Amino Acid Substitutions of the NH_2-Terminal Ala^1 of Porcine Pancreatic Phospholipase A_2: a Monolayer Study†

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ABSTRACT: Previously it has been shown that the binding of porcine pancreatic phospholipase A_2 to lipid–water interfaces is governed by the pK of the α-NH_3^+ group of the N-terminal alanine. Chemically modified phospholipases A_2 in which the N-terminal Ala has been replaced by D-Ala or in which the polypeptide chain has been elongated with DL-Ala no longer display activity toward micellar substrate. The activity of DL-Ala^{-1}-, [D-Ala^1]-, and [Gly^1]phospholipases A_2 on substrate monolayers, which allow a continuous change in the packing density of the lipid molecule, was investigated. At pH 6 [Gly^1]phospholipase A_2 behaves like the native enzyme on lecithin monolayers. DL-Ala^{-1}- and [D-Ala^1]phospholipases A_2, although they are active in this system, showed a weaker lipid penetration capacity at this pH. Studies on the pH and Ca^{2+} ion dependency of the pre-steady-state kinetics and of the activity of these radiolabeled proteins showed that [D-Ala^1]phospholipase A_2 does not possess a second low-affinity site for Ca^{2+} ions in contrast to the native phospholipase A_2. This second low-affinity Ca^{2+} binding site, which is also absent in [Gly^1]phospholipase A_2, is induced in the latter enzyme by the presence of lipid–water interfaces.

Pancreatic phospholipase A_2 specifically catalyzes the hydrolysis of the 2-acyl ester linkage of naturally occurring phosphoglycerides. The pancreatic enzymes have an absolute requirement for Ca^{2+} ions which bind in a 1:1 molar ratio to the enzyme at the catalytic or high-affinity site. The high affinity and activity of porcine pancreatic phospholipase A_2 toward lipid–water interfaces have been attributed to a specific region of the enzyme molecule distinct from the active site: the so-called interface recognition site (IRS)[1] (Verger et al., 1973; Pieterson et al., 1974). It has been demonstrated that the IRS embraces the NH_2-terminal sequence of the polypeptide chain Ala^1-Leu-Trp-Gln-Phe-Arg-Ser-Met^8 (van Dam-Mieras et al., 1975; van Wezel et al., 1976) and also Tyr-69 and Leu-19 (H. Meyer, unpublished experiments). The interaction of the enzyme with lipid–water interfaces is governed by the pK of the α-NH_3^+ group of Ala^1, which most probably forms an ion pair with a buried carboxylate group (Abita et al., 1972; Slotboom & de Haas, 1975). The pK of this α-NH_3^+ function is shifted from 8.4 to 9.3 upon binding of a second Ca^{2+} ion to a low-affinity site, most probably located close to Trp-3 of the protein, enabling the enzyme to penetrate lipid–water interfaces at basic pH (Slotboom et al., 1978a). Recently, Slotboom & de Haas (1975) showed by specific modification of the NH_2-terminal Ala^1 of ϵ-amidinated phospholipase A_2 (AMPA) that chain shortening as well as chain elongation with one amino acid residue yielded proteins retaining enzymatic activity toward monomeric substrates comparable to that of the "native" AMPA. However, these modified proteins no longer display enzymatic activity toward micellar substrates. Moreover, replacing L-Ala^1 by its enantiomer D-Ala also abolished the micelle binding capacity of the protein (Slotboom et al., 1977). Table I depicts the various substitutions and additions of amino acids at the N terminus of AMPA and their respective micellar and monomer activities. On the basis of these results, one might conclude that the presence or absence of an IRS is an "all or none" property of these proteins. It should be realized, however, that the use of micellar substrate limits the possibilities of changing the parameters of the lipid–water interface. The packing of monomers in the micelle is dictated by the hydrophobic–hydrophilic balance of the substrate used.

