Structure and Thermodynamic Properties of the Complexes between Phospholipase A_2 and Lipid Micelles†

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ABSTRACT: The interaction between porcine pancreatic phospholipase A_2 and a homogeneous population of micelles of the subtrate analogue *n*-hexadecylphosphorylcholine containing 155 lipid monomers was studied by light scattering, equilibrium gel filtration, and isothermal calorimetry. From the detergent/protein molar ratio and the equivalent "molecular weight" of the resulting complex it is concluded that insertion of the enzyme into the detergent micelle results in a protein–detergent complex containing two phospholipase A_2 molecules and 80 lipid monomers at 25 °C. The affinity constants and complex composition have been determined at different temperatures, allowing calculation of the thermodynamic parameters of the binding process. It is concluded that the interaction of phospholipase A_2 with micellar lipids is predominantly hydrophobic.

Many membrane-bound proteins display enzymatic activity only when they are in close contact with certain organized phospholipid structures (De Pierre & Ernster, 1977). However, the number of reports on successful reconstitution experiments involving highly purified apoproteins and pure synthetic phospholipids is very limited, and often the enzymic activities of such reconstituted complexes are lower than those of the native lipoprotein systems. Considerable improvement of our understanding of the binding forces between lipids and proteins in natural membranes has been obtained by extensive studies on model systems such as serum albumin and serum apolipoproteins and phospholipids (Morriset et al., 1977; Haberland & Reynolds, 1975). Because most reconstitution experiments using the latter systems cannot be monitored by a sensitive bioassay, we are interested in lipolysis as a possible model for studying specific lipid–protein interactions. The secretory enzymes such as the mammalian pancreatic (phospho)lipases are known to behave as usual esterases if they act on soluble *monomeric* lipids and they display normal Michealis kinetics (Entressangles & Desnuelle, 1968; Wells, 1974; Pieterson et al., 1974).

In the presence of certain *organized* (phospho)lipid–water interfaces, however, such as micelles, liposomes, or monomolecular surface layers, these enzymes display catalytic activities which are three or four orders of magnitude higher than found with monomeric substrate molecules (Verger & de Haas, 1976). It has been proposed (Verger et al., 1973) that a particular surface region of phospholipase A_2, the so-called interface recognition site (IRS),[1] is involved in the specific interaction with membranelike phospholipid structures and that this interaction is accompanied by a lipid-induced minor conformational change in the enzyme resulting in an optimization of the fine architecture of the active center. Although the IRS and the catalytic site, where monomeric substrates are bound and hydrolyzed, are topographically distinct (van Dam-Mieras et al., 1975), recent results from 360-MHz NMR studies (Slotboom et al., 1978) and high-resolution X-ray crystallography (Dijkstra et al., 1978) strongly indicate that the two lipid-binding regions cannot be far apart. While some progress has been made in the identification of amino acid side chains involved in the binding of phospholipase A_2 to interfaces (van Dam-Mieras et al., 1975), so far little work has been done to obtain more quantitative information on the nature of the interaction forces and the composition of the lipid–protein complexes.

The purpose of this study is to investigate the stoichiometry of the complexes formed between pancreatic phospholipase A_2 and micelles of the substrate analogue *n*-hexadecylphosphorylcholine (C_{16}-PN) and to determine the thermodynamic parameters of the interaction process.

Materials and Methods

Phospholipase A_2 was isolated from pig pancreas as its zymogen and converted into the active enzyme by limited proteolysis as described previously (Nieuwenhuizen et al., 1974). C_{16}-PN was synthesized as described elsewhere (van Dam-Mieras et al., 1975). [*N-methyl*-^{14}C]-C_{16}-PN was prepared by the procedure of Kamp et al. (1977). The final product (specific activity 266 180 dpm/μmol) was purified by high-pressure liquid chromatography on a Knauer reversed-phase silica column operated at 120 atm; the eluant was methanol–hexane (95:5 v/v). In all experiments 2× glass-distilled water was used throughout, and solutions were prepared in 50 mM sodium acetate buffer, pH 6.00, containing 100 mM NaCl. All pH values were adjusted using a Radiometer 62 pH meter equipped with a glass combination electrode type GK 2321C. The pH meter was standardized with pH 7 and 4 reference buffers before each measurement.

Phospholipase activity was measured as described previously (Nieuwenhuizen et al., 1974). Protein concentrations were determined from the absorbance at 280 nm using an $E_{1cm}^{1\%}$ of 13.0 (van Dam-Mieras et al., 1975). Phospholipase concentrations in the presence of C_{16}-PN micelles were measured either from the enzymatic activity of the samples (as in equilibrium gel filtration experiments) or by the absorbance at 280 nm using as blank a C_{16}-PN solution containing the same concentration (as in samples from the microcalorimeter). In both cases values obtained agreed within 2% of the expected numbers. C_{16}-PN concentrations were determined by the

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[1] Abbreviations used: C_{16}-PN, *n*-hexadecylphosphorylcholine; [*N-methyl*-^{14}C]-C_{16}-PN, *n*-hexadecylphosphorylcholine in which one of the choline methyl groups is substituted by $^{14}CH_3$; IRS, interface recognition site.

