Experimental report

29/01/2022

Proposal: 8-02-914 **Council:** 4/2020

Title: Assessing the contribution of active site accommodation of glycerophospholipid in regulating the specificity of iPLA2γ

Research area: Biology

This proposal is a new proposal

Main proposer: Giacomo CORUCCI

Experimental team: Giovanna FRAGNETO
Giacomo CORUCCI

Local contacts: Giovanna FRAGNETO

Samples: (1) d-16:0/h-18:1-PC; (2) d-16:0/h-18:2; (3) d-16:0/h-18:3; (4) d-16:0/h-20:4; (5) d-16:0/h-22:6 phosphocholine

Instrument	Requested days	Allocated days	From	To
D17	3	0		
FIGARO	3	3	19/02/2021	22/02/2021

Abstract:

The project is the latter part of a previously accepted proposal that attempts to define the complexities underlying how GPLs and the mitochondria-integral phospholipase iPLA2-gamma interact in a complex relationship. The proposed experiments involving the structural characterization of lipid bilayers interacting with the enzyme can give a better understanding of the principles underlying the substrate specificity of the human iPLA2-gamma by allowing us to assess the contribution of the active site accommodation of the substrate.
Through the proposed measurements we will assess the contribution of unique acyl chains (as a function of degree of unsaturation) on the rate of hydrolysis by iPLA2-γ thus allowing us to shed light on the active site accommodation.

Report for the Proposal: 8-02-914

Phospholipases, a biophysical view; investigation of the factors regulating the substrate specificities of phospholipases

In collaboration with the University-Grenoble Alpes and Oxford University.

Supervisors: *Giovanna Fragneto (UGA and ILL), Krishna Batchu (ILL), Robert Jacobs (Oxford supervisor)*

INTRODUCTION

My main PhD project is focused on the investigation of factors regulating the substrate specificities of phospholipase, employing biophysical techniques, like neutron reflectivity, small angle Xray/neutron scattering, quartz microbalance, ellipsometry and mass spectrometry. There are mainly 5 classes of phospholipases in nature and in this project, we are interested on the class A phospholipases. Phospholipases A (PLAs) are a very important class of lipolytic enzymes, that hydrolyze phospholipid substrates (GPLs) at specific ester bonds (sn1 or sn2). PLAs are involved in several pathways, from lipid bilayer remodeling, to energetic metabolism and cell signaling (for example inflammation). Some of those PLAs are also involved in some neurodegenerative diseases that include Alzheimer and Parkinson's. Therefore, a deeper understanding of their dynamics and kinetics can possibly be very crucial for a future "drug design" to either block or alleviate the activity of the PLA related to the specific disease.

At the moment, some proposed hypothesis of kinetic molecular mechanisms from previous studies have already been published, but a more commonly accepted model emerging from those studies is as following (see also *figure 1*):

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;
3) Hydrolysis of the specific ester bond takes place, and the products are released from the enzyme.

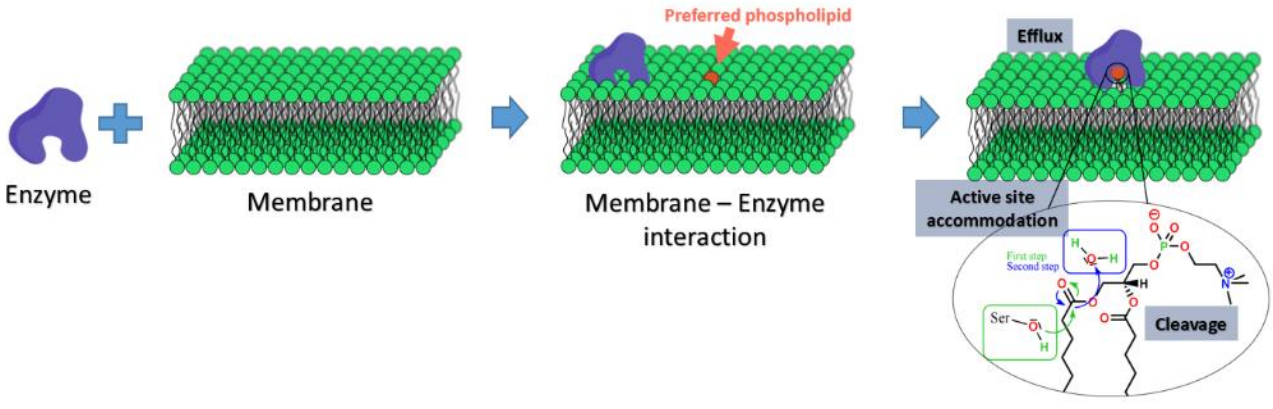

Figure 1 - Schematics of the accepted molecular mechanism

According to this model, there are two bottlenecks in the reaction mediated by phospholipases:

- Propensity of the phospholipids to efflux from the bilayer;
- Accommodation of GPL acyl chains in the active site cavity of the phospholipase enzyme.

The phospholipid efflux process is defined as the ability of a phospholipid molecule to leave the lipid membrane, in other words is the energy required to pull away the phospholipid from the membrane. The active site accommodation is how well a phospholipid fits the active site pocket of the enzyme. Ones the reaction passes those two bottlenecks, the enzyme cleaves the proper ester bond, giving as a product of the reaction a free fatty acid (FFA) and a lyso phospholipid (a phospholipid with one missing fatty acid).