Table I: Some N-Terminally Modified AMPA Analogues and Their Enzymatic Activities toward Substrates Present as Micelles (L-di-C8-PC) and Monomers (L-di-C6-PC) (Slotboom & de Haas, 1975; Slotboom et al., 1977)

	micellar act.	monomer act.
AMPA	+	+
[Gly^1]AMPA	+	+
[D-Ala^1]AMPA	−	+
DL-Ala^{-1}-AMPA	−	+

Much more versatile is the monolayer technique (Verger et al., 1973; Verger & de Haas, 1976), where a monomolecular surface film of substrate can be compressed or expanded, allowing a continuous change in the packing density of the molecules at the interface. By use of this technique it is possible to measure pre-steady-state kinetics (Verger et al., 1973). In the first paper of this series (Pattus et al., 1979a), we confirmed that the pre steady state, visualized by the induction time of the kinetic curves, is due to the rate-limiting reversible penetration step of the enzyme in the interface. This penetration is dependent on lipid packing. In paper two of this series the effect of Ca^{2+} ions and pH on the penetration and activity of AMPA acting on lecithin monolayers was investigated (Pattus et al., 1979b). The loss of the penetrating capacity of the enzyme shown by the increase of the lag time of the kinetics upon raising the pH correlates quite well with the deprotonation of the α-NH_3^+ group of L-Ala^1 in the same pH range. Furthermore, the effect of the low-affinity site for Ca^{2+} ions on the pK of the α-NH_3^+ group could also be visualized in the monolayer study. Using this technique Slotboom et al. (1977) showed that all N-terminally modified enzymes were able to act on lecithin monolayers at low surface pressure. On the other hand, the zymogen as well as phos-

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[1] C_{16}-P-N stands for *n*-hexadecylphosphocholine. For a list of other abbreviations see the first paper in this series (Pattus et al., 1979a).

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pholipase A_2 derivatives having a blocked α-NH_3^+ function was unable to attack surface films even at lower surface pressures. Therefore, it is evident that some of the modified proteins, notwithstanding their inability to hydrolyze micellar substrates, still possess a certain lipid-penetrating capacity and should have an IRS, albeit of lower efficiency than that of AMPA.

The purpose of this study was to quantitate the penetrating capacity of these modified enzymes and to study the effect of Ca^{2+} ion and pH on these proteins. Using ^{125}I-labeled proteins, we also measured the influence of the modifications of the IRS on the equilibrium surface concentration and the specific activity of the enzyme.

Materials and Methods

Enzymes. Porcine (pro)phospholipase A_2 was prepared as described previously (Nieuwenhuizen et al., 1974). The exact enzyme concentration in solution was determined spectrophotometrically at a wavelength of 280 nm ($E_{1cm}^{1\%} = 13.0$). ϵ-Amidinated phospholipase A_2 (AMPA), DL-Ala^{-1}-AMPA, [D-Ala^1]AMPA, and [Gly^1]AMPA were prepared as described previously (Slotboom & de Haas, 1975). Iodination reactions with ^{125}I (Radiochemical Centre, Amersham) were carried out according to Slotboom et al. (1978b). The specific radioactivity of the proteins was 6.5×10^7, 7.9×10^6, and 7.8×10^6 cpm/mg of protein for AMPA, [Gly^1]AMPA, and [D-Ala^1]AMPA, respectively.

Monolayer Technique. The surface barostat and the "zero-order trough" are identical with those described by Verger & de Haas (1973). Film recovery, film counting, and technical precautions have been described (Rietsch et al., 1977; Pattus et al., 1979a). A standard buffer (10 mM Tris–acetate in 0.1 M NaCl) was used at various pH values. The short-chain 1,2-didecanoyl- and 1,2-dioctanoyl-*sn*-glycero-3-phosphocholines were prepared as described by Cubero Robles & van den Berg (1969).

Equilibrium Dialysis. The technique used and the treatment of the data have been described by Slotboom et al. (1978a).

Enzymatic Assay. Kinetics measurements using micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine were performed as described before (de Haas et al., 1971).

