Department of Biomedical Engineering and Computational Science

PLA2 Interfacial Activation on Lipid Interfaces Promoting Fibril Formation

Christian Code

Aalto University

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Christian Code

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Abstract

Phospholipase A2 is a widely studied protein found ubiquitously in nature. The protein interacts with the lipid membrane cleaving the phospholipid at the sn-2 position. The interfacial activation mechanism is characterized by a low activity on monomeric substrates but a greatly enhanced activity on aggregated substrates. Several of our papers have elucidated the complex interfacial activation mechanism of this protein from different angles in two very diverse scientific frames: from the view of the lipid and the view of the protein. In this thesis the interfacial activation mechanism of PLA2 is understood to behave in a temporal sequence of events first by binding, dimer formation, oligomer formation and finally by the formation of amyloid-like fibrils.

Keywords Liposome, PLA2, Peptide, Phospholipid, Amyloid, Protein-Lipid Interaction, Dimer, Temporin B, Heat-Shock Protein, Oxidized Phospholipids

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II. Code C, Domanov YA, Killian JA, Kinnunen PK. Activation of phospholipase A(2) by temporin B: Formation of antimicrobial peptide-enzyme amyloid-type cofibrils. Biochim Biophys Acta. 2009 May;1788(5):1064-72.

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*equal contribution

Author Contributions

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Christian Code conducted the experiments and prepared the figures. He also wrote the second draft of the manuscript and was responsible for most of the referencing. Christian Code wrote the very first draft of the comments for reviewers.

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Christian Code contributed to the design and execution of the experiments, analysis of the data, prepared the figures, wrote the very first draft of the manuscript, and was responsible for most of the referencing. Christian Code wrote the very first draft of the comments for reviewers.

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Christian Code contributed to the early phase of the study and the writing of the very first draft of the manuscript.

Abbreviations

ATP	Adenosine-5' triphosphate
DIC	Differential interference contrast
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine (Lamellar, L)
FFA	Free fatty acid
FRET	Förster resonance energy transfer
FTIR	Fourier transform infrared spectroscopy
γ	Lag time
IBS	Interfacial Binding Site
LUV	Large unilamellar vesicles
lysoPC	1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (HI Lipid)
PA	Palmitic acid (Free Fatty Acid)
PEG	Polyethylene Glycol
PKC	Protein Kinase C
PLC	Phospholipase C
PLA2	Phospholipase A2
PLA2A	PLA2 labeled with Alexa568 (acceptor)
PLA2D	PLA2 labeled with Alexa488 (donor)
PoxnoPC	1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine
proPLA2	Zymogen of porcine pancreatic PLA2
OA	Oleic Acid (Free Fatty Acid)
RFI	Relative Fluorescence Intensity
r	Anisotropy
τ	Fluorescence lifetime
T_m	Phospholipid main phase transition temperature
temB	Temporin B
ThT	Thioflavin T

All amino acids are abbreviated either using one letter or three letter code system.

1. Introduction

Phospholipase A2 (PLA2) hydrolyzes sn-2 fatty acyl ester bonds of phospholipids to release free fatty acids and lysolipids (Six,D.A. 2000;). This ubiquitous enzyme is present in both intracellular, extracellular and secreted forms and resides for example in the pancreas and in the venoms sacs of insects and animals (Six,D.A. 2000;). The central role of the secreted phospholipase from venoms is to destroy the membrane, producing lysolipids and free fatty acids. One characteristic of PLA2 is the interfacial activation whereby the protein's activity is dramatically enhanced on phospholipid interfaces when compared to monomeric substrates (Pieterson,W.A. 1974; Verger,R. 1973;).

The interfacial activation mechanism of PLA2 was studied in the present work using fluorescence spectroscopy and microscopy techniques. We showed interfacial activation on DPPC membranes, where PLA2 forms dimers and higher order oligomers, with oligomers being inactive functional amyloid-like fibers. The literature review briefly describes 1) the lipid and lipid products obtained after this reaction 2) the protein and briefly, 3) functional amyloid formation. Finally, the factors known to specifically affect the PLA2 interfacial activation mechanism are covered in more detail and are a central theme in this thesis.

2. Review of Literature

2.1 Overview of biological membranes and interaction with proteins

Phospholipase A2 is an ubiquitous enzyme that cleaves one lipid chain from phospholipid to remodel biological membranes. Cell membranes are two-dimensional arrays of glycoproteins, glycolipids, embedded proteins and an astounding number of different kinds of lipids (Singer & Nicolson, 1972) with some forming domains (Fig. 2.1). Phospholipids contribute a great deal to the structure and function of the bilayer assembly (Reinert,J.C. 1970;) and proteins like PLA2 liberate free fatty acids and change the membrane architecture.

As lipids are a main and dynamic component of the plasma membrane and a component of the membranes of organelles they are of great interest to study. Lipids are amphipathic and do not form covalent links with other lipids to make a bigger structure. Only a small amount (10^{-10}M) of lipids is needed to make supramolecular assemblies like bilayers when present in aqueous solution (Tanford, C. 1980;). These supramolecular assemblies display a wide array of polymorphism depending on the size and shape of the lipid (Cullis,P.R. 1979;). Lipids order themselves in an array showing the broadest range of polymorphism of any known class of biological molecules viz. liquid crystalline, gel, and liquid disordered, liquid ordered phases as well as sponge, cubic phases and HII phases (for review see Gruner,S.M. 1985 and Caffrey,M. 1989;).

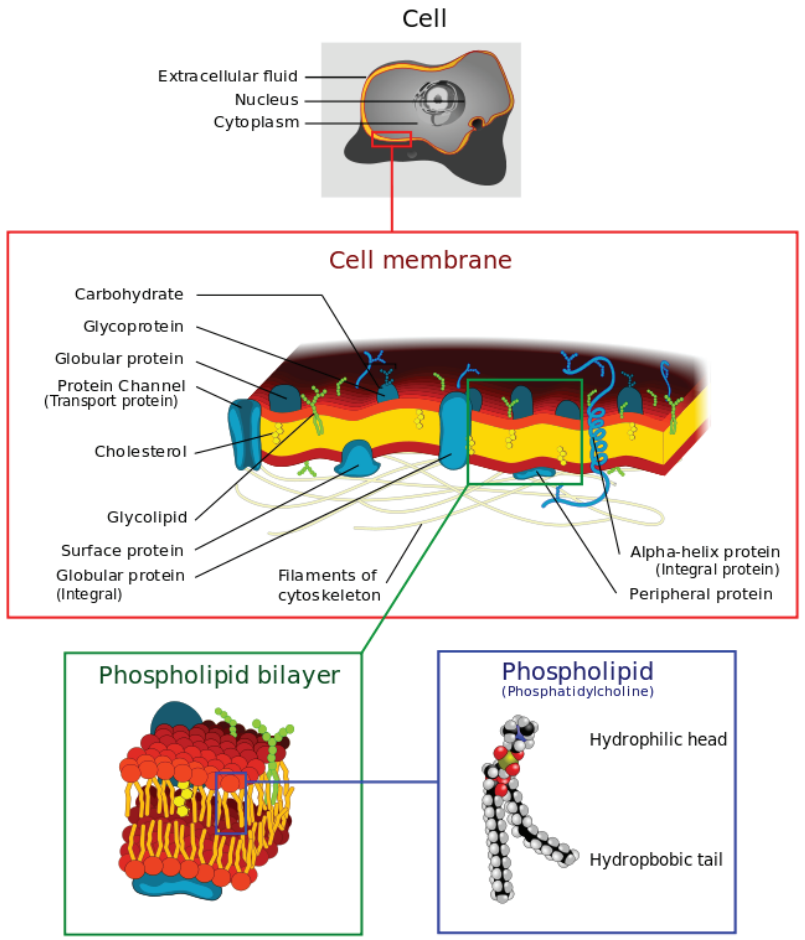

Figure 2.1 View of biological membrane with different lipids, cholesterol, membrane proteins and cytoskeleton from the cell to an individual phospholipid. (Image provided with creative commons license: Cell_membrane_detailed_diagram_3.svg licensed with Cc-by-sa-3.0 2008-06-13T14:41:45Z Dhatfield)

Dynamic modifications occur on the cell membrane with new lipids, proteins and other molecules added, removed or changed in the bilayer interface. Water soluble enzymes viz. lipase, sphingomyelinase, phospholipase modify the lipid membrane by breaking the lipid into smaller components at the interface. Phospholipase A2 (PLA2), the protein that is used in this work cleaves the phospholipid at the sn-2 chain ester bond into lysolipid and free fatty acid. PLA2 is found in almost every cell and is present in

venoms and other secretions and contains many different isoforms sharing structural or genomic homology (Six,D.A. 2000). The hydrolytic function and specificity to particular phospholipids slightly changes depending on the PLA2 enzyme being discussed. The ubiquity of this enzyme shows the importance of a modification of the membrane in biology.