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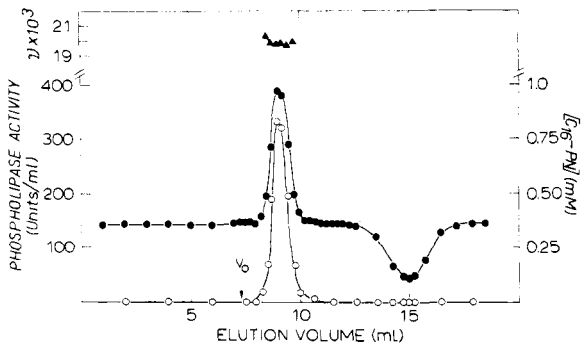

FIGURE 1: Elution pattern of a 0.8 μmol sample of [*N-methyl*-^{14}C]-C_{16}-PN micelles on a Sephadex G-100 column previously equilibrated with phospholipase A_2 (9.7×10^{-6} M): (●) phospholipase A_2, (○) C_{16}-P. ν represents the number of phospholipase A_2 molecules bound per molecule of detergent.

measurement of the lipid phosphorus as described by Mandersloot et al. (1978).

Light-Scattering Measurements. For light-scattering measurements, the buffer was filtered under pressure through Millipore filters (25 nm). Solutions of C_{16}-PN, phospholipase A_2, and mixtures of both were centrifuged at 20 000 rpm for 1 h to remove dust present in C_{16}-PN or phospholipase A_2. All glassware used was made dust-free by cleaning with condensing acetone. Light-scattering measurements were made at 25 °C with a FICA light-scattering photometer at angles between 30 and 150° with light of 546 nm. For all the measured solutions of C_{16}-PN, phospholipase A_2, and mixtures of both protein and detergent, the light-scattering intensity was not a function of the scattering angle. The intensity of the light scattering at 90° was linear with the concentration (see Figure 2). From these results, it appears that C_{16}-PN and phospholipase A_2 are monodisperse systems with a constant particle size throughout the concentration range studied.

Refractive-index increments were measured with a Rayleigh interferometer (λ 546 nm) at 25 °C and were found to be 0.132 ± 0.002 and 0.203 ± 0.007 mL/g for C_{16}-PN and phospholipase A_2, respectively.

From the values of the light-scattering intensity at 90° and the refractive-index increment, the molecular weight can be determined using the equation for the light scattering of dilute solutions of monodisperse particles (Stacey, 1956). For C_{16}-PN micelles, the measured value of the molecular weight was 65 000 ± 1000 which gives an aggregation number of 155 ± 3.

Diffusion coefficients were determined from quasi-elastic light scattering (Berne & Pecora, 1976) using an argon ion laser (λ 514 nm). With this technique, one monitors the fluctuations in the scattered-light intensity caused by the Brownian movement of the scattering particles and relates these fluctuations to the diffusion coefficient to be determined. With the Stokes–Einstein relation a hydrodynamic radius, r_h, can be calculated from these diffusion coefficients. For C_{16}-PN micelles r_h was found to be 34 ± 2 Å.

Gel Filtration. Equilibrium gel filtration was conducted essentially as described by Pieterson et al. (1974). Columns (0.9 × 30 cm) were packed with Sephadex G-100 superfine and equilibrated in buffer containing phospholipase A_2 and [*N-methyl*-^{14}C]-C_{16}-PN at concentrations usually 10% below the critical micelle concentration. A typical elution profile is shown in Figure 1. A sample of [*N-methyl*-^{14}C]-C_{16}-PN (usually between 0.3 and 1.3 μmol) was dried under vacuum, redissolved in the column buffer, and passed through the column. Phospholipase A_2 concentrations in the fractions (~250 μL) were determined from the activity measured and C_{16}-PN concentrations were determined from the countings obtained in a Searle Isocap 300 scintillation counter. Counting vials contained 50 μL of the fractions and 5 mL of instagel (Packard).

The critical micelle concentration of C_{16}-PN in the buffer was determined by the Wilhelmy plate method (Davies & Rideal, 1961) to be 10.3 ± 0.15 μM. This value was constant in the buffer at NaCl concentrations between 100 and 250 mM.

Gel filtration for molecular weight estimation was conducted in a column (0.9 × 60 cm) packed with Sephadex G-100 and operated with upward flow elution. Volumes of the fractions were measured by weight. The column was calibrated using a Boehringer protein calibration kit. The elution volumes of the standard proteins did not change after passing a lipid sample through the column. Phospholipase A_2–C_{16}-PN complex elution volumes were measured while operating the column in equilibrium conditions as described above.

