Regulation of the Interaction of Pancreatic Phospholipase A_2 with Lipid–Water Interfaces by Ca^{2+} Ions: a Monolayer Study†

F. Pattus,‡ A. J. Slotboom, and G. H. de Haas*

ABSTRACT: In addition to the Ca^{2+} ion bound to the active site of porcine pancreatic phospholipase A_2, it is known that Ca^{2+} binds to a second, lower affinity site on the enzyme. This latter binding influences the interaction of phospholipase A_2 with lipid–water interfaces by shifting the pK of the α-NH_3^+ group of the N-terminal Ala residue from 8.4 to 9.3 [Slotboom, A. J., Jansen, E. H. J. M., Vlijm, H., Pattus, F., Soares de Araujo, P., & de Haas, G. H. (1978) *Biochemistry 17*, 4593–4600]. The effects of Ca^{2+} ion and pH on the pre-steady-state kinetics and on the activity of porcine phospholipase A_2 acting on lecithin monolayers were investigated. At basic pH there is a profound decrease of the penetration capacity of the enzyme in the lipid interface as shown by the increase of the induction time of the kinetics. This increase of the induction time with pH is shifted to a higher pH value by increasing Ca^{2+} ion concentration. There is a good correlation between the change in the pK of the NH_3^+ of Ala[1] and the effect seen in monolayer studies. The surface concentration of the enzyme is independent of pH until the pH region where deprotonation of the α-NH_3^+ group of Ala[1] occurs and then decreases. At pH 6, a further lowering of Ca^{2+} ion concentration below the saturation of the high-affinity site of phospholipase A_2 decreases the penetration rate of the enzyme to the lipid interface without changing its equilibrium surface concentration. This indicates an effect of the catalytic Ca^{2+} binding site on the activation energy of the binding process. At pH 9, Ca^{2+} ions bound to the low-affinity site are required for the binding to interfaces. At this pH, removal of Ca^{2+} ions from the subphase produces a fast expulsion of the enzyme from the interface. From Lineweaver–Burk plots of the specific activity of the enzyme on substrate monolayers as function of Ca^{2+} ion concentration, it was concluded that the low-affinity site not only is indispensable for the penetration at basic pH but also affects the turnover of the enzyme.

Phospholipase A_2 (EC 3.1.1.4) catalyzes the specific hydrolysis of fatty ester bonds at the 2 position of 3-*sn*-phosphoglycerides (de Haas et al., 1968). There is good evidence that, in addition to the catalytic site, the pancreatic enzymes possess a site topographically distinct which is responsible for the penetration of the enzyme into the lipid phase: the so-called interface recognition site (IRS)[1] (Verger et al., 1973; Pieterson et al., 1974a). The N-terminal Ala[1] is one of the key residues of the IRS (Slotboom & de Haas, 1975). The interaction of the porcine pancreatic enzyme with organized lipid–water interfaces is governed by the pK of the α-NH_3^+ group of this alanine residue (Pieterson et al., 1974a; van Dam-Mieras et al., 1975). The enzyme shows an absolute requirement for Ca^{2+} ions which bind in a 1:1 molar ratio to the active site of the protein (Pieterson et al., 1974b; Volwerk et al., 1974). In addition to the Ca^{2+} ion bound in the active site, this enzyme has been shown to possess a second metal ion binding locus, also specific for calcium which enables the enzyme to interact with the lipid–water interface at alkaline pH (van Dam-Mieras et al., 1975). Recently, using ^{13}C NMR and proton titration during tryptic activation of the zymogen, Slotboom et al. (1978) showed that the binding of this latter Ca^{2+} ion increases the pK of the α NH_3^+ of the N-terminal Ala[1] from 8.4 to 9.3. Furthermore, these authors found by equilibrium dialysis that, at pH 8.0, phospholipase A_2 binds two Ca^{2+} ions per mol of enzyme and that there is a strong synergistic effect of micelles of the substrate analogue *n*-hexadecylphosphocholine on the binding of both Ca^{2+} ions. Ultraviolet difference spectroscopy suggested that the second Ca^{2+} binds close to the single Trp-3 residue which is part of the IRS. From these studies it was concluded that the two Ca^{2+} ions which bind to the enzyme have two distinct functions. The high-affinity site is absolutely required for the hydrolysis reaction; the lower affinity site is important in the lipid binding process at alkaline pH.