The secreted PLA2 first binds to the membrane and is coordinated with calcium. PLA2 is known to be optimally activated on aggregated substrates through an interfacial activation mechanism whereby the activation could proceed through a change in conformation of the enzyme (enzyme model) (Verger, 1973) or a change in the state of the aggregated interface (substrate model) (Wells 1974; Verger 1973). The physicochemical properties of the interface are key determinants in understanding the mechanism of interfacial activation in the latter model.

Studies describing the interfacial activation mechanism of PLA2 are discussed from different view points of the lipid membrane and from the enzyme. This work makes use of several biophysical techniques to understand the overall mechanism of PLA2 interfacial activation.

2.1.1 DPPC and PLA2 Lipid Products

Several soluble proteins in nature modify the membrane such as PLA2, PLD, PLC, and sphingomyelinase, where their properties are designed to interact with the membrane and subsequently modify it. An interesting attribute of the lipid modifying enzymes is that they are water soluble but modify water insoluble substrates which are often aggregated to make a 2-dimensional interface (Roberts,M.F. 1996;). In order to understand the interfacial activation mechanism of PLA2, it is necessary to first understand the properties of the lipids they modify and the properties of the products that are produced upon this modification.

In order to study closely the temporal sequence of events involved in the kinetics of PLA2, we used DPPC as a model interface, as its physicochemical properties are well characterized and it can be used in the laboratory around physiological temperatures (Heimburg, 1998). A DPPC bilayer in liposomes allowed us to also understand the lag-burst kinetics (covered in 2.3) which is tied to this phase transition temperature with a shortening of the lag phase that occurs closer to the phase transition temperature (Apitz-Castro, 1982; Lichtenberg 1986; Hoyrup, 2001;).

The products of PLA2 hydrolysis of DPPC are lysolipids and free fatty acids (structures are shown in Fig. 2.2). In addition to the polymorphic changes these structurally diverse lipids induce on the membrane, the products of PLA2 have potent effects on the cell. LysoPC for example, with its long chains (C18:0) has been proposed to act as second messenger for the cytokine interleukin 6 (IL-6) from the rat anterior pituitary gland (Spangelo,B.L. 1996;). On the other hand, the other products, the free fatty acids (FFA), have a different physiological role. Long chain fatty acids e.g. arachidonate and linoleic acid, are the precursors to prostaglandins (twenty-carbon fatty acid derivatives containing a five-carbon ring), thromboxanes (six membered ether ring) and leukotrienes (with three conjugated double-bonds). These compounds, eicosanoids, are local hormones involved in a number of processes including: stimulation of inflammatory responses, inhibition of lipolytic effects, regulation of blood flow, and modulation of synaptic transmission (Smith,W.L. 1991;).

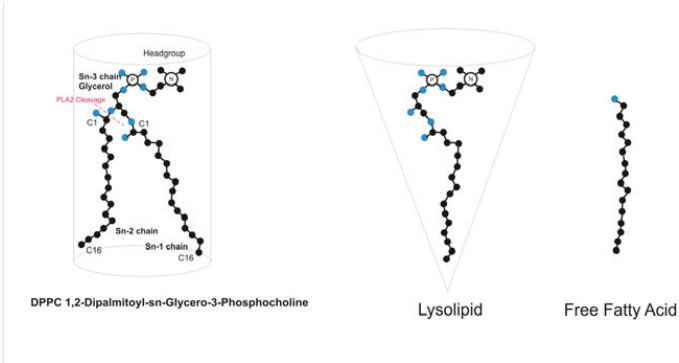

Figure 2.2 Structure of DPPC and the products of PLA2 hydrolysis Lysolipid and Free Fatty Acid. The cylindrical shape of DPPC with equal diameter head and tail moieties allows it to spontaneously form lamellar bilayers. The cone shape of lysolipids makes them form microdomains at the surface of liposomes or micelles.

2.1.2 Use of Liposomes as a Biomembrane Mimetic

Several different interfaces have been used to understand the nature of the interfacial activation of PLA2 including large unilamellar vesicles (LUV) (Bell 1989), monolayers (Verger, 1973), SUV (Menashe, 1986) , GUV (Sanchez, 2002) and micelles (Hille, 1983). In this work we used liposomes as a model system. Understanding the function of PLA2 in nature with a large number of lipids, multiple proteins and various conditions is hampered by many confounding effects. For a good understanding of models and model systems for lipid research some well laid out thoughts of membrane models are shown in Chapter 7.1 of Life as a Matter of Fat (Mouritsen,O.G. 2005;).

A LUV can serve as a membrane model. A good model can be compared with the physicochemical properties of a living system's membrane like the phase transition (Reinert,J.C. 1970;) or the effect of lipid order (Heiner,A.L. 2008;). In order to find direct evidence of protein-protein interaction, model membrane LUVs were employed in this work to provide a simplified and efficient tool for experiments.

Multilamellar vesicles are formed spontaneously after hydration of the dry lipid film (Mayer,L.D. 1986;). The unilamellar vesicles provide a mimetic for the cell membrane as they enclose an intravesicular aqueous environment with only one surrounding bilayer (Bangham,A.D. 1965;). Liposomes can be prepared efficiently by extrusion through 100 nm filters using syringe pistons giving a homogeneous monodispersed size of 50-200 nm (Mayer,L.D. 1986; Hunter,D.G. 1998;). The size can simply be assessed by techniques like dynamic light scattering (DLS) (Hunter,D.G. 1998;) (as we used). Also CryoEM can be used to assess the size, shape and unilamellarity of the liposomes (Almgren. M 1996).

Several different types of aggregated lipid systems could have been used to understand the mechanism of PLA2, however, LUVs proved to be the best choice. The effects of surface charge, packing, hydration, lipid specificity and phase transition temperature play a role when comparing the different systems (Kensil,C.R. 1981; Reynolds,L.J. 1991;). A LUV system containing only DPPC or one other lipid allows for better control of the system's parameters.

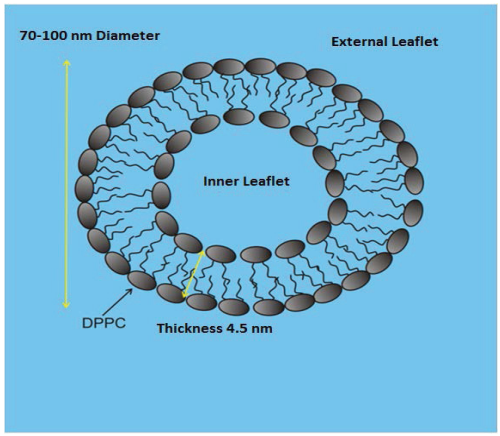

Figure 2.3 Liposome composed of DPPC. The bilayer thickness at physiological temperature (full review of DPPC liposome's in Heimburg T. 1998) and the diameter of the liposomes after extrusion in buffer (measured with DLS in the Kinnunen lab after extrusion (data not shown))

2.2 Phospholipase A2

The venom PLA2s are readily available and exist in various forms among the various species of snakes and insects. Bee Venom PLA2 from the European Honey Bee (Apis mellifera) can be bought in large quantities. Although its form is similar to other groups in the classification superfamily of PLA2s, bee venom phospholipase is unique in both its structure and sequence (Kuchler,K. 1989; Six,D.A. 2000;). The bee venom PLA2 is stored in the sacs of honey bees with citrate which keeps it in an inactive form (Fenton,A.W. 1995;). After a bee sting the protein and the antimicrobial peptide melittin (Habermann E. & Neumann 1957) are secreted and subsequently activated in the presence of calcium and a phospholipid membrane to yield lysolipid and free fatty acid.

