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EVIDENCE THAT THE ZYMOGEN OF PHOSPHOLIPASE A_2 BINDS TO A NEGATIVELY CHARGED LIPID-WATER INTERFACE

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Evidence is presented that the zymogen of porcine pancreatic phospholipase A_2 (prophospholipase A_2) interacts with a lipid-water interface provided that the interface has a net negative surface charge. Fluorescence spectroscopy and non-equilibrium gel filtration indicate that binding of prophospholipase A_2 (proPLA) to mixed detergent micelles is dependent on the presence of an anionic detergent. Prophospholipase binding is accompanied by a change in the environment of the single tryptophan residue qualitatively similar to that observed when the active enzyme, phospholipase A_2 (PLA), binds to micelles. In addition, the rate of tryptic activation of prophospholipase is significantly reduced in the presence of negatively-charged mixed micelles, whereas no change in rate occurs when neutral micelles are present. These observations suggest that the lack of catalytic activity of the zymogen toward organized substrates carrying a negative surface charge cannot be explained by a failure to bind at the lipid-water interface.

Keywords: prophospholipase A_2; phospholipase A_2; zymogen; interfacial binding; surface charge.

Introduction

PLA (EC 3.1.1.4) catalyzes the stereospecific hydrolysis of fatty acid ester bonds at the 2-position of 3-*sn*-phosphoglycerides in a Ca^{2+}-dependent reaction. Presently the amino acid sequences of more than 30 PLA from various sources have been determined [1] and the crystal structures of the bovine [2] and porcine [3] pancreatic enzymes as well as the enzyme from *Crotalus atrox* venom [4] have been elucidated. In mammals PLA is secreted by the pancreas in the form of a zymogen, proPLA, which is converted into the active enzyme by trypsin catalyzed cleavage of the N-terminal arg^{-1}-ala^1 bond and release of a heptapeptide [5].

Abbreviations: PLA, porcine pancreatic phospholipase A_2; proPLA, porcine pancreatic prophospholipase A_2; $C_{16}PN$, *n*-hexadecylphosphocholine; SDS, sodium dodecyl sulfate; CMC, critical micellar concentration; TPCK, tosyl-phenylalanine chloromethylketone.

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One of the most intriguing aspects of the study of PLA catalyzed lipolysis is the so-called interfacial activation displayed by this enzyme. Although both PLA and proPLA hydrolyze monomolecularly dispersed substrates at a fairly slow rate, only the active enzyme shows a rate enhancement of several orders of magnitude when the substrate is present as a micellar aggregate [6]. Various hypotheses have been formulated but up to now no generally accepted explanation of this phenomenon has been given [7].

For the pancreatic enzyme experimental work has been guided by the interface recognition site hypothesis, which postulates the presence on the enzyme surface of one or more specific regions, distinct from the active site, important for binding of the protein to the organized lipid substrate [6,8]. So far, however, kinetic and direct binding studies supporting this hypothesis have been performed largely using either phosphatidylcholines as substrates or substrate analogs carrying the phosphocholine polar head group [7]. Thus the possible importance of lipid surface charge in the mechanism of action of PLA has remained obscure in spite of the fact that, in retrospect, there are clear indications in the older literature [9], that lipid-protein charge interactions might play a role in PLA function.

In the course of our investigations aimed at characterization of the role of charge interactions in the interfacial binding and catalysis of pancreatic PLA [10] and identification of the individual amino acid residues involved in these interactions, we obtained, quite unexpectedly, evidence that the porcine pancreatic zymogen, proPLA, apparently binds to a lipid-water interface provided a negative surface charge is present. Since it has been assumed until now that the lack of activity of proPLA on organized substrates is due to its inability to bind to the lipid-water interface it became essential to examine the interfacial binding of the zymogen more closely.

In this paper we present data suggesting that interfacial binding of proPLA is surface charge-dependent. Formation of the lipid-protein complex influences the environment of the single tryptophan of the protein and leads to a significant reduction of the rate of tryptic activation of the zymogen.

Experimental Procedures

Materials

PLA and proPLA were obtained as described previously [11]. Tosyl-phenylalanine chloromethylketone (TPCK)-treated trypsin was from Worthington and *n*-hexadecylphosphocholine (C_{16}PN) was prepared as reported by Van Dam-Mieras et al. [12]. Sodium dodecyl sulfate (SDS) and lauric acid were from Matheson, Coleman and Bell, and Mann Research Laboratories, respectively. Sephadex G-75 was from Pharmacia.

