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Activation of interfacial enzymes at membrane surfaces

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Abstract

A host of water-soluble enzymes are active at membrane surfaces and in association with membranes. Some of these enzymes are involved in signalling and in modification and remodelling of the membranes. A special class of enzymes, the phospholipases, and in particular secretory phospholipase A_2 ($sPLA_2$), are only activated at the interface between water and membrane surfaces, where they lead to a break-down of the lipid molecules into lysolipids and free fatty acids. The activation is critically dependent on the physical properties of the lipid-membrane substrate. A topical review is given of our current understanding of the physical mechanisms responsible for activation of $sPLA_2$ as derived from a range of different experimental and theoretical investigations.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

In the actively functioning state of the biological membrane, the lipid bilayer component is subject to a range of non-equilibrium processes involving signal and energy transduction and transport of material as well as remodelling involving local and global topological

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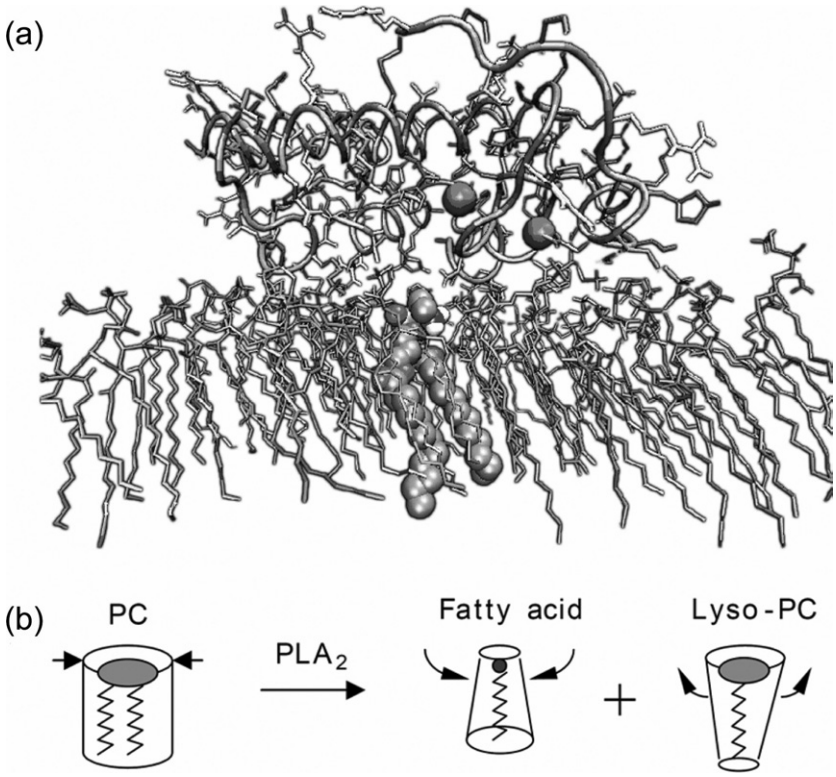

Figure 1. (a) Molecular representation of a $sPLA_2$ molecule bound at the interface of one of the monolayers of a lipid bilayer. The picture is obtained from an atomistic model of the system studied by molecular dynamics calculations. A single phospholipid molecule prone to attack is highlighted. This molecule is near the active site of the enzyme where the hydrolytic cleavage of the lipid takes place. (Adapted from [4] and reprinted with permission from the Theoretical and Computational Biophysics Group at the University of Illinois at Urbana-Champaign.) (b) Schematic illustration of the $sPLA_2$-assisted cleavage of a phosphatidylcholine (PC) lipid molecule into a fatty acid and a lyso-PC lipid emphasizing the change in molecular shape. The change in shape leads to an effective shift in the interfacial pressures as illustrated by the arrows.

changes [1–3]. A host of enzymes are involved in these processes. The action and activation of the enzymes are in many cases controlled by an intricate interplay between the enzyme and its conformation on one hand and the chemical and physical properties of the lipid bilayer on the other hand.

In the present paper we shall address a particular class of small 14–18 kD enzymes, the secretory phospholipases A_2 ($sPLA_2$), whose mechanism of activation is closely controlled by the physical properties of the lipid substrate. $sPLA_2$ is only active at organized structures of lipids, cf figure 1(a), and inactive towards monomeric lipids in solution. $sPLA_2$ is found in large amounts in, for example, venom, pancreatic, and cancerous fluids [5, 6]. It is involved in lipid metabolism, membrane remodelling, endocytosis, inflammation, and cell death [7].

The action of $sPLA_2$ is to catalyse the hydrolysis of the *sn*-2 acyl ester linkage of *sn*-glycero-3-phospholipids [5]. The enzyme requires Ca^{2+}-ions as a cofactor for activation. The cleavage products are 1-acyl-lysolipid fatty acids and free fatty acids, cf figure 1(b). Being amphiphiles, the cleavage products accumulate in the lipid bilayer, and due to their propensity for forming non-lamellar phases the bilayer properties are strongly altered by their existence.