In the present study further insight into the role of Ca^{2+} was obtained by using the monolayer technique. With this technique it is possible to measure pre-steady-state kinetics and to determine the amount of protein at the interface in a well-defined system (Verger et al., 1973). In the preceding paper we showed that the induction time (τ) of the kinetics of hydrolysis is a good experimental parameter to evaluate the penetration capacity of an enzyme acting at an interface (Verger et al., 1976; Pattus et al., 1979). This penetration is lipid-packing dependent. Any variation of the lag time induced by pH or calcium ion concentration will reflect modifications of the penetration process.

It is the purpose of this study to demonstrate that the "catalytic" Ca^{2+} ion, bound to the high-affinity site, also influences the lipid binding process and that the presence of a second Ca^{2+} ion on the low-affinity locus changes the turnover of the enzyme. This result indicates that, although the interface recognition site and the active site are topographically distinct in the primary structure of the enzyme, there must be a regulation mechanism between these sites when the tertiary structure is formed.

Materials and Methods

Enzymes. Porcine phospholipase A_2 and its zymogen were prepared as described previously (Nieuwenhuizen et al., 1974). The exact enzyme concentration in solution was determined spectrophotometrically at a wavelength of 280 nm. Values of 13.0 and 12.3 for $E_{1cm}^{1\%}$ were used for phospholipase A_2 and the zymogen, respectively. ϵ-Amidinated prophospholipase A_2 (AMPREC) and phospholipase A_2 (AMPA) were prepared

† From the Laboratory of Biochemistry, State University of Utrecht, Transitorium 3, "De Uithof", Padualaan 8, Utrecht, The Netherlands. *Received November 15, 1978.* These investigations were carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with the financial aid from the Netherlands Organization for the Advancement of Pure Research.

‡ Present address: Institut für Biochemie, Universität Bern, 3012 Bern, Switzerland.

[1] For a list of abbreviations see the preceding paper.

0006-2960/79/0418-2698$01.00/0

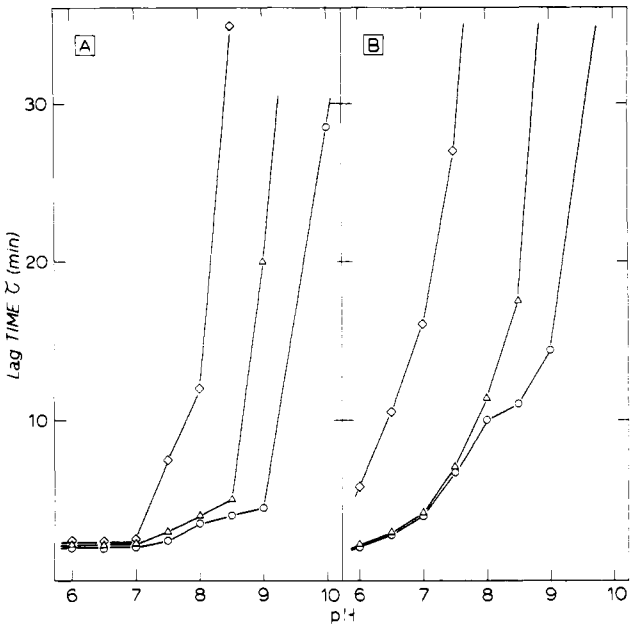

FIGURE 1: Effect of pH on the induction time τ of the kinetics of hydrolysis of di-C10-PC monolayers by [^{125}I]AMPA at a surface pressure of 10 dyn/cm (A) and 12 dyn/cm (B). Standard buffer; $CaCl_2$, 20 or 5 mM (○); $CaCl_2$, 0.5 mM (△); $CaCl_2$, 0.05 mM (◇). [^{125}I]AMPA (15 μg) was injected.

as described previously (Slotboom & de Haas, 1975). [^{3}H]AMPA and [^{125}I]AMPA were obtained as described in the preceding paper (Pattus et al., 1979). Their specific radioactivities were 1.6×10^7 and 6.5×10^7 dpm/mg of enzyme for [^{3}H]- and [^{125}I]AMPA, respectively. Enzymatically inactive [^{3}H]AMPA was prepared by blocking the active site residue His-48 with 1-bromo-2-octanone as described by Volwerk et al. (1974) and Slotboom et al. (1978).