The molecular weight of phospholipase A_2 was estimated from a sample passed through the calibrated column. This molecular weight could also be measured by the position where the trough eluted in a column operated under equilibrium conditions (see Figure 1). The elution profile was followed by the absorbance of the fractions at 280 nm, by the measurement of phospholipase A_2 activity in the fractions, and by counting the radioactivity of [*N-methyl*-^{14}C]-C_{16}-PN. The elution volumes were plotted either as described by Andrews (1965) or by Laurent & Killander (1964) with an excellent agreement with the published values for the standard proteins.

Calorimetric Measurements. Heats of binding of phospholipase A_2 to C_{16}-PN micelles were measured at 15 ± 0.3, 25 ± 0.3, and 35 ± 0.3 °C using an LKB 2107 batch microcalorimeter equipped with gold cells. A Keithley 150B microvoltmeter coupled with an LKB 2066 recorder equipped with a ball and disk integrator were used to amplify and record the output from the calorimeter. All measurements were performed in the 10- or 30-μV range of the voltmeter and always the same cell was used as the reaction cell. Electrical calibration heaters were checked by measuring the precision of the heat of dilution of sucrose (Gucker et al., 1939). Heats of dilution of phospholipase A_2 and C_{16}-PN micelles were measured in separate experiments and were always insignificant for all the protein and lipid concentrations used. Heats of binding were measured in the reaction cell against matching volumes of buffer and C_{16}-PN micelles in the reference cell. Occasionally, small shifts in the base line occurred during the mixing cycle of the reaction, and the best results were obtained by using the averages of the leading and following base lines, the same procedure being used in the friction runs and in the electrical calibrations. For each experiment three friction runs and usually one electrical calibration were performed. Electrical calibrations were always adjusted to produce a heat effect within ±20% of that observed for the reaction mixture. A reaction was assumed to be complete after the return to base line, which always occurred in less than 20 min after the mixing. The calorimeter cells were filled volumetrically using Gilson P-5000 precision pipets, individually calibrated by weight to deliver 2 or 4 mL of each solution. In all experiments phospholipase A_2 and C_{16}-PN concentrations were measured in the samples removed from the cells. In general the heats of binding were measured in duplicate. Every curve contained at least 15 points. Series of experiments were conducted at constant phospholipase A_2 concentration (~0.035 mM final concentration) and the C_{16}-PN concentration varied up to 7

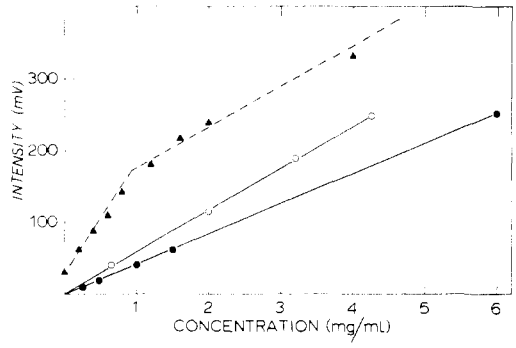

FIGURE 2: Light-scattering intensity at 90° as a function of the concentration in the samples: (●) phospholipase A_2, (○) C_{16}-PN micelles, and (▲) mixtures of phospholipase A_2 (5×10^{-5} M) with increasing concentrations of C_{16}-PN micelles. The break corresponds to a phospholipase A_2/C_{16}-PN ratio of $(25 \pm 4) \times 10^{-3}$.

mM (final concentration). In other series, the C_{16}-PN concentration was kept constant (~2 mM final concentration) and the phospholipase concentration varied up to 0.25 mM (final concentration). The excellent agreement of these experiments was taken as an indication that the model used for the calculation of the results is correct (Bolen et al., 1971).

The basic assumption made to obtain the binding parameters is that C_{16}-PN micelles contain N' independent and identical sites for binding phospholipase A_2. If each micelle contains M monomers there will be $N = N'/M$ sites per lipid monomer. At full saturation the heat effect is given by

$$Q_{max} = cNL_T \quad (1)$$

where L_T is C_{16}-PN total concentration and c is a constant. Below saturation the heat effect observed is

$$Q = cnL_T \quad (2)$$

where n is the fractional saturation of N given by

$$n = \frac{NP_f}{K'_D + P_f} \quad (3)$$

where P_f is the free phospholipase A_2 concentration and K'_D is the apparent dissociation constant (i.e., the free phospholipase A_2 concentration necessary for transfer of half of the phospholipid to the final complex). Free phospholipase concentrations were calculated from

$$P_f = P_T - nL_T \quad (4)$$

where P_T is the total phospholipase concentration. From eq 2–4 it follows that

$$Q = \frac{1}{2}c\left[(NL_T + P_T + K'_D) \times \left(1 - \sqrt{1 - \frac{4NP_TL_T}{(NL_T + K'_D + P_T)^2}}\right)\right] \quad (5)$$

Equation 5 was fitted to the actual calorimetric data by means of an iterative least-squares program using three adjustable parameters c, N, and K'_D, which was based on the program of Fletcher & Powell (1963).