Fluorescence

Tryptophan emission spectra of PLA and proPLA were recorded between 310 and 400 nm using a SPEX FLUOROLOG fluorimeter. Excitation was at 295 nm

with excitation and emission slitwidths of 20 and 5 nm, respectively. The protein concentration was 0.2 mg/ml in 0.1 M Tris–HCl (pH 8.0), containing 0.1 M NaCl and 10 mM $CaCl_2$. Emission spectra were recorded after addition of small aliquots from 75 mM stock solutions of detergent in distilled water. Background fluorescence in the absence of protein was negligible.

Gel filtration

A column of Sephadex G-75 (28 × 1.5 cm) was equilibrated in 0.1 M Tris–HCl (pH 8.0) containing 0.1 M NaCl, 10 mM $CaCl_2$ and 10 μM $C_{16}PN$. In the presence of this concentration of $C_{16}PN$, which is the CMC reported for this detergent [13], tailing of the detergent peak was reduced considerably. The void volume of the column, determined with dextran blue, was 13.5 ml, whereas proPLA eluted at 19 ml. Binding of proPLA to the mixed detergent micelles was studied by loading onto the preequilibrated column 0.2 ml portions of a solution containing 1 mg/ml protein and 10 mM detergent (total) in the same buffer, and measuring the absorbance at 280 nm in the eluted fractions. Fractions (1.3 ml) were collected at a flow-rate of 17 ml/h. Although the column was run at room temperature, no activation of proPLA took place as determined by phospholipase activity assay (see below). When the $C_{16}PN$/SDS (molar ratio, 1 : 1) mixture was dissolved in buffer, a small amount of precipitate, presumably of Ca^{2+}-SDS salt, developed with time. This occurred both in the absence and presence of protein. The sample containing mixed micelles at the 1 : 1 molar ratio was therefore loaded on the column immediately after mixing. No precipitation was noticed in any of the column fractions and recovery of material, as judged by the elution pattern, was essentially complete.

Zymogen activation

The time course of the trypsin-catalyzed conversion of proPLA to PLA was followed in the absence and presence of detergents by measuring the increase in PLA activity as a function of time. Zymogen activation was carried out at room temperature and pH 8.0 in 0.1 M Tris–HCl, 0.1 M NaCl and 10 mM $CaCl_2$. At temperatures below 4°C, which are usually employed for zymogen activation [11], precipitation of the $C_{16}PN$/SDS mixture occurred. Activation was initiated through addition of a 10-μl aliquot of a 0.1 mg/ml trypsin solution to 1.0 ml buffer containing 0.1 mg proPLA. A stock solution of 1.0 mg/ml trypsin was prepared in distilled water acidified with a few drops of acetic acid (pH 4) and frozen in 100-μl aliquots. Immediately before each experiment one of these aliquots was thawed and diluted 10-fold. In this way autocatalytic degradation of trypsin was minimized, while the trypsin concentration was kept the same in each run. The experiment with the $C_{16}PN$/lauric acid mixture was done in the absence of Ca^{2+} to avoid precipitation of the fatty acid-Ca^{2+} salt. Phospholipase activity was determined on 10-μl aliquots withdrawn from the activation mixture using the egg-yolk lipoprotein assay as described previously [11].

Results

The increase in emission intensity of the single tryptophan of PLA and proPLA as a function of the concentration of neutral and negatively charged detergent micelles is shown in Figs. 1A and 1B, respectively. The data show that, as expected, PLA binds both to the neutral zwitterionic interface of the $C_{16}PN$ micelles and to the negatively charged interface formed by incorporation of SDS molecules. The affinity of PLA for the latter is markedly increased over the affinity for the neutral micelles, as indicated by the steeper rise in the curve, while the maximum value of the relative fluorescence increase obtained at saturating total detergent concentrations is similar for both types of micelles (Fig. 1A). For both the zwitterionic $C_{16}PN$ micelles and the negatively charged mixed micelles of $C_{16}PN$ and SDS, binding of PLA at the interface is accompanied by a blue-shift in the wavelength of the emission maximum of 8–10 nm. Qualitatively similar results were obtained with co-micelles of two other neutral detergents (lauryl maltoside and Zwittergent 314) and SDS.

