor of his 75th birthday. We gratefully thank L. C. Mey-Brants for synthesizing the substrate analogue, R. Dijkman for determining the cmc value, and Dr. A. J. H. Donné for drawing the figures. Thanks are due to Drs. R. Verger (Marseille, France) and C. Pathmamanoharan for stimulating discussions. We thank H. J. Mos for the laserbeat measurements, the group Lyophilic Colloids of the van't Hoff Laboratory for Physical and Colloid Chemistry for the permission to use their light-scattering apparatus, and Professor Dr. P. Desnuelle for measuring time allowed on the analytical ultracentrifuge at the CNRS, Marseille, France.

Appendix

Binding of proteins like phospholipase A_2 to micellar lipids may generally be written as

$$mE + L_n \overset{K}{\rightleftharpoons} pE_rL_s \qquad (5)$$

where E and L_n are the concentrations of free enzyme and

micelles, respectively, n being the number of lipid molecules in the free micelles. E_rL_s are the concentrations of the enzyme–lipid complexes formed, the latter consisting of r enzyme and s lipid molecules. Mass conservation requires that $p = m/r = n/s$.

Binding of a series of substrate analogues [C_{14}PN (Donné-Op den Kelder et al., 1981), C_{16}PN (de Araujo et al., 1979), and C_{18}PN (this paper)] to porcine phospholipase A_2 generally resulted in a decrease of the number of lipid molecules in the micelles; i.e., the number of lipid molecules in the free micelles (n) was approximately halved after complex formation with enzyme ($s = n/2$). A previously undiscussed consequence of this finding is that in general binding curves as a function of lipid or protein will not be simply hyperbolic. For a single step as shown in the above equilibrium (eq 5), Hill constants obtained from either lipid or protein titrations will not be equal to 1. However, as was shown in Figure 5A,B, both lipid and protein titrations yielded linear Hill plots with slopes close to 1 over the investigated concentration ranges.

Therefore it seems more likely that the lipid–protein interaction is considered as a multistep process, e.g., as indicated in Scheme I. The same symbols are used as for the single step equilibrium 5. The dissociation constants K_r and K_r' relate the concentrations of the reaction components via

$$K_r = E(E_{r-1}L_n)/(E_rL_n) \qquad K_r' = E(E_{r-1}L_s)/(E_rL_s) \qquad (6)$$

Stepwise binding of enzyme to micelles having a constant amount of lipid molecules (vertical set of equilibria in Scheme I) may give rise to Hill constants being close to 1. This requires that the binding sites on the micelles are independent. The single binding constant then obtained for this whole process (K_d) is related to the dissociation constants for the individual steps via

$$K_d = \frac{(r - i + 1)}{i} K_r \qquad (i = 1, 2, ..., r) \qquad (7)$$

where the terms $(r - i + 1)/i$ are statistical factors involved in the binding steps.

It is possible that only part of the total number of interactions is being observed under the experimental conditions, such that indeed the number of lipid molecules in the complexes remains constant (N). Thus, by use of the parameters K_d and N, the very complex set of equilibria mentioned above can, in fact, virtually be written as

$$E + L_N \overset{K_d}{\rightleftharpoons} EL_N \qquad (8)$$

where L_N are the combined sets of lipid molecules that provide one binding site for the enzyme molecules E.

For equilibrium 8, eq 3 mentioned earlier was derived

$$K_d = \frac{(E_T - X)[(L_T/N) - X]}{X} = \frac{(E)(L_N)}{(EL_N)}$$

and binding data were fitted by using iterative nonlinear regression methods.

When complex formation is measured as a function of the enzyme concentration, it is also possible to obtain the parameters N and K_d by graphical tests without the use of a computer. For example, eq 3 can be written as

$$1/X = C/Q_{obsd} = \frac{N}{L_T}\left[1 + \frac{K_d}{E_T - X}\right] \qquad (9)$$

where C (the UV absorption difference extinction coefficient) is found by adding enzyme to a large excess of lipid.

However, as was already discussed, titrations of enzyme with lipid cannot be analyzed by graphical tests, because the stoichiometry factor N has to be known beforehand:

$$C/Q_{obsd} = (1/E_T)[1 + NK_d/(L_T - NX)] \qquad (10)$$

Only when $L_T \gg NX$ is a reliable value for NK_d obtained.

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