Results

Figure 2 shows the results of light-scattering experiments. From the upper curve representing mixtures of phospholipase A_2 and increasing concentrations of C_{16}-PN, it appears that, contrary to the pure compounds, the intensity of the scattered light is not a simple linear function of lipid concentration. Under the conditions of the experiment shown in Figure 2, the light-scattering intensity increases more than theoretically predicted from both lower curves up to 0.9 mg/mL of C_{16}-PN. Above this value the increase in scattering intensity remains linear with lipid concentration, but the slope becomes equal to that of the pure C_{16}-PN micelles. The rapid initial increase in light scattering upon addition of C_{16}-PN micelles is interpreted as due to complex formation; above 0.9 mg/mL saturation is reached and further increase of C_{16}-PN concentration leads only to an increased number of pure lipid micelles. From the concentrations of phospholipase A_2 and C_{16}-PN micelles that correspond to the break in the upper curve in Figure 2, a protein/lipid ratio of $(25 \pm 4) \times 10^{-3}$ is calculated. This value agrees with the number (N) found by microcalorimetry at the same temperature (see Table II).

Table I: Estimation of Molecular Size by Gel Filtration and by Calculation

	K_{av}[a]	r_S[a] (Å)	measured M_r[b]	calcd M_r[c]
phospholipase A_2	0.57	17	14 000	
C_{16}-PN micelles at 25 °C	0.14	40	90 000	96 000
complex at 15 °C	0.14	40	90 000	86 000
complex at 25 °C	0.17	37	78 000	76 000
complex at 35 °C	0.20	34	66 000	60 000

[a] K_{av} (partition coefficient) and r_S (equivalent Stokes radius) were calculated from the elution volumes of a Sephadex G-100 column as described by Laurent & Killander (1964). [b] Measured "molecular weights" were obtained from elution volumes as described by Andrews (1965). [c] "Molecular weights" were calculated assuming at all temperatures a constant hydration of the phospholipid of 12 water molecules per lipid monomer as described in the text. The values for C_{16}-PN micelles were calculated from the aggregation number of 155 (see Materials and Methods section); values for the complexes were calculated from the N values (Table II) and 14 000 as the molecular weight of phospholipase A_2, according to the dimeric model proposed.

Taking into account the equivalent "molecular weight" of the hydrated lipid micelles as determined by gel filtration (Table I), the number of 155 monomers per lipid micelle (see Materials and Methods) indicates that every C_{16}-PN monomer is surrounded by 10 strongly bound water molecules. Calculation of the extent of hydration using the radius of the lipid micelle given by quasi-elastic light scattering (34 Å) and the length of the extended lipid molecule (27.5 Å) yields a value of 12 ± 3 water molecules per lipid monomer. Using the above stoichiometry found from light-scattering experiments and assuming that the hydration of the polar head groups in the complex is the same as in the protein-free micelle, we calculate the minimum "molecular weight" of the complex to be 38 000. From Table I it can be seen that the hydrated complex at 25 °C has a "molecular weight" of 78 000 as measured by gel filtration. This result indicates that at 25 °C the complex consists of two enzyme molecules and about 80 lipid monomers. Information on the stability of the complexes was obtained by equilibrium gel filtration. The slopes of the Scatchard plots at three different temperatures (Figure 3) yield apparent dissociation constants, K'_D, which vary from 5.0×10^{-6} to 7.0×10^{-6} M.

More accurate values for the stoichiometry of the complexes at different temperatures were obtained by microcalorimetry. Using the N values shown in Table II and a constant hydration extent of 12 water molecules per lipid monomer, we can calculate the "molecular weight" of the complex. As shown in Table I, these values are in good agreement with the numbers determined experimentally by gel filtration for the case of a dimeric structure. Information about the stability

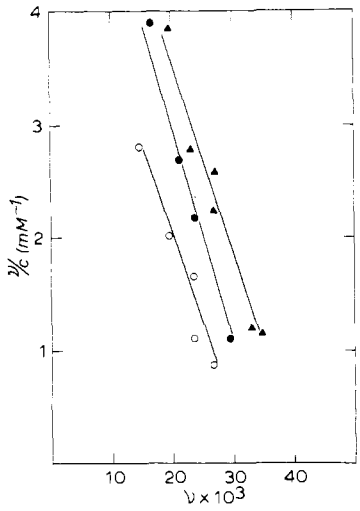

FIGURE 3: Scatchard plots for binding data between phospholipase A_2 and C_{16}-PN micelles. ν values were obtained from the fractions over the peak, as shown in Figure 1, for different free-enzyme concentrations (c) ranging from 3.1×10^{-5} M to 4.4×10^{-6} M: (○) experiments at 35, (●) 25, and (△) 15 °C.