These alterations augment the further activation of the enzyme and may ultimately lead to break-down of the bilayer structure [8].

The enzymology of $sPLA_2$ is complicated [4, 6] and involves a number of steps. In order to become active the enzyme first has to bind to the lipid bilayer. Since $sPLA_2$ is positively charged the binding to anionic lipids is stronger than to zwitterionic lipids. Once bound, the enzyme undergoes a scooting lateral motion across the surface. The structure of the enzyme–bilayer complex is unknown. The entrance to the active site is situated at the so-called docking surface that is presented to the bilayer in the bound state. The docking surface is rather flat and the active site is located roughly 1.5 nm above the membrane [6]. While the docking surface is flat, it does exhibit irregularities, among them slits connecting the active site to the periphery [4]. Finally, the kinetics of $sPLA_2$ activation is not of the usual Michaelis–Menten type. In contrast, it depends on the morphology and physico-chemical properties of the bilayer [9]. Often, the initial rate of hydrolysis is fairly low but can suddenly increase by two to three orders of magnitude (lag-burst behaviour) over a very short period of time [10–12].

A coherent picture of which physical factors enter the mechanism of activation of $sPLA_2$ has gradually appeared. These factors include product accumulation and formation of membrane defects, lipid–molecule protrusion modes and electrostatic lipid–enzyme interactions, as well as membrane undulations. Several of these factors are interrelated and may be interpreted in terms of macro- and mesoscopic phenomena like phase equilibria, membrane mechanics, and membrane thermodynamics. Below we shall review the evidence from experimental and theoretical studies that has led to this coherent picture. Unless explicitly mentioned, all of the experimental data presented below pertain to snake venom $sPLA_2$.

2. Activation by vesicular bilayers in the bulk

A very large amount of work has been done on $sPLA_2$ action on vesicular substrates in bulk solution, either unilamellar or multi-lamellar samples, and all conceivable aspects of the relationships between substrate quality and reaction conditions on the one side and enzyme activation and turnover on the other side have been investigated [13–15]. We will here focus on some of the physical factors that appear to control the activation measured in terms of the so-called lag time, τ, which is the time it takes from applying the enzyme until a burst of activity is observed as illustrated in figure 2(a). By changing the reaction conditions, τ can for some systems be varied from seconds to hours. It should be noted that the definition of τ is not always straightforward and the burst region for some lipid substrates is fairly broad [16].

In figure 2(b) are shown data that illustrate the temperature dependence of the activation of $sPLA_2$ for three different vesicular lipid-bilayer substrates, specifically DMPC, DPPC, and DSPC[8]. For all three cases, there is a pronounced minimum in τ at a temperature that corresponds to the phase transition temperature of the lipid bilayer in question [11, 16, 17]. Furthermore, the minimum is deeper and wider for shorter lipid acyl chains. This striking anomalous behaviour near the phase transition correlates directly with the thermal density fluctuations. The phase transition in question is the so-called main phase transition that takes the bilayer from a solid phase to a liquid (fluid) phase [3]. The shorter the chains, the stronger the fluctuations, and the more rapid the activation. Computer simulation calculations have revealed [11, 17, 18] that the lag time for all the data in figure 2(b) can be related semi-quantitatively to the degree of micro-heterogeneity in the corresponding lipid bilayer. The micro-heterogeneity in turn is related to the density fluctuations, which in turn are related

[8] Abbreviations: DMPC, di-palmitoyl PC; DMPG, di-myristoyl PG; DPPC, di-myristoyl PC; DSPC, di-stearoyl PC; SMPC, stearoyl–myristoyl PC; PC, phosphatidylcholine; PG, phosphatidylglycerol; POPC, palmitoyl–oleoyl PC.

