Binding of Porcine Pancreatic Phospholipase A_2 to Various Micellar Substrate Analogues. Involvement of Histidine-48 and Aspartic Acid-49 in the Binding Process†

Gabriëlle M. Donné-Op den Kelder, Jan D. R. Hille, Ruud Dijkman, Gerard H. de Haas, and Maarten R. Egmond*

ABSTRACT: The interaction of porcine pancreatic phospholipase A_2 (PA_2) with micelles of various single-chain phospholipid analogues was studied by ultraviolet absorption difference spectroscopy and light-scattering measurements. The phospholipids used were either substrate analogues or products, varying in hydrocarbon chain lengths and polar head groups. The results indicate that the enzyme forms a stable complex over a wide range of enzyme and lipid concentrations. From the equivalent "molecular weight" and from the lipid to enzyme molar ratio (N) of the micelle–enzyme complex, it can be calculated that complexes containing saturated hydrocarbon chain lipids generally consist of two enzyme molecules and half of the number of lipid monomers present in free micelles. The interaction forces between the enzyme and lipid monomers bound in the complex are mainly hydrophobic. Stronger binding is found when the essential cofactor Ca^{2+} is bound to the enzyme. pH-titration studies on the binding of native PA_2 to aggregated lipid structures showed that at least one group with a pK_A value of 6.25 is involved in the interaction with lipid micelles. At acidic pH, micelle binding is stronger than at neutral or alkaline pH. Alkylation of the active site residue His^{48} resulted in a shift of the pK_A value to 4.6, while addition of Ca^{2+} appears to stabilize the micelle-binding conformation of both native and modified enzymes over a broad pH range (pH 4–9.5). From these observations it is suggested that both the Ca^{2+} binding residue Asp^{49} [Fleer, E. A. M., Verheij, H. M., & de Haas, G. H. (1980) *Eur. J. Biochem. 113*, 283–288] and His^{48} control micelle binding of the native enzyme. For optimal binding in the absence of Ca^{2+}, a long-distance hydrogen bond between these two residues is required; this can be established via a water molecule. It is assumed that it is a proton of this "H bond" which is titrated with a pK_A value of 6.25. When the "H bond" is absent, as in the alkylated enzymes, Asp^{49} alone controls micelle binding with a pK_A of 4.6. These results, together with the effect of Ca^{2+} on micelle binding, indicate that it is not the "hydrogen bridge" between His^{48} and Asp^{49} which is of main importance for an optimum binding conformation of the enzyme but the effective charge in the microenvironment of Asp^{49}. It is proposed that a negative charge on this carboxylate causes a conformational change of the enzyme which leads to a protein conformation lacking an active micelle binding site. Binding of Ca^{2+} or reprotonation neutralizes this negative charge and restores the enzyme's ability to bind micelles.

Until recently, little work has been done to obtain quantitative information on the composition of micelle–phospholipase A_2 complexes and on the nature of the interaction forces between the components of these structures. Considerable improvement of our understanding of the binding forces between aggregated lipid structures and proteins has been obtained by de Araujo et al. (1979). He investigated the stoichiometry of the complexes formed between porcine pancreatic phospholipase A_2 and micelles of the substrate analogue $C_{16}PN$[1] by light scattering, equilibrium gel filtration, and isothermal calorimetry. He concluded that insertion of the enzyme into micelles resulted in a complex consisting of 2 enzyme molecules and 80 lipid monomers. Furthermore, it was concluded that the interaction forces were predominantly hydrophobic. However, it was not possible at that time to attribute an exact meaning to K_D, the dissociation constant of the complex. A recent study on the substrate analogue $C_{18}PN$ (Hille et al., 1981) led to a further insight into the meaning of this binding parameter. UV absorption difference spectroscopy was introduced as a fast and accurate technique to determine the binding parameters of complex formation. The advantages of this spectroscopic technique compared with equilibrium gel filtration and isothermal calorimetry are outlined in this study on $C_{18}PN$.

