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Role of lipid protrusions in the function of interfacial enzymes

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Abstract Secretory phospholipase A_2 ($sPLA_2$) is a class of interfacially active enzymes that selectively hydrolyze lipid molecules organized at interfaces like membranes. We present a simple theoretical model that relates the $sPLA_2$ action to the protrusions of the lipid molecules. The model explains (1) the observed enhancement of enzymatic activity by lipids with flexible, neutral, water-soluble polymers linked to their head groups and (2) the lag-burst kinetics of $sPLA_2$. It yields qualitative predictions of the effect of the initial composition of the membrane, the molecular weight of the polymer, and the composition of the hydrolysis products.

Keywords

Secretory phospholipase A_2 ($sPLA_2$) comprises a large family of small, 14–18-kDa, water-soluble enzymes that are found, for example, in venom, pancreatic, and cancerous fluids (Berg et al. 2001; Six and Dennis 2000; Singer et al 2002). $sPLA_2$ catalyzes the hydrolysis of diacyl glycerophospholipids in the *sn*-2 position, breaking the diacyl phospholipids down into lysolipids and free fatty acids. $sPLA_2$ is only active at the interface between water and organized assemblies of lipids, such as membranes. This suggests that the enzyme is sensitive to the physical properties of its substrate (Bell and Biltonen 1991; Høyrup et al. 2002; Raudino 1998). However the relevant properties and the mechanism of formation of an active lipid–$sPLA_2$ complex are yet to be identified.

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In this letter we suggest that the mechanism involves protrusion of lipid molecules from the surface of the lipid bilayer (Israelachvili and Wennerström 1990; Lipowsky 1995). Scattering data (White and Wiener 1996) as well as atomic-scale molecular dynamics simulations (Tieleman et al. 1997; Høyrup et al. 2004) show that lipid molecules make vertical excursions of up to 0.2 nm on the time scale of tens of picoseconds. We propose that these protrusions allow the lipid substrate to access the active site of the enzyme. The protrusion-based model rationalizes many experimental observations, including the distinctive "lag-burst" kinetics of $sPLA_2$ and the enhancement of enzymatic activity in the presence of lipids functionalized by the water-soluble polymer poly(ethylene glycol) (PEG). It leads, as we shall discuss, to predictions concerning the composition of lysis products, the effect of the molecular weight of PEG chains and of the initial composition of the membrane. Our discussion involves an analysis of the protrusions of PEG lipids. As we shall see, these differ from the protrusions of nonfunctionalized lipids in being equilibrium features rather than fluctuations. In turn, this gives rise to a modification of the density profile of the membrane that can be studied by neutron reflectometry. The protrusion hypothesis is of fundamental interest since it relates membrane physics to enzymology. It is also of practical interest in the optimization of the design of "stealth" liposomes (Lasic and Papahadjopoulos 1998), incorporating PEG lipids, to achieve controlled release of drugs by utilizing the fact that $sPLA_2$ is upregulated in cancer tissue (Jørgensen et al. 2002).

The enzymology of $sPLA_2$ is complex (Berg et al 2001; Zhou and Schulten 1996). The following items are essential:

1. To become active the enzyme has to bind to the membrane. Since $sPLA_2$ is positively charged the binding to anionic lipids is stronger than to zwitterionic lipids (Singer et al. 2002). The bound enzyme covers a patch comprising 30-40 lipids.

2. Once bound, the enzyme undergoes a "scooting" lateral motion across the surface.
3. The structure of the enzyme–membrane complex is currently unknown. On the basis of crystallographic studies, $sPLA_2$ is described as a small kidney-shaped protein ($2.2\times3.0\times4.2$ nm^3 for the case of bovine pancreatic $sPLA_2$). The "entrance" to the active site is situated at the "docking surface" or "i-face" that is presented to the membrane in the bound state. The docking surface is rather flat and the active site is located roughly 1.5 nm above the membrane (Berg et al. 2001). While the docking surface is flat, it does exhibit irregularities, among them slits connecting the active site to the periphery (Zhou and Schulten 1996).
4. The enzymatic kinetics deviates strongly from the familiar Michaelis–Menton scheme. Typically, the $sPLA_2$ displays lag-burst behavior, characterized by a lag time, τ, during which the enzymatic activity is low, followed by a burst of high activity. The value of τ depends on the reaction conditions, membrane composition and temperature (Bell and Biltonen 1991; Høyrup et al. 2002).