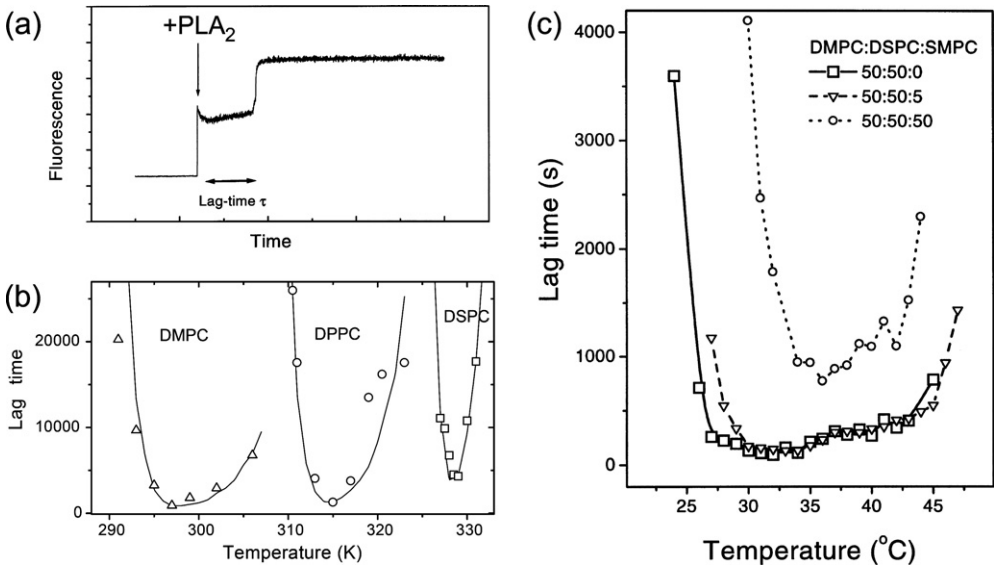

Figure 2. (a) Schematic illustration of the lag-burst behaviour for the activation of $sPLA_2$ on a lipid bilayer. The rapid burst occurs after a characteristic time, τ, the lag time. The activation is here monitored by the intrinsic Trp fluorescence of the enzyme. (b) Lag time of $sPLA_2$ activation on bilayers of three different lipids, a short chain lipid (DMPC), an intermediate chain lipid (DPPC), and a long chain lipid (DSPC), as a function of temperature in the neighbourhood of their respective lipid phase transitions. Symbols denote experimental data and the solid lines results from computer simulation calculations. (c) Lag time versus temperature for mixtures of DMPC–DSPC with varying amounts of a third intermediate lipid species, SMPC.

to thermodynamic and thermo-mechanic response functions, like specific heat, mechanical modules, and permeability. All these response functions display a similar anomalous behaviour at the phase transitions with a systematics similar to that shown in figure 2(b) [3]. This suggests that $sPLA_2$ is sensitive to micro-heterogeneity and specifically defect lines and boundaries between domains of the two phases.

The same kind of reasoning carries over to vesicular substrates with more than one kind of lipid species where the phase equilibria dictated by the underlying thermodynamic phase diagram are reflected in the activation of $sPLA_2$ as illustrated by the data in figure 2(c) for a mixture of DMPC and DSPC. τ now displays two minima corresponding to the position of the solidus and liquidus phase boundaries in the binary phase diagram. It is noted that the activity remains high in the phase coexistence region, possibly indicating the presence of micro-domains and defect lines in this region. By the further introduction of a third intermediate species, in this case SMPC, which can mediate boundary regions between the short (DMPC) and the long (DSPC) chain lipid, the lag time is seen to be progressively prolonged. This may indicate that SMPC is interfacially active and modulates the domain boundaries between DMPC and DSPC rich regions in a way that makes these regions less prone to attack of the enzyme [19].

3. Activation by supported bilayers

In order to investigate directly the spatial modulation of the lipid substrate under the action of $sPLA_2$, the enzymatic process has been imaged on well defined interfaces, such as single

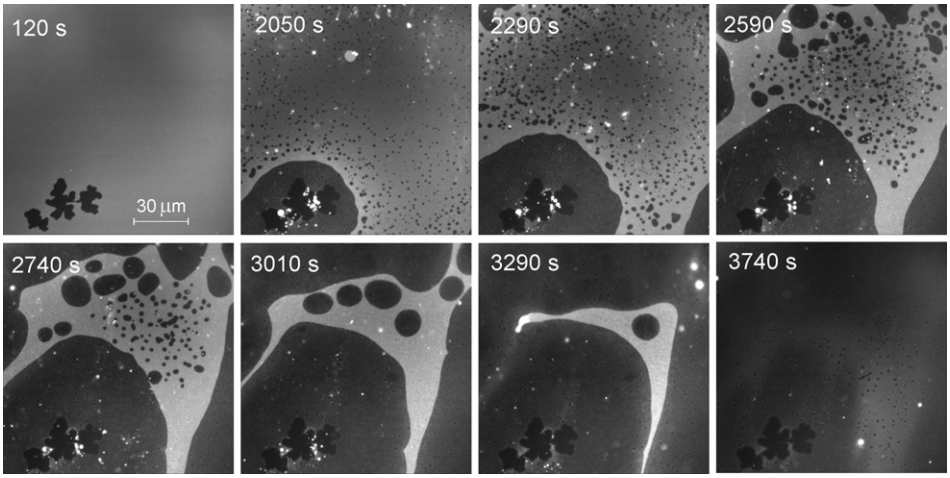

Figure 3. Time resolved fluorescence microscopy images of the lateral structure of a fluid POPC solid-supported lipid bilayer in water subject to $sPLA_2$ action. The initially smooth, defect-free bilayer with a few large holes (dark) is subjected to $sPLA_2$, a lag phase follows with very little action, followed by a burst of activity that appears to be spawned by the pre-existing large holes (bottom left) and newly formed smaller holes. Eventually the whole bilayer is digested with some unidentified remains of the products and fluorescent probes at the surface of the support. The frames are 120 μm × 120 μm.

giant unilamellar vesicles [20, 21], Langmuir films on air–water interfaces [22–24] and solid-supported bilayers [10, 25–29] formed by vesicle fusion, spin coating or Langmuir–Blodgett techniques. We shall here report on recent advances in the use of time-resolved imaging techniques that have been used to analyse the enzyme kinetics and the morphological changes of the bilayer under the influence of the enzyme.