Bee venom PLA2 is a 14 kDa, 134 aa protein that contains one domain. It is a glycoprotein containing one asparagine-linked oligosaccharide and contains five disulfide bonds (Shipolini,R.A. 1971;). More information on the catalytic sites is available on Swiss Prot Expasy server (Appel, 1994).

In addition to its catalytic activity, bee venom PLA2 may have myotoxic or neurotoxic effects likely due to a hydrophobic region of amino acid residues near the C-terminus of the protein {{368 Kini,R.M. 1986; }}. These regions have been implicated in the binding to N-type (neuronal type) and M-type (found in smooth muscle) receptors (reviewed by Lambeau,G. 1999;). The N-type receptors are proposed to mediate the physiological, pathophysiological, and toxic effects. They are special because they bind bee venom PLA2 but not pancreatic nor inflammatory type PLA2s (Nicolas,J.P. 1997;).

Studies have shown bee venom PLA2 to prevent infections viz. inhibiting the malaria parasite development (Moreira,L.A. 2002;), suppressing adenovirus (Mitsuishi,M. 2007;), and inhibiting the entry and thereby replication of human immunodeficiency virus (HIV) in host cells. These effects may be independent of its catalytic activity and

are most likely related to bee venom PLA's ability to bind to N-type receptors (Fenard,D. 1999;218 Fenard,D. 2001;).

The main structural features pertaining to the hydrolytic action of the protein are: 1) the interfacial binding site (IBS) 2) the calcium binding loop and 3) the Asp-His dyad catalytic site (Scott, 1990). Other interesting parts of the PLA2 protein are the elapid loop, the C-terminal sequence and potential phospholipid binding sites. The whole protein will not be covered in this thesis but for a more detailed view of bee venom PLA2 one can consult Scott (Scott,D.L. 1990;) and for a structural comparison with other PLA2s the review by Arni et al. (Arni, 1996).

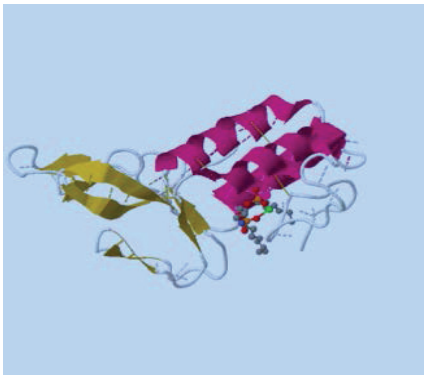

Figure 2.4 Crystal Structure of Bee-Venom PLA2 In a Complex With A Transition-State Analogue (1-O-Octyl-2-Heptylphosphonyl-Sn-Glycero-3-Phosphoethanolamine). The one domain bee venom phospholipase (apis mellifera) from group III is structurally similar to group I and II but different in sequence (Six, 2000, Scott 1990). Bee venom PLA2 has five disulphide bonds, two antiparallel alpha-helices held together by hydrogen bonds (residues 25-37 and residues 61 -74), which make a hydrophobic channel, and two antiparallel beta sheets (101-105 & 113-116) that make a hydrophobic pocket (Nicolas,J.P. 1997). The functional portions are a calcium binding loop (8-12) and the catalytic region containing a His-Asp diad (34, 64) with Tyr-87. The C-terminus is unstructured and proposed to have functional consequences if deleted (Kini, 1986). Image from the RCSB PDB (www.pdb.org) of PDB ID 1POC (D.L. Scott, 1990)

2.3 Phospholipase A2 Interfacial Activation Mechanism

The binding of PLA2 to the lipid interface is known to occur rapidly (Menashe, G. 1986; Lichtenberg 1986) and the lipid interface may modulate effective binding. These conditions also vary depending on the PLA2 enzyme used. Bee venom PLA2 binds with a non-electrostatic mechanism (Bollinger, J.G. 2004; Ghomashchi,F. 1998; }} and may not insert itself in the membrane like other PLA2s (Pande 2006). It is generally accepted that 10-20 amino acids of PLA2 bind to tens of phospholipids on the surface of the membrane (Lin 1998, Bollinger 2004).

Two non-exclusive complementary models explain the interfacial activation mechanism. The enzyme model implies that activation of the enzyme involves changing the conformation of the enzyme (Verger, 1973). On the other hand, the substrate model states that the binding is dependent upon the organization of the substrate which largely depends on the physicochemical properties of the interface (Op den Kamp 1974). This initial rate of activation is dictated by dynamic structural fluctuations associated with the vesicle being in the vicinity of its gel-liquid crystalline phase transition region. These 'cracks' between the gel phases allow the protein to penetrate easily into the lipid surface (Op den Kamp,J.A. 1975; Op den Kamp,J.A. 1974;). The lipid composition and physicochemical properties of the bilayer affect the poorly defined 'quality of the interface'. The interface is affected by: membrane fluidity (Op den Kamp, 1974), curvature (Bell,1989; Leidy, 2004), lipid packing densities (Lehtonen, 1995), surface charge (Thuren, 1987;), phase (Burack 1993; Hoyrup 2001), which are key determinants in understanding the mechanism of interfacial activation.

Additionally, PLA2 is said to undergo conformational changes, together with the physical state of the substrate, causing an augmented catalytic activity (Tatulian 1997, Tatulian 2001, Tatulian 2003). This augmented activity could result from dimerization or higher order oligomers which is suggested by Hille et al. with a concomitant increase in PLA2 activity and molecular weight of the protein-substrate complex on micellar substrates (Hille,J.D. 1983; Hille,J.D. 1983b;). These aggregates characterized by Hille

et al. contained six enzyme molecules and 40 detergent molecules (Hille,J.D. 1983;). Other studies showed higher order aggregates both on monolayers (Grainger,D.W. 1990; Maloney,K.M. 1996;) and on liposomes (Hazlett,T.L. 1985; Pluckthun 1985, Hazlett,T.L. 1989;). However, the functional necessity for a dimer or higher order aggregate for catalytic activity of PLA2 has not been assessed.

Furthermore, the activity is greatly influenced by the phase state of the substrate. This is pronounced and observed when PLA2 is present on a bilayer like a liposome (Apitz-Castro, 1982), or supported bilayer (Nielsen, 1999) which undergoes a peculiar lag-burst phenomenon (Apitz-Castro 1982; Burack 1994). Generally, groups have sought the lag-burst phenomenon by changing the phase transition temperature (Bell 1991, Hoyrup 2002), introduction of lipid protrusions (Halperin, 2005), or by creating a change in curvature by the presence of other lipids like the products (Burack, 1994). The lag-burst phenomenon is characterized by a kinetic rate of activity with a 'lag phase' where there is a low intrinsic activity of the enzyme (Bell 1989, Burack 1995, Jackman, 2009), to reach the formation of a critical fraction of products (Burack, 1993; Bent, 1995) to a 'burst phase' where rapid hydrolysis occurs. It was thought that the bilayer undergoing a change in the physiochemical state was controlling the action of the phospholipase.

In this thesis we firstly, aim to clarify if there is an interfacial activation mechanism operating at the burst phase. Secondly, we wish to identify the observed aggregated state and its role in the catalytic mechanism. An introduction to the latter is covered in the next section.

2.4 Amyloid Fibril introduction

The previous section reviewed the steps in which PLA2 is thought to become activated on the surface of the lipid membrane. Also the properties of both the membrane and the protein that may control this activity were reviewed. It was proposed in this thesis work that PLA2 could form amyloid like fibrils at the end of the reaction. Here such fibrils are introduced briefly.

2.4.1 Overview of Fibrils

Proteins are linear polymers of peptide bonds with 20 different amino acid side chains. The nature of the protein allows it to form secondary, tertiary and quaternary structures. The tertiary and quaternary structures are necessary for a protein or enzyme to function properly. Protein folding (and protein denaturing followed by refolding) can be understood by Levinthal's paradox with proteins forming through a desired interaction via the most favored energy path to achieve folding from an unstructured high energy state to one where it is in the 'correct' conformation in a lower energy state that is kinetically accessible ('native' state). However, this kinetically accessible energy state might not necessarily be the overall lowest energy state (Levinthal 1969). Along these lines another energy state that is even lower than the native state can exist with a high order of molecular interactions to form a new native like intermediate (Levinthal 1969) possibly existing to form a highly active molten globule third equilibrium state (Jahn & Radford, 2005). With the correct amino acid sequence and under the right conditions, particularly in the presence of mildly denaturing pH or with certain types of lipids a hydrophobic collapse may occur towards fibrillization of the protein (Nguyen 2004, Zanuy 2004).

