Rapport d'évaluation du mémoire de thèse / Evaluation report of the PhD thesis

Doctorant **PhD student**	Nom prénom / Full name	Corucci Giacomo
	Ecole Doctorale / Doctoral School	PHYS - Physique
	Titre thèse / PhD Title	Analysis of the Factors Regulating the Activity of the

PLA1-1 isoform: A Neutron Reflectivity and Mass-Spectrometric study

Rapporteur **Reviewer**	Nom prénom / Full name	Carrière Frédéric
	Etablissement / Institution	CNRS
	Statut, fonction / Status, position	Directeur de Recherche

Qualité du mémoire : structuration, rédaction & illustrations / Thesis quality, style & illustrations
(A titre indicatif/For information : Exceptionnel = top 5%, Très bon/very good = top 25 %)

➡ Insatisfaisant / Unsatisfactory [ ]   Satisfaisant / Satisfactory []   Bon / Good [ ]

Très bon / Very good [X]   Exceptionnel [ ]

Commentaires/comments :

The manuscript is well written and organized, apart from a very brief state of the art section. Methods and techniques, especially regarding recombinant protein production and characterization, and biophysical studies are well described in terms of principle, choice of parameters, validation, and results obtained. Illustrations of results are very clear. The Introduction section is however missing some illustrations in my oipinion. Most importantly, many valuable results were obtained, well presented and discussed. They validate new tools for studying the evolution of a lipid substrate in the course of enzymatic lipolysis. I am convinced they can be applied to the study of many other enzymes and a better understanding of their mechanism of action.
The development of the CoruxFit Program by the candidate was instrumental for the analysis of NR data. My overall evaluation is therefore very positive although the state of the art could have been covered more thoroughly. See my specific comments below.

Contexte/ collaborations, background : état de l'art / state of the art :

➡ Insatisfaisant / Unsatisfactory [ ]   Satisfaisant / Satisfactory [ ]   Bon / Good [X]

Très bon / Very good [ ]   Exceptionnel [ ]

Commentaires/comments :

Regarding the Introduction section, I found many relevant and basic informations regarding phospholipids and phospholipases. The text is well written and relevant references are given. The section on Phospholipases A2 is rather complete with the various subfamilies described, as well as some structural properties. Since the PhD work was mostly about a phospholipase A1 from fungal origin, I was expecting a more complete section on PLA1s. Although these enzymes, often associated with lipases, have not been reviewed in litterature like PLA2s, many of them, often referred first as lipases, are known, including 3D structures and substrate specificity, and various biophysical approaches have been used for studying their interaction with lipids. This is some how missing. Illustrations are also missing with only 3 figures in this section and none regarding PLA structures.

Regarding what is called "State of the art" section (half a page), this is not a state of the art (while the previous section is). It mainly indicates very briefly what has been done in the PhD work such the development of a high-throughput mass spectrometry assay for studying phospholipase activity on various lipids and novel methods to study PLA-membrane bilayer interactions by utilizing neutron reflectivity and mass spectrometry in parallel. A state of the art in that case would have been the description of previous phospholipase assays with various substrates and biophysical techniques. There are so many assays described over the last 50 years using micelles, liposomes, monolayers or supported bilayers, especially for characterizing PLA2.

This is my main criticism regarding the thesis. I will provide further reading to the candidate that can be discussed during the defence and used for improving the state of the art section. I understand that the candidate mainly focused on phospholipases A2 during his literature search and missed the comparison with lipases displaying PLA1 activity.

Qualité scientifique : méthodologie, expérimentations, validation
Scientific quality, methodology, experiments, validation

➡ Insatisfaisant / Unsatisfactory [ ]   Satisfaisant / Satisfactory [ ]   Bon / Good [ ]

Très bon / Very good [X]   Exceptionnel [ ]

Commentaires/comments :

After producing and characterizing a recombinant phospholipase A1 of fungal origin, a combination of various biophysical techniques was used to study lipid organization within a bilayer submitted to enzymatic lipolysis. All techniques including neutron reflectivity (NR) and MS analysis for high throughput lipidomics are well described, including principles and parameters used with ILL instruments. Methods for observing interfacial enzymes like phospholipases in interaction with their organized substrates (here lipid bilayers) are particularly important to better understand the mechanism of action of these enzymes and their interaction with the subtrate in a dynamic environment where its organization changes. This is challenging and this thesis is an excellent contribution with that respect.