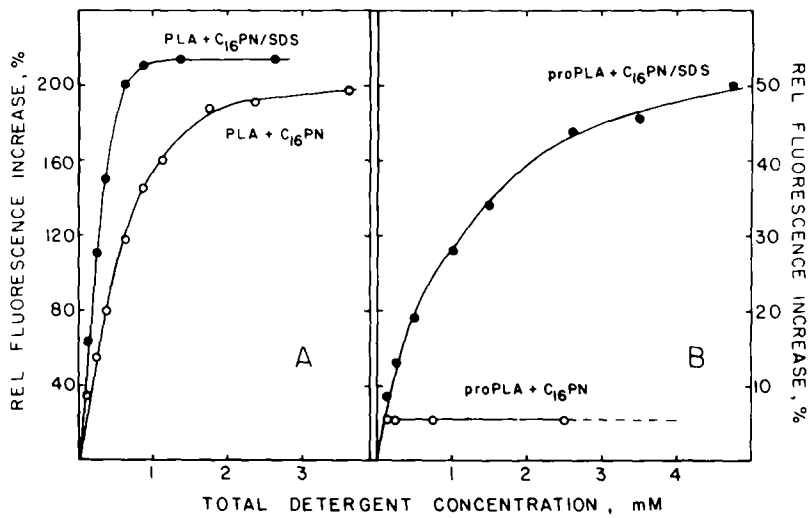

Fig. 1. Comparison of the effect of neutral micelles and negatively charged mixed detergent micelles on the tryptophan fluorescence of (A) PLA and (B) proPLA. For both proteins the increase in the fluorescence emission intensity at 346 nm is plotted as the relative fluorescence increase, defined as $100(F - F_0)/F_0$, where F and F_0 are the emission intensities in the presence and absence of detergent, respectively, with different scales on the ordinates. Fluorescence emission spectra were recorded with excitation at 295 nm after addition of small aliquots of a stock solution containing 75 mM total detergent to a solution containing 0.2 mg/ml (0.014 mM) protein in 0.1 M Tris–HCl, 0.1 M NaCl and 10 mM $CaCl_2$ (pH 8.0). A: data for phospholipase obtained with pure $C_{16}PN$ micelles (○) and mixed micelles of $C_{16}PN$ and SDS (9:1, molar ratio) (●), respectively. B: data for the zymogen obtained under the same conditions.

As shown in Fig. 1B, there is a much more dramatic difference in the effect of the negatively charged versus the neutral micellar interface on the fluorescence of the zymogen than is observed for the active enzyme. In the absence of a negative surface charge, proPLA showed no apparent affinity for the lipid-water interface. The very small and concentration independent increase in emission intensity observed with pure $C_{16}PN$ (Fig. 1B) is not accompanied by a blue-shift in the emission maximum and is probably due to binding of a detergent monomer in the active site [14]. However, when proPLA is titrated with $C_{16}PN$ micelles containing 10 mol% SDS, a substantial and concentration dependent increase in the tryptophan emission intensity is observed (Fig. 1B), accompanied by a blue-shift in the emission maximum of about 3–5 nm. This suggests that the presence of a negative surface charge on the detergent micelle is required for binding of the zymogen.

An idea of the relative binding affinities can be obtained by comparing in Figs. 1A and 1B the detergent concentrations at which half maximal saturation is obtained. For proPLA and the $C_{16}PN$/SDS mixture the value for the relative fluorescence increase at saturating detergent concentrations, estimated by linear extrapolation in a double reciprocal plot of relative fluorescence increase versus total detergent concentration, gives a value of 60(±5)%. This means that about 80% of the protein is bound at the micellar interface at the highest detergent concentration shown in Fig. 1B, and the affinity of proPLA for the negatively charged interface is comparable to the affinity of PLA for neutral micelles. Similar results were obtained when the binding of proPLA to micelles of the neutral detergent lauryl maltoside and to mixed micelles of lauryl maltoside and 10 mol% SDS were compared. In the presence of SDS the relative fluorescence increase leveled off at about 30%, while the affinity of proPLA for the neutral detergent micelles was undetectable.