2.4.2 Lipids involved in Nonpathogenic Amyloid Formation

All the proteins in the human body can make amyloid under the correct conditions and with enough time, but could they form amyloid as part of their normal function? Mature amyloids may serve as a protective adaptation to the disease in A-β (Lee,H.G. 2004;). The membrane may influence the behavior of proteins and peptides to arrange into nonpathogenic amyloid like structures. For example, antimicrobial peptides aggregate on the membrane surface to form lipid protrusions, pores and amyloid. Such peptides can form pores with both zwitterionic and acidic phospholipids, however, amyloid fibers only form with the latter. Temporin B (Sood,R. 2007,), temporin L, magainin, indolicidin (Zhao,H. 2005;), and plantaracin (Zhao,H. 2006;) formed amyloid fibrils with acidic membranes. Nevertheless, antimicrobial amyloid fibril formation has been

proposed as a more general mechanism of how amyloid destroys cells (Mahalka & Kinnunen, 2009).

PLA2 is a dangerous enzyme and few inhibitors are known work specifically on it. PLA2 as well as other interfacial proteins need a strict level of control for the safety of the organism. Formation of inactive nonpathogenic amyloid fibers on the surface of membranes may offer a novel solution to achieve such control. The formation of these oligomers can be stopped by the addition of a molecular chaperone which restores the PLA2 function.

3. Aims of the Study

i) To investigate the molecular mechanism underlying the interfacial activation of PLA2 on a phospholipid membrane.

ii) To define the molecular events involved in the lag-burst behavior of PLA2 acting on DPPC.

iii) Based on the results, to develop a new model for the mechanism of control of PLA2 activity.

4. Material and Methods

All the details about the Materials and Methods used are described in the published papers. A summary of the methods used is shown in Table 4.1 below. Further details of the methods not described in the papers are briefly mentioned in the rest of the section.

Table 4.1 Summary of Methods with Referenced Papers

Section	Method	Referenced Paper(s)
4.1 Materials		
4.2 Preparation of labeled Protein and Peptide		
4.2.1	Preparation of labeled PLA2	OPI, II, III, IV
4.2.2	Preparation of Labeled Peptide	**OPII**
4.3	Preparation of large unilamellar vesicles.	**OPI, II, III**
4.4 Kinetic Assays for Monitoring PLA2 Hydrolysis		
4.4.1	Kinetic Assay Using DPPC LUV to Assess the lag-burst behavior in PLA2 reaction	**OP I, II, III**
4.4.2	Kinetic Assay Using Pyrene Labeled Lipids to Assess the reaction rate and number of products of PLA2 hydrolysis	OP III & IV
4.5 Assays for Determination of PLA2 Structural Change, Oligomerization and Fibrillization		
4.5.1	FRET	OPI & II
4.5.2	Fluorescence anisotropy	OPI
4.5.3	Determination of Fluorescent Lifetimes	OPI
4.5.4	Thioflavin T fluorescence	OPI & II
4.5.5	Light Microscopy	OPI & II
4.5.6	Electron Microscopy	OPIII
4.5.7	CIrcular Dichroism	OPII

4.1 Materials

All reagents and materials used are specified in the original papers. Key experiments were carried out using the premises of HBBG.

4.2 Preparation of Labeled Proteins and Peptides

4.2.1 Preparation of labeled PLA2 (OPI, II, III, IV)

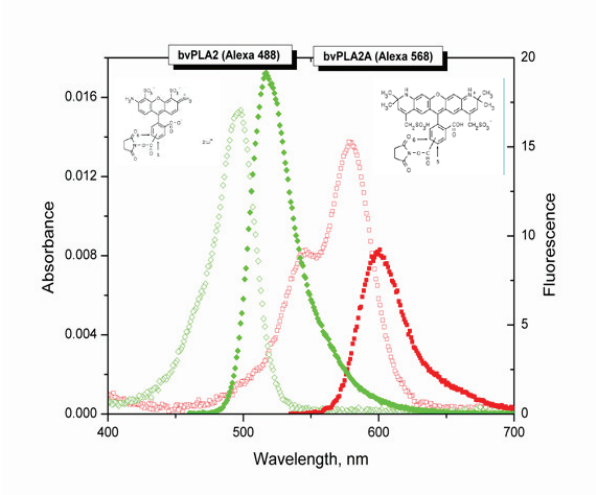

Figure 4.1 Absorbance (left axis) and Emission Spectra (right axis) of bvPLA2D (Alexa 488; Donor) and bvPLA2D (Alexa 568; Acceptor) dyes attached to bee venom PLA2 proteins in the absence of lipid. The absorbance and emission wavelengths shows that it is an effective FRET pair as the Stokes shift between the Donor and Acceptor is large enough.

4.4.1 Assessment of the lag-burst behavior in PLA2 reaction (OP I-III)

PLA2 releases lysolipids, free fatty acid and a proton during its reaction. We used an increase in the acidification of the medium to monitor reaction rate. Other techniques like the use of fluorescent phospholipids analogues (Thuren, Virtanen, Lalla, & Kinnunen, 1985; Thuren, Tulkki, Virtanen, & Kinnunen, 1987), base titration (using pH stat) (Bell & Biltonen, 1989), displacement of a fluorescent fatty-acid probe

(Wilton,D.C. 1990) among other methods could also have been used to observe the reaction rate. The one used in this thesis was used because: 1) its ease of use, 2) fluorescent lipid analogues change the reaction kinetics, 3) we only wanted to monitor the change in fluorescence of the protein. The time it takes for the protein to be activated and the time preceding this, the lag time γ, was measured using a pH electrode inserted into the cuvette (Microelectrodes, Bedford, New Hampshire, USA) so that it did not disrupt the light path. A script was made for the Varian Carey by Dr. Juha Matti Alakoskela to run the pH and different excitation and emission parameters simultaneously. Prior to starting the reaction three buffers were used to calibrate the system. The reaction was started with the substrate and a baseline was maintained before adding protein. For the assays involving FRET donor and acceptors the protein or peptide materials were mixed in an Eppendorf tube prior to adding to the cuvette.

4.4.2 Assay for PLA2 (OP III & IV)

Assays for determining PLA2 kinetics were the same as specified in the research articles. Since the pyrene labeled lipids are in SUVs, and these lack a phase transition temperature in the 15-45 C (being in the liquid disordered phase) there is no lag time present in the interfacial activation mechanism (Thuren et al. 1987).

4.5.1 FRET studies (OP I & II)

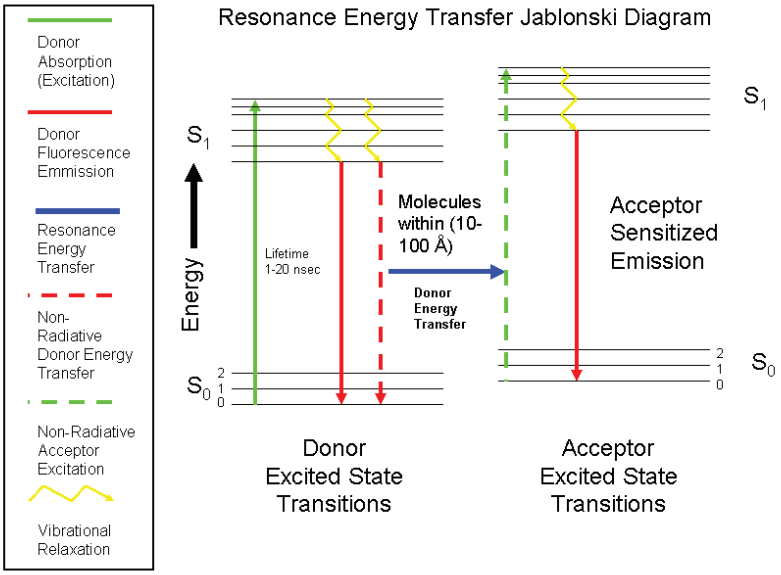

Figure 4.2 The Jablonski diagram shows the different electronic states and transitions for a molecule and the transitions between them. The different states are arranged by energy vertically and grouped horizontally. For Förster Resonance Energy Transfer (FRET) to occur between paired components like Alexa 488, and Alexa 560 or Texas Red, non-radiative transitions need to occur. When a photon is absorbed by one fluorophore a radiative and non-radiative transition can occur. If the latter occurs forming non-radiative dipole-dipole coupling (blue line) between the paired fluorophores then FRET is observed as in OPI & OPII. FRET is extremely sensitive to small distances so the fluorescent species attached to PLA2 or tempB need to be close as the radius of interaction of the two fluorophores is much smaller than the wavelength of light emitted. (Modified from Lakowicz, 1999).