Results

Figure 1 shows the lag time profile (A) and apparent activity profile (B) of AMPA, [D-Ala^1]AMPA, DL-Ala^{-1}-AMPA, [Gly^1]AMPA, and their ^{125}I-labeled analogues acting on L-di-C10-PC monolayers at pH 6. The penetration capacity of [Gly^1]AMPA measured by the lag time profile (Figure 1A) is identical with that of AMPA at this pH. Substitution of L-Ala^1 by D-Ala or elongation of the chain (DL-Ala^{-1}-AMPA) dramatically decreases the penetrating power of the enzyme as shown by the critical surface pressure where the modified proteins can no longer penetrate the lipid–water interface. As shown by the dotted lag time profiles of Figure 1A, introduction of one molecule of iodine on Tyr-69 increases the penetration capacity of the modified enzymes as it was found for AMPA (Pattus et al., 1979a). Not only the penetration capacity but also the apparent enzymatic activity of [Gly^1]AMPA are identical with those of AMPA (Figure 1B). The activities of DL-Ala^{-1}-AMPA and [D-Ala^1]AMPA at 8 dyn/cm are 15 and 50% of the activity of AMPA, respectively. However, these values are apparent specific activities, because only a small amount of injected protein is present in the interface. Therefore, we used radioactively ^{125}I-labeled [D-Ala^1]- and [Gly^1]AMPA. Figure 1B (dotted curves) shows that the

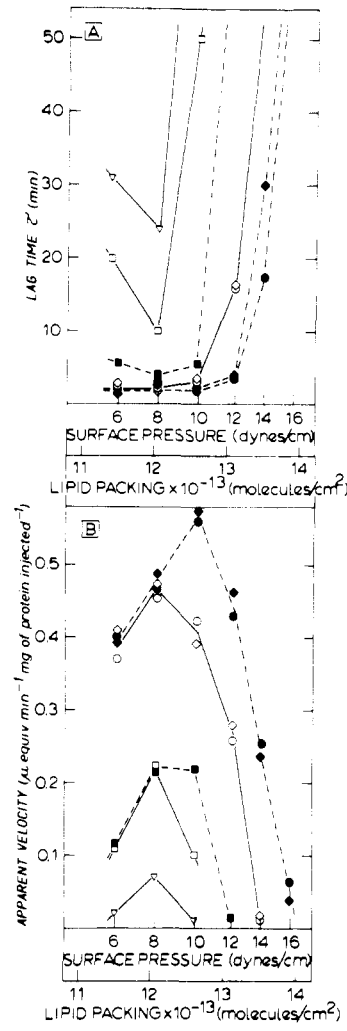

FIGURE 1: Influence of surface pressure, c.q. lipid packing on the lag time (A) and apparent activity (B) of the kinetics of hydrolysis of L-di-C10-PC monolayers at pH 6 (20 mM $CaCl_2$) by AMPA (○), [Gly^1]AMPA (◇), [D-Ala^1]AMPA (□), DL-Ala^{-1}-AMPA (▽), and their iodinated analogues (●, ◆, and ■, respectively). Proteins (12–15 μg) were injected.

Table II: V_{max} Values[a] of AMPA, [D-Ala^1]AMPA, and Their Iodinated Analogues on L-Di-C8-PC at pH 6.0

	V_{max}
AMPA	1120
[^{125}I]AMPA	1120
[D-Ala^1]AMPA	50
[^{125}I]-[D-Ala^1]AMPA	145

[a] Expressed as microequivalents per minute per milligram of protein.

apparent activity profiles of the iodinated enzymes are shifted to higher surface pressure. In the first paper of this series, we demonstrated that the specific activity of [^{125}I]AMPA is identical with the specific activity of ^{3}H-labeled AMPA. The shift of the apparent activity profile is exclusively due to the better penetrating capacity of the iodinated proteins, which allows the enzymes to bind to the interface at higher surface pressure. [D-Ala^1]AMPA is inactive on micellar substrate (Table I) while AMPA binds with a high affinity to micellar interfaces. From Figure 1A it is clear that the introduction of iodine in [D-Ala^1]AMPA reinforces the penetrating power of the protein. Although its penetration capacity is still somewhat weaker than that of AMPA, it was of interest to investigate whether this enzyme would be able to hydrolyze

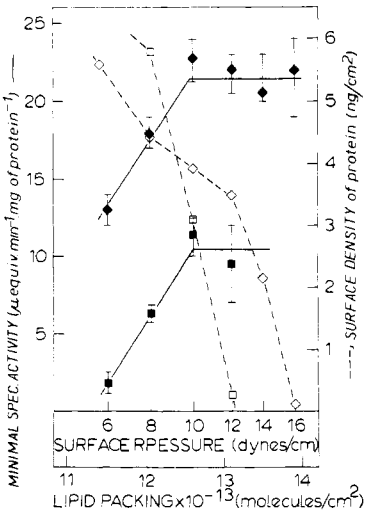

FIGURE 2: Influence of surface pressure, c.q. lipid packing on the specific activity (filled symbols) and steady-state surface density (open symbols) of [^{125}I]-[Gly^1]AMPA, (♦) and (◇), and of [^{125}I]-[D-Ala^1]AMPA, (■) and (□), acting on L-di-C10-PC monolayers at pH 6, 20 mM $CaCl_2$. Proteins (15 μg) were injected.