Film Constituent. 1,2-Didecanoyl-*sn*-glycero-3-phosphocholine was prepared as described by Cubero Robles & van den Berg (1969).

Monolayer Technique. The surface barostat and the "zero-order trough" were identical with those described by Verger & de Haas (1973). The film was recovered as described previously (Rietsch et al., 1977), and radioactivity was counted in a Searle Isocap 300 scintillation system (Nuclear Chicago Division) by using 10 mL of a liquid scintillation cocktail (Packard Instagel). The washing of the trough after each experiment has been described and commented on in the preceding paper (Pattus et al., 1979).

In all assays, the aqueous subphase was composed of a Tris (10 mM)–acetate buffer adjusted at a given pH in 0.1 M NaCl (standard buffer). Ca^{2+} ion concentration was adjusted with 2 M $CaCl_2$ solution.

Results

Influence of pH and Calcium on the Penetration Step. Figure 1 shows the lag time profile at different concentrations of Ca^{2+} ions as function of pH of [^{125}I]AMPA acting on L-di-C10-lecithin monolayers at a surface pressure of 10 dyn/cm (A) and 12 dyn/cm (B), respectively. At a Ca^{2+} concentration of 20 or 5 mM and a surface pressure of 10 dyn/cm, the lag time τ is slightly affected from pH 6 to 9 and increases abruptly between pH 9 and 10, indicating a loss of the penetration capacity of the enzyme in this range of pH. Decreasing Ca^{2+} ion concentration shifts this abrupt increase to lower pH values. At 12 dyn/cm (Figure 1B) a similar shift is observed. However, one can also see a less pronounced increase in τ between pH 6 and 8.5 which remains unchanged

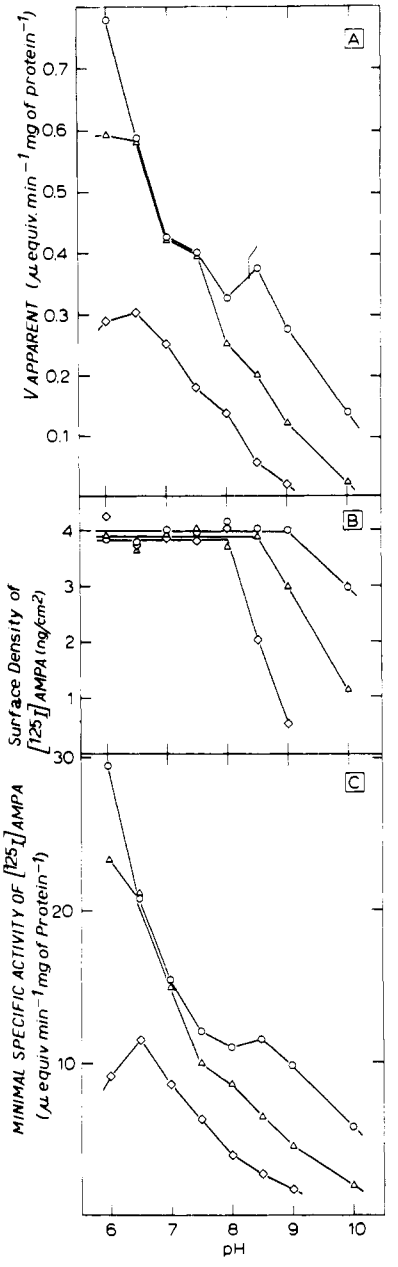

FIGURE 2: Effect of pH on the apparent velocity (A), surface density (B), and minimal specific activity (C) of [^{125}I]AMPA acting on di-C10-PC monolayers at a surface pressure of 10 dyn/cm. Standard buffer; $CaCl_2$, 20 mM (○); $CaCl_2$, 0.5 mM (△); $CaCl_2$, 0.05 mM (◇).

when Ca^{2+} concentration is decreased from 5 to 0.5 mM. This kind of titration curve is also measurable at 10 dyn/cm (Figure 1A). A Ca^{2+} concentration of 0.05 mM is far below the concentration required to saturate the high-affinity site of the enzyme (see Table I). At this concentration there is a higher lag time at a surface pressure of 12 dyn/cm than at 10 dyn/cm even at pH 6 where no second site for Ca^{2+} ions is detectable. This effect has to be attributed to the high-affinity site.