The aim of the present paper is to extend these studies to the interactions of PA_2 with various micelles, using UV absorption spectroscopy to determine the dissociation constant K_D and the enzyme to lipid molar ratio of the complex formed. Light-scattering measurements were used to determine the composition of the enzyme–micelle complex. The phospholipids studied were either substrate analogues or products of the enzymatic action of PA_2, varying in hydrocarbon chain length or polar head group. Furthermore, we have studied the effects of Ca^{2+}, pH, and irreversible active-site modifications on the binding of the porcine enzyme to lipid–water interfaces in order to reveal some aspects of the mechanism that controls micelle binding.

Experimental Procedures

Materials and Methods. Prophospholipase A_2 was purified from porcine pancreas and converted into phospholipase A_2 by limited proteolysis as described by Nieuwenhuizen et al. (1974). 1-Bromo-2-octanone was synthesized essentially according to the procedure described by Visser et al. (1971) and

† From the Biochemical Laboratory, State University of Utrecht, University Center "De Uithof", 3508 TB Utrecht, The Netherlands. *Received October 17, 1980.* This study was partly carried out under the auspices of the Netherlands Foundation of Chemical Research (SON) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

[1] Abbreviations used: $C_{14}PN$, *n*-tetradecylphosphocholine; $C_{16}PN$, *n*-hexadecylphosphocholine; $C_{18}PN$, *n*-octadecylphosphocholine; $C_{18:1}PN$, (*cis*-9-*n*-octadecenyl)phosphocholine; C_{14}lyso-PC, 1-tetradecanoyl-*sn*-glycero-3-phosphocholine; C_{16}lyso-PC, 1-hexadecyl-*sn*-glycero-3-phosphocholine; $C_{18:1}$lyso-PC, 1-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphocholine; C_{16}-PDME, *N*,*N*-dimethyl-*n*-hexadecylphosphoethanolamine; PA_2, phospholipase A_2; cmc, critical micelle concentration; IRS, interface recognition site; Tris, tris(hydroxymethyl)aminomethane; Hepes, 2-[4-(2-hydroxymethyl)-1-piperazine]ethanesulfonic acid.

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Mangold (1973). By using 1-bromo-2-octanone, it was shown that after complete inactivation of phospholipase A_2, 1 mol of the inhibitor/mol of enzyme was incorporated with the concomitant loss of one His residue (Verheij et al., 1980). Just as *p*-bromophenacyl bromide (Volwerk et al., 1974) reacts specifically with His^{48}, so does 1-bromo-2-octanone.

By using *p*-nitrobenzenesulfonate, it has been shown that the N-1 nitrogen atom of the imidazole ring of His^{48} was exclusively methylated (Verheij et al., 1980), as was found for the reaction with bromo ketones. Protein concentrations of native and modified phospholipase A_2 were determined from the absorbance at 280 nm, using an $E^{1\%}_{1cm}$ of 13.0.

1-Acyl-*sn*-glycero-3-phosphocholines (lysolecithins) were prepared from the corresponding diacyl compounds by catalytic action of PA_2. *n*-Alkylphosphocholines were synthesized as described by van Dam-Mieras et al. (1975), *N*,*N*-dimethyl-*n*-hexadecylphosphoethanolamine was synthesized by the procedure of Kamp et al. (1977).

Critical micelle concentrations were determined by using the Wilhelmy plate method (Davis & Rideal, 1961). Lipid concentrations were determined by weight. In all experiments, glass-distilled water was used. For all light-scattering measurements, buffer solutions were used containing 50 mM sodium acetate, 100 mM sodium chloride, and 25 mM calcium chloride at pH 6 and 25 °C. In the measurements with C_{14}lyso-PC, however, 100 mM barium chloride was added instead of 25 mM calcium chloride.