Fluorescence microscopy is a useful method to visualize the lateral structure of solid-supported lipid bilayers in water, provided that suitable non-perturbing fluorescent lipid-analogue probes are used to highlight the structure. In figure 3 is shown an example of a fluid POPC bilayer that is being subjected to the action of $sPLA_2$. The lag phase is clearly observed, followed by a dramatic burst in activity, apparently controlled by pre-existing defect lines and newly formed holes in the bilayer [27].

The enzyme kinetics of such time-resolved images can be analysed quantitatively [27, 28], as illustrated in figure 4, where the total membrane area and its rate of change with time are shown for a POPC bilayer. Four distinct regimes can be discerned with a clear lag phase and a burst region. After a region of constant activity, there is a linear decrease of area, corresponding to a simple rate equation, $\mathrm{d}A/\mathrm{d}t \sim -\sqrt{A}$, consistent with an assumption of the enzyme mostly being active at the perimeter of the membrane patches.

More details of the enzymatic action can be revealed by using atomic force microscopy imaging [10, 25, 30, 31]. Such investigations have also revealed the lag phase and furthermore shown explicitly that the enzyme is mostly active at defect lines, such as the rim of holes or at induced defects caused by the accumulation of hydrolysis products. In figure 5 is shown an example of the sub-micron morphological changes of a solid supported DPPC lipid bilayer in water under the influence of $sPLA_2$ [30].

4. Activation by membrane ripples

A particular kind of well defined small-scale defect structure can be induced in certain lipid bilayers by bringing them into the so-called ripple phase, that for PC lipids persists over a

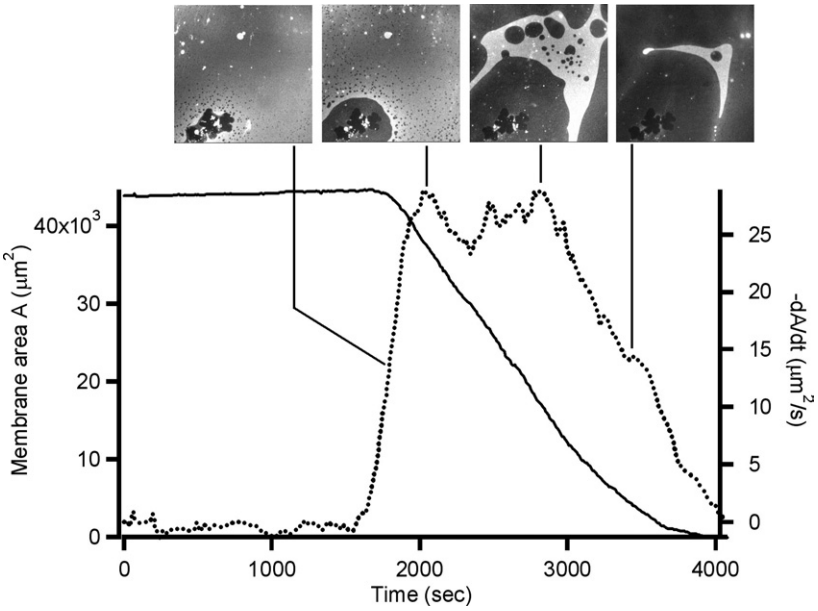

Figure 4. Time evolution of the intact bilayer area (solid line) shown together with the rate of area change (dotted line) of a fluid POPC lipid bilayer subject to the action of $sPLA_2$. The kinetics is characterized by four distinct regimes: a lag phase, a burst region, a region of constant activity; and a linear region where the area diminishes proportionally to the perimeter of the bilayer domains. Typical 120 μm $\times$ 120 μm images of the lateral structure in the different regions are also shown.

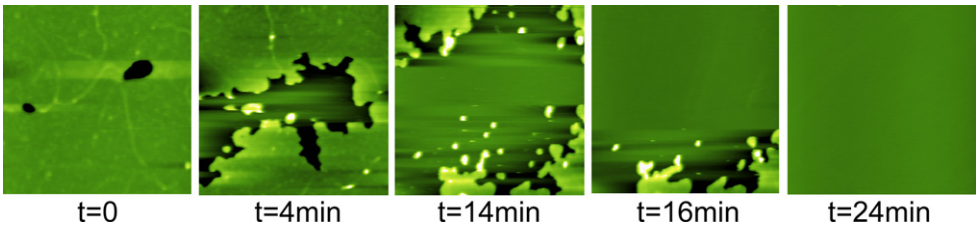

Figure 5. Time resolved atomic force microscopic imaging of the action of $sPLA_2$ on solid supported DPPC bilayers at room temperature where the lipids are in a solid phase. The initial bilayer has two holes that are provoked by exerting a large force on the bilayer by the tip of the atomic force microscope. After addition of $sPLA_2$ to the bilayer, the enzyme is immediately activated at the rim of the holes and there is no lag phase. The frames are 2 μm $\times$ 2 μm.