Influence of Ca^{2+} Ions and pH on the Activity and the Equilibrium Surface Concentration of [^{125}I]AMPA. Figure 2 shows the pH dependency of apparent velocity (A), surface density (B), and specific activity (C) during steady-state hydrolysis of di-C10-lecithin monolayer by [^{125}I]AMPA at a surface pressure of 10 dyn/cm and at different Ca^{2+} concentrations. One can see that the second optimum of activity at pH 8.5 in the presence of high concentration of Ca^{2+} ions which was reported earlier (Verger et al., 1973) is only a

Table I: Dissociation Constants of Porcine Phospholipase A_2 Binding Sites for Ca^{2+} Ions from Pieterson et al. (1974b) and Slotboom et al. (1978)

n-hexadecylphosphocholine micelles	pH 6		pH 8	
	K_1 (mM)	K_2 (mM)	K_1 (mM)	K_2 (mM)
−	3.6		0.5	20
+	0.5		0.1	3

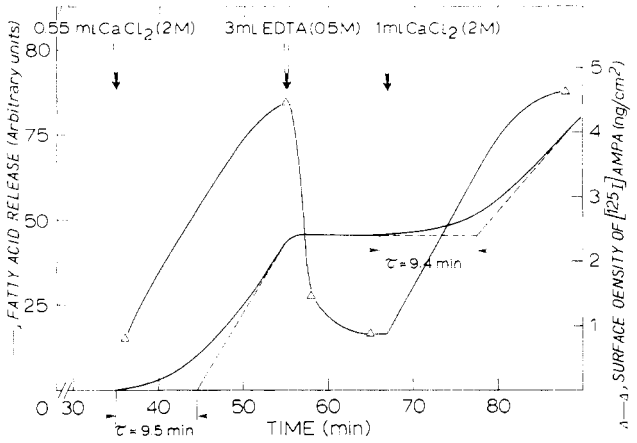

FIGURE 3: Effects of Ca^{2+} ions on the penetration of [^{125}I]AMPA in di-C10-PC monolayers at pH 9. Surface pressure was 12 dyn/cm. [^{125}I]AMPA (24.4 μg) was injected. Standard buffer; 0.5 mM EDTA. (—) Fatty acid release; (△) surface density of [^{125}I]AMPA. The dotted lines are the asymptotes of the kinetic curves. The continuous line of the adsorption kinetics is derived from the slope of the advancement curve at any time which gives the velocity of the reaction, based on the fact that the specific activity remains constant during the adsorption process (see preceding paper). The desorption line is experimentally determined.

shoulder in the activity profile of Figure 2A. We checked that these results are identical with those of either ^{125}I-labeled porcine phospholipase A_2 or noniodinated porcine phospholipase A_2. This discrepancy can be due to the better accuracy of the present data made possible by the improved washing procedure of the trough after each experiment (see preceding paper). However, this shoulder decreases as Ca^{2+} ion concentration is decreased. The amount of enzyme at the interface during steady-state kinetics is constant up to the pH value where the lag time increases sharply at basic pH (Figure 1). It remains identical even when the high-affinity site for Ca^{2+} ions is not saturated and the lag time is high at intermediate pH (Figure 1B and π = 12 dyn/cm; data not presented). The pH-dependent specific activity profile (Figure 2C) was obtained by dividing apparent velocity by surface density. Again, the second pH optimum at pH 8.5 is visible as a shoulder only at high Ca^{2+} concentration where the low-affinity metal ion binding site is saturated.