Ultraviolet Absorption (Difference) Spectroscopy. Ultraviolet difference spectra were recorded on an Aminco DW-2A spectrophotometer equipped with a MIDAN data analyzer. The titration technique used was as described in the preceding paper in this issue (Hille et al., 1981). Both lipid and enzyme titrations were carried out. The spectroscopic data obtained were used as input for an iterative least-squares program on the basis of the method of Fletcher & Powell (1963). In this nonlinear regression program, the spectroscopic data are fitted to a quadratic equation in X, the bound enzyme concentration. Output parameters of the program are the molar extinction coefficient, ϵ, the number of bound lipid monomers per enzyme molecule, N, and the dissociation constant of the complex, K_D [see Hille et al. (1981)].

It is known (van Dam-Mieras et al., 1975) that the UV difference spectra arise mainly from Trp^3 perturbation upon complex formation. However, when phospholipase A_2 concentration is high relative to the lipid concentration, the characteristic features of these spectra change due to a large contribution of tyrosine signals. Previously, it was found that lipid monomers only perturb tyrosine residues in porcine PA_2 (van Dam-Mieras et al., 1975). Even in the case of C_{18}PN, where the cmc is low (1 μM), bound lipid monomers were observed at high enzyme to lipid molar ratios. Generally, either no observations were included under those conditions or the absorption readings were corrected for this contribution.

Light-Scattering Measurements. For light-scattering measurements, a FICA 50 photometer was used. All experiments were done at a wavelenth of 546 nm and at 25 °C. The lipid to protein molar ratios of the various comlexes were determined as described by de Araujo et al. (1979). The molecular weights of micelles and micelle–enzyme complexes were determined by the method described by Hille et al. (1981). The light-scattering intensities at 90° were corrected for pure solvent scattering, and the lipid concentrations were corrected for the monomer concentration by substracting the cmc value from the total lipid concentration. The light-scattering intensities of solutions containing phospholipase A_2 and/or micellar phospholipid compounds were independent of the scattering angle. There was one exception: solutions containing micellar C_{16}-PDME showed angle-dependent scattering intensities, indicating formation of large nonspherical aggregates. The molecular weight of these particles could be obtained from a Zimm plot (Zimm, 1948). For all measured solutions, the intensities of the light scattering at 90° were linear with concentration.

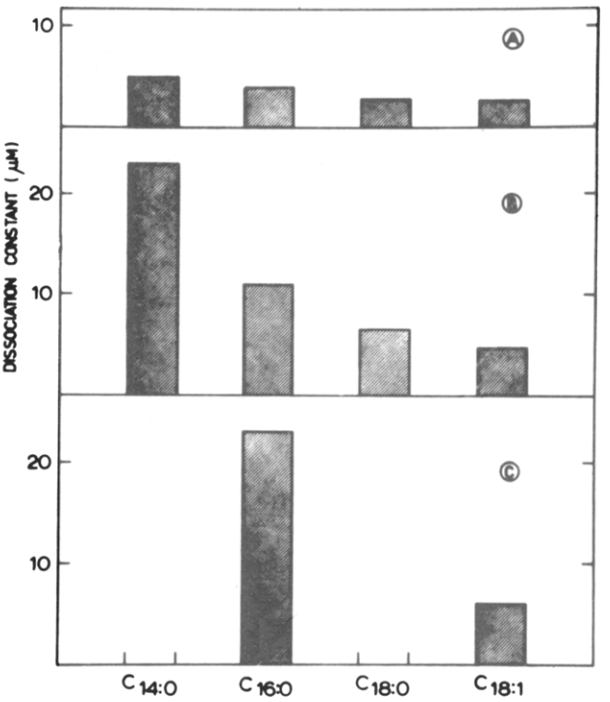

FIGURE 1: Binding constants of porcine pancreatic phospholipase A_2 for various micellar substrate analogues. (A) A homologous series of *n*-alkylphosphocholines in the presence of 100 mM Ca^{2+}; (B) the same series of phospholipids now in the absence of Ca^{2+}; (C) two corresponding lysolecithins in the presence of 100 mM Ca^{2+}. All spectroscopic data were obtained from protein titrations. Conditions used are 50 mM sodium acetate, pH 6, containing 100 mM sodium chloride; the lipid concentration ranges from 500 to 1000 μM; total protein concentrations range from about 2 to 70 μM. The standard deviation in all determined K_D values is about 5–7%.