4.5.4 Thioflavin T fluorescence (OP I & III)

Thioflavin T is a fluorescent probe with a weak excitation and emission at 342 and 430 nm (Rochet & Lansbury 2000, Khurana et al., 2005). Above the CMC of 6 μM it can bind to amyloid fibers showing an observed red shift with an excitation at 450 nm and a strong emission at 482 nm. ThT is pH sensitive and base can increase the intensity of the emission at 482 nm (Khurana et al., 2005).

4.5.7 Circular Dichroism (OP II)

CD spectra were measured with a Jasco J-500A spectropolarimeter (Jasco, Tokyo, Japan) courtesy of Antoinette Killian's laboratory in the Department of Chemical Biology & Organic Chemistry (CB&OC), University of Utrecht, Netherlands.

5. Results

5.1 Protein binding and slow accumulation of products during the lag time (OP I, II, III)

In order to further investigate the interfacial activation mechanism of PLA2 we employed methodology which helped us to monitor the fast kinetics of the interfacial activation. Firstly, we used DPPC in liposomes. As we know that PLA2 is activated between the gel and liquid phase transition (Op Den Kamp 1974 & 1975, Honger 1996), a better experimental control of the membrane interface can be obtained with DPPC with its high phase transition temperature. This allowed us to control the kinetics by increasing or decreasing the lag to observe specific kinetic events. The second thing that helped us monitor the fast kinetics was that we used singly labeled PLA2. The high quantum yield of the fluorescent label (as calculated in OPI) allowed us to monitor enzyme binding to lipid and observe some conformational changes in the enzyme. Lastly, we monitored the fluorescence and hydrolysis simultaneously. Using this experimental setup we first benchmarked the difference the label makes in comparison to the unlabeled enzyme to validate our approach.

Once the enzyme is added it binds rapidly to the surface of the liposome as indicated by emission anisotropy (Fig. 3, OPI). Bee venom PLA2 binds without preference for negatively charged lipids over zwitterionic membranes (Bollinger,J.G. 2004;189 Ghomashchi,F. 1998;). This binding could be helped by heterogeneities at the lipid interface that affect the PLA2 activity (Burack et. al 1994). These heterogeneities can, for example, arise upon the addition of PoxnoPC to the DPPC membranes which does affect several properties including the lateral organization (Megli,2005; Sabatini, 2006) , lipid packing (Volinsky, 2012), and passive transmembrane diffusion (flip-flop) (Volinsky, 2011). Non-bilayer structures can also be induced from the addition of temB which is known to change lipid structure through tubular lipid protrusions or segregating negatively charged lipids (Zhao 2001, Zhao

2002). The binding for these two lipid interfaces likely takes place as fast, or faster than on DPPC liposomes.

Following the binding we show a nominal increase in product formation (Fig. 5.1). Poorly understood changes in the membrane occurs when there is a small increase in the products mixing with the substrate slightly before the burst. From our observations the light scattering during most of the lag time is similar than the level of individual liposomes so it is unlikely that there is any significant aggregation of the liposomes. In light of this, a drop in scattering occurs slightly after the burst phase (5.1 panel A). With PoxnoPC on DPPC liposomes there is a similar slight increase in scattering before the burst phase (OPIII Fig. 1). Nevertheless, without more structural information on the lipid phase during this time it is difficult to predict the cause of the change in light scattering.

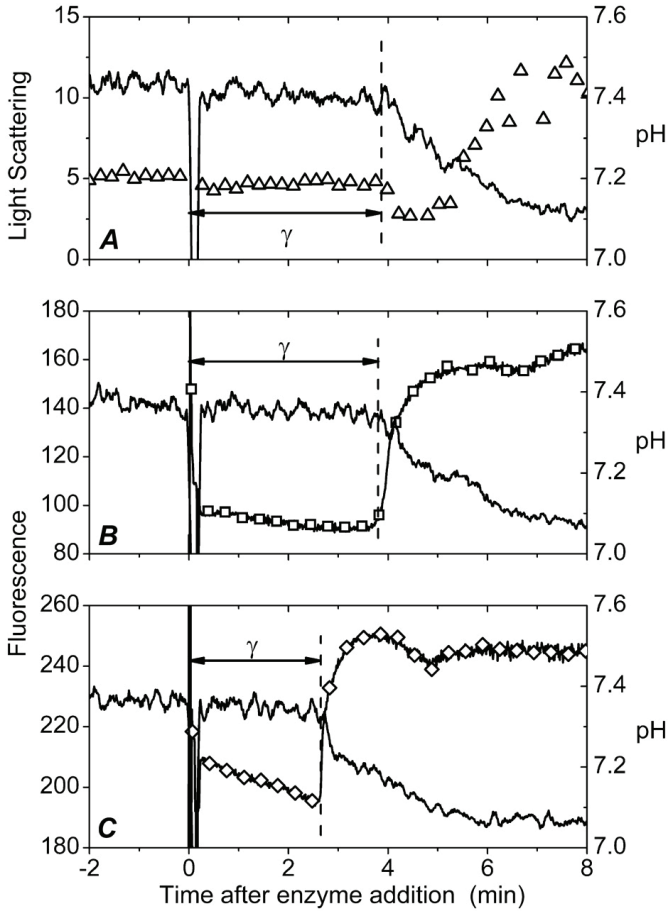

Figure 5.1 The reaction of unlabeled and singly labeled PLA2 and DPPC LUVs. Reaction was monitored by its pH (solid line) in the presence of unbuffered water with 2mM $CaCl_2$ and 75 nM of PLA2. The activity of the PLA2 reaction was observed by 90 degree light scattering of unlabeled PLA2 (Panel A, △), fluorescence of the PLA2 labelled FRET acceptor at emission 600 nm (Panel B, □) and PLA2 labelled FRET donor at emission at 520 nm (Panel C, ◊). The PLA2 was added at time zero and the start of the reaction was observed from an increase in light scattering or fluorescence and subsequent decrease in pH. γ is the lag time shown as the time between the burst in catalytic rate.

5.2 Slow Dimerization Occurs During the Lag Phase (OP I, II)

With the singly labeled FRET pair PLA2D and PLA2A we observed that a slow dimerization of the enzyme takes place. In this thesis the FRET technique was utilized to ensure that the interaction between the PLA2D and PLA2A (Alexa488 and Alexa568 labeled enzymes) could be captured within the fast kinetic timeframe. To our knowledge this study was the first to utilize singly-labelled PLA2 to show that a dimer exists leading to the point of the burst phase. As seen in Figure 5.2, Panel A, a pronounced decrease in the PLA2D is observed following a pronounced increase in the PLA2A. This change in fluorescence occurs simultaneously at the point where there is a large decrease in pH. Panel B shows the FRET efficiency of the two probes interacting at the same time point. As mentioned in the Materials and Methods of OPI these two probes have to be 54 Å apart for there to be energy transfer from one fluorophore to the other (also see supplementary section of OPI). These initial results using FRET show that a homodimer exists during the lag phase with a progressive increase in energy transfer leading up to the burst phase.