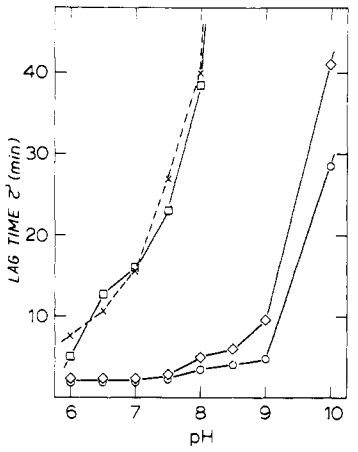

FIGURE 3: Influence of pH on the lag time of the kinetics of hydrolysis of L-di-C10-PC monolayers at a surface pressure of 10 dyn/cm by [^{125}I]AMPA (○), [^{125}I]-[Gly^1]AMPA (◇), and [^{125}I]-[D-Ala^1]AMPA (□); 20 mM $CaCl_2$. Proteins (15 μg) were injected. (×) Lag time profile of the kinetics of hydrolysis of di-C10-PC monolayers by [^{125}I]AMPA at a Ca^{2+} ion concentration of 0.05 mM and a surface pressure of 12 dyn/cm.

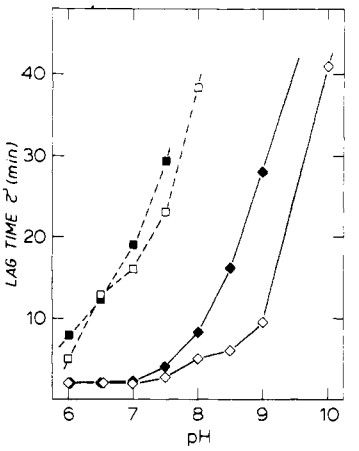

FIGURE 4: Influence of Ca^{2+} concentration on the pH dependency of the lag time; L-di-C10-PC monolayers at a surface pressure of 10 dyn/cm. [^{125}I]-[Gly^1]AMPA: (◇) 20 mM $CaCl_2$; (♦) 0.5 mM $CaCl_2$. [^{125}I]-[D-Ala^1]AMPA: (□) 20 mM $CaCl_2$; (■) 0.5 mM $CaCl_2$.

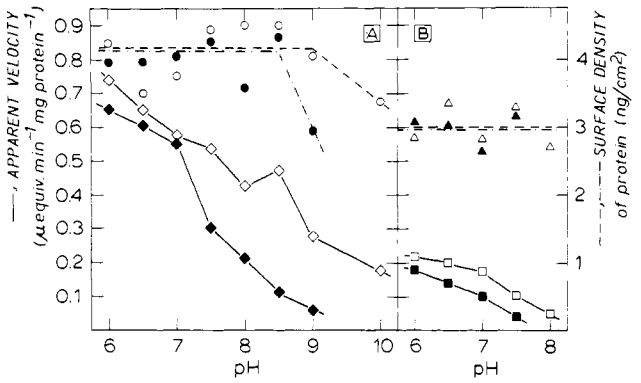

FIGURE 5: Influence of pH and Ca^{2+} ion concentration (open symbols, 20 mM $CaCl_2$; filled symbols, 0.05 mM $CaCl_2$) on the apparent velocity and steady-state surface concentration of protein during hydrolysis of an L-di-C10-PC monolayer at 10 dyn/cm catalyzed by the following enzymes. [^{125}I]-[Gly^1]AMPA (A): (◇) and (♦), apparent velocity; (○) and (●), surface density. [^{125}I]-[D-Ala^1]AMPA (B): (□) and (■), apparent velocity; (△) and (▲), surface density.

micellar substrates. Table II gives the V_{max} values of [^{125}I]AMPA, AMPA, [D-Ala^1]AMPA, and [^{125}I]-[D-Ala^1]-AMPA activity on L-di-C8-PC micelles at pH 6. Most of the activity found with [D-Ala^1]AMPA has to be attributed to monomeric activity. [^{125}I]-[D-Ala^1]AMPA is at least 3 times more active than [D-Ala^1]AMPA.