Refractive Index Increment Measurements. The refractive index increments were measured with a Rayleigh interferometer (Aus Jena, DDR) at a wavelength of 545 nm and at 25 °C. For micelles of the substrate analogues C_{14}PN, C_{16}PN, and $C_{18:1}$PN, the values 0.123 ± 0.002, 0.129 ± 0.003, and 0.144 ● 0.003 mL/g, respectively, were found. For micelles of C_{14}lyso-PC, C_{16}lyso-PC, and $C_{18:1}$lyso-PC, the values 0.121 ± 0.002, 0.123 ± 0.002, and 0.126 ± 0.003 mL/g, respectively, were obtained. For C_{18}PN and PA_2, the values from the preceding paper in this issue (Hille et al., 1981) were used. The refractive index increment for C_{16}-PDME was determined to be 0.128 ± 0.003 mL/g.

Results

Interaction of Porcine PA_2 with Various Phospholipids. The results obtained with various substrate analogues and products, using UV difference spectroscopy, are presented in Figure 1. As is shown in Figure 1B, the micellar dissociation constant K_D gradually decreases with a constant factor of about 2 in a homologous series of *n*-alkylphosphocholines. Introduction of a double bond (18:1, Δ^9) results again in a small decrease in K_D. Figure 1A shows the effect of 100 mM Ca^{2+} on the binding of the enzyme to lipid–water interfaces. A Ca^{2+} concentration of 100 mM was chosen in order to saturate the Ca^{2+} binding site of the enzyme (Pieterson et al., 1974). The

Table I: Physicochemical Parameters for Binding of Various Phospholipids to Porcine Pancreatic Phospholipase A_2 at 25 °C and pH 6

			parameters of the lipid–protein complex[f]					
compound	cmc (μM)	no. of lipid monomers per micelle[a]	M_r[a] of the complex (anhydrous) ($\times 10^3$)	no. of enzyme molecules in the complex[a]	no. of lipid monomers per enzyme molecule, N[c]	micellar dissociation constant, K_D (μM)[d]	[RT ln (K_d)]/N (kcal mol^{-1})	[RT ln (cmc)]/N (kcal mol^{-1})
C_{14}PN	120	106	40.0	2	33	23	−0.19	−0.016
C_{16}PN	10	155	61.0	2	42	11	−0.16	−0.016
C_{18}PN	1	196	77.0	2[b]	53	6.6	−0.13	−0.015
$C_{18:1}$PN	7	144	91.5	3	33	4.7	−0.17	−0.021
C_{14}lyso-PC	53	ND[g]	60.0	2	33	122	−0.16	−0.018
C_{16}lyso-PC	6	180[e]	70.5	2	42	23	−0.15	−0.017
$C_{18:1}$lyso-PC	5	180[e]	97.0	3	42	6	−0.17	−0.017
C_{16}-PDME	5	11 000	ND	ND	42	2.2	−0.18	−0.017

[a] Values obtained from light-scattering measurements. [b] Results also obtained from ultracentrifugation measurements (Hille et al., 1981). [c] Averaged values from UV difference absorption spectroscopy and light-scattering measurements. [d] Results obtained from UV difference absorption spectroscopy. [e] Lewis & Gottlieb (1971). [f] The standard deviation of all determined values is about 10%. [g] ND, not determined.

dissociation constants K_D of the lipid–protein complexes decrease considerably for the series of substrate analogues due to the presence of Ca^{2+}, but the differences between the K_D values become very small.

As shown in Figure 1C, micellar lysolecithins show a weaker binding to the enzyme as compared with the corresponding alkylphosphocholines. This is most clearly seen for the shorter chain length phospholipids (see also Table I). No reliable data could be obtained for C_{18}lyso-PC because of the limited solubility under the conditions used. In the presence of Ca^{2+}, C_{14}lyso-PC slowly hydrolyzes due to the high PA_2 concentration and therefore failed to give reliable binding parameters. However, no hydrolysis of C_{14} lyso-PC was observed when 100 mM Ba^{2+} was added. The dissociation constant found was 122 ± 16 μM. An additional protein titration of micellar C_{14}PN in the presence of 100 mM $BaCl_2$ demonstrated that Ba^{2+} facilitates binding of PA_2 to the lipid micelles (K_D = 11 ± 0.6 μM). However, this effect is less pronounced for Ba^{2+} than for Ca^{2+}.