Influence of the Low-Affinity Site for Ca^{2+} Ions on the Binding of [^{125}I]AMPA to the Lipid–Water Interface at Basic pH. Figure 3 shows a kinetics of hydrolysis of di-C10-lecithin monolayer at a surface pressure of 12 dyn/cm at pH 9. In the absence of Ca^{2+} ions no activity is observed. After 35 min only a small amount of enzyme is found in the interface. After injection of calcium ions (5 mM final concentration), hydrolysis starts. The kinetics of hydrolysis is identical with the kinetics obtained when enzyme and calcium are injected at the same time. When steady-state kinetics is reached, an excess of EDTA is injected. The hydrolysis stops and the AMPA desorbs rapidly from the surface. A new injection of Ca^{2+} ions in the subphase restores activity with a kinetics identical with the previous one (τ = 9.5 min). In order to

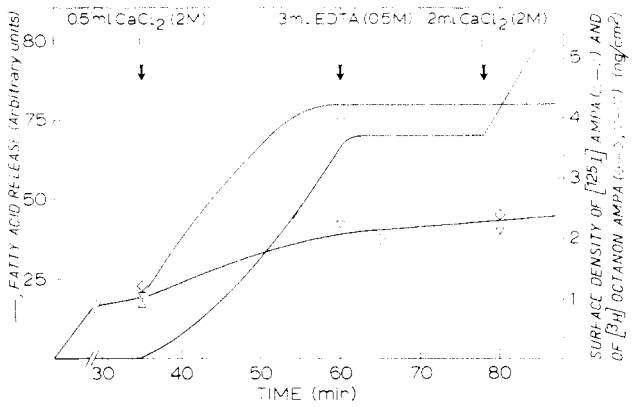

FIGURE 4: Effect of Ca^{2+} ions on the penetration of [^{125}I]AMPA on di-C10-PC monolayer at pH 6. Surface pressure was 14 dyn/cm. The continuous line of the adsorption curve was constructed as indicated in Figure 3. Standard buffer; 0.5 mM EDTA. (—) Fatty acid release; (△) surface density of [^{125}I]AMPA; (◇) surface density of octanone–[^{3}H]AMPA, same conditions as those for [^{125}I]AMPA; (▽) surface density of octanone–[^{3}H]AMPA, without additions of Ca^{2+} ions. [^{125}I]AMPA (12.2 μg) was injected.

demonstrate that these effects are due to the low-affinity binding site for Ca^{2+} ions, we used [^{3}H]AMPA blocked on its active site residue His–48 by 1-bromo-2-octanone. This modified protein no longer shows hydrolytic activity and has lost its high-affinity site for Ca^{2+} ions. However, the IRS and the low-affinity Ca^{2+} site are preserved (Pieterson et al., 1974a; Volwerk et al., 1974; Slotboom et al., 1978). Also, the penetration of octanone–[^{3}H]AMPA into the lecithin film at alkaline pH requires Ca^{2+} ions, and upon subsequent injection of excess EDTA, the protein rapidly desorbs from the monolayer. The adsorption kinetics of "native" AMPA and its octanone-modified derivative indicate a slower penetration of the latter protein in the film.

Influence of the High-Affinity Site for Ca^{2+} Ions on the Binding of [^{125}I]AMPA to the Lipid–Water Interface. Figure 4 shows a similar type of experiment as Figure 3 but at pH 6 and at a surface pressure of 14 dyn/cm. At this pH value no second site for Ca^{2+} ion is detectable in the enzyme (Slotboom et al., 1978), and any effect of Ca^{2+} ions has to be attributed to the high-affinity site. In the presence of EDTA, 35 min after the injection of the enzyme 25% of the equilibrium surface concentration of [^{125}I]AMPA in the presence of Ca^{2+} ions has been reached. The kinetics obtained after injection of Ca^{2+} ions in the subphase shows a lag time which is shorter than when the divalent ion and the enzyme are injected simultaneously (7.5 min instead of 10.5 min). A subsequent addition of EDTA stops the hydrolysis reaction. However, AMPA does not leave the film as observed at pH 9. Addition of an excess of Ca^{2+} ions restores activity without a pre steady state and the kinetics is directly linear. Octanone–AMPA which has no detectable affinity for Ca^{2+} ions at this pH is not influenced by the different additions of Ca^{2+} and EDTA. Its adsorption rate, independent of the absence or presence of Ca^{2+} ions, is smaller than the adsorption rate of unmodified AMPA in presence of Ca^{2+} ions. As shown in Figure 5, at lower surface pressure the [^{125}I]AMPA adsorption is faster. At a surface pressure of 10 dyn/cm, the enzyme has already reached the equilibrium surface concentration before injection of Ca^{2+} ions. These experiments indicate that the high-affinity site for Ca^{2+} ions is not absolutely required for the binding of phospholipase A_2 to the interface but increases the adsorption rate of the enzyme.