range of temperatures just below the main phase transition. These ripples can be imaged by atomic force microscopy provided that the bilayer is sufficiently decoupled from the influence of the solid support, e.g. by sitting on top of another bilayer [26]. In figure 6(a) is shown an image of a 1:1 DMPC–DSPC bilayer in the ripple phase. A clear spatial corrugation can be discerned of ripples with a periodicity around 26–30 nm and an amplitude around 2–3 nm.

Upon exposure to $sPLA_2$, the ripples are progressively broadened and their amplitude is increased until a critical point, where the bilayer collapses into a flat state. It appears from the data that $sPLA_2$ can exhibit a short-ranged directed movement while digesting one leaflet of a membrane [26]. This phenomenon appears to be linked to the intrinsic structure of the membrane as the directed motion is only observed in the solid and ripple phases of a bilayer. It is likely that the lipids most prone to attack are those that reside at the top of the ripples.

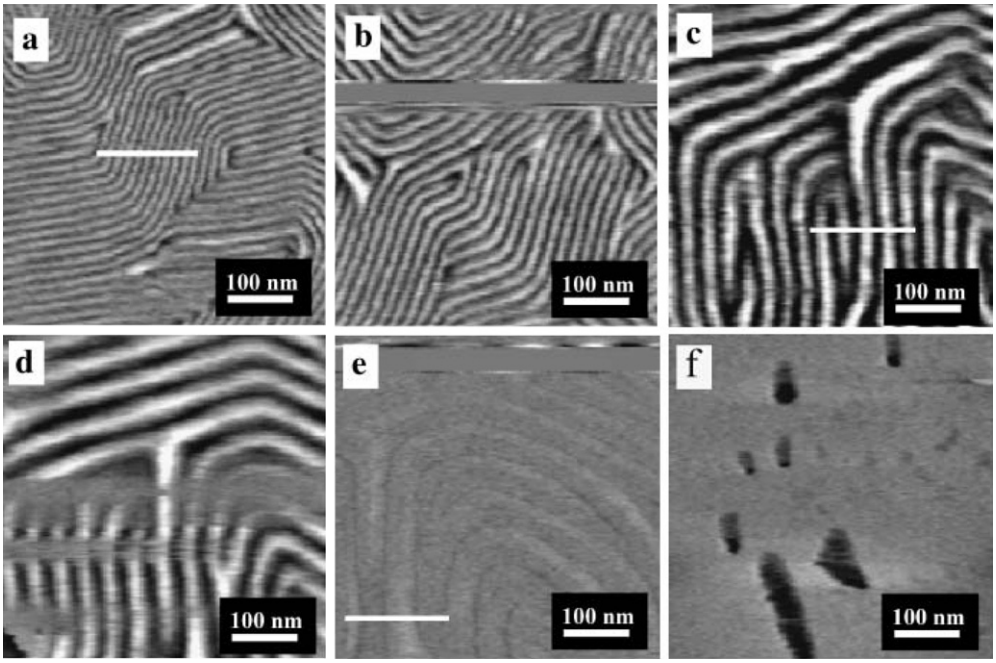

Figure 6. (a)–(f) Time resolved atomic force microscopic imaging of the action of $sPLA_2$ at room temperature on solid supported 1:1 DMPC–DSPC double bilayers at room temperature, where the lipids are in the ripple phase. The time lapse after adding the enzyme is, going from (a) to (f), 0, 64, 77, 82, 84, and 91 min. The grain structure in the ripple pattern is due to the solid (crystalline) nature of the bilayer.

5. Activation by charged lipids

$sPLA_2$ is positively charged and the binding to the lipid substrate will hence be enhanced if there is a negative surface charge density at the lipid bilayer surface, e.g. due to charged lipid species. Some types of $sPLA_2$ require anionic lipids in order to be activated. An example is human type-IIA $sPLA_2$ [32]. The question now arises as to what surface charge density is required and whether a high small-scale local charge density can activate the enzyme, despite the global surface charge density being lower that the critical value. This question is of particular relevance in connection with inflammatory response in wounded cells or the function of $sPLA_2$ as an anti-bacterial agent in e.g. tear fluid.

A recent study of human type-IIA $sPLA_2$ action on vesicular lipid bilayer substrates made by mixtures of anionic DMPG and DSPC has shown that whereas a certain critical threshold of a uniform global surface charge density is required to activate the enzyme a lower global surface charge density can activate the enzyme provided that the lipid bilayer is structured in small domains that are enriched in charged species (DMPG) [32]. Such a domain structure can be induced by positioning the mixture in appropriate regions of the phase diagram where phase coexistence and domain formation are expected. This effect is illustrated in figure 7 where the activity of human type-IIA $sPLA_2$, as measured by the leakage of an encapsulated fluorescent compound through unilamellar DMPG–DSPC vesicles of various compositions, is shown as a function of temperature across the binary phase diagram.