Table II: Thermodynamic Parameters of the Binding of Phospholipase A_2 to C_{16}-PN Micelles[a]

temp (K)	$N \times 10^3$	$K'_D \times 10^3$ (M)	$\Delta H \times 10$ (kcal/mol of C_{16}-PN)	ΔG (kcal/ mol of C_{16}-PN)	ΔS [cal/(deg mol of C_{16}-PN)]
288	21.4 ± 1.0	6.5 ± 1.6	−2.7 ± 0.1	−6.8 ± 1.7	+24.6 ± 6.3
298	25.9 ± 1.4	10.6 ± 3.2	−3.6 ± 0.3	−6.8 ± 2.0	+23.9 ± 7.6
308	38.7 ± 1.2	13.3 ± 2.4	−5.2 ± 0.3	−6.9 ± 1.2	+23.9 ± 4.5

[a] Values are presented as the mean ± SD. N, K'_D, and ΔH values were obtained from an iterative least-squares procedure as described in the Materials and Methods section. Free energy changes were calculated from the association constants expressed in L/mol using the relationship $\Delta G = -RT \ln 1/K'_D$. Entropy changes were computed from the classical relationship $\Delta S = (\Delta H - \Delta G)/T$, where T is temperature in K.

of the complexes was also obtained by microcalorimetry. This technique allows a direct determination of the thermodynamic parameters ΔG, ΔH, and ΔS of the binding process. In Figure 4 a typical thermal saturation curve is shown for experiments at 25 °C.

The inset of Figure 4 shows the double-reciprocal plot of ΔH against phospholipase A_2 free concentration. Such a linear relationship had to be expected from eq 2 and 3 and supports the model used. The results of the calorimetric measurements at three different temperatures are compiled in Table II.

Similar experiments have been conducted at 25 °C in the buffer containing higher NaCl concentration (300 mM). Under these conditions K'_D was found to be 2.8×10^{-6} M, which is nearly four times smaller than the value reported in Table II, measured at 100 mM NaCl.

Figure 5 shows the dependence of ΔH on temperature. The results were fitted to the equation

$$\Delta H = a + bT + cT^2 \qquad (6)$$

(Donnér et al., 1976), where a, b, and c are constants with the numerical values of −27.731, 0.196, and −0.00035, respectively, and T is temperature expressed in K. ΔC_p obtained from the derivative of eq 6 at 298 K is $\Delta C_p = -12.5$ cal K^{-1} (mol of C_{16}-PN)$^{-1}$.

Discussion

In an attempt to obtain information on the nature of the binding forces between lipolytic enzymes and lipid–water

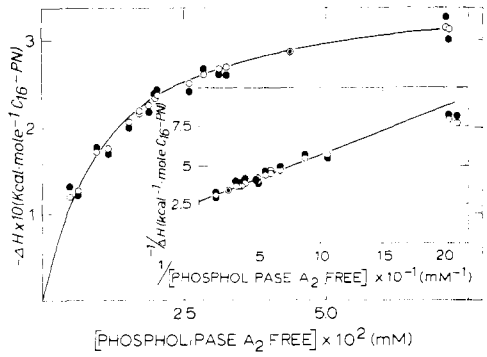

FIGURE 4: The measured enthalpy changes for the interaction of phospholipase A_2 and C_{16}-PN micelles as a function of the phospholipase A_2 free concentration. In the inset, a double-reciprocal representation of the results is given. Experiments were conducted at 25 °C. Experimental details are described in the text. The solid lines were calculated from eq 5: (●) measured values, (○) best fit values obtained by least-squares iterative program.

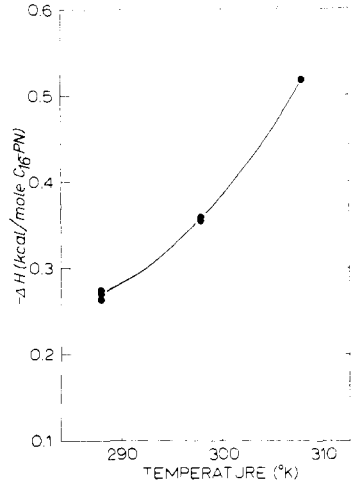

FIGURE 5: ΔH for the binding of phospholipase A_2 as a function of temperature. The results have been fitted to the equation $\Delta H = a + bT + cT^2$, where a, b, and c are constants and T is the temperature in K. $\Delta C_p = b + 2cT$ and at 298 K ΔC_p has the value of −12.5 cal/(K/mol of C_{16}-PN).

interfaces, we determined thermodynamic parameters of the interaction process between porcine pancreatic phospholipase A_2 and micelles of the substrate analogue *n*-hexadecylphosphorylcholine. Like lysolecithin, this single chain "phospholipid" forms micellar solutions in water at the low critical micelle concentration of 10.3 μM. Light-scattering measurements on aqueous solutions of this zwitterionic detergent showed a homogeneous population of most probably spherical micelles composed of 155 monomeric lipid molecules. In light-scattering experiments, the absence of angle dependence of the light-scattering intensity is an indication of a spherical or quasi-spherical shape of the particles. Our results showing that between 30 and 150° the light-scattering intensity is independent of the scattering angle indicates that phospholipase A_2, C_{16}-PN micelles, and the detergent–protein complex are all most probably spherical.