If the change in the tryptophan fluorescence in the presence of a negatively charged lipid-water interface is due to zymogen binding, the elution pattern of a mixture of micelles and protein from a gel filtration column would be expected to depend on the micellar composition. We tested this and the Sephadex gel filtration of mixtures of protein and detergents (see Experimental Procedures) gave the results shown in Fig. 2. As expected for the mixture of proPLA and pure $C_{16}PN$, the detergent micelles elute in the void volume of the column (13.5 ml) while proPLA elutes at the elution volume of the pure protein (19 ml). The void volume fraction contained no protein, as judged by phospholipase assay before or after addition of trypsin. Incorporation of 10 mol% SDS in the detergent micelles results in a broadening of the protein peak and a reduction of the elution volume with proPLA eluting at about 17.5 ml. This effect, which signifies binding of proPLA to the negatively charged mixed micelles eluting in the void volume, is much more pronounced in the presence of $C_{16}PN$/SDS (1 : 1, molar ratio) mixed micelles. Here the detergents and protein essentially co-elute in the void volume indicating a rather strong interaction between proPLA and the negatively charged mixed micelles. These results are consistent with the conclusion drawn from the

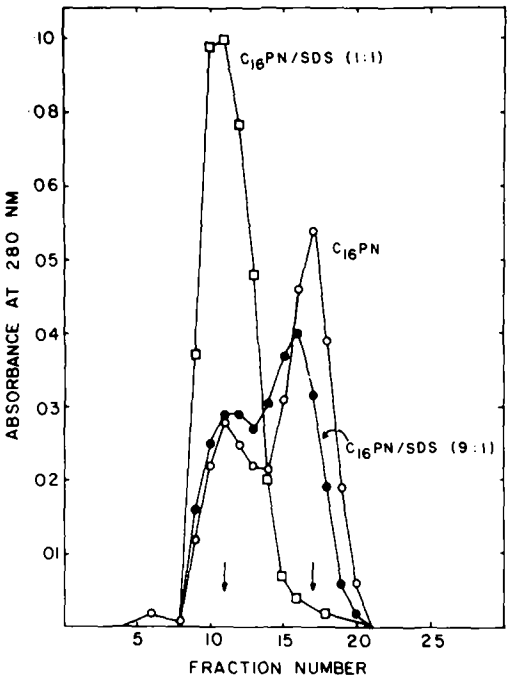

Fig. 2. Gel filtration of mixtures of proPLA and neutral or negatively charged detergent micelles. Aliquots (200 μl) of a solution containing 1 mg/ml protein and 10 mM detergent (total) were loaded on a column (28 × 1.5 cm) of Sephadex G-75 pre-equilibrated with a buffer (pH 8.0) containing 0.1 M Tris–HCl, 0.1 M NaCl, 10 mM $CaCl_2$ and 10 μM $C_{16}PN$. ○, proPLA + $C_{16}PN$; •, proPLA + $C_{16}PN$/SDS (9:1, molar ratio); □, proPLA + $C_{16}PN$/SDS (1:1, molar ratio). The absorbance at 280 nm indicates both the presence of detergent micelles, through light scattering, and the presence of protein, through light absorption. Arrows indicate the void volume (left) and the elution volume of proPLA (right), respectively.

fluorescence data described above, i.e., the results can be most readily interpreted in terms of binding of proPLA to the lipid-water interface when a net negative surface charge is present.

The fairly strong and charge-dependent binding of proPLA to detergent micelles alters the environment of the single tryptophan, which is located in position 3 of the primary sequence close to the trypsin-labile arg^{-1}-ala^{1} bond. This suggests the possibility that the trypsin-dependent conversion of the zymogen into the active enzyme could be affected by zymogen binding to the lipid-water interface. In Fig. 3, data obtained describing the time course of the tryptic activation of proPLA in the absence and presence of various detergent mixtures are plotted. Under the conditions used here zymogen activation, without any added detergent, is optimal after about 1 h and enzyme activity levels off at about 800 μmol/min mg. Addition of $C_{16}PN$ has no influence on the initial rate of the trypsin catalyzed zymogen activation (dashed lines, Fig. 3). In contrast, in the presence of 10 mol%