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. Clearly, effects which increase the amplitude of the protrusions are expected to enhance the activity of the enzyme. An example of such effects are thermal fluctuations close to phase transitions that soften the bilayer rigidity (Lemmich et al 1995; Chen et al. 1997) resulting in larger protrusions (Lipowsky 1995). Experimental observations and simulations of $sPLA_2$ are consistent with this picture (Høyrup et al. 2002; Hønger et al. 1996).

A more stringent test of the protrusion hypothesis concerns the hydrolysis of lipids incorporated into stealth liposomes. These liposomes contain lipids with a head group linked to a PEG chain. The presence of the lipopolymers prolongs the circulation time of the stealth liposomes in the blood, thus improving their performance as drug delivery vehicles (Lasic and Papahadjopoulos 1998; Jørgensen et al. 2002). The beneficial effect of the PEG lipoproteins is attributed to a repression of protein adsorption leading to slower clearing by the immune system. Accordingly it was expected that the PEG coat on these liposomes should screen the surface from attack by $sPLA_2$. Surprisingly, the presence of the lipopolymers actually enhanced the enzymatic activity (Vermehren et al. 1998), thus leading to two independent questions. First, why does the PEG layer not repress the $sPLA_2$ activity? Second, why does it enhance the $sPLA_2$ activity? The answer to the first question is straightforward. Brushes of terminally anchored PEG chains may repress protein adsorption because of the osmotic penalty incurred by the protein in the immediate vicinity of the surface (Halperin 1999). However, in the systems considered here this penalty is smaller than k_BT and is thus negligible. This is due to two factors: $sPLA_2$ is rather small and the surface concentration of the PEG chains corresponds to the "mushroom" regime i.e., below the overlap threshold. To explain the enhancement of the $sPLA_2$ activity by PEG lipids we need to invoke protrusions and their stabilization by the PEG chain.

A lipid may protrude out of a planar surface thus incurring a penalty αz, where z is the distance that the head group extends out of the surface and α is the corresponding energy penalty per unit length. The density of protrusions of distance z is $\rho(z) = \rho(0)\exp(-\alpha z/k_BT) = \rho(0)\exp(-z/\lambda)$, where $\lambda = k_BT/\alpha$ is the protrusion decay length. α varies between 1.5×10^{-11} and 5×10^{-11} J m^{-1} at 25°C, i.e., roughly $10k_BT$ per nanometer. The corresponding λ varies from 0.08 to 0.3 nm, e.g., $\lambda = 0.14$–0.26 nm for dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine and $\lambda = 0.17$–0.34 nm for egg phosphatidylcholine (Israelachvili 1992). The average protrusion height is $\langle z\rangle = \int_0^\infty z\exp(-z/\lambda)\mathrm{d}z \Big/ \int_0^\infty \exp(-z/\lambda)\mathrm{d}z = \lambda$. The situation is qualitatively modified when some of the lipids serve as anchors for water-soluble polymers. It is then necessary to distinguish between two populations: simple lipids and lipopolymers. The simple lipids undergo protrusion fluctuations as described before. In marked contrast, the lipopolymers are expected to protrude in equilibrium as illustrated in Fig. 1a.

