Principles underlying the substrate specificity of the phospholipase iPLA$_2$-γ: neutron study of the effect of lipid composition in model membrane systems

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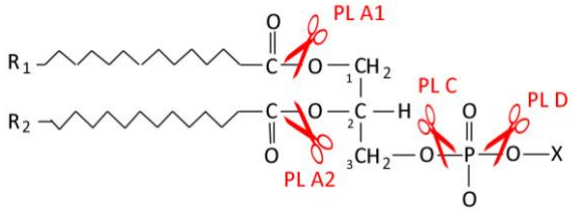
INTRODUCTION

Phospholipases A$_2$ (PLA$_2$s) which form a ubiquitous class of enzymes are hydrolases that are divided into PLA$_1$s and PLA$_2$s, that catalyze the hydrolysis of fatty acids present at the *sn*-1 or *sn*-2 position of the glycerophospholipid (GPL) glycerol moiety, respectively, thus producing a lysophospholipid (LPL) and a free fatty acid (FFA) (see Figure 1, *from* https://fr.wikipedia.org/). Several groups of PLA$_2$s other than the secretory PLA$_2$s (sPLA$_2$) are known, but among these only the group IV Ca^{2+}-dependent cytosolic phospholipase (cPLA$_2$-α) and group VI Ca^{2+}-independent phospholipases (iPLA$_2$-β) & (iPLA$_2$-γ), are thought to be the ones that play a key role in various cellular processes that include membrane remodeling and GPL homeostasis (Ramanadham, et al, 2015). Of these PLA isoforms, the focus for several years has been mainly on cPLA$_2$-α and iPLA$_2$-β for obvious reasons (implication of the previous in a number of inflammatory signaling cascades and the later in GPL homeostasis). Studies on the mitochondria-localized enzyme, iPLA$_2$-γ encoded by the gene 'PNPLA8' have been largely elusive although it has been shown to play a key role in integration of lipid and energy metabolism (Mancuso, DJ, et al, 2000). Deficiency of iPLA$_2$-γ in mice led to a mitochondrial neurodegenerative disorder characterized by degenerating mitochondria, autophagy, and cognitive dysfunction that was shown to be associated with alterations in GPL compositions (PCs, PEs, oxidized PEs) and also a shift in cardiolipins from long to short-chained molecular species (Mancuso, DJ, et al, 2009). In addition, Rauckhorst and colleagues observed significant reduction in the release of polyunsaturated fatty acids (PUFAs) in uncoupled mitochondria isolated from iPLA$_2$-γ knockout (KO) animals while no decrease in the membrane-sensitive release of fatty acids upon iPLA$_2$-β KO was noticed (Rauckhorst, et al, 2015). Interestingly, iPLA$_2$-γ KO mice derived mitochondria also displayed a substantial decrease in membrane potential sensitive iPLA$_2$-activity compared to those from wild type (WT) mice suggesting that iPLA$_2$-γ is the predominant membrane potential sensitive activity. In contrast, cardiac-specific over expression of iPLA$_2$-γ presented multiple phenotypes that included reductions in myocardial GPLs, accumulation of TGs and acute hemodynamic dysfunction accompanied by loosely packed and disorganized mitochondrial cristae and an increase in arachidonic acid (AA) or docosahexaenoic acid (DHA) containing lyso-phosphatidylcholine (LPC) species (Mancuso, DJ, et al, 2005).



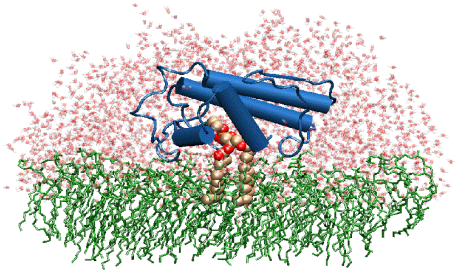


Fig2: From Sergey Stepaniants, Sergei Izrailev, and Klaus Schulten. Journal of Molecular Modeling, 3:473-475, 1997