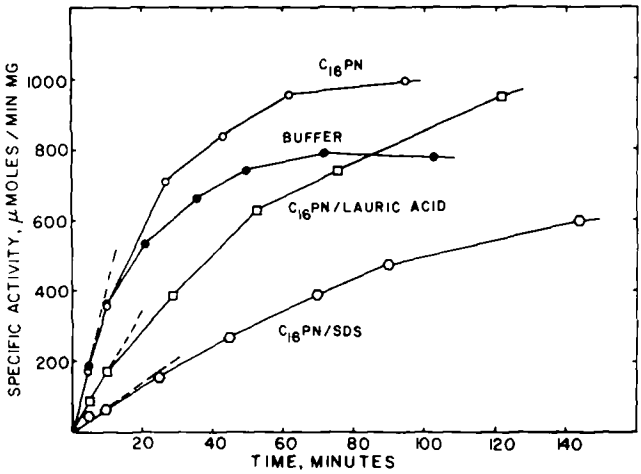

Fig. 3. Time-course of the tryptic activation of proPLA in the presence and absence of detergent. The reaction was carried out at room temperature in 0.1 M Tris–HCl (pH 8.0) containing 0.1 M NaCl and 10 mM $CaCl_2$ except for the $C_{16}PN$/lauric acid mixture where $CaCl_2$ was omitted. ProPLA concentration was 0.1 mg/ml and the activation was initiated by addition of 1 μg/ml trypsin. •, buffer alone; ○, 9 mM $C_{16}PN$; ○, 8.1 mM $C_{16}PN$ and 0.9 mM SDS; =, 8.1 mM $C_{16}PN$ and 0.9 mM lauric acid.

of either a short chain fatty acid (lauric acid) or SDS, both of which form mixed micelles with $C_{16}PN$, the initial rate of zymogen activation is significantly reduced. This slower rate is consistent with the interpretation that the arg^{-1}-ala^{1} bond, which is hydrolyzed when the zymogen is converted into the active enzyme, must be at least partially shielded against tryptic hydrolysis in the proPLA-charged micelle complex. In addition, the data presented in Fig. 3 indicate that the optimal specific phospholipase activity reached under these conditions is significantly higher when tryptic activation is allowed to proceed in the presence of $C_{16}PN$ micelles or $C_{16}PN$/lauric acid mixed micelles. This phenomenon can probably be explained by binding of the newly formed enzyme to both the netural and the charged detergent micelles, and this binding protects the protein against further tryptic digestion and inactivation.

Discussion

The binding of pancreatic PLA at the lipid-water interface has been studied extensively using a variety of techniques including gel filtration, spectroscopy and hydrodynamic measurements [13–15]. When detergent-like *n*-alkylphosphocholines are used as substrate analogs the stoichiometry of the lipid-protein complex formed between PLA and the zwitterionic detergent micelles varies from 30–40 detergent molecules per protein molecule with 2–3 proteins per particle, and hydrophobic interaction between protein and lipids has been implicated as

the main stabilizing factor [14]. A combination of spectroscopic, semisynthetic and chemical modification studies [7], together with X-ray crystallography [16], has identified apolar residues on the protein surface that might be involved in interfacial binding, including the single tryptophan located in position 3 in the amino acid sequence of all pancreatic PLAs [7]. Interestingly, inspection of the 3-dimensional structure also suggests several positively charged amino acid residues in a position favorable for interaction with any negatively charged groups in the interface [16].

Two decades ago it was reported that hydrolysis of acidic phospholipids was more complete than hydrolysis of zwitterionic phospholipids, as judged by chromatographic analysis of the mixture after incubation with human PLA [9]. This apparent preference of pancreatic PLA for anionic phospholipids has been confirmed more recently by kinetic studies on monomolecular surface films [8,17]. In addition, studies aimed at a characterization of the role of charge interactions in phospholipase function have shown that the activity of pancreatic PLA is greatly improved when the substrate micelle is negatively charged either by including an anionic substrate or by including an anionic detergent [10]. Therefore, lipid surface charge is implicated in the interfacial binding and catalytic activity of the active enzyme. Charge interactions might play an important role in the formation of a functional enzyme-substrate complex and the interface binding surface might include positively charged residues, i.e., lysine and arginine [18], as well as apolar residues. A related observation is that of Hille et al. [19,20], who found that both porcine pancreatic PLA and its zymogen bind in a two-step process to negatively charged monomeric substrates containing a sulfate group as the polar head. In a concentration range far below the CMC both proteins first bound two sulfate molecules, followed by formation of a well-defined lipid-protein complex consisting of 4–6 protein molecules and about 40 sulfate monomers. In this 'pseudo-micellar' complex the enzyme but not the zymogen exhibits enhanced hydrolysis rates.