For phospholipase A_2, this indication agrees very well with the three-dimensional structure of the prophospholipase that has been elucidated recently (Drenth et al., 1976); for C_{16}-PN micelles, it correlates well with the proposed spherical shape of other detergent micelles (Ekwall & Stenius, 1975).

Quasi-elastic laser light scattering yielded a Stokes radius for the hydrated micelles of 34 Å which compares favorably with the value (34 Å) reported by Haberland & Reynolds (1975) for 1-hexadecanoylglycero-3-*sn*-phosphorylcholine.

Also, the earlier findings of Saunders (1966), concerning shape and dimensions of lysolecithin micelles which structurally closely resemble C_{16}-PN, are in agreement with the present results. Recently, the extent of hydration of lecithin molecules organized in bilayer structures (Lindberg et al., 1978) and, earlier, the extent of hydration of other detergent micelles (Ekwall & Stenius, 1975) have been reported. The number of 12 H_2O molecules per C_{16}-PN monomer, calculated by two different approaches in the present study, agrees with the previously reported results.

A surprising result of the present study is the decrease in particle size (and equivalent "molecular weight") upon addition of phospholipase A_2 to the micelles; whereas the protein-free hydrated micelles at 25 °C possess a "molecular weight" of 90000, the detergent–protein complex at that temperature was shown to have a particle weight of only 78000 (Table I). This finding would suggest that binding is not a simple additive process but that the enzyme is inserted into the micelle and as a result reorganization of the detergent monomers occurs. Taking into account the detergent–protein stoichiometry (N) determined by light scattering (Figure 2) and microcalorimetry (Table II) and assuming an identical hydration extent of the detergent molecules in the complex (12 water molecules per mol of C_{16}-PN), the calculated particle weights of the complexes given in Table I are in rather good agreement with the experimentally measured values. Preliminary ultracentrifugation results (not shown here) also indicated a dimeric phospholipase structure in the presence of C_{16}-PN, thus providing corroborative evidence by yet another independent technique of the results presented here.

The binding reaction of phospholipase A_2 with C_{16}-PN micelles is exothermic and takes place very rapidly as shown by microcalorimetry. From binding isotherms such as given in Figure 4, numerical values have been calculated for the stoichiometric relation N, the free enthalpy of binding ΔH, and the apparent dissociation constant K'_D (see Table II). While ΔH can be clearly defined as the free enthalpy change upon transfer of 1 mol of C_{16}-PN from the pure micelle to the detergent–protein complex, it is more difficult to attribute an exact meaning to K'_D. Nevertheless, a suitable working definition of K'_D is the following: the free enzyme concentration necessary for transfer of half of the detergent from the micelle into the detergent–protein complex. In this respect it should be noted that a completely different technique, equilibrium gel filtration, yields apparent K'_D values which are in good agreement with the ones obtained by microcalorimetry (compare the K'_D numbers from the Scatchard plots of Figure 3 with the values given in Table II). Although the measured heat effects certainly are the result of a complex series of reactions, which could include conformational changes in the protein, changes in the extent of hydration of both micelles and the protein, monomer rearrangement, the ΔH observed upon interaction of phospholipase A_2 with C_{16}-PN micelles is attributed mainly to the binding process. This attribution is supported by the fact that no measurable heat effects are observed for the zymogen of phospholipase A_2 (de Haas et al., 1978); moreover, the contribution of the lipid rearrangement should be minimal since ΔH of demicellization is extremely low (Rosseneu et al., 1974).

The low ΔH values, combined with the positive entropy changes, point to a hydrophobic interaction between the enzyme and the detergent molecules. It is clear from Table II that the transfer of 1 mol of detergent from the C_{16}-PN micelles to the detergent–protein complex is accompanied by changes in ΔS, which are temperature independent. On the other hand, this transfer involves a change in ΔH, which is a function of temperature.

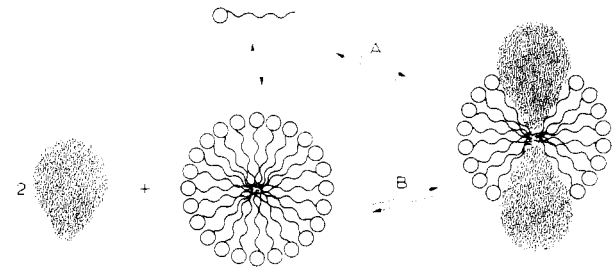

FIGURE 6: Schematic view of the pathways for the formation of a complex between phospholipase A_2 and C_{16}-PN micelles.