Several PLA_2s display significant specificity towards the GPL acyl chain(s) *in vitro*. Hydrolysis of GPLs by PLAs has been studied extensively for more than 4 decades. **A commonly accepted model emerging from those studies is that 1) the enzyme associates peripherally with the macrosubstrate surface, 2) the GPL substrate moves upward and engages with the active site cavity of the enzyme, and 3) hydrolysis of the *sn*2 ester bond takes place, and the products are released from the enzyme (Scott et al, 1990)** (see Figure 2). Based on this model, the key factors determining the substrate specificity can be considered to be 1) accommodation of the GPL acyl chain(s) in the active site cavity of the enzyme and 2) propensity of the GPL substrate to efflux from the bilayer (see Figure 3). Although active site accommodation might be a key player, it might also be necessary that the GPL substrate effluxes out of the membrane for it to be hydrolyzed by the PLA (*which is based on the assumption that soluble PLAs may not be able to penetrate the membrane significantly and therefore the catalytic site remains well above the interface* (Gelb, et al, 1995)). In addition, recent studies employing hydrogen/deuterium (H/D) exchange and Molecular Dynamic (MD) simulations have proposed that the substrate molecule has to be extracted (i.e. *efflux*) from the membrane in order for it to reach the active site for its hydrolysis (Hsu, YH et al, 2009).

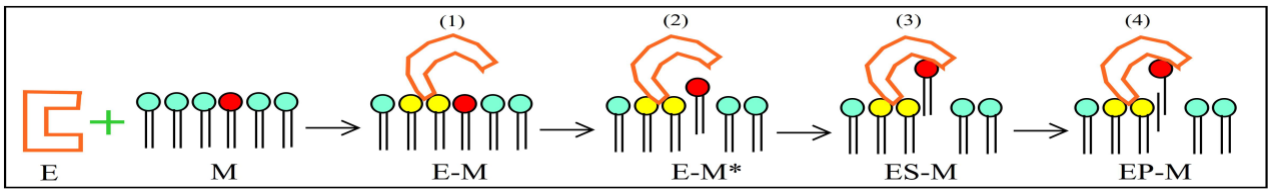


Fig3: Accepted model of the catalytic cycle of soluble PLAs (1) Enzyme-membrane association [E-M], (2) Efflux of the substrate [E-M*], (3) Accommodation of substrate in the catalytic site [ES-M], (4) Hydrolysis of the substrate [EP-M].

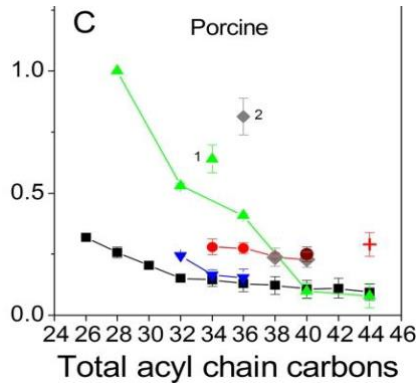
Although several studies had been undertaken to understand acyl chain accommodation in the active site of a PLA_2, this has been greatly hampered by the lack of crystal structures of PLAs complexed with physiological substrates. Crystal structures were obtained for some PLAs with a bound non-cleavable short-chain substrate analogue (Thunnisen et al, 1990, Scott, DL, et al, 1990), but since these analogues have truncated acyl chains, they provide very limited information on the factors underlying acyl chain specificity. Further there is uncertainty with such non-cleavable lipid analogues since these unnatural moieties may greatly increase their affinity for the enzyme (Yu L, et al, 1992), thus potentially masking the effects of acyl chain structure. Even if crystal structures with bound natural substrates were to be obtained, it will not help us reveal how efflux of the GPL substrate from the bilayer contributes to PLA specificity. Assessing the contribution of efflux is crucial since lipid-lipid interactions influence the vertical movement of the GPL, a