Effect of the Low-Affinity Site for Calcium on the Turnover of the Enzyme at Basic pH. In Figure 6 are presented

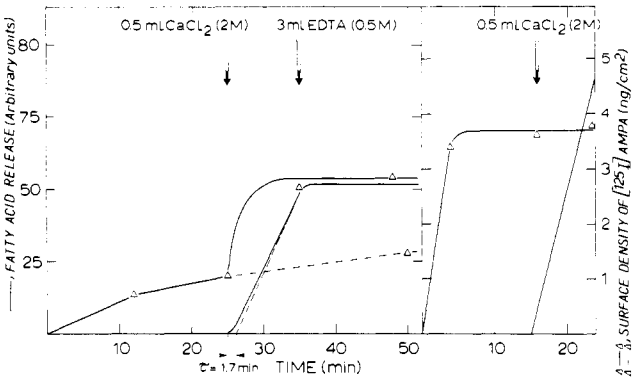

FIGURE 5: Effect of Ca^{2+} ions on the penetration of [^{125}I]AMPA on di-C10-PC monolayers at pH 6 and a surface pressure of 10 (right) and 12 dyn/cm (left). Standard buffer; 0.5 mM EDTA. (—) Fatty acid release; (Δ—Δ) surface density of [^{125}I]AMPA; (Δ---Δ) surface density of [^{125}I]AMPA without addition of Ca^{2+} ions. [^{125}I]AMPA (12.2 μg) was injected.

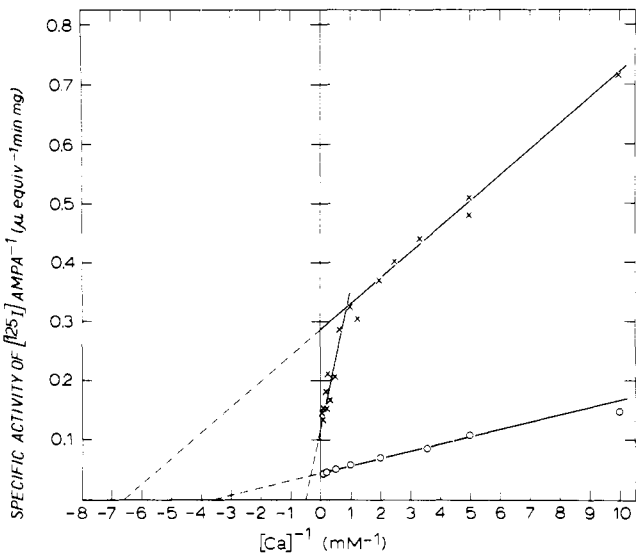

FIGURE 6: Lineweaver–Burk plots of the specific activity of [^{125}I]-AMPA acting on L-di-C10-lecithin monolayers as function of Ca^{2+} ion concentration at pH 6 and a surface pressure of 10 dyn/cm (○) and pH 9 and a surface pressure of 12 dyn/cm (×). At each concentration of Ca^{2+} ion, activity and surface radioactivity were measured as described under Materials and Methods. Specific activities were calculated from the apparent velocity and the amount of enzyme in the interface. Standard buffer. [^{125}I]AMPA (15 μg) was injected.