The combined used of various lipids and their mixtures, as well as their deuteration allows maximizing the output of NR.

The development of the CoruxFit Program was essential for the analysis of NR data. It has allowed the beautiful plots of results within the result section.

MS allowed measuring the production of lysophospholipids and confirmed tfat these molecules could be further hydrolyzed by a PLA1.

Apports personnels : originalité, valorisation, perspectives
Personal contributions : originality, exploitation and application of results, prospects.

➡ Insatisfaisant / Unsatisfactory [ ]   Satisfaisant / Satisfactory [ ]   Bon / Good [ ]

Très bon / Very good [X]   Exceptionnel [ ]

Commentaires/comments :

The most important and original result obtained using NR is the possibility to characterize the lipid bilayers and they evolution during lipolysis by measuring simultaneously the cleavage rates of ester bonds at sn-1

versus sn-2 positions, phospholipid flip-flop from the inner to outer leaflet to compensate for membrane asymmetry and variation in mean molecular area per molecule. NR also allows observing other structures/lipid organizations formed upon lipolysis, such as the appearance of a micellar layer above the bilayer depending on the lipolysis rates. This method allows comparing various lipids and their mixture. This approach could be used to study other lipolytic enzymes and their substrate to better understand their interactions at interfaces.

There were only few report on the use of X-ray or Neutron reflectivity for studying lipolytic enzyme interaction with lipids. The present study is a critical step forward to go beyond using NR. The use of NR for studying the cleavage of acyl chains at both sn-1 and sn-2 positions and possible acyl chain migration is very interesting. Mass spectrometry analysis of lipolysis products confirmed that lysophospholipids were further hydrolyzed by phospholipase A1, indicating that either both ester bonds qat sn-1 and sn-2 positions could be cleaved or that sn-2 to sn-1 acyl migration occurred before the second ester boind hydrolysis at sn-1 position.

As can be noticed in figure 4.31, the higher the number of double bonds harbored in the acyl chain of a phospholipid,the higher its hydrolysis rate.

MS allowed determining the relative rates of hydrolysis of various PC molecules in a complex mixture, allowed measuring the appearance of lysophospholipids and confirmed their further hydrolysis by the same enzyme (PLA1).

The development of the CoruxFit Program by the candidate allowed a unique analysis of NR data. I am not an expert in NR and software development but I understand that this development was instrumental to really valorise the NR data. I now have a better idea of what can be done with NR regarding lipolytic enzymes and I thank the candidate and supervisors for inviting me to review this thesis

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Conclusions du rapporteur / Reviewer's conclusions
Commentaires/comments :

Overall, this is a very good PhD work. The manuscript would have been excellent with a better state of the art section. The candidate obtained original results on lipase-lipid interactions that validate his biophysical approaches. NR in particularly may now be applied for studying other lipolytic enzymes thanks to the development of a specific program to fit the data. I therefore recommend the defence of this work and will be happy to discuss his results with the candidate.

Avis du rapporteur / Reviewer's opinion :
Défavorable à la soutenance / Unfavorable to the defence [ ]   Favorable [X]

Date   13 février 2023   Signature

Visa du directeur de l'école doctorale :

Rapport détaillé, commentaires libres, questionnements
Detailed report, free comments, questions

I listed here the important points I read in the manuscript. This is a kind of summary that will help me discussing with the candidate.

Regarding the Introduction section, I found many relevant and basic informations regarding phospholipids and phospholipases. The text is well written and relevant references are given. The section on

Phospholipases A2 is rather complete with the various subfamilies described, as well as some structural properties. Since the PhD work was mostly about a phospholipase A1 from fungal origin, I was expecting a more complete section on PLA1s. Although these enzymes, often associated with lipases, are less described in literature than PLA2s, Many of them are known, including 3D structures and substrate specificity. This is some how missing. Illustrations are also missing with only 3 figures in this section and none regarding PLA structures.