factor that is thought to be necessary for its binding to the active site of the enzyme (Blow, D, et al, 1991, Burke JE et al, 2008). In conclusion, the relative contributions of the PLA active site accommodation and efflux propensity to GPL hydrolysis are poorly understood at present, therefore such information is necessary to fully understand the mode of action of PLAs. Earlier substrate preference studies, suggest that iPLA$_2$-γ potentiates release of both saturated and monounsaturated fatty acids (MUFA) at equal rates from the *sn*-1 or *sn*-2 position in diacyl PC substrates (Yan, et al, 2005). Albeit, experimental evidence from other studies indicated that iPLA$_2$-γ also has the ability to cleave a PUFAs specifically AA from an intact GPL (Murakami, et al, 2005), there is little information on its substrate preference at the molecular species level including factors that dictate such specificity. Studies employing biochemical techniques, showed that efflux of the GPL from the macro substrate rather than the catalytic active site plays a key role in regulating its substrate specificity of 'iPLA$_2$-β' and 'sPLA$_2$' (Haimi, P, et al, 2009).

Neutron reflectometry is a non-destructive tool for characterizing the structure of biomimetic systems at scales ranging from tenths to tens of nanometers (Fragneto-Cusani, 2001). Sensitive to light elements (H, C, O, N, P…), the unique benefit of neutrons comes from the possibility to perform isotopic substitution in order to highlight the parts of interest in the systems studied. Indeed, hydrogen and its isotope deuterium have similar chemical properties, but they have different interaction potential with neutrons. Specular neutron reflectivity probes the structure of a bilayer in the direction perpendicular to its plane down to about half a nanometer and gives access to the thickness, composition, and roughness of uniform layers normal to the substrate.

Previously, Vacklin and colleagues had used neutron reflectivity to study sPLA$_2$-catalyzed hydrolysis of model membrane bilayers (Vacklin et al, 2005). In a very elegant experiment, planar lipid monolayers or bilayers from lipids possessing one hydrogenous and one deuterated chain were prepared. Authors monitored the composition of the supported phospholipid bilayers during PLA$_2$ hydrolysis using specular neutron reflection and ellipsometry. Earlier, it has been shown that porcine pancreatic PLA$_2$ shows a long lag phase of several hours during which the enzyme binds to the bilayer surface, but only 5 ± 3% of the lipids react before the onset of rapid hydrolysis (Haimi et al, 2009). The amount of PLA$_2$, which resides at the water-bilayer interface, as well as its depth of penetration into the membrane, increase during the lag phase, the length of which is also proportional to the enzyme concentration. Hydrolysis of a single-chain deuterium labelled d$_{31}$-POPC revealed for the first time that there is a significant asymmetry in the distribution of the reaction products between the membrane and the aqueous environment. The lyso-lipid leaves the membrane while the number of PLA$_2$ molecules bound to the interface increases with increasing fatty acid content. These results constituted the first direct measurement of the membrane structure and composition, including

the location and amount of the enzyme during hydrolysis. These are discussed in terms of a model of fatty-acid mediated activation of PLA_2.



In this project, we intend to employ a similar strategy to study the effect of acyl chain length and unsaturation of GPLs on their rate of hydrolysis. Increasing the chain length (right from the shortest (12 carbons) to the longest (22 carbons) at either the *sn*-1 or the *sn*-2 position will allow us to know the extent of the acyl chain interaction with the active site. Next, varying the degree of unsaturation (4 to 6 double bonds) in the acyl chains will not only give us information on the efflux rates but also the active site accommodation properties. Thus, all such data is necessary to better understand the mode of action of $iPLA_2$-γ as well as to establish why it preferentially hydrolyses certain GPL species.

PRELIMINARY RESULTS

We have successfully extracted and separated hydrogenated and deuterated PC, PE and PS pools comprising of molecular species of varying chain lengths and degrees of unsaturation (from *Pichia pastoris*). In addition, we have also been successful in separating a pool of deuterated PE, CL and PG from *Escherchia Coli*. Characterization and fatty acid compositional analysis was done by subjecting the purified fraction to a GC system while GPL purity and identification analysis was carried out in collaboration with Juliette Jouhet (CEA) on a mass spectrometer (MS) *via* direct infusion. Thus, in the very near future, we could make bilayers by using a combination of different molecular species across different classes that shall be adequately separated on such a purification system.