There is a small accumulation of products at the interface which occurs during the lag phase. This can result in product accumulation on the outer leaflet. A critical mole fraction of reaction products prior to the burst was observed by Burack et. al 1995 (Burack, 1995) resulting in increased recruitment of PLA2 at the membrane with the possibility that there is an inactive membrane bound state. We further explored the topic with a surface active labeled antimicrobial peptide, temB, at the same concentration of products to determine if there was hetero-oligomer formation. The rationale leading to this was from previous reports which showed that a) there was activation of PLA2 by temB and other antimicrobial peptides (Mingarro, 1995; Zhao, 2003), and b) with the formation of amyloid fibrils from both the antimicrobial peptide temB (Sood, 2007; Mahalka, 2009) and the PLA2 (Code, 2008) there was the possibility of hetero-oligomer formation. With a concentration of 8 mol % of the PLA2 products on an inactive liposomal membrane substrate we found FRET between PLA2D and TRC-temB (Fig. 5.3). Similar to the homodimers found in OPI, we found that

heterodimers are formed during the lag phase before the burst. Furthermore, a small amount of products and formation of PLA2 dimers seem to be key factors in the interfacial activation mechanism leading from the long lag phase to a burst phase. This dimer formation may be required for the burst in activity. Several research groups have proposed that a dimer or higher order PLA2 oligomer may be present on lipid interfaces (Bell & Biltonen, 1989; Hazlett 1990). One drawback of these studies is that they did not show that this dimerization is intimately tied with the burst phase within the interfacial activation mechanism. Our results indicate that an increasing amount of PLA2 homo-dimers occur at a critical mole fraction of products during the lag phase right before the burst point.

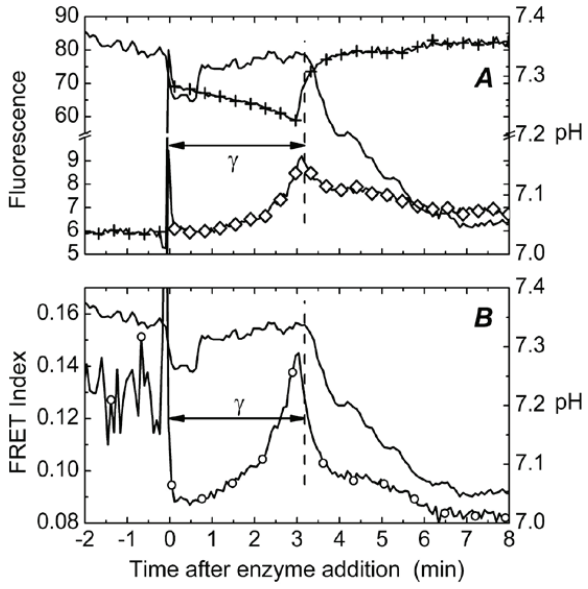

Figure 5.2 FRET observed between singly labeled PLA2 donor and acceptor. Panel A shows a decrease in the relative fluorescence of the donor (panel A, +) and an increase in the acceptor (panel A, ◊) pronounced where the lag time (γ) ends and the burst in hydrolysis begins as observed by a decrease in pH (shown by a vertical dotted line). Panel B shows the relative FRET efficiency (panel B, ○). Conditions were described in OPI or in figure 5.1.

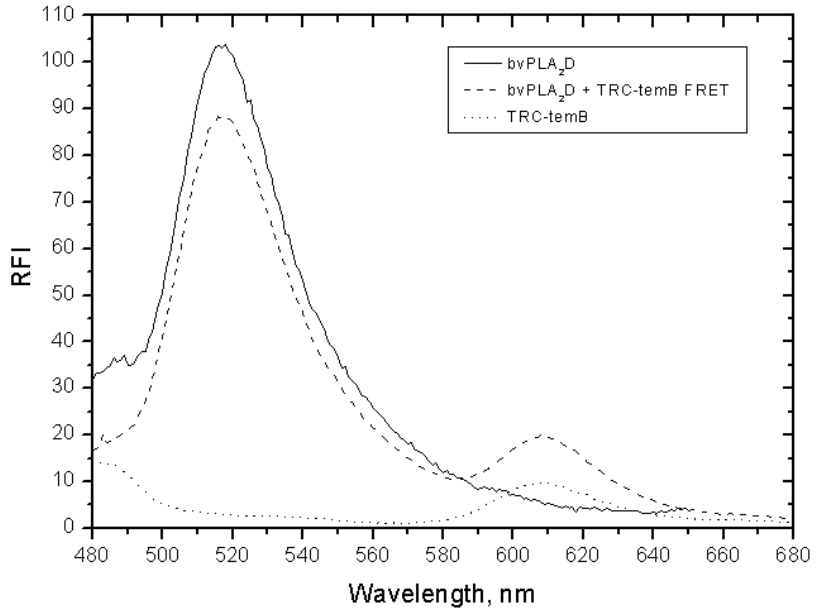

Figure 5.3 FRET of TRC-temB and PLA2D shown as Relative Fluorescence Intensity (RFI) over the emission wavelength of the donor and acceptor. FRET observed between FRET donor labeled PLA2 and acceptor labeled temB on D-DPPC liposomes shows an association between the two species (PLA2D and TRC-temB).

5.3 PLA2 Dimer Changes Into Highly Reactive Conformation at the Burst (OP I, III & IV)

Near the end of the latency period a slow decrease in the emission anisotropy becomes apparent (OPI, Fig. 3). A change in the emission anisotropy is indicative of the rotational time of the fluorophore that is attached to the molecule, and a decrease in anisotropy could potentially be associated with a protein conformational change (Eftink, 1991), although further studies are needed to show this with bee venom PLA2. Bee venom PLA2 has five disulphide bonds and is a robust protein. However, conformational changes and segmental motions in the protein are known to occur after substrate binding and before the burst (Tatulian 1997, Tatulian 2001). Other

conformational changes in the protein are reported with a more alpha-helical and beta-sheet structures observed using CD when FFA is bound in the active site which enhances the activity of the protein (Ahmed & Price 1996). Further understanding of this conformation change right before the burst was characterized by Tatulian et al. (2001) who showed that there is an increase in the amount of products which induces a more flexible helical structure of PLA2. In this case the interfacial activation was more pronounced at higher negative charge densities. Overall, with a decrease in anisotropy it is clear that there is a conformational change in the enzyme before the burst but it is unclear what this intermediate might be.

In order to understand this intermediate before the burst point, we tried to trap this protein in an active conformation using PoxnoPC. This lipid has one reactive aldehyde that is known to readily form Schiff bases or Michael addition with primary amino groups on proteins (Ahmed, 2003; Sayre 2006). It was earlier found that the treatment of the enzyme at specific sites with oleic acid and glutaraldehyde (Lawrence, A.J., 1975;) or transglutaminase can produce irreversible activation (Cordella, 1990). An aldehydic oxidized phospholipid (PoxnoPC) was initially thought to induce a similar effect. This oxidized lipid was previously demonstrated to make covalent adducts with lysine residues on polypeptides. With bee venom PLA2 having 11 lysines on the surface of its interfacial activation site this reaction is likely to take place. A reaction could occur on an exposed portion of the enzyme to form an intermediate. With the absence of scattering in the presence of methoxyamine (Fig. 4, OPIII) we showed that a Schiff base or Michael addition may occur between the PoxnoPC and PLA2. The Poxno-PLA2 reaction product showed a pronounced increase over the monomer when acting on pyrene labeled lipids (Fig. 3, OPIII).

Further evidence was sought that the molten oligomer intermediate could be the predominant reactive species leading to the burst. The intermediate right before the burst could be further understood using a molecular chaperone. We speculated that segmental motions and a loosening of the structure occur as the PLA2 goes closer to the burst. These segments can be explored with a molecular chaperone which can bind and

stabilize specific regions on proteins that are in a more open molten globule state (Hayer-Hartl 1994; Ewbank 1995) and reverse amyloid formation (Hartl, 2002). We used the ATP dependent molecular chaperone, HSP70, which likely can bind to specific exposed regions of PLA2 as discovered using proteomics tools (Reumers, 2008). We showed that HSP70 in the presence of ATP activates PLA2 which complies with the scenario proposed in (OPI) to control the activity of the enzyme. This scenario is shown with a larger quantity of products in the reaction (Fig. 1, OPIV).

5.4 Higher Order Oligomers are Present After the Burst (OPI, II, III)

As observed from the literature there might be higher order oligomers present at some point during the interfacial activation mechanism of PLA2 (see literature review of this thesis). In our own research we showed through several independent experiments that these oligomers likely occur after the burst.