Although the zymogen, proPLA, possesses a fully functional active site, its catalytic activity remains low both when short-chain micellar lecithins [6] or when the negatively charged mixed micelles of long-chain phospholipids and deoxycholate employed in the standard phospholipase assay [11], are used as substrates. Since none of the binding techniques referred to above has indicated significant binding of proPLA to a *neutral* micellar interface, the straightforward conclusion has been that lack of activity of the zymogen towards lipid substrate aggregates is explained by the inability of this protein to bind to these structures.

Our results, however, show that porcine pancreatic proPLA apparently does bind to a lipid-water interface that carries a net negative surface charge. Our results are consistent with observations that proPLA binds to negatively charged mixed micelles of *n*-octadecylphosphocholine and *n*-tetradecylphosphate using UV difference spectroscopy and microcalorimetry (P. Soares de Araujo, unpublished observations). The most likely interpretation of the results presented here is charge-facilitated binding of the zymogen to the mixed micellar interface. This assumes

the thermodynamically favorable mixing of SDS and $C_{16}PN$ into mixed micelles. Since all our experiments were done in the presence of excess $C_{16}PN$ at concentrations far above the CMC of this compound, it is unlikely that protein/SDS 'pseudo-micelles' [19] and $C_{16}PN$/SDS mixed detergent micelles would occur in the same solution without equilibrating to a ternary complex. Our fluorescence data for proPLA when titrated with the $C_{16}PN$/SDS mixture (Fig. 1B) or the lauryl maltoside/SDS mixture present no evidence for binding according to the two-step process described by Hille et al. [19] and this also tends to exclude pseudo-micelle formation.

Micellar binding of the active enzyme involves the single tryptophan side chain of the protein [12]. Interestingly, we see a qualitatively similar effect on the tryptophan fluorescence for proPLA. Our results show that for both proteins binding to the interface there is a resulting increase in the fluorescence quantum yield accompanied by a blue-shift in the emission maximum. For PLA itself, these effects have been previously interpreted as a change in the environment of the tryptophan fluorophore towards a more hydrophobic environment [12]. The same interpretation appears valid for proPLA and the fluorescence data presented here, although the effects are less pronounced (compare Figs. 1A and 1B). An important question that remains to be answered is the relative contribution of hydrophobic and of charge interactions to the stability of the lipid-protein complex formed by PLA or proPLA and various neutral or charged organized substrates.

An interesting corollary of the fluorescence and gel filtration data presented here is our observation that the presence of a negatively-charged mixed detergent micelle leads to a significant reduction of the rate of zymogen activation (Fig. 3). This lower rate is probably due to a protective shielding of the arg^{-1}-ala^{1} bond in the detergent-zymogen complex rather than a direct effect of the charged detergent on the trypsin activity. The SDS concentration used here (0.9 mM) is far below the concentration range normally employed for protein denaturation (30 mM) and a reduction of the activation rate is also observed with lauric acid, which is not known as a protein-denaturing agent. Conceivably the reduced rate of activation of proPLA in the presence of negatively charged mixed micelles might have significance for the in vivo mechanism or regulation of intestinal zymogen activation.

In summary we have presented evidence supporting the new and unanticipated observation that porcine pancreatic proPLA binds to a lipid-water interface carrying a net negative surface charge. This interaction is not accompanied by the dramatically enhanced hydrolysis rates seen when the active enzyme binds to the lipid-water interface. In several hypotheses (reviewed in Refs. 1 and 7) a particular conformation or a decreased hydration of the lipid molecules at the interface is held responsible for the interfacial activation of lipolytic enzymes. Our observation that the zymogen of PLA, which has a functional active site [6], binds to a negatively charged lipid-water interface together with the known fact that proPLA is not activated even when the substrate aggregate carries a negative surface charge [11], seems to throw some doubt on the validity of these hypotheses. Apparently inter-

facial binding per se cannot explain the phenomenon of interfacial activation and this suggests that the latter involves a structural rearrangement in the enzyme upon binding to the lipid-water interface, and this rearrangement apparently cannot occur in the zymogen.

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