Regarding what is called "State of the art" section (page 43), this is not a state of the art (while the previous section is). It mainly indicates very briefly (half a page) what has been done in the PhD work such the development of a high-throughput mass spectrometry assay for studying phospholipase activity on various lipids and novel methods to study PLA-membrane bilayer interactions by utilizing neutron reflectivity and mass spectrometry in parallel. A state of the art in that case would have been the description of previous phospholipase assays with various substrates and biophysical techniques, and there are so many assays described over the last 50 years using micelles, liposomes, monolayers or supported bilayers, especially for characterizing PLA2.

Regarding the Materials and Methods section, all techniques including NR and MS are well described, including principles and parameters used with ILL instruments.

Regarding the Results and Chapter 3 on Protein and lipids production, the protein (*Aspergillus orizae* PLA1-1) of 30kDa with phospholipase activity was well produced and purified. A 3D homology model was built, including using Alphafold. This lipase shows a high sequence and 3D structure homology with well known fungal lipases with PLA1 activity, like Thermomyces lanuginosus lipase tTLL), and its is a pity that it is not mentioned husing ere. Asa model lipase, TLL and its interaction with lipids have been extensively characterized using various biophysical techniques.
The characterisation of lipids consisted of (a) the lipidomics profiling, finding the relative abundance of the acyl chains for the threeclasses of GPL molecules purified, and (b) the structural characterisation of flat membranes containing the purified GPLs.
Various bilayers of mixed, deuterated or not, phospholipids were characterized avec vesicle fusion or Langmuir Blodgett / Langmuir-Schaefer double transfer. No structural differences between H and D membranes.

Regarding the Results and Chapter 4 on Protein-lipid interactions: After the deposition of the SLBs followed by their structural characterisation, PLA1-1 was injected into the cell. The right amount of protein per lipid was assessed by employing ellipsometry. NR provided information on the substrate preference and the structural changes of the model membrane upon reaction, by utilising mono-species SLBs constituted by the synthesised partially deuterated PC molecules. MS experiments were carried out, to investigate the substrate specificity using vesicles constituted by a multitude of PC molecules in the same vesicle sample.

Using partially deuterated phospholipids, with the sn1 chain deuterated and the sn2 hydrogenous, it is possible to track where the FFA or the lyso go during the course of the kinetics, since the two have a different SLD value. Once, the FFA orthe lyso leaves the membrane, its SLD will vary over the hydrolysis reaction. With neutron scattering technique it is also possible to follow structural changes of the membrane due to the activity of the enzyme.
With DSPC, NR data show the appearance of asymmetry in the bilayer upon lipolysis and no flip-flop mechanism. With DPPC, NR data clearly show degradation of the bilayer, where the outer leaflet of the lipid bilayer undergoes degradation first followed by degradation of the inner leaflet as well as a flip-flop mechanism to compensate for bilayer assymetry. kinetics of degradation can be estimated from NR using CoruxFit program. They reveal a slow lipolysis process, maybe revealing that the bilayer organization is not the best substrate presentation for this type of phospholipase. NR data also suggest the formation of lipolysis product micelles over the bilayer. When the lipid bilayer has been degraded by more than 0.15, the micelles start appearing in the bulk solution (enough FFA to be above their CMC). It was found experimentally with NR, that those micelles, over time, attached back to the lipid membrane, creating a diluted layer on the top of the lipid membrane.
With long chain unsaturated lipids NR shows lower thickness of bilayer due to fluidity. Lipolysis is also faster and results in a large degradation of the bilayer. The cleavage of both acyl chain after sn-2 to sn-1 position is proposed, what fits with what is known for other PLA1.
With h16:0 | h20:4 PC, **Cleavage rates of sn-1 versus sn-2 positions, flip-flop and variation in mean molecular area per molecule were estimated simultaneously (most important results)**. Various PC species could be compared. The substrate preference seems dependent on the ability of the PC molecules to efflux from the membrane, since shorter chain length and higher number of double bonds in the acyl chain lead to higher degradation. It could also be due to the preferential formation of micelles and solubilisation of

lipolysis products avoiding enzyme inhibition by products. Charged mixed bilayers with PS or PG mixed with PC are also degraded more rapidly.