The equilibrium surface densities and specific activities of ^{125}I-labeled [D-Ala^1]- and [Gly^1]AMPA are given in Figure 2. As it was found for the lag time profile and apparent activity profile, the steady-state surface concentration and specific activity of iodinated [Gly^1]AMPA are identical with the steady-state concentration and specific activity of AMPA (not shown in Figure 2). The equilibrium surface concentration of ^{125}I-labeled [D-Ala^1]AMPA decreases sharply at a lower surface pressure than that of [^{125}I]AMPA and [^{125}I]-[Gly^1]AMPA, confirming the weaker penetration of this modified protein. Its specific activity is half of the specific activity of AMPA in this system, indicating that modification of the N terminus also has an effect on the catalytic turnover of the enzyme. As was found for AMPA, the specific activities of the modified proteins increase up to a surface pressure of 10 dyn/cm and then remain constant. The relatively large errors in the specific activities at the highest surface pressures are due to inaccuracies in the determination of the low amount of enzyme in the interface. Moreover, minor variations in surface pressure give rise to large changes in the surface density of penetrated protein. Figure 3 shows the lag time profile as a function of pH of iodinated [D-Ala^1]AMPA, [Gly^1]AMPA, and AMPA acting on L-di-C10-PC monolayers at a surface pressure of 10 dyn/cm and a Ca^{2+} ion concentration of 20 mM. AMPA shows a loss of penetrating capacity between pH 9 and 10, which corresponds to the deprotonation of the α-NH_3^+ group of Ala^1. [^{125}I]-[Gly^1]AMPA gives a similar profile. [D-Ala^1]AMPA shows a completely different pattern. This modified enzyme loses its penetrating capacity at considerably lower pH. It lag time profile is quite similar to the lag time profile of AMPA when both sites for Ca^{2+} ions are not saturated [dotted line, 0.05 mM $CaCl_2$; cf. Pattus et al. (1979b)]. In order to determine whether the modified proteins are affected upon binding of Ca^{2+} to the low-affinity site as observed for AMPA, we measured the lag time profile as a function of pH at a Ca^{2+} ion concentration of 0.5 mM (Figure 4). As shown in this figure, decreasing Ca^{2+} ion concentration from 20 to 0.5 mM shifts the ascending part of the lag time profile of [Gly^1]AMPA to lower pH values as it does to native AMPA (Pattus et al., 1979b). This effect was not observed

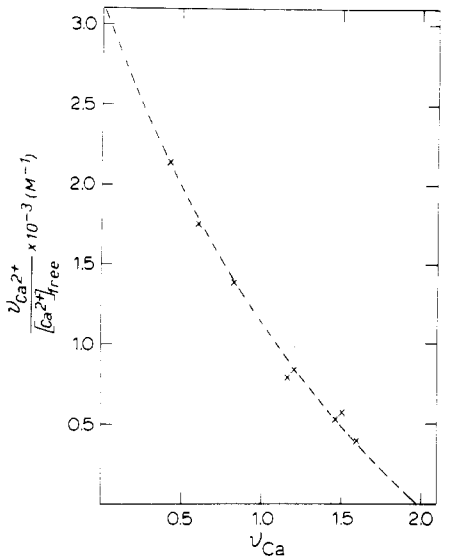

FIGURE 6: Scatchard plot of the binding of Ca^{2+} ions to [Gly^1]AMPA (4 mg/mL) in the presence of *n*-hexadecylphosphocholine micelles (30 mg/mL) measured by equilibrium dialysis. Tris-HCl buffer (50 mM), pH 8; NaCl (0.1 M).