The main question that we intend to answer through this study is what dictates the specificity of a phospholipase for different kind of phospholipids. Given a scenario where-in a PLA is presented with a mixture of GPL molecular species that differ from one another in terms of their physical structure (as a function of their chain length or degree of unsaturation), *what is it that drives the enzyme in preferring one molecule as its substrate over the others*. For instance, phospholipases prefer phospholipids where the efflux is easier or the ones that fit its active site pocket; the reaction could also be also a combination between the two points, depending on the lipid that is consider. In this project we are also interested on the structural changes of the lipid membrane during the kinetic of the PLA, *analyzing where the products of the reaction are going, and if there is a structural change of the membrane*.

RESULTS

The present study employs neutron reflectivity, including other physical/chemical techniques, to better understand the principles underlying the substrate specificity of the PLA. We had started with assessing the factors that regulate substrate specificity of the *Aspergillus oryzae* – PLA1-1. This isoform that belongs to the PLA1 class, has been shown to hydrolyse the acyl chain from the sn-1 position of a phospholipid. This enzyme was expressed in E coli cells, and purified.

Ones we had the enzyme ready, afterwards, we started the chemical synthesis of the partially deuterated substrates, to analyze the enzymatic reaction using neutron scattering techniques. The main neutron tool used is reflectivity. The idea with this technique is to make flat silicon supported lipid bilayers (using the synthetized partially deuterated phospholipids), mimicking the real cell membrane, and after the structural characterization of the membrane, the PLA was injected and the kinetic followed. To fit those data, we design an ad-hoc python program (CoruxFit), with also a plug-in for PLAs kinetics data (kinetic model plug-in). With CoruxFit we were able to fit the structure of the lipid membrane before injecting the phospholipase, and using the kinetic plug-in the reflectivity data recovered during the kinetic (after the PLA injection). In *figure 4* an example of an analysis of partially deuterated DPPC kinetic (d-sn1, h-sn2).

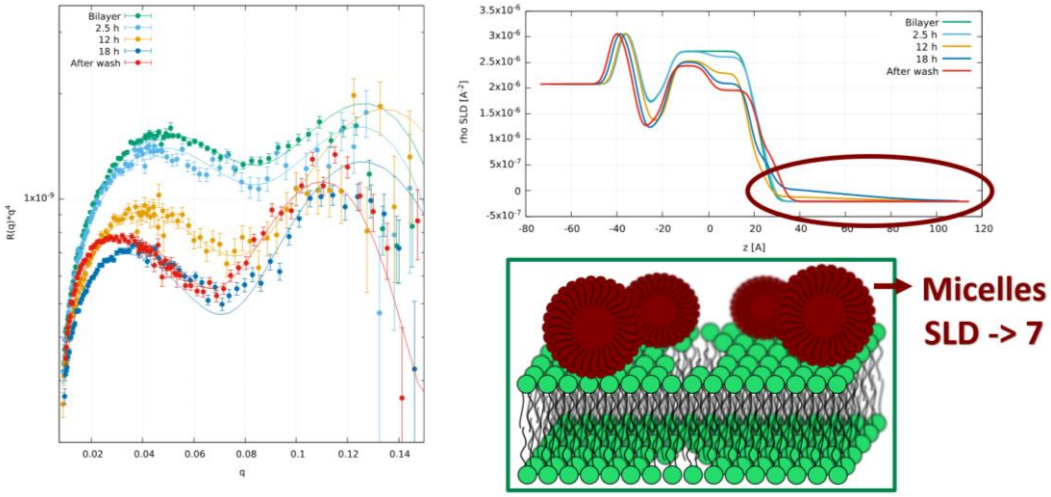

Figure 4 - DPPC Kinetics fitted using CoruxFit - kinetic model

From the analysis in *figure 4*, it is possible to notice a decreasing of the reflectivity signal over the time after the PLA1-1 injection, which is caused by the cleaving activity of the enzyme. The activity lead to an SLD decreasing of the hydrophobic layer, caused by a losing of cleaved deuterated fatty acids from the lipid bilayer to the bulk solution. The structure of the membrane is also changing over the time, with an area per lipid that decreases, with an increase of defects in the bilayer. We can also confirm that the material removed from the lipid membrane by the enzyme, is rearranging itself in micelles or vesicles, and this material from the bulk goes and sticks again to the membrane, creating a layer of deuterated material on the top of it (like in the cartoon in *figure 4*). This kind of experiment was carried out also using other phospholipids, and in *figure 5* is plotted the degradation graph calculated from the fitted neutron reflectivity data.

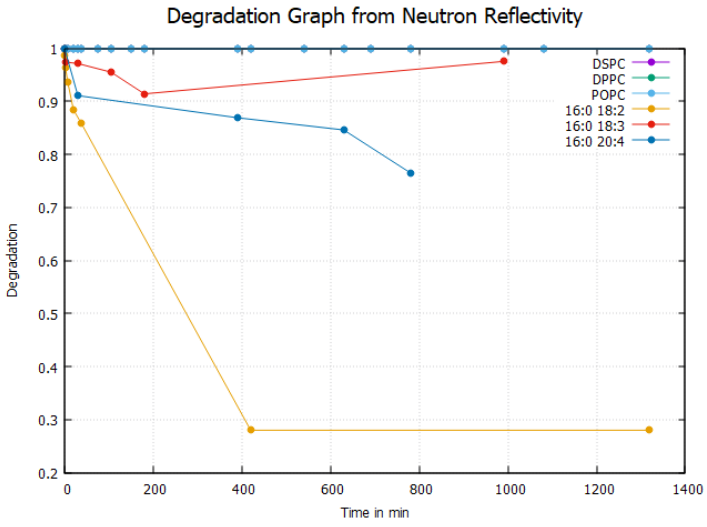

Figure 5 - Degradation graph, data from neutron reflectivity

We are carrying out other lab experiment using QCM-D microbalance, and ellipsometry, to prove and confirm the reflectivity data. Still a lot of experiments need to be done with those two techniques.