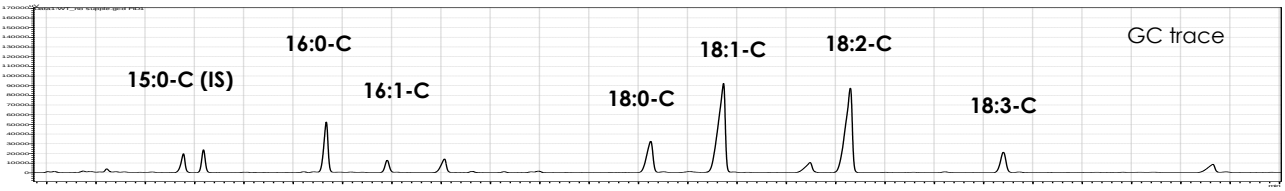


Figure 4: A Gas Chromatography trace of total yeast lipid extract under deuterated conditions

PROPOSED EXPERIMENTS AND EXPERIMENTAL DATA

The main goal of the proposed experiments is to get a better understanding of the principles underlying the substrate specificity of the human $iPLA_2$-γ overexpressed in *Sf9* insect cells on a recombinant baculovirus system. Enzyme purification will be done by affinity chromatography on an AKTA-FPLC system. Further activity assays will be done by incubating the purified enzyme with unlabelled GPL mixtures followed by Mass spectrometric analysis of the products formed.

Planar lipid bilayers will be prepared by Langmuir-Blodgett Langmuir-Schaefer techniques []. While complementary techniques will be employed to optimise the sample preparation the main tool of investigation

will be neutron reflectometry carried out either at the Institut Laue-Langevin (Grenoble, FR) or the ISIS facility at the Rutherford Appleton Laboratory (Didcot, UK). A wide range of bilayer systems will be tested by incubating them with the purified iPLA$_2$-γ. The bilayer systems that we propose to investigate are: **1)** a single GPL (D-12:0/12:0 PC); **2)** a single GPL (D-14:0/14:0 PC); **3)** a single GPL (D -16:0/16:0 PC); **4)** a single GPL (D-18:0/18:0 PC); **5)** a single GPL (D-20:0/20:0 PC); **6)** a single GPL (D-22:0/22:0 PC); **7)** a double GPL bilayer (H-16:0 + D-16:0/20:4 PC) ; **8)** a double GPL bilayer (H-16:0 + D-20:4/16:0 PC); **9)** a double GPL bilayer (H-16:0 + D-16:0/22:6 PC) and **10)** a double GPL bilayer (H-16:0 + D-22:6/16:0 PC). Systems 1 to 6 will allow us to test the degree of acyl chain hydrophobicity thus allowing us to know if substrate efflux plays a key role in enzyme activity regulation and it will also give us information on the active site length. Systems 7 to 10 (isomeric GPLs) will help assess the contribution of individual acyl chains (isomeric specificity) on the rate of hydrolysis of iPLA$_2$-γ allowing us to shed light on the active site accommodation. Such experiments shall help study the effect of the acyl chain length and unsaturation of GPLs on their rate of hydrolysis in membrane bilayers. After characterization of the bilayer all these systems will be incubated with the purified PLA$_2$. The kinetics of the interaction is of the order of several minutes and up to hours on solid substrates and can be followed easily on the available neutron reflectometers. The use of deuterated lipids will help in the localization of the enzyme that has a scattering length density similar to that of hydrogenous lipids. In addition, it will be possible to do the kinetics measurements in light water (in heavy water the mechanism is slowed down considerably) and follow up removal of deuterated lipids. Systems 1 to 4 will allow us to test the degree of acyl chain hydrophobicity thus allowing us to know if substrate efflux plays a key role in enzyme activity regulation and it will also give us information on the active site length. Systems 5 and 6 will give us information on the specificity of the enzyme in terms of the degree of unsaturation, which will help shedding light on the active site accommodation. The final system will give us information on the influence of the head group (that we shall compare with system 2). Protein concentration for the interaction with lipids is around 0.15mg/mL.