for [D-Ala^1]AMPA. Figure 5 shows the apparent velocity and equilibrium surface concentration, as a function of pH, of [^{125}I]-[Gly^1]AMPA (A) and of [^{125}I]-[D-Ala^1]AMPA (B) acting on L-di-C10-lecithin monolayers at a surface pressure of 10 dyn/cm and at a Ca^{2+} ion concentration of 20 and 0.5 mM, respectively. [Gly^1]AMPA shows a second basic optimum as a shoulder in the activity profile at a Ca^{2+} ion concentration of 20 mM. As in the case of AMPA this optimum disappears when the Ca^{2+} ion concentration is lowered to 0.5 mM. [D-Ala^1]AMPA has only one pH optimum at pH 6 at high and low Ca^{2+} concentration. The steady-state surface concentration of [Gly^1]AMPA remains constant up to a pH value corresponding to the deprotonation of the α-NH_3^+ of the IRS (Slotboom et al., 1978c). Also, the surface concentration of [D-Ala^1]AMPA remains constant in the range of pH studied. Higher pH values could not be investigated because of the extremely low activity of the enzyme and the long lag times of the kinetics. In order to determine the number of calcium binding sites on [Gly^1]-AMPA, we carried out equilibrium dialysis studies in the presence of lipid–water interfaces. Figure 6 shows a Scatchard plot of the binding of Ca^{2+} ions to [Gly^1]AMPA in presence of micellar *n*-hexadecylphosphocholine at pH 8. From this data it can be concluded that [Gly^1]AMPA indeed possesses two binding sites. Two binding constants could be obtained by treatment of the data according to Klotz & Hunston (1971). These dissociation constants (K_{D_1} = 0.4 mM and K_{D_2} = 2 mM) are quite similar to the Ca^{2+} ion dissociation constants of AMPA (Pattus et al., 1979b).

Discussion

In the first paper of this series (Pattus et al., 1979a), it was shown that the precursor of phospholipase A_2 which has an active site but no IRS adsorbs aspecifically to substrate monolayers at surface pressures below 10 dyn/cm. However, no activity could be detected. In micellar systems [D-Ala^1]AMPA and DL-Ala^{-1}-AMPA behave like the zymogen, and only a weak monomer hydrolysis is observed. From Figure 1 it is evident that in monomolecular surface films these proteins are different from the zymogen. These proteins do possess an interface recognition site but it is of lower efficiency than that of AMPA. Iodination of Tyr-69 in [D-Ala^1]AMPA increases the penetrating capacity of this modified enzyme as it does for AMPA. However, no effect in the penetration capacity of the zymogen was observed upon iodination of Tyr-69. This specific effect of iodination on both L- and D-[Ala^1]AMPA indicates that [D-Ala^1]AMPA also possesses an IRS in contrast with the zymogen. Comparing the critical surface pressure where these proteins can no longer penetrate a lecithin monolayer (Figure 1A), it is tempting for us to speculate that the micellar interfaces investigated have a lipid packing similar to that of an L-di-C10-PC monolayer between 10 and 12 dyn/cm which corresponds to a lipid surface density of 12.8×10^{13} molecules/cm^2 (78 Å^2/molecule). The lag time profile of [^{125}I]-[D-Ala^1]AMPA indicates that this enzyme might possess sufficient penetrating power to bind to micellar interfaces. The experiment using L-di-C8-PC micelles confirmed the observation of the monolayer study. However, the turnover of ^{125}I-labeled [D-Ala^1]AMPA in the system is quite lower than the activity of AMPA. As shown in Figure 2, the specific activity of [^{125}I]-[D-Ala^1]AMPA on substrate monolayers is also lower than the activity of AMPA, indicating that modification of the N terminus of the protein also has an effect on the turnover of the enzyme. This confirms the previous findings that the IRS and the catalytic site of phospholipase A_2 are not far apart in the three-dimensional structure of the protein (Pattus et al., 1979b). Figures 3 and 4 demonstrate that [Gly^1]AMPA is rather similar to AMPA in its response to Ca^{2+} and pH, indicating that [Gly^1]AMPA also binds a second Ca^{2+} ion to a low-affinity site as does AMPA. However, no second calcium binding site could be detected either by ultraviolet difference spectroscopy or by nuclear magnetic resonance spectroscopy (Slotboom et al., 1978c). To explain these discrepancies one has to assume that the high-affinity site is induced in [Gly^1]AMPA upon binding to the lipid–water interface. This is confirmed by the equilibrium dialysis studies in the presence of micellar interfaces which show that [Gly^1]AMPA binds two Ca^{2+} ions/molecule of enzyme with the same affinity as AMPA. From Figures 4 and 5B it is evident that Ca^{2+} has no effect on the penetration capacity of [D-Ala^1]AMPA and that this modified protein has no second optimum of activity. As shown in Figure 3 the lag time profile as a function of pH is quite similar to the lag time profile of AMPA at very low Ca^{2+} ion concentration (0.05 mM). These results indicate that [D-Ala^1]AMPA has no low-affinity site for Ca^{2+} ions. Unfortunately, this could not be confirmed by equilibrium dialysis studies since [D-Ala^1]-AMPA does not bind micellar lipids.