In the case of a uniform substrate, the critical surface charge density is found to correspond to a composition of about 38% PG head groups. For the 1:1 mixture in figure 7(a) this

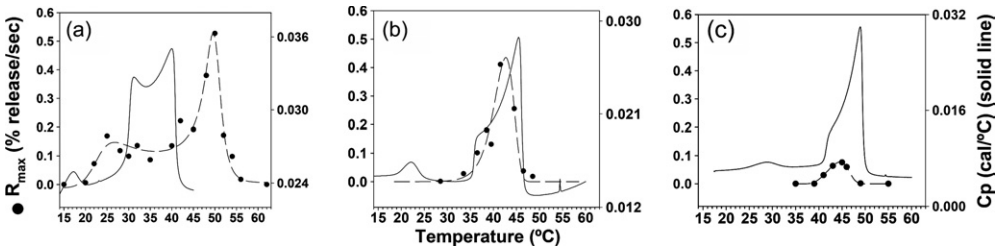

Figure 7. Enzyme activation (solid points and dashed lines), measured in terms of release of a fluorescent probe out of unilamellar vesicles, as a function of temperature for three different mixtures of DMPG–DSPC. (a) 1:1 DMPG–DSPC; (b) 30:70 DMPG–DSPC; (c) 20:70 DMPG–DSPC. The release data are compared to the specific heat profiles, C_P (solid lines).

limit is crossed and there is a steady increase of activation for increasing temperatures. The rapid decrease in activity above 50 °C is due to denaturation of the enzyme. For the other two mixtures in figures 7(b) and (c), the global surface charge density is below the critical value. Still, a strong activity is seen to occur for temperatures where the mixture is near the phase boundaries and in the coexistence region. This finding suggests that the bilayer mixture is heterogeneous and possibly forms domains and that the enzyme can sense this domain formation. Hence, local accumulation of the charged DMPG lipids in domains locally exceeds the critical limit, and the enzyme becomes activated.

Although $sPLA_2$ clearly requires Ca^{2+} ions as a cofactor, the calcium affinity is complicated and may even change during binding and activation of the enzyme at the lipid membrane interface [33]. When working with anionic lipid substrates, it is furthermore of importance to use low Ca^{2+}-ion concentrations in order to minimize structural changes in the lipid membrane substrate [34].

6. Activation by polymers

Somewhat surprisingly it was discovered some time ago that the introduction of lipopolymers, i.e. lipids with covalently attached PEG (poly-ethylene glycol) polymers as shown in figure 8(a), led to an enhancement of the action of $sPLA_2$ towards lipid vesicles [35, 36]. Subsequently, it was observed that part of this effect is due to a stronger binding of the enzyme to vesicles with lipopolymers that have a charge due to the use of phosphatidylethanolamine head groups in the lipopolymers. Still there was a remaining enhancement effect that scales with the length, N, of the polymer chain. This observation is substantiated in figure 8(b), which shows that the lag time of $sPLA_2$ for vesicles with the same composition of lipopolymers, i.e. with the same surface charge density, decreases for increasing polymer index N. Hence, longer polymers induce stronger activation.

A general theory was recently put forward to explain this phenomenon [37] in terms of the entropy driven process of enhancing the protrusions of lipids and lipopolymers out of the bilayer interface. The lipids are pulled out due to the gain in entropy of the polymer when it gets displaced from the confining membrane surface.

7. Activation as a trigger for drug release

The finding of enhanced $sPLA_2$ activation at lipid bilayer surfaces covered by polymers has been exploited in a new principle of liposome based drug delivery. Liposomes with