Firstly, we observed that after the burst there appeared to be an increase in emission anisotropy. This indicated a lower amount of rotational freedom of the fluorophore and aggregation (Fig. 3 A&B, OPI) which could be caused by binding to a larger group (liposome or aggregation) or by a decrease in the fluorescence lifetime of the fluorophore. However, the lifetime was confirmed with time-resolved fluorescence to be unchanged at various stages of the activation process (Fig. 4, OPI) indicating that aggregation was indeed the cause.

Secondly, in light of the anisotropy data, we were tempted to follow the aggregation of PLA2 which occurred after the burst point. Thioflavin T, ThT, is a molecular probe used extensively by amyloid researchers to study the degree of amyloid formation (Rochet & Lansbury 2000; Khurana 2005). During the lag phase the ThT emission remained constant. However, after the burst point there was a clear increase in ThT emission reaching a plateau at 2-4 minutes and showing an intensity several fold higher than the background (Fig. 5 A&B, OPI).

After PLA2 aggregates were shown to increase the ThT emission, we showed that fibrils can be viewed using light and EM microscopy. We found long amyloid fibrils at the end of the reaction that had characteristic ThT fluorescence after incubating with ThT or red-green birefringence after Congo Red staining (OPI, Fig. 6). Fibers consisting of labeled PLA2 and TRC-temB were found to exhibit FRET with a higher intensity found in the red channel (Fig.9, OPIII). Lastly, we confirmed by electron microscopy that PLA2 forms long twisted amyloid-like fibrils at submicellar PoxnoPC concentrations. With our results we redefined previous research that showed PLA2 to form oligomers (Hazlett,T.L. 1990;), aggregates (Grainger,D.W. 1990;) or supermolecular structures (Hille et al. 1983), which were now shown to represent amyloid and to be inactive.

6. Discussion

6.1 Protein binding and slow accumulation of products lead up to the burst (OP I, II, III)

The fast binding of the PLA2 to the membrane interface is essential for the interfacial activity. As bee venom PLA2 does not have a preference for negative lipids over zwitterionic lipids, it is bound through a nonelectrostatic mechanism (Bollinger,J.G. 2004;189 Ghomashchi,F. 1998;) and we would likely see fast binding at the interface. Although the lipid head group might not be essential in the case of this particular PLA2, other factors may affect the quality of the interface. This interface was changed with pure DPPC liposomes at the phase transition temperature which can affect the binding of PLA2. This interface was furthermore changed by addition of PoxnoPC and by the antimicrobial peptide temB. The former changes the phase transition (Sabatini, 2006), lipid packing (Volinsky, 2012), and passive transmembrane diffusion (flip-flop) (Volinsky, 2011). The latter, temB, causes enhanced acyl chain order (Zhao et al. 2002) and tubular protrusions spanning from the bilayer (Domanov et. al 2006). Although we did not look into the binding mechanism in particular it is expected that PLA2 binds quickly to the interface in OPII & OPIII because there is a greater reduction of the lag time with the temB and Poxno respectively than with DPPC alone.

Once the PLA2 is bound to the membrane a noticeable lag takes place where we propose a modest catalytic activity and buildup of products. The monomeric enzyme shows some intrinsic activity when bound (Lombardo & Dennis 1985) and a small amount of product is known to accumulate at the interface (Bell,J.D. 1989;102 Burack,W.R. 1993;). Our results are consistent with those of Burack et. al who showed that the bulk of the enzyme is recruited at the membrane interface well before the catalytic reaction occurs. Lateral phase separation may induce a change in membrane curvature and defects in the membrane structure (Burack 1997). This small buildup of products at the interface affects the interfacial activation by helping to sequester more PLA2 in these microdomains. Along these lines, the

membrane curvature and outcroppings like the pronounced structures in the ripple phase (Leidy 2004) or addition of polyethylene glycol attached to lipids (Jorgensen et. al 1999) may seem to be an important regulator for PLA2 activation. In our case, the buildup of lysolipids on the membrane dramatically changes the curvature, makes the substrate phospholipids more accessible and affects the activity (Sheffield 1994). Similarly, the change in the membrane upon addition of PoxnoPC likely makes the PLA2 more accessible to the membrane. This result would agree with the observed activation of PLA2 on peroxidized lipid vesicles (Salgo, 1993).

6.2 Slow Dimerization Occurs During the Lag Phase (OP I, II)

A slight decrease in the emission anisotropy occurs toward the end of the latency period before the burst (Fig. 3, OPI). We rationalize this decrease as a loosening of the tertiary structure of PLA2 (Tatulian, 2001) and augmented segmental motions of the regions where the fluorophore is coupled to the terminal amine of PLA2. Overall conformational changes of the structure occur before the burst as indicated by a higher alpha-helical and beta-sheet content (Price & Ahmed) and more flexible helixes before the interfacial activation step (Tatulian 1997).

To add to this we propose that the N-terminal region may likely be loosening. This N-terminal segment of this unique group of phospholipases (specifically group III; Six, 2000) has been shown to be necessary for interfacial activation and it was suggested by Valentin et. al that this region may be involved in dimerization among other things (Valentin, 2000). The rate of formation of the dimers is limited to a critical mole fraction of the products at the interface as observed in OPII. There appears to be a delicate balance between the mean charge and the hydrophobicity of globular monomeric proteins (Uversky, 2002) where they may form a partially folded state and stabilize as dimers (Neet & Tim 1994; Mei, 2004).

Many PLA2 enzymes function as dimers. It was earlier shown that dissociation of the old world snake venom Crotalus adamanteus PLA2 leads to enzyme inactivation (Wells 1971). It is also shown that PLA2 from porcine pancreas and new world snake venoms can also function as dimers (Hazlett 1985), which lends more evidence for models such as the dual phospholipid model (Roberts 1977; Reynolds 1995). Along these lines the formation of dimers, as we have found in our research, seems to be a requirement for interfacial activation. As shown in this thesis, a number of dimers need to be made concomitantly on the membrane surface with a critical mole fraction of products before a burst in the enzyme activity takes place. Dimerization occurs with many proteins and there seems to be a near linear correlation for small proteins, approximately the size of PLA2, to form homodimers which give the protein more structural stability (Neet & Tim 1994).

6.3 PLA2 Dimer Changes Into Highly Active Conformation at the Burst (OP I, III & IV)

For PLA2 and many other secreted proteins the hydrophobic interior of the protein is stabilized on the membrane allowing water to maximize the entropy thus lowering the total energy of the system (Jahn & Radford, 2005). On the other side of the protein favorable energetic contacts would give PLA2 both conformational stability and increase its catalytic activity through dimerization. This conformational change lowers the protein's total free energy and may result in the formation of molten oligomers.

The formation and stabilization of a highly active conformation was further shown when PLA2 was added to oxidized phospholipids with reactive side chains. Adducts from 9-oxononanoic acid, the aldehydic moiety of Poxno, and lysine residues, corresponding to the catalytic site and the carboxyl terminal end loop, were found recently using mass spectrometry (Mahalka, 2012). We understood that this formation of a Schiff base resulted in a highly active PLA2 conformational state. The finding that oxidized lipids attenuate the active state of this enzyme may have implications in acute toxicity and inflammatory diseases (Schroder, 1980; Schoenberg, 1989).

With the results from OPIV we further rationalize that HSP70 can revert or stabilize the PLA2 in a highly active molten oligomer state for longer resulting in more extensive substrate hydrolysis. In this case, we chose HSP70, a chaperone, that was studied in our laboratory previously (Kirkegaard, 2010). PLA2 has a binding site for HSP70 discovered using the WALTZ algorithm (Reumers et al., 2008). Hypothetically, this hydrolysis could last for long periods of time or until the HSP70 or the ATP is exhausted. This type of mechanism may be present in breast and other types of cancer where both PLA2 (human, type II) (Yamashita, 1994) and HSP70 are expressed in vast amounts (Daugaard, 2007). This chaperone may also act to rescue other lipid-activated enzymes the activity of which is controlled with a mechanism similar to PLA2 (Code et al., 2008).