The partition function of an ideal chain anchored to a point located at altitude z above an impenetrable planar surface is $Z_a = q_N\mathrm{erf}(z/2R_0) \approx q_N(1/\pi)^{1/2}z/R_0$ (Eisenriegler 1993), where the chain is inscribed on a lattice with coordination number q. N is the degree of polymerization and $R_0 \approx aN^{1/2}$ is the span of the ideal chain. The approximation is valid when $z \ll R_0$ and $\mathrm{erf}(y) \approx 2y/\sqrt{\pi}$. In our case, this limit is obtained because the maximum value of z is the length of the lipid tail. The loss of entropy of a chain at altitude z is accordingly $\Delta S = k_B \ln Z_a/Z = k_B \ln z + \mathrm{const}$, where $Z = q^N$ is the partition function of an unconstrained chain. ΔS reflects the reduction in the number of allowed

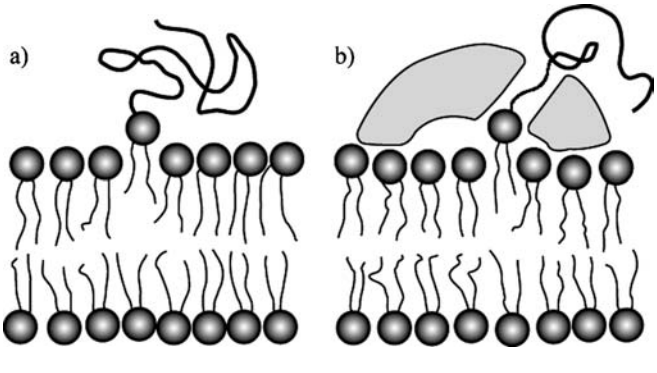

Fig. 1 Schematic illustration of a protruding lipopolymer incorporated in a lipid bilayer before (**a**) and after (**b**) binding of a secretory phospholipase A_2 ($sPLA_2$) enzyme to the membrane surface. After binding and the venture of the lipid into the active site of the enzyme, part of the polymer moiety is confined to a narrow slit or "chimney" in the enzyme

chain configurations by the proximity of the impenetrable membrane. The equilibrium protrusion height is thus specified by the minimum of the free energy $F \approx \alpha z - k_B T \ln Z_a/Z \approx \alpha z - k_B T \ln z + \text{const}'$. The minimum condition $\partial F/\partial z = 0$ yields the equilibrium height $z_{eq} \approx k_B T/\alpha = \lambda$, where λ is identical to the protrusion decay length of simple lipids. The equilibrium character of the protrusions of PEG lipids is expected to modify the density profile of membranes on a solid support. This effect can be studied by neutron reflectometry.

The previous argument concerns the effect of PEG chains on protrusions from a "free membrane", in the absence of bound $sPLA_2$. However, the effect of the PEG lipids on the kinetics of $sPLA_2$ involves lipids within the patch covered by the bound enzyme. In this situation, the PEG chains are confined by the bound $sPLA_2$ and our earlier analysis requires modification. A full discussion of this case requires detailed knowledge of the structure of the enzyme–membrane complex that is currently lacking. To obtain a rough idea of the essential physics we recall that the docking surface contains slits connecting the binding site to the periphery of the complex. This suggests that the PEG chain can escape confinement by the docked enzyme by entering such a chimney (Fig.1b). To be specific, we picture the chimney as a narrow cylindrical capillary of radius r and length $R_p < R_0$. The PEG section close to the head group is thus confined to the capillary, while the distal part dangles out. As before we consider PEG as an ideal chain and the confined section is viewed as a sequence of n_b penetrable blobs of size $r \approx g^{1/2} a$ each comprising g monomers (Rubinstein and Colby 2003). The n_b blobs undergo a one-dimensional random walk within the capillary i.e., $R_p \approx n_b^{1/2} r$ or $n_b \approx \left(R_p/r\right)^2$. The confinement free energy, as obtained by assigning $k_B T$ to each blob, is $F_{conf}/k_B T \approx 1 + \left(R_p/r^2\right)$, where the 1 accounts for the single blob formed by the distal, dangling segment. The confinement energy as given by the previous equation does not allow for the effect of the impenetrable surface presented by the membrane and the bound enzyme. Since the dangling segment comprises $N - n_b g$ monomers and is anchored at a height asymptotically equal to R_p above the membrane, a rough estimate of this effect is $-T\Delta S = -k_B T \ln \left[R_p \Big/ \left(Na^2 - R_p^2\right)^{1/2}\right] +$ const' . We now argue that a protrusion of height z displaces the proximal end of the chain a distance z along the capillary, thus lowering the confinement penalty to $F_{conf}/k_B T \approx 1 + \left(R_p - z^2\right)/r^2$. The equilibrium value of z is determined by $\partial F/\partial z = 0$ with $F = \alpha z + k_B T \left(R_p - z\right)^2 \Big/ r^2 - k_B T \ln \left[R_p \Big/ \left(Na^2 - R_p^2\right)^{1/2}\right] +$ const' . The confinement increases the amplitude of the protrusions to $z_{eq} \approx R_p - r^2/2\lambda$.