In Table I physicochemical parameters of the phospholipids investigated are shown. The first column gives the cmc values of these compounds. As found previously (van Dam-Mieras et al., 1975), the cmc values for the lysolecithins and the corresponding *n*-alkylphosphocholines are of the same order of magnitude. It should be noted that the cmc values of $C_{18:1}$PN and C_{16}PN are close, while the numbers of bound lipid monomers per enzyme molecule in the lipid–protein complexes also agree rather well. The numbers of monomers in the respective micelles are also nearly identical. This is probably due to a similar packing of both phospholipids in the free micelles as in the enzyme–micelle complexes.

The stoichiometry of the complex can be derived from the enzyme to lipid molar ratio and from the "molecular weight" of the lipid–protein complex. It can be concluded that micelles of saturated phosphocholine compounds form complexes composed of two enzyme molecules and about half of the number of lipid molecules as present in the free micelles. The free energy change of the binding process, expressed per lipid monomer, is also indicated in Table I. In the homologous series of phosphocholines, N increases and K_D decreases when the length of the alkyl chain is increased. This leads to a free-energy change which is roughly constant over the series when expressed per lipid monomer.

Table I also includes physicochemical parameters for the phospholipid C_{16}-PDME. This allows a comparison of two phospholipids with identical alkyl chains, but with different polar head groups. Even though the molecular structures of C_{16}PN and C_{16}-PDME are close, their physical behavior is very different. C_{16}-PDME forms micellar structures which are no longer spherical. Most probably rodlike, hexagonal structures are formed. This is confirmed by light-scattering measurements which show an angle-dependent scattering intensity and which give a value of about 11 000 lipid monomers per particle. The existence of rodlike structures is also supported by dielectric relaxation time measurements of solutions containing micellar C_{16}lyso-PC, C_{16}PN, or C_{16}-PDME (Kaatze et al., 1980).

Although both phospholipids form different "micellar" structures, the enzyme to lipid molar ratio of the enzyme–C_{16}-PDME complex suggests that the same type of complex is formed as found for other saturated substrate analogues or products. However, no reliable "molecular weight" could be determined for the complex by the light-scattering technique, because the system appeared to be unstable under the conditions used. It should be noted that C_{16}-DPME is much less soluble in water than C_{16}PN. UV experiments could be carried out at much lower lipid concentrations, allowing determination of the binding parameters.

pH Dependence of Micelle Binding. Figure 2 shows the effect of pH on the binding of phospholipase A_2 to C_{18}PN micelles. The micellar dissociation constant K_D and the stoichiometry factor N are obtained from the binding data by means of a nonlinear regression analysis. The factor N was found to be independent of pH, giving an approximate value of 50 (±10%) phospholipid molecules per enzyme molecule bound in the comlex.

The pH dependence of the binding constant K_D, shown as a Dixon plot (Dixon & Webb, 1962) in Figure 2, reveals that in the native enzyme the group(s) that controls (control) micelle binding in the absence of Ca^{2+} has (have) a pK_A value of 6.25 and a Hill coefficient around 0.95. Figure 2 also shows that in the presence of 100 mM Ca^{2+}, optimal binding is obtained over the whole pH range studied (pH 4–9.5).