This implies a change in the heat capacity ΔC_p of the system, a change which can be calculated from Figure 5. Although on a detergent molar base this ΔC_p value is very low, the formation of a complex between two enzyme molecules and 80 detergent monomers at 25 °C (Figure 6) corresponds to an overall ΔC_p value of −1 kcal K^{-1}. This is a very negative value which again supports the idea that mainly hydrophobic interactions determine the stability of the phospholipase A_2–lipid complex. The lower K'_D values measured by microcalorimetry at higher ionic strength support a previously reported dependence of kinetically determined K_m numbers on NaCl concentration. These latter values were found to decrease by a factor of 4.6 upon increasing the salt concentration from 0 to 3 M (de Haas et al., 1971). Such behavior can be understood only if hydrophobic binding forces play a predominant role in the interaction between phospholipase A_2 and micellar aggregates. A highly schematic drawing of a complex formation, which is in agreement with the stoichiometries found by light scattering and microcalorimetry, is given in Figure 6. At 25 °C, the complex between phospholipase A_2 and C_{16}-PN micelles consists of two protein molecules and about 80 detergent monomers.

As regards the pathway along which the final complex is built up, at least two possibilities can be considered as discussed by Robinson & Tanford (1975): (A) the mode of association of the enzyme with lipid monomers being similar to the micelle formation occurring in the protein-free system; this means that the interaction between the monomeric detergent and the enzyme binding site(s) is slightly favored over the micelle formation; (B) direct insertion of a hydrophobic surface area of the protein into the core of the micelle, followed by a rearrangement of the complex into the thermodynamically most favored structure.

Although the comicellization mechanism (pathway A) has been proposed for some water-soluble proteins containing several high-affinity sites such as serum albumin (Makino et al., 1973) and serum apolipoproteins (Haberland & Reynolds, 1975; Rosseneu et al., 1976), in the case of pancreatic phospholipase A_2 we strongly favor pathway B for the following reasons.

(1) It has been shown that pancreatic phospholipase A_2 possesses a so-called interface recognition site (IRS), a hydrophobic surface area of the protein, which involves at least the N-terminal sequence of the polypeptide chain + Tyr^{69} (van Dam-Mieras et al., 1975). The natural zymogen of the enzyme in which the polypeptide chain is N-terminally extended with a heptapeptide lacks this IRS and does not bind to micelles.

A functionally active IRS has been demonstrated to depend critically on protonation of one single amino acid, L-Ala[1]. Even the very minor change, namely, substitution of L-Ala[1] by D-Ala[1] in phospholipase A_2, switches off the IRS and prevents binding of the protein to micellar substrates (Slotboom et al., 1977).

(2) Under conditions where no monomeric lipids are present in solution, which is the case with monomolecular surface films at the air–water interface, the active phospholipase A_2 interacts with the lipid film, whereas the zymogen is unable to penetrate. Also, the findings of Op den Kamp et al. (1974, 1975) that the enzyme can hydrolyze liposomes of dimyristoyllecithin only at the transition temperature but not below or above this temperature point to a direct insertion of the enzyme into the lipid bilayer at the phase transition.

(3) Although the interaction of phospholipase A_2 with micellar substrate analogues is accompanied by local, small perturbations of aromatic amino acid side chains (van Dam-Mieras et al., 1975), no gross conformational changes in the protein occur as was shown by circular dichroic measurements (Slotboom, A. J., personal communication).

(4) Direct binding studies of the enzyme and the zymogen with substrates or substrate analogues at concentrations below the critical micelle concentration have shown that both proteins contain only one high-affinity binding site, which is the active center of the protein. Switching off the active center in phospholipase A_2 by irreversible active-site-directed inhibitors such as *p*-bromophenacyl bromide yields a protein which is no longer able to bind a monomeric substrate in its active site but which fully retains its affinity for micellar lipid–water interfaces (Pieterson et al., 1974).

Finally, it should be emphasized that, in contrast to several venom phospholipases A_2 (Wells, 1973; Roberts et al., 1977), the dimeric structure of pancreatic phospholipase A_2 in the complex shown in Figure 6 should not be interpreted to mean that an enzyme dimer is functionally active in catalysis. We have no indication whatsoever that the enzyme in lipid-free solutions has any tendency to dimerize. On the contrary, even in the presence of monomeric C_{16}-PN the Sephadex experiments yield a molecular weight of 14000, a value which is in close agreement with the known amino acid sequence (13800). Moreover, the light-scattering studies up to protein concentrations of 6 mg/mL do not indicate dimer formation.

Similar studies with other substrate analogues differing in polar head group and acyl chain length are in progress in order to determine whether the structure found for phospholipase A_2 and C_{16}-PN is general or peculiar to this particular detergent.

Acknowledgments

The authors gratefully acknowledge R. Dijkman and W. S. M. Geurts van Kessel for the synthesis and purification of the ^{14}C-labeled *n*-hexadecylphosphorylcholine. They are indebted to H. J. Mos for his assistance with the laser experiments and to Professor Dr. A. Vrij and Dr. H. Feinaut for helpful discussions.