In the two situations considered, free lipopolymers and lipopolymers confined to a finite capillary, the polymer favors protrusions so as to lower the loss of configurational entropy. The real situation may well be intermediate between the two extreme situations considered. However, in both cases the protrusions of the lipopolymers are characterized by an *equilibrium* position $z_{eq} \geq \lambda$ above the membrane. In contrast, for simple lipids the equilibrium state corresponds to $z_{eq} = 0$ though fluctuations lead to an average $\langle z \rangle = \lambda$. The two situations differ in the population distributions. Most lipopolymers would protrude to $z_{eq} \approx \lambda$, while the fraction of simple lipids that protrude to λ is $1/e \simeq 0.37$. The kinetics of the enzymatic reaction is determined by the population distribution of the protrusions, not the average value of z. Furthermore, the lifetime of the protrusion of simple lipids is in the range of tens of picoseconds (Høyrup et al. 2004), thus limiting their accessibility to enzymatic attack. The protrusions of PEG lipids, being equilibrium phenomena, do not suffer from this dynamic problem. Within our model these features explain the enhanced $sPLA_2$ activity in the presence of lipopolymers. A qualitative prediction that follows immediately is that the reaction products will contain PEG lysolipids.

To understand the origins of the lag-burst kinetics of $sPLA_2$ it is necessary to consider the time dependence of the membrane composition. We further assume that (1) the lysis products are retained within the membrane and their fraction within the layer, ϕ, increases up to a saturation value $\phi_{max} \ll 1$ (Høyrup et al. 2001) and (2) the protrusion penalty α decreases with increasing ϕ. In time, the hydrolysis products accumulate within the membrane. This enhances the protrusions of the simple lipids, thus rendering them more susceptible to attack by the enzyme. To implement this assumption we choose the simplest mean-field form $\alpha = \alpha_0(1-\phi) + \alpha_h \phi = \alpha_0 - \phi\Delta\alpha$, where α_0 corresponds to the pure unhydrolyzed layer. $\alpha_h < \alpha_0$ is the coefficient for the effect of small amounts of hydrolyzed product and $\Delta\alpha = \alpha_0 - \alpha_h > 0$. In our model $sPLA_2$ reacts with protruding lipids when $z > z_c$, where z_c is related to the position of the hydrolyzable bond in the lipid with respect to the active site. This picture recovers the observed sigmoid form of the rate of the enzymatic reaction. Within the picture the number of reactive lipids is roughly $\rho(z_c) = \rho(0)(1-\phi)\exp\left[-(\alpha_0 - \phi\Delta\alpha)z_c/k_B T\right]$. It is thus possible to distinguish three regimes as ϕ increases: (1) initially, while $\phi\Delta\alpha z_c/k_B T \ll 1$, the reaction is very slow; (2) an onset of the burst occurs when $\phi\Delta\alpha z_c/k_B T \approx 1$; and (3) late-time plateau behavior when ϕ tends to ϕ_{max} and the hydrolyzed fraction within the layer saturates. Note that the local concentration of lysis products may initially exceed ϕ. This effect is not included in our mean-field argument and may play a significant role at short times scales. The mean-field picture is in qualitative accordance with experimental observations (Bell and Biltonen 1991; Høyrup et al. 2002). It is also consistent with the observation that the lag phase can be eliminated if the bilayer is preincubated with hydrolysis products (Bell and Biltonen 1991). It suggests that the lag time τ can be tuned by preparing membranes containing a varying fraction of lysis products.