Possible candidates for the group(s) that controls (control) micelle binding in the absence of Ca^{2+} are histidine residues, as judged from the pK_A value of 6.25. Also, carboxylate groups of Asp and Glu residues can be involved (see Discussion). For further investigation of which residues are involved, His^{48}-modified enzymes were prepared and tested on their micelle binding properties. One such modification involves methylation of the N-1 nitrogen atom of the imidazole ring of His^{48}. The binding of the essential cofactor Ca^{2+} and

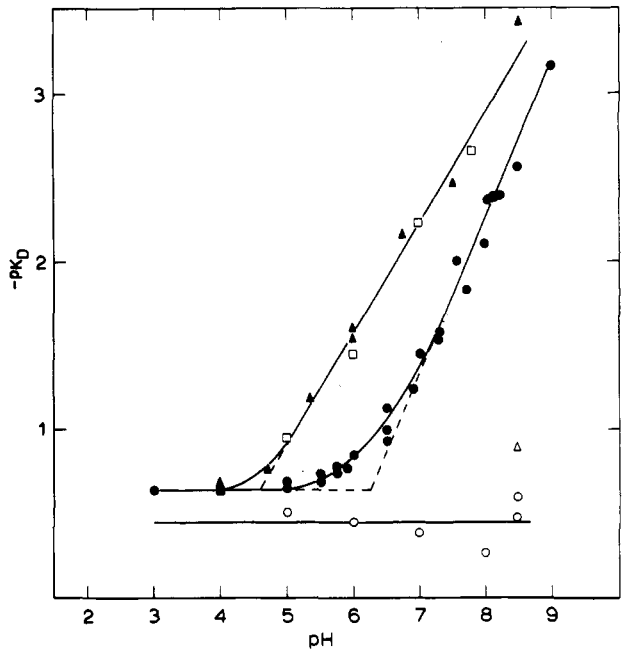

FIGURE 2: Dixon plots of the effect of pH on the interaction of porcine phospholipase A_2 with micelles of *n*-octadecylphosphocholine. Native phospholipase A_2 in the absence (●) and in the presence of 100 mM Ca^{2+} (○); [1-MeHis[48]]phospholipase A_2 in the absence of Ca^{2+} (□); [1-(2-oxooctyl)-His[48]]Pa_2 in the absence (▲) and in the presence of 100 mM Ca^{2+} (△). Experimental conditions are 50 mM sodium acetate or Hepes or Tris containing 100 mM sodium chloride. K_D values (expressed in μM) were obtained from UV difference spectroscopy by means of either lipid or protein titrations. The standard deviation in K_D varies from about 2% (low pH region) to 15% (high pH region).

monomeric lipid molecules is only slightly affected (Verheij et al., 1980). Another modification involves covalent linking of an octanone group to the N-1 nitrogen atom (Verheij et al., 1980). This modified enzyme fails to bind monomeric substrates, while Ca^{2+} binding is extremely poor. However, in the presence of aggregated lipid structures, binding of Ca^{2+} to the enzyme seems to be restored, as is shown in Figure 2.

Figure 2 also shows the pH titration of both modified enzymes in the absence of Ca^{2+}. The curves are overlapping to a large extent and show a decrease in the p*K* value to 4.6, as judged from the pH dependence of pK_D. Ca^{2+} restores the micelle binding at alkaline pH.

Discussion

As shown in Figure 1B,C, the affinity of porcine pancreatic phospholipase A_2 for micellar substrate analogues and products increases with increasing chain length. This points to mainly hydrophobic interactions involved in the binding process. In the presence of Ca^{2+}, the affinities for the different micellar compounds are further improved, most strongly for the shorter chain compounds (Figure 1A). This probably indicates that Ca^{2+} stabilizes the conformation of the enzyme that has optimum micelle binding properties. According to Scheme I, improved micelle binding in the presence of Ca^{2+} requires that micelles should improve Ca^{2+} binding. Following the principle of microreversibility, the relation $K_D K_{Ca}^* = K_D^* K_{Ca}$ must hold, thereby giving $K_{Ca}^* < K_{Ca}$ for $K_D^* < K_D$. An improved affinity for Ca^{2+} in the presence of micelles was indeed observed (van Dam-Mieras et al., 1975). The competitive inhibitor Ba^{2+} mimics the action of Ca^{2+}, although the effects are smaller (see Results).