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Enzymatic Resynthesis of the Hydrolyzed Peptide Bond(s) in Ribonuclease S†

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ABSTRACT: Bovine pancreatic ribonuclease S was incubated with subtilisin at pH 6.2 in partially nonaqueous solutions of 0–95% (v/v) glycerol. The solutions were monitored at various times for the presence of ribonuclease A‡ (a mixture of ribonuclease A, des-Ser^{21}-ribonuclease A, and possibly Ser^{21A}-ribonuclease A) by enzymatic assay in the presence of 40% (v/v) dioxane (where ribonuclease S is inactive) and by sodium dodecyl sulfate gel electrophoresis. In all cases, the concentration of ribonuclease A‡ first increased and then declined. In water the maximal amount of synthesis was only 4.3%; it smoothly increased with increasing glycerol concentration until in 90% (v/v) glycerol 50% synthesis was attained. The increase in synthesis with glycerol concentration is in rough quantitative agreement with model studies on peptide bond hydrolysis equilibria in simple peptides [Homandberg, G. A., Mattis, J. A., & Laskowski, M., Jr. (1978) *Biochemistry 17*, 5220]. From the data a very rough value of $K_{hyd}°$ for the Ala^{20}–Ser^{21} peptide bond in water can be estimated as 20; it is roughly unity in 90% (v/v) glycerol. Addition of organic cosolvents other than glycerol was ineffective in promoting synthesis. The ribonuclease A‡ obtained from the synthesis in 90% (v/v) glycerol was isolated and subjected to 25 cycles of Edman degradation in an automatic sequencer. The results showed it to be a mixture of ribonuclease A, des-Ser^{21}-ribonuclease A, and possibly Ser^{21A}-ribonuclease A.

The discovery by Richards & Vithayathil (1959) that subtilisin specifically hydrolyzes the Ala^{20}–Ser^{21} bond in bovine pancreatic ribonuclease A is one of the milestones in protein chemistry. The product of this hydrolysis, ribonuclease S,[1] is a noncovalent complex of the S-peptide and S-protein and retains enzymatic activity. However, after low pH dissociation both isolated components are devoid of activity but regain it when they are allowed to recombine. These observations had important implications for our understanding of the nature of enzymatic active sites and of the effect of noncovalent interactions upon protein conformation and folding. Furthermore, they allowed for a large number of investigations of the functional and structural role of individual amino acid residues in ribonuclease since S-peptide or S-protein analogues could be prepared and their recombination with the other component and the resultant enzymatic activity of the complex could be studied. Many such studies are still going on. Earlier work was reviewed by Richards & Wyckoff (1971). However, to the best of our knowledge all such studies were conducted with noncovalently bound complexes. No attempt to resynthesize the hydrolyzed peptide bond between the S-peptide and S-protein has been reported.

In a recent paper (Homandberg et al., 1978) we have shown that addition of large concentrations of organic cosolvents significantly raises the value of the equilibrium constant for peptide bond synthesis and yet does not abolish the catalytic activity of proteinases. We have also shown that the reactive site peptide bond in modified soybean trypsin inhibitor (Kunitz) can be more efficiently resynthesized in the presence of large amounts of glycerol. It appeared that enzymatic resynthesis of the split peptide bond in ribonuclease S would serve as a test of the applicability of the enzymatic resynthesis to proteins other than proteinase inhibitors.

However, the problem was more complex than that of dealing with soybean trypsin inhibitor (Kunitz), where the limited proteolysis is very strictly limited and no other bonds aside from Arg^{63}–Ile are hydrolyzed even after prolonged exposure to massive amounts of trypsin and where significant resynthesis can be carried out even in the absence of organic cosolvents (Mattis & Laskowski, 1973). For ribonuclease S the value of K_{syn} in water was not known,[2] and therefore we could not calculate a priori how large an increase in K_{syn} was required in order to carry out efficient synthesis. More importantly the proteolysis giving rise to ribonuclease S is not strictly limited.

First, subtilisin hydrolyzes not only the Ala^{20}–Ser^{21} but alternatively also the Ser^{21}–Ser^{22} bond (Gross & Witkop, 1967). Therefore, ribonuclease S may well be a mixture of four components: (1–20, 21–124), (1–20, 22–124), (1–21, 20–124), and (1–21, 22–124) (Doscher & Hirs, 1967). This

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[1] Nomenclature used: ribonuclease S [presumed mixture of noncovalent complexes (1–20, 21–124), (1–20, 22–124), (1–21, 21–124), (1–21, 22–124)] obtained by incubation of bovine pancreatic ribonuclease S with subtilisin; ribonuclease A‡ (presumed mixture of ribonuclease A, des-Ser^{21}-ribonuclease A, and Ser^{21A}-ribonuclease A) obtained by enzymatic resynthesis of the hydrolyzed peptide bond(s) in ribonuclease S; ribonuclease S′ obtained by recombining S-peptide and S-protein.

[2] However, in a personal communication Dr. I. M. Chaiken suggested that at equilibrium in water we may well have 5% of ribonuclease A, a value very close to the one reported here. In an earlier report on this work (Homandberg & Laskowski, 1978) we have mistakenly concluded, by examining the data at too long a time of incubation (see Figure 1), that the equilibrium amount of ribonuclease A would be much lower (0.1%), and thus we believed that the added glycerol had a much larger effect on K_{syn} than reported here.

0006-2960/79/0418-0586$01.00/0