Lineweaver–Burk plots of the specific activity of [^{125}I]AMPA acting on L-di-C10-lecithin monolayers at pH 9 and 6, respectively. At pH 6, a straight line is obtained with a dissociation constant of 0.3 mM and a maximal specific activity at infinite Ca^{2+} of 23 (μM/min)/mg of protein. At pH 9, the plot is biphasic as was found in bulk assays using lecithin micelles (Pieterson, 1973). Two dissociation constants can be calculated (K_1 = 0.12 mM; K_2 = 2 mM). These values are in good agreement with the values found by equilibrium dialysis in the presence of substrate analogue micelles (Slotboom et al., 1978). Two specific activities at infinite calcium concentration can also be calculated (3.5 and 8 (μM/min)/mg of protein, respectively). These results indicate that both binding sites for Ca^{2+} ions are influencing the turnover of the enzymatic hydrolysis.

Discussion

It has been shown that the IRS which is responsible for the affinity of phospholipase A_2 to lipid–water interfaces owes its

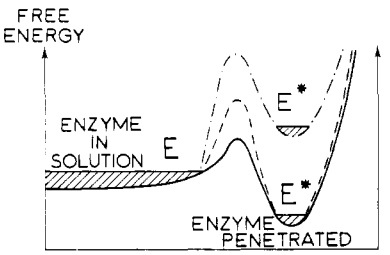

FIGURE 7: Schematic diagram showing the effect of Ca^{2+} ion binding to the low- and high-affinity sites of porcine phospholipase A_2 on the free energy states of the penetration process. (—) Ca^{2+} ions, binding sites saturated; (- - -) pH 6 in absence of Ca^{2+} ions; (····) basic pH, no Ca^{2+} ion bound to the low-affinity site. At pH 6 the high-affinity site is the only site for Ca^{2+} ion on phospholipase A_2. At basic pH both sites exist.

stability most probably to an internal salt bridge between the α-NH_3^+ group and a buried carboxylate function.[2] The pK of the α-NH_3^+ group of Ala^1 in porcine phospholipase A_2 is 8.4. Therefore, at alkaline pH values deprotonation of the NH_3^+ group occurs, the salt bridge is disrupted, and the IRS is destroyed. Under these conditions, the enzyme is not able to penetrate into organized lipid–water interfaces. It has been demonstrated that, in solution, the pK of the N terminus gradually shifts from 8.4 to 9.3 as Ca^{2+} ion concentration increases. This shift is due to a second binding site for Ca^{2+} ion of low affinity (20 mM at pH 8) located close to the Trp-3 residue of the N-terminal part of the protein (Slotboom et al., 1978). The loss of penetrating capacity of AMPA acting on substrate monolayers at basic pH (Figure 1) which is shifted to higher pH values as Ca^{2+} ion concentration is increased correlates quite well with the shift of the pK of the N-terminus Ala^1 determined in the absence of interfaces. As shown in Figure 2B, there is a parallel decrease of the amount of enzyme bound to the monolayer of substrate. The fact that lower concentrations of Ca^{2+} ions (5 mM instead of 50 mM) are already sufficient to produce a maximal effect confirms a previous finding that the affinity of the enzyme for Ca^{2+} ions increases in the presence of substrate monolayers (Slotboom et al., 1978). Between pH 6 and 8 the lag time profiles show a kind of titration curve which corresponds to a slight decrease of the penetration capacity of AMPA (Figure 1B). This suggests that the N-terminal Ala^1 is not the only residue which regulates the binding of the enzyme to the lipid–water interface. In the same range of pH there is a decrease in the specific activity of the enzyme (Figure 2C) without any change in its surface concentration (Figure 2B). This decrease in the specific activity and increase of the lag time between pH 6 and 8 cannot yet be explained. Figure 3 demonstrates clearly that Ca^{2+} ions are required at basic pH to allow the enzyme to bind to the interface. This binding is reversible. As Ca^{2+} ions are removed from the subphase, AMPA desorbs rapidly from the monolayer. Under the same conditions octanone–AMPA, which lacks the high-affinity site for Ca^{2+} ions, behaves similarly although the penetration velocity is slower. This indicates that the Ca^{2+} ion bound to the low-affinity site is responsible for the stabilization of the IRS at basic pH.