The results summarized in Table I demonstrate that the model of lipid–protein interaction as presented by de Araujo et al. (1979) is generally valid for saturated hydrocarbon chain

Scheme I

$$\begin{array}{ccc} E + \text{Micelle} & \overset{K_D}{\rightleftharpoons} & E(\text{Micelle}) \\ K_{Ca} \downarrow\uparrow & & \uparrow\downarrow K_{Ca}^{*} \\ E(Ca^{2+}) + \text{Micelle} & \underset{K_D^{*}}{\rightleftharpoons} & E(Ca^{2+})(\text{Micelle}) \end{array}$$

compounds; i.e., two enzyme molecules bind to micelles consisting of half the number of lipid monomers present in free micelles.

It is a matter of importance to mention in this respect that not all lipid monomers can be in direct contact with the enzyme. This can simply be derived from the size of the hydrophobic region of phospholipase A_2 (called the IRS) with respect to the number of lipid monomers constituting an enzyme binding site. Model building studies further argue against direct protein–protein interactions in the enzyme–micelle complexes, as these would destabilize the lipid–protein interaction.

The results obtained for unsaturated chain compounds (Table I) show that the above model is not restricted to complexes containing two enzyme molecules. A final note on the lipid–protein interactions as given in Table I concerns the near constancy of the average free energy change per hydrocarbon chain on complex formation. Since the quantity *RT* ln (cmc) per lipid monomer in the free micelles is also roughly constant, one may conclude that the same type of stabilizing forces determines the size of free micelles and of enzyme–micelle complexes. Previous UV difference absorption spectroscopy studies (van Dam-Mieras et al., 1975) using the substrate analogue $C_{16}PN$ demonstrated that micelle binding to the porcine enzyme in the absence of Ca^{2+} is controlled by one or more groups in the enzyme with an apparent pK_A value around 8. However, there were several reasons to reinvestigate the effect of pH upon binding of the enzyme to lipid–water interfaces. One is that apparent binding constants were used to determine the above pK_A value. These apparent binding constants contain both the stoichiometry factor *N* and the micellar dissociation constant K_D which may vary differently with pH. Furthermore, the graphical tests used to obtain the binding parameters (double-reciprocal plots) are unsuitable for lipid titrations [see Hille et al. (1981)]. Another reason to reinvestigate the enzyme–micelle interaction was the observed contribution from lipid monomer binding to the UV difference signal, which had not been corrected for in earlier studies. For minimization of the contribution from lipid monomers, the substrate analogue $C_{18}PN$ was used. The results obtained from our present pH-titration studies seem to contradict our previous conclusions.

The group(s) that controls (control) $C_{18}PN$ micelle binding is (are) found to have a pK_A value of 6.25. This value is clearly too low to be assigned to the pK_A of the N-terminal α-amino group of phospholipase A_2 (van Dam-Mieras et al., 1975). However, other studies (Slotboom & de Haas, 1975) have demonstrated that a protonated amino group of Ala[1] is required for micelle binding. Thus it must be concluded that several groups in the enzyme are involved in the micelle binding process.

More likely candidates for groups having a pK_A value of 6.25 are His residues and possibly also carboxylate groups of Asp and Glu residues. The fact that Ca^{2+} restores micelle binding at high pH values points to the active-site residue His[48] (Volwerk et al., 1974, 1979) and Asp[49] (Fleer et al., 1980) as the most likely candidates involved in the lipid binding

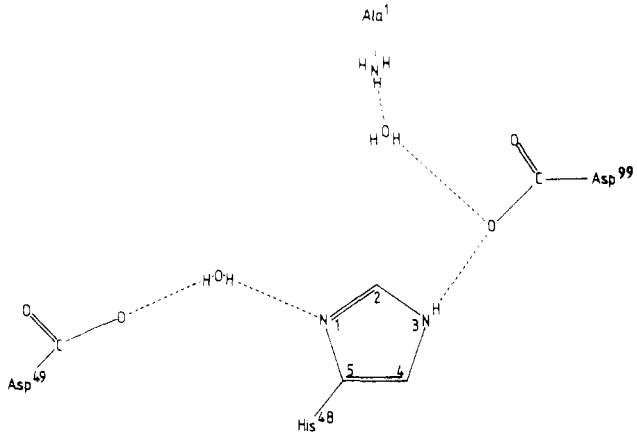

FIGURE 3: Schematic presentation of the groups involved in the micelle binding process and their mutual interactions. This scheme is based upon X-ray data obtained from a recent refinement analysis of the three-dimensional structure of bovine pancreatic phospholipase at 1.7-Å resolution (Dijkstra, 1980).