From the data presented in this paper it can be concluded that the stereospecific adjustment of L-Ala^1 in the three-dimensional structure of porcine phospholipase A_2 is extremely important not only in the lipid binding process but also in the binding of Ca^{2+} ion to the low-affinity site. This second Ca^{2+} binding site must be in close proximity to the N-terminal sequence of the protein. In the foregoing paper, it was shown that there is a regulation between the IRS and the active site of phospholipase A_2 (Pattus et al., 1979b). The lower specific activity of [D-Ala^1]AMPA as compared to AMPA also indicates that such interactions occur. The induction of a Ca^{2+} binding site on [Gly^1]AMPA by membranelike structures is an example of the regulation potential of interfaces on protein function.

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Histone Gene Switch in the Sea Urchin Embryo. Identification of Late Embryonic Histone Messenger Ribonucleic Acids and the Control of Their Synthesis†

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ABSTRACT: During embryogenesis in the sea urchin *Strongylocentrotus purpuratus*, there is a shift from one histone mRNA population to another. The early and late embryonic histone mRNAs, previously shown to differ considerably in sequence from each other by hybrid melting studies, are shown here to differ also in electrophoretic mobility on polyacrylamide gels as the positions of the early and late mRNAs are completely noncoincident. The various species of both early and late samples are identified as particular histone mRNAs by hybridization to cloned histone DNAs containing part of the early-type repeat unit or to restriction enzyme fragments derived from these units. Four bands in the early mRNA sample are identified as H1, H3, H2A + H2B, and H4 mRNA while at least 10 bands can be seen in the late mRNA preparation with unambiguous identification of H1, H2B, and H4 mRNAs. A cluster of late species is shown to contain both H3 and H2A mRNA. When a polysomal RNA preparation from the 26-h embryo is hybridized to the histone DNA, eluted, and then translated in vitro in a wheat germ system, the histone products migrate in the position of late histones when subjected to electrophoresis on Triton X–urea gels. Using DNA which contains genes for H2A + H3 or H2A alone, we demonstrate the specificity of the early-type DNA probes for these two late histones. Therefore, by hybridization of newly synthesized RNAs and translation of the total polysomal RNA present in the late embryo, it is shown that mRNAs for all five histone classes may cross-react with the cloned early-type DNA. The hybrids formed, however, are much less stable than those formed with the early histone mRNA. In vitro translation of total cytoplasmic RNA from various embryonic stages indicates that transition between the two classes occurs during most of the blastula period.

Two widely diverged classes of histone mRNA, each coding for all five histone types, are synthesized at particular times during embryogenesis in the sea urchin *Strongylocentrotus purpuratus* (Kunkel & Weinberg, 1978). In this organism, one class of histone gene transcripts ("early" RNA) is synthesized during cleavage and blastula stages. Another class ("late" RNA) begins to be made during blastulation and represents the vast bulk of the histone mRNA synthesized at the subsequent mesenchyme blastula. The two mRNA classes have been distinguished by properties of hybrids formed between cloned *S. purpuratus* histone DNA and each of the two transcript populations (Kunkel & Weinberg, 1978) and by the difference in histone subtypes which result from in vitro translation of mRNAs obtained from the two stages (Weinberg et al., 1977; Newrock et al., 1978). The sequences of the early mRNAs are very similar, if not identical, to the vast bulk of the several hundred histone gene repeats in the *S. purpuratus* genome (Kunkel & Weinberg, 1978). These genes consist of clustered 6–7 kilobase units, each of which contains genes coding for the five histone classes (see Figure 4) (Kedes et al., 1975; Weinberg et al., 1975; Cohn et al., 1976; Wu et al., 1976; Holmes et al., 1977; Overton & Weinberg, 1978). The late

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