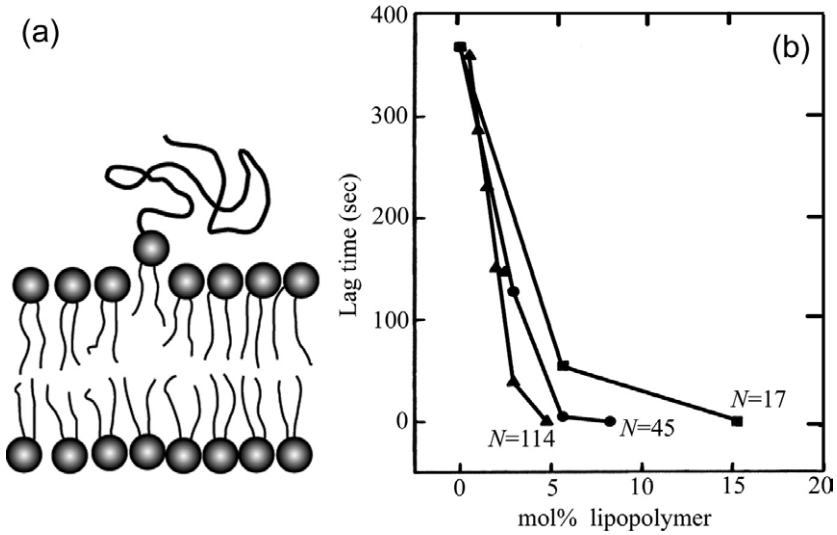

Figure 8. (a) Schematic drawing of a lipid bilayer with a single lipopolymer. (b) Lag time of $sPLA_2$ activation on DPPC lipid bilayers in the solid phase just below the phase transition temperature. Results are shown for different concentrations of lipopolymers of varying chain length, N.

lipopolymers are known to exhibit improved circulation time due to their screening from the macrophages [38, 39]. These so-called 'stealth liposomes' can therefore be poised to tune in at a particular $sPLA_2$ activity at a particular site in the body, provided that the relationship is known between enzyme activation, the molecular composition of the liposomal drug carrier, and the physico-chemical properties prevailing at the target.

It turns out that $sPLA_2$ is strongly upregulated in certain cancers and that this fact can be used as a trigger mechanism of unloading drugs from liposomes precisely at the tumour [40]. This principle is illustrated in figure 9, where the drug-loaded liposome (figure 9(a)) can be triggered by $sPLA_2$ to release its drug load. The superiority of this principle in drug release and reduction in cancer cell growth is documented in figures 9(b) and (c).

8. A unified picture of PLA_2 activation

The unifying picture that emerges from the range of experimental studies, some of which were reviewed above, is one where the physical properties of the lipid bilayer substrate is in control of the activation of $sPLA_2$. This is a highly unique situation for a case of enzymatic activity since it suggests that the chemistry of the involved molecular species is not the only controlling factor but the collective properties of the lipid assembly are at least as important.

In particular, the heterogeneous and small-scale lateral structure of the lipid bilayer appears to be important for the activation. Once this is established it holds promise for rational control of enzyme action by manipulating the properties of the lipids, e.g. via thermodynamic variables. It is noteworthy that this manipulation can be performed for a range of parameter values, e.g. temperature, where the intrinsic properties of the enzyme molecule are not expected to change. Finding correlations between enzyme activation on the one hand and different mesoscopic bilayer properties, defect patterns, fluctuations, domain formation, and heterogeneity on the other hand renders it conceivable that a single or few microscopic modes or underlying mechanisms have key responsibility for the activation.

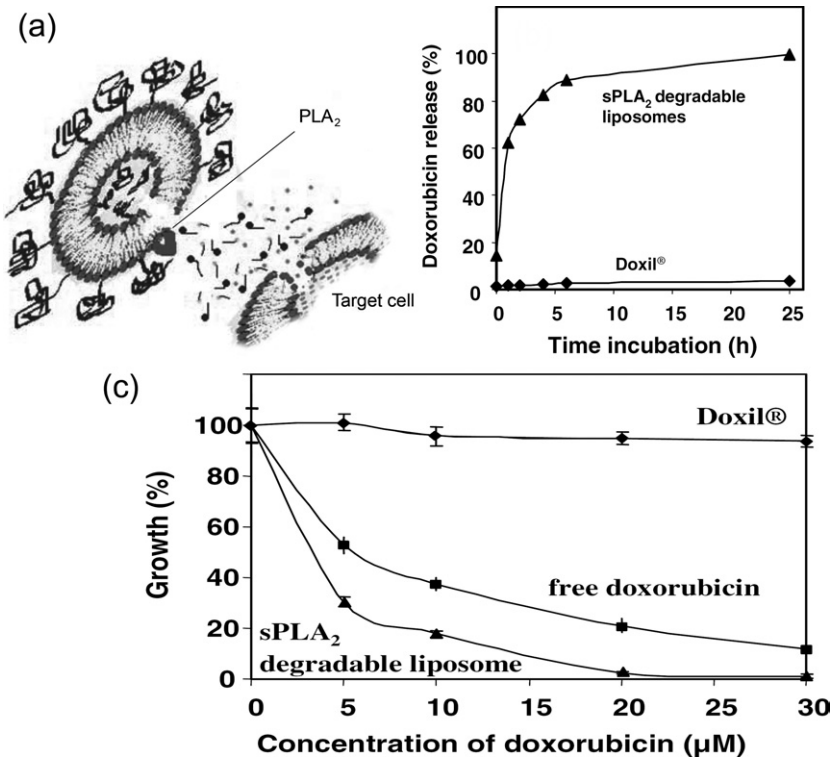

Figure 9. (a) Polymer coated liposome being attacked by $sPLA_2$ leading to a break-down of the liposome, unloading of the encapsulated drug, and release of hydrolysis products that furthermore can act as permeability enhancers at the target cell. (b) Data which show the superior release of drugs (in this case the anti-cancer drug doxorubicin) from liposomes designed to be sensitive to $sPLA_2$ action compared to conventional liposomes (Doxil). (c) *In vivo* efficacy of liposomes (designed to be sensitive to endogenous $sPLA_2$) for reducing the growth of colon cancer cells compared to the free drug (doxorubicin) and a conventional liposomal formulation (Doxil).