process apart from the N-terminal Ala^1 residue. This conclusion is based on previous UV difference spectroscopy (Pieterson et al., 1974), which showed that His^{48} is perturbed by Ca^{2+}, and on recent NMR pH titrations (Aguiar et al., 1979) and fluorescence measurements (Verhey et al., 1980), which showed that the pK_A value of His^{48} is affected when Ca^{2+} is bound to the enzyme. Further studies demonstrated that Asp^{49} is one of the ligands of the bound Ca^{2+} (Fleer et al., 1980; Dijkstra, 1980).

For further investigation of this hypothesis, the micelle binding properties of His^{48}-modified phospholipase A_2 were studied. Both in [1-MeHis[48]]- and in [1-(2-oxooctyl)-His[48]]-modified enzymes, a pK_A value of 4.6 was found for the group (groups) that controls (control) binding to $C_{18}PN$ micelles. Addition of Ca^{2+} at high pH values again restores the micelle binding properties as it did for the unmodified enzyme. Thus, most probably in the modified enzymes the group having a pK_A of 4.6 should be assigned to Asp^{49}.

In this respect, it is important to mention that it is not necessary to postulate a second (regulatory) binding site for Ca^{2+} (van Dam-Mieras et al., 1975) even when different affinities are found for Ca^{2+} in the absence and presence of micelles, respectively. As mentioned above, a synergistic effect of Ca^{2+} and lipid binding was observed for native PA_2 as well as for modified enzymes. Binding of micelles may lead to a conformational change which in turn can give rise to an improved affinity for Ca^{2+}. The Ca^{2+} binding site can then still be the same as already identified, containing Asp^{49} as one of its ligands. In Figure 3 a schematic drawing of the active-site region extending to the N-terminal α-amino group of Ala^1 is given in order to summarize the results.

It has been shown that either the protonated form of the enzyme or the Ca^{2+}-containing enzyme is able to bind to $C_{18}PN$ micelles. The above results point to His^{48} and Asp^{49} as important groups involved in lipid binding, indicating a requirement to neutralize a negatively charged Asp^{49} in order to exhibit maximum micelle binding capacity. When the N-1 atom of His^{48} is alkylated, the pK_A value of the group that controls micelle binding drops from 6.25 to 4.6, the latter value being normal for a carboxylate group of an Asp or Glu residue in a protein. This group was assigned to Asp^{49}. As indicated in Figure 3, the higher pK_A value of 6.25 in the native enzyme may be due to the proximity of His^{48}.

A strong hydrogen bond exists between the N-3 nitrogen atom of His^{48} and the carboxylate group of Asp^{99}, which is not broken after modification of His^{48} by alkylating the N-1 nitrogen atom. The N-3 hydrogen atom of [1-MeHis[48]]PA_2 was previously found to titrate at very high pH values, having a pK_A value of 8.9 (Verheij et al., 1980). The Asp^{99} residue in its turn is coupled to the N-terminal Ala^1 residue via a H_2O molecule. The importance of the α-NH_3^+ group of Ala^1 has already been emphasized (Slotboom & de Haas, 1975). Thus, it seems that the groups depicted in Figure 3 are all involved in the micelle binding process.

It is not likely that the system functions as a charge relay system, because N-1 modification of His^{48} would disrupt a charge relay system while micelle binding is still observed for the His^{48}-modified enzymes. Probably a positive charge on Asp^{49} induces a conformational change of the enzyme by which micelle binding is optimized. Further studies are in progress to obtain information about such a conformational change in phospholipase A_2.

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