Besides the appearance of a micellar layer, NR data also suggest the formation of other structures on the top of the bilayer with the appearance of a Bragg peak (Cubic phase formed by lipolysis products?).

MS analysis: In this section, the kinetics results recorded through employing a mass spectrometer (MS) technique are shown and discussed. MS analysis was performed on bulk vesicle solutions, in order to assess the preferential interaction of PLA1-1 with the various phospholipid species present in the same vesicle sample.
The kinetics performed using mixA and displayed in figure 4.25 (A) showthat when the purified PLA1-1 is incubated with the LUV solution composed of the saturated, unsaturated and polyunsaturatedphospholipid molecules, the short-chained saturated series up to 14:0 | 14:0 PC (DMPC) areeasily hydrolysed. Specifically 12:0 | 12:0 PC (DLPC), looked to be the best substrate for PLA1-1 in this mixture, followed by DMPC.
From the fit of MS data, the 'degradation units' (D.U.) per minute are obtained, describing the amount of lipid degraded per minute. This value is then multiplied by the amount of lipid used for the sample (2 nmol), thus obtaining the degradation expressed in nmol/min. Knowing the amount of enzyme used in the assay, specific activities (IU= µmolesper min per mg of enzyme) could therefore be determined and compared to those in previous literature. Did the candidate do that?

It could be noticed that the trend of the lysos is different and did not increase during the whole course of the reaction. An analogous behavior was also recorded with neutron reflectivity as well as during the biochemical assays that were visualised by TLCs, that showed an increase in lyso production upon phospholipid degradation, up to a certain level, above which they remain constant or decrease over time. Lyso-PL are therefore further hydrolyzed by PLA1.
It was noted that the vesicle size was critical for the rate of the hydrolysis reaction.not really surprizing since the activity of lipolytic enzymes depends ion substrate surface concentration.

Discussion: A the beginning of the discussion, I found some repeats of the Introduction but with additional references that could have been used for writing the state of the art.

It is started here (page 151 (127)), that he reaction mediated by the phospholipase enzymes is characterised by the following steps: (1) phospholipase - membrane association, (2) efflux of the GPL molecule, (3) active site accommodation of the molecule and finally (4) cleavage of the substrate. Since the PLA1 studied here is a lipase with a lid controlling the access to the active site, an additional step of conformational change upon interaction with lipid should also be considered. The candidate could have exploited here the 3D model of the PLA1-1 he built.

Justification of using NR here: NR pioneering experiments with one-chain deuterated and one-chain hydrogenous lipids were performed by Wacklin and coworkers [237] using PLA2 enzymes. NR experiments on porcine pancreatic PLA2 showed a long lag phase of several hours, after which the degradation started. The long lag phase is usually due to a poor affinity of pancreatic sPLA2 for bilayers. Preference for micelles. However the slow release of fatty acids favours enzyme adsorption. This could have been discussed

The use of NR for studying the cleavage of acyl chains at both sn-1 and sn-2 positions and possible acyl chain migration is very interesting.

Overall, the discussion is very interesting and will deserve further discussion with the candidate. Since a 3D model of PLA1-1 was made and could give some additional informations on the mode of action of the enzyme, I would have like to see some discussion about it. I understand that the candidate mainly focused on phospholipases A2 during his literature search and missed the comparison with lipases.

CoruxFit Program : CoruxFit is a python based program for neutron and X-ray reflectivity data analysis and simulation. program developed during the thesis by the candidate. Based on four principal parameters (see figure 6.9): molecular area variation, SLD variation (lipolysis with cleavage of acyl chain), defects formation and flip-flop. It is difficult for me to evaluate the time within the thesis preparation devoted to this development but I understand it was instrumental for treating and using the NR data. It has allowed the beautiful plots of results within the result section, This helped me to better understand the potentiality of NR for studying lipases at interfaces in interaction with their substrate;