A functioning enzyme must be properly docked and have a lipidic phosphate at its active site. The topology of the enzyme thus identifies the substrate as a protrusion. A protrusion would be expected to enhance the activity since it facilitates the recruitment of the lipid molecule into the active site of the enzyme. Hence, a potential candidate for a key mechanism of activation is the protrusion of lipids out of the bilayer surface [16, 37], cf figure 10(a). Such protrusion modes have indeed been detected by molecular dynamics calculations as illustrated in figure 10(b) [16] and by dissipative particle dynamics simulations as illustrated in figure 10(c) [41].

Clearly, effects that increase the amplitude of the protrusions are expected to enhance the activity of the enzyme. An example of such effects is thermal fluctuations close to phase transitions and coexistence regions which tend to soften the bilayer rigidity, resulting in larger protrusions [42]. The range of experimental observations and simulations of $sPLA_2$ reported in the present review is consistent with this picture. Protrusions will be enhanced near phase transitions and when defects and heterogeneities occur. Moreover, the entropy driven polymer extraction of the lipids from the bilayer surface pictured in figure 8(a) is in itself a protrusion which will further enhance the protrusion modes of neighbouring lipids. Finally, the gradual accumulation of hydrolysis products in the bilayer also enhances the protrusions and the roughness of the bilayer surface as illustrated in figure 10(c).

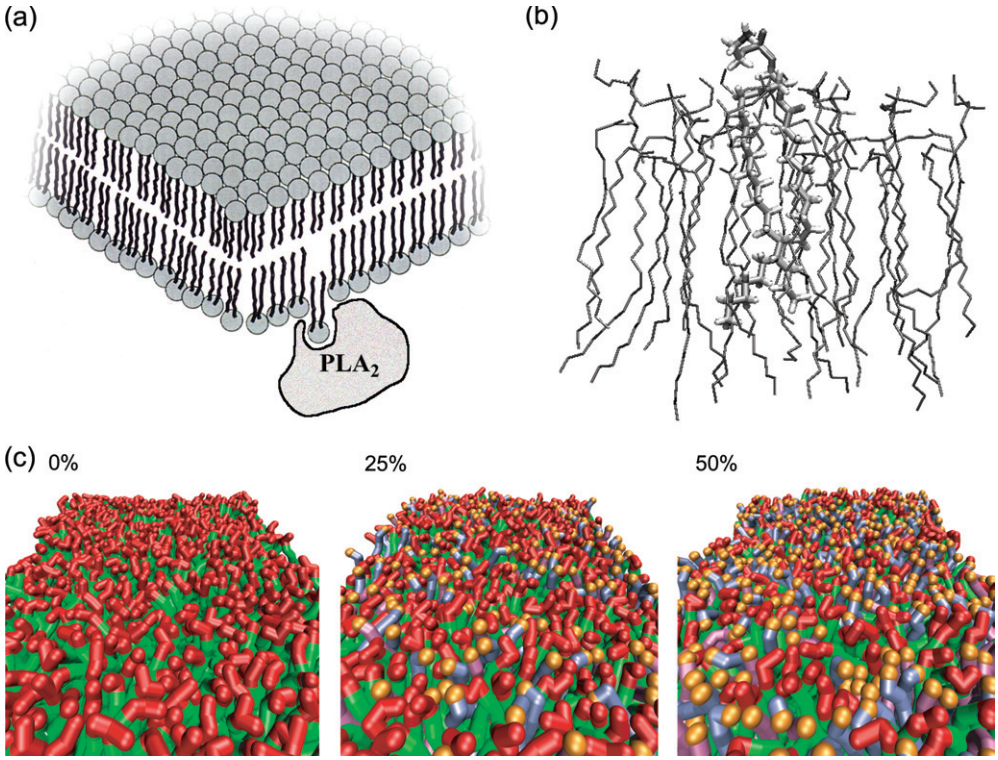

Figure 10. (a) Schematic drawing suggesting that a limiting step for activation of $sPLA_2$ is a protrusion of a lipid molecule into a pocket of the enzyme containing the active site. (b) Protrusion of a single lipid molecule as captured in an atomic-scale molecular dynamics simulation. (c) Surface structure of a lipid bilayer with varying amounts (molar per cent) of hydrolysis products in the upper monolayer leaflet. The results are obtained from dissipative particle dynamics simulations on a coarse-grained model.

All these observations and their relation to the measured activation of $sPLA_2$ strongly suggest that the $sPLA_2$ is activated by lipid protrusion modes.

Acknowledgments

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