6.4 Higher Order Oligomers are Present After the Burst (OPI, II, III)

Amyloid-like fibers are formed at the end of the PLA2 reaction on a membrane. To the authors knowledge this is the first study showing that an enzyme becomes inactive during its normal sequence through the formation of amyloid aggregates. In line with our research, also some other groups have shown fibrillar structures upon membrane hydrolysis with PLA2. Chiu et al showed a triangular fibrillar structure that appears on top of the membrane after one hour (Chiu et al. 2009). We also speculate that fibrillar-like structures are apparent in several AFM studies of PLA2 reacting on DPPC membranes although this is not stated in the following studies. These structures identified as edge-on flattened bilayer discs (Callisen & Talmon,1998) or steadily growing 3-D structures that appeared precisely at the burst point (Balashev, 2013) could be interpreted as the same structures we have observed.

Overall, our findings widen the scope of functional amyloid fibers and suggest that they are involved in the regulation of lipid-bound enzymes. Furthermore, we have speculated on potential amyloidgenic region on the PLA2 dimers. A sequence exists in bee venom

at the N-terminus (Kini & Iwanaga, 1986) which is said to be the most toxic portion of the protein and is present as a separate helix in the tertiary structure of most phospholipases. The aa 76-91 helix mentioned by Nicolas et. al (Nicolas et al., 1997) is implicated both in binding to N-type receptors and in neurotoxicity. Taking a sequence from this region may lead to substantial inhibition of the protein through similar noncovalent docking to block the dimerization process of the enzyme as shown by Cordella-Miele with uteroglobin (Cordella-Miele et. al 1987, Facchiano, 1992). This region can also be made to dock by taking a peptide made from the primary sequence (Church, 2001) and further fine-tuned to act as a class-specific inhibitor (Mahalka et al 2013). The sequence could be probed using computer algorithms like PASTA (Trovato, 2007), Aggrescan, TANGO and WALTZ (Conchillo-Sole et al., 2007;) to see whether there is a likelihood that it can aggregate specifically.

Understanding that amyloid could form as part of the normal functional cycle of an enzyme is a key point in this work. Why would a protein fold into an amyloid? Pathological proteins like those found in Alzheimer's and Parkinson's disease and type II diabetes form amyloid fibrils which are maintained by extensive beta-sheets. These structures represents the minimum free energy occurring in the aggregated proteins, and the amyloid formation is a major hallmark of these diseases (Rochet & Lansbury, 2000). One theory is that these conditions may be a physiological protective mechanism whereby the toxic protofibrils might be eventually trapped into macroscopic and inert aggregates (Chiti & Dobson, 2006). Some amyloids may also serve nonpathological or protective functions. Proteins like PLA2 may have evolved to fall into this category of nonpathological inactive amyloid as a way to control and stop membrane hydrolysis. The enzyme is not consumed in the reaction but it forms a non-functional state after the reaction which we speculate may be necessary for it to be processed by the cell. It is suggested that the PLA2 enzyme in its active form is shielded from proteolytic cleavage (Camero 1985). Along these lines it is found that many proteins have isoforms which are resistant to proteolytic cleavage (Park 2007). If PLA2 is resistant to cleavage during its active state formation of inactive amyloid may help it be processed by cells through a ubiquitin-proteasome pathway or an autophagy-mediated pathway to be degraded.

These pathways are used to dampen the neurotoxicity of amyloid aggregates (Sharma et. al 2006; Cuervo 2004). We speculate that the advantage of PLA2 forming these insoluble aggregates is two-fold: 1) this might be a self-inhibitory allosteric mechanism to stop the activity of a very toxic protein, 2) The protein in its amyloidgenic state may be packaged for destruction in the proteosome of the cell.

Overall these findings widen the functional perspectives to amyloid-type protein aggregates and suggest that this might also be a mechanism involved in control of other lipid bound enzymes viz. protein kinase C (Bazzi 1992). Moreover, it might help in the understanding of the nature of enzymes that associate with negatively charged lipids, e.g. Glyceraldehyde 3-phosphate dehydrogenase (Zhao 2004, Cortez 2010), to form amyloid like fibrils or in-vivo Lewy bodies (Tsuchiya, 2005).

6.5 Summary of PLA2 Interfacial Activation

We tried to understand the full extent of the interfacial activation of PLA2 on lipid membranes. There is a lag time that is highly dependent on the substrate and the conformational changes of the enzyme. To control for the latter we used DPPC liposomes that can be controlled by changing the temperature to allow us to view the overall interfacial activation kinetics in slow motion snapshots.

The published papers showed a lag-burst behavior and interfacial activation from different perspectives, and a mechanistic sequence is illustrated (Fig. 6.1) and summarized briefly in this section: i) The enzyme rapidly binds as a monomer to the substrate interface. This binding is observed by the large increase in anisotropy when the labeled protein comes into contact with the much larger liposome limiting its motion (OPI). When the enzyme is bound only a minor catalytic activity is observed (Burack, Gadd, & Biltonen, 1995). ii) From this minor catalytic activity microdomains of the products form (Burack, 1994; Callisen, 1998). Similar microdomains could also be formed from oxidized phospholipids. With microdomains on the membrane surface the proteins are bound and are concentrated in close proximity whereby a conformational

change takes place. This conformational change in the enzyme leads to dimers or potentially higher order oligomers which likely stabilize the enzyme and its interfacial activity. The dimers were observed as homoFRET between two or more PLA2 molecules (OPI & Fig. 5.1 above) or heteroFRET between PLA2D and TRC-temB (OPII & Fig. 5.4 above). iii) The protein changes its conformation to a highly active state, which we speculate could be a molten oligomer, and a sudden pronounced increase of activity is shown as evident in OPI. The formation of this highly active state was further induced by oxidized phospholipids in OPIII or stabilized by a molecular chaperone in OPIV. iv) Finally, the highly active PLA2 conformation is converted to amyloid fibrils.

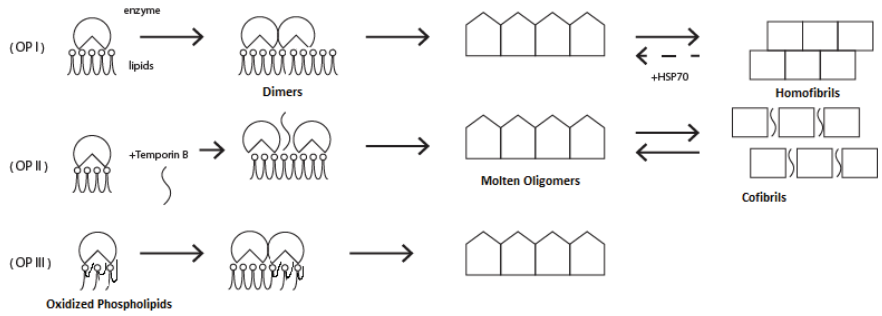

Figure 6.1 Summary diagram of PLA2 action on membranes. Monomers act on membranes coming in close proximity to form dimers. A change in the amount of products in the membrane changes the enzyme conformation to generate a highly reactive protein conformation (shown here as potential molten oligomers), which is finally converted into insoluble inert fibrils (left to right).

7. Conclusion

In order to study the interfacial activation mechanism of PLA2 further we used singly labeled PLA2, antimicrobial peptides, oxidized lipids and a chaperone protein using biophysical techniques viz. fluorescence, absorbance and microscopy techniques. PLA2 rapidly binds to the membrane interface. Slow activation of monomeric PLA2 leads to an accumulation of products within the interface during the lag phase. Dimers are formed at a critical concentration of products during the lag phase leading up to interfacial activation. At the burst point a change of protein conformation occurs and a highly active protein state is shown to act constitutively on the substrate. At the end of reaction the highly active PLA2 dimers or oligomers changes in conformation resulting in amyloid-like aggregates devoid of activity.

8. Acknowledgements

I would like to thank my supervisor, the head of the Helsinki Biomembrane and Biophysics Group, Professor Paavo Kinnunen for his enthusiasm with this work. His dedication to science, new ideas and discussion has been thought provoking.

Secondly, I would like to thank my friend, and colleague Dr. Yegor Domanov. He has been both inspirational and skeptical of my work both of which are needed for good science. Yegor's dedication has been motivating through the thick and the thin. It has been a pleasure working, traveling and drinking with him.

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