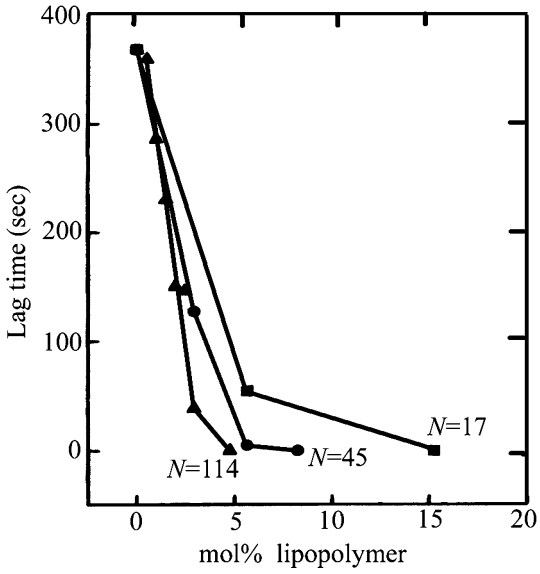

Fig. 2 Experimental data for the activity of snake venom $sPLA_2$ on fluid-phase lipid bilayers composed of dipalmitoyl phosphatidylcholine incorporated with various amounts of negatively charged lipopolymers dipalmitoyl phosphatidylethanolamine–poly(ethylene glycol)$_N$ for three different polymer lengths, $n = 17$, 45 and 114. The activity is measured in terms of the lag time, τ, shown as a function of the molar concentration of lipopolymer. Adapted from Jørgensen et al. (1999)

Altogether, the activity of $sPLA_2$ requires two ingredients: binding to the membrane and protruding lipid molecules. The first condition is promoted by negative charges on the membrane, while the second is enhanced by the presence of lipolymers. The two contributions are coupled when the head group of the lipopolymer is negatively charged. Within our model, the activity of $sPLA_2$ is expected to be independent of N for a constant fraction of lipopolymers and constant membrane charge. This trend is indeed observed experimentally for negatively charged lipopolymers (Jørgensen et al. 1999) when N is sufficiently large to ensure $z/R_0 \ll 1$ (Fig. 2).

Use of lipopolymers with neutral headgroups results in lower $sPLA_2$ activity compared with the same system with negatively charged head groups (Andresen et al. 2002). This demonstrates the crucial role of electrostatic interactions in promoting binding to the membrane. Finally, in optimizing the conditions for $sPLA_2$ function it is important to note that the maximal attainable fraction of lipopolymers decreases as N increases (Davidsen et al. 2001).

Hitherto, lipid protrusions were used to explain mesoscopic membrane properties, like interlamellar forces (Israelachvili and Wennerström 1990) and bilayer elastic bending modules (Lipowsky and Grotehans 1993). However, neither the biochemical role of protrusions nor their enhancement in lipopolymers was considered previously. In this letter these two ingredients were combined in a simple theoretical model explaining the distinctive kinetics of the interfacial enzyme $sPLA_2$ and their modification by PEG lipids. It yields a variety of predictions concerning the effect of the molecular weight of the PEG chains, the composition of the reaction products and the initial composition of the membrane. Our discussion also predicts a modification of the density profile of membranes incorporating PEG lipids. The model, while speculative, is able to explain a wide range of experimental data. In addition to the effects discussed, it can also explain the kinetics of $sPLA_2$ acting on membranes on a solid support (Kaasgaard et al. 2001; Granbois et al. 1998; Nielsen et al. 1999). A full quantitative understanding of $sPLA_2$ kinetics requires the clarification of the structure of the bound enzyme, the equilibrium and kinetics of enzyme docking, the dynamics of lateral motion of the bound enzyme, and its coupling to the hydrolysis reaction.

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