CONCLUSION

The project attempts to define the complexities underlying how GPLs and the mitochondria-integral phospholipase 'iPLA$_2$-γ' interact in a complex relationship. Trying to understand these phenomena, shall provide novel information on factors determining the substrate specificity of iPLA$_2$-γ that should be helpful for basic research attempting to resolve its biological structure and functions. Clinical research is this direction is relevant since recent reports have implicated iPLA$_2$-γ in Chagas disease a condition where cardiac myocytes are infected causing release of inflammatory mediators such as eicosanoids (Sharma, J, et al, 2013). Further, iPLA$_2$-γ-deficiency provided resistance to thromboembolism, raising the possibility of targeting iPLA$_2$-γ for antithrombotic drug development (Yoda, E, et al, 2014). In view of such developments we conclude that results emanating from our use of GPLs with acyl chain lengths varying in their degree of

hydrophobicity and unsaturation and their respective isomers interacting with iPLA$_2$-γ shall shed light on the molecular dimensions of the catalytic active site in addition to iPLA$_2$-γ's substrate specificity profile that shall assist in studies (Molecular Dynamic simulations/molecular docking) attempting to design novel drugs/inhibitors targeting the enzyme.

PHD PLAN

The PhD student will be enrolled at the University of Grenoble Alpes and will be based at the Institut Laue-Langevin. Short stays at the University of Oxford are foreseen.

1st year – Production of PLAs and work on secretary PLAs to prove the principle with mass-spec, QCM-D, spectroscopy and other characterization measurements available both in Grenoble and Oxford.

2nd year – Optimization of planar lipid bilayer preparation; purification of PLAs; biochemical activity assays; neutron reflectometry experiments at ILL and ISIS.

3rd year – Completion of neutron experiments and data analysis, writing up of PhD manuscript and publications on neutron results.

REFERENCES

Blow D (1991) *Nature* **351**, 444–445.
Burke JE, et al; (2008) *Biochemistry* **47**, 6451–6459
Fragneto-Cusani G. - Journal of Physics Condensed Matter Vol. 13 pp. 4973-4989, 2001
Gelb, et al; (1995) *Biochemistry* **34**, 7807-7818.
Haimi, P, et al; (2009) *Journal of Biological Chemistry* **285**, 751-760.
Hsu, YH, et al; (2009) *Journal of Biological Chemistry* 284, 23652-61.
Mancuso, DJ, et al; (2000) *Journal of Biological Chemistry* **275**, 9937–9945.
Mancuso, DJ, et al; (2009) *Journal of Biological Chemistry* **284**, 35632–35644.
Mancuso, DJ, et al; (2007) *Journal of Biological Chemistry* **282**, 9216–9227
Manusco, et al; (2009) *Journal of Biological Chemistry* **284**, 35632-44.
Murakami, et al; (2005) *Journal of Biological Chemistry* **280**, 14028-41.
Ramanadham, et al; (2015) *Journal of Lipid Research* **56**, 1643-1668.
Rauckhorst, et al; (2015) *Febs Letters* 589, 2367-2371.
Scott, DL, et al; (1990) *Science* **250**, 1563-1566.
Sharma, J, et al; (2013) *Infect. Immun.* 81: 2278–2287.
Thunnissen M. M, et al; (1990) *Nature* 347, 689–691.
Vacklin, et al; (2005) *BBA Biomembranes* **1768**, 1036-1049.
Yan, et al; (2005) *Journal of Biological* Chemistry **280**, 26669-26679.
Yoda, E, et al; (2014) *PLoS ONE*. **9**: e109409.
Yu L, et al; (1992) *J. Am. Chem. Soc.* **114**, 8757–8763.