As shown in Figure 1B, at the very low Ca^{2+} ion concentration of 0.05 mM at pH 6, the value of the lag time is greater than that when the high-affinity site is saturated. However, there is no effect on the surface concentration of AMPA during

[2] The fact that this ion pair was not detected so far in the X-ray structure (Dijkstra et al., 1978) might be related to the fact that the protein crystals were obtained from a medium different from the present one.

steady-state hydrolysis of the substrate (Figure 2B). At this pH, as the enzyme is bound to the interface, removal of Ca^{2+} ions from the subphase does not produce a desorption of the enzyme from the interface as it does at pH 9. This effect of the catalytic binding site for Ca^{2+} ions on the penetration is only a kinetic effect. Figure 7 describes schematically the free energy state of the penetration process. The kinetics of binding to the interface is governed by the activation energy while the surface concentration of enzyme at the interface is a function of the difference in the free energy between enzyme in solution and enzyme penetrated in the lipid–water interface. The present data suggest that the binding of Ca^{2+} to the high-affinity site, which is required for the hydrolysis reaction to occur, also decreases the activation energy of the binding process while, at basic pH, the binding of Ca^{2+} to the low-affinity site decreases at the same time the activation energy of the binding and the free energy state of the enzyme bound to the lipid–water interface.

The dissociation constants calculated from the Lineweaver–Burk plots of the specific activity of AMPA acting on lecithin monolayers (Figure 6) are in good agreement with those found by equilibrium dialysis in the presence of *n*-hexadecylphosphocholine micelles. This confirms the synergistic effect of the binding of the enzyme to interfaces and the binding of the divalent cations to both sites of the protein. Furthermore, this plot shows that Ca^{2+} ion bound to the low-affinity site increases the catalytic turnover of the enzyme. These results are in agreement with the kinetic data on di-C8-lecithin micelles (Pieterson, 1973). However, in the latter case all enzyme molecules were assumed to be bound to the micellar interface, but this was not experimentally verified. This implies that, although the two binding sites for Ca^{2+} ions in pancreatic phospholipase A_2 are located far apart in the primary structure of the enzyme (one in the IRS; one in the active site), they must be rather close in the enzyme–substrate complex.

References

Cubero Robles, E., & van den Berg, D. (1969) *Biochim. Biophys. Acta 187*, 520–526.

de Haas, G. H., Postema, N. M., Nieuwenhuizen, W., & van Deenen, L. L. M. (1968) *Biochim. Biophys. Acta 159*, 103–117.

Dijkstra, B. W., Drenth, J., Kalk, K. H., & Vandermaelen, P. J. (1978) *J. Mol. Biol. 124*, 53–60.

Niewenhuizen, W., Kunze, H., & de Haas, G. H. (1974) *Methods Enzymol. 32B*, 147–154.

Pattus, F., Slotboom, A. J., & de Haas, G. H. (1979) *Biochemistry* (first paper of three in this issue).

Pieterson, W. A. (1973) Ph.D. Thesis, State University, Utrecht.

Pieterson, W. A., Vidal, J. C., Volwerk, J. J., & de Haas, G. H. (1974a) *Biochemistry 13*, 1455–1460.

Pieterson, W. A., Volwerk, J. J., & de Haas, G. H. (1974b) *Biochemistry 13*, 1439–1445.

Rietsch, J., Pattus, F., Desnuelle, P., & Verger, R. (1977) *J. Biol. Chem. 252*, 4313–4318.

Slotboom, A. J., & de Haas, G. H. (1975) *Biochemistry 14*, 5394–5399.

Slotboom, A. J., Jansen, E. H. J. M., Vlijm, H., Pattus, F., Soares de Araujo, P., & de Haas, G. H. (1978) *Biochemistry 17*, 4593–4600.

van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14*, 5387–5394.

Verger, R., & de Haas, G. H. (1973) *Chem. Phys. Lipids 10*, 127–136.

Verger, R., Mieras, M. C. E., & de Haas, G. H. (1973) *J. Biol. Chem. 248*, 4023–4034.

Verger, R., Rietsch, J., van Dam-Mieras, M. C. E., & de Haas, G. H. (1976) *J. Biol. Chem. 251*, 3128–3133.

Volwerk, J. J., Pieterson, W. A., & de Haas, G. H. (1974) *Biochemistry 13*, 1446–1454.