Experimental report

09/09/2022

Proposal: 8-02-918 **Council:** 4/2020

Title: Role of molecular players in endocytosis revealed by neutron scattering: the Clathrin Adaptor CALM

Research area: Biology

This proposal is a resubmission of 8-02-893

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Samples: Protein
LIPIDS

Instrument	Requested days	Allocated days	From	To
FIGARO Adsorption troughs	2	2	10/03/2021	12/03/2021
D22	1	0		
D11	1	1	24/05/2021	25/05/2021

Abstract:

Clathrin-mediated endocytosis (CME) is the main mechanism by which eukaryotic cells internalize and recycle most membrane proteins (termed cargo). It is driven by different Adaptor Proteins which directly link the clathrin scaffold to cargos, and modulated by accessory proteins. Simplifying the system down to its core elements is enabling us to address a number of important questions.

The focus on this proposal is on the clathrin-adaptor CALM. The protein possesses an amphipathic Alpha helix that inserts into membranes curvature-induced defects, driving the formation of clathrin-coated pits as well as influencing clathrin-coated vesicle size. However, the modality of the binding and the spatial arrangement of CALM upon lipid interaction is still unclear. A combined Neutron Reflectometry (NR) and Small Angle Neutron Scattering (SANS) approach studying each steps of this process, including the effect of the accessory SNARE protein, will provide a complete physical and thermodynamic description of the collective mechanisms involved.

The proposed experiments will form part of the PhD of Andreas Santamaria.

Experimental report of #8-02-918 on FIGARO and D11

Role of molecular players in endocytosis revealed by neutron scattering: the Clathrin Adaptor CALM

Scientific Background

Clathrin-mediated endocytosis (CME) is the main endocytic pathway used by cells to the internalize membrane proteins (termed cargoes). Molecular biology and proteomic approaches have been essential to understand the function and structure of the main proteins involved in such pathway, as CALM, which can bind the membrane and recognize the cargoes. However many questions remain unanswered concerning the interaction between proteins and lipids, which represents the very first steps of the CME. For example, although it is clear that the main interaction driving CALM towards the inner leaflet of the plasma membrane is electrostatic, due to the presence of Phosphatidylinositol 4,5-bisphosphate (PIP_2), information is missing, regarding the orientation of these proteins with respect to the lipid interface, as well as their eventual insertion in the membrane.

CALM (clathrin assembly lymphoid myeloid leukemia protein) is one of the most abundant clathrin adaptors[1]. It possesses a relatively small, compact, stacked-helical domain (ANTH domain), with large, natively unstructured, C-terminal tails[2]. The single PIP_2 binding site is located in the ANTH domain, Lysines 28, 38 and 40[2]. Moreover, CALM is characterized by a membrane-inserting amphipathic helix at the N-terminus (thus called amphipathic helix 0, AH0), whose 3D orientation is thought to change upon membrane binding. The absence of AH0 leads to the formation of flat clathrin lattices on lipid monolayers and not invaginated clathrin-coated pits[2], [3]. Indeed, it was shown that CALM senses and induces membrane curvature[4], [5], being also able to drive pearlized liposomes tubulation, thanks to the insertion of AH0 in the membrane[4]. Although the insertion of the helix in the membrane was hypothesized, it was not experimentally proved[4]. Questions remain unanswered concerning the interaction between CALM and lipids, which represents the very first steps of the CME, such as the protein insertion and orientation with respect to the membrane. The main aims of this work is to exploit neutron reflectometry (NR) to determine whether CALM inserts in the membrane and to unravel its orientation with respect to the plane of the membrane (by using the software SLDMOL), and also to investigate the influence of CALM on the size and shape of PIP_2-containing liposomes by exploiting small-angle neutron scattering (SANS). In order to assess the insertion of the AH0 in the membrane, the wild type ANTH domain of the protein (hereinafter CALM wild type, $CALM_{wt}$) and the mutant missing the AH0 ($CALM_{\Delta AH0}$) were compared. Both constructs were kindly provided by Dr. Owen (Cambridge University).

Results

The experiments performed during these beamtimes on FIGARO and D11, with lipid monolayer and liposomes, respectively, were used to complete studies on CALM, and were included in Andreas Santamaria's thesis. They are now being readied for publication.

Neutron reflectometry on FIGARO

A composition of DPPC:DPPE:PIP_2, in molar ratio 7:2:1, was employed. The lipid monolayer were characterized in three isotopic contrasts: 100% D_2O *v/v*, 60% D_2O *v/v*, and 8.1% D_2O *v/v* (ACMW). The NR data obtained (**Figure 1**) were modelled through a multilayer slab model that takes into account lipid tails- and headgroups- layers, and three extra layers for the protein, considered underneath the monolayer. $CALM_{wt}$ was found inserted in the headgroups-layer as expected (**Figure 1 C**), with a total surface coverage of 13% *v/v*, which is similar to the coverage obtained by Miller et al with PIP_2 containing liposomes (16%[4]). Afterwards, after data analysis, the software SLDMOL (available on the online SASSIE package platform (https://sassie-web.chem.utk.edu/sassie2) was employed to orient the crystallographic structure of CALM on the membrane and obtain its 3D orientation upon binding. Moreover, the binding to lipid monolayers of $CALM_{\Delta AH0}$ was investigated. The same multilayer model, in terms of number of layers as

well as thickness, was exploited. Thus obtaining similar total thicknesses for $CALM_{wt}$ and $CALM_{\Delta AH0}$ (57±4 Å and 63±4 Å, respectively), as well as similar volume fraction percentage underneath the monolayer (12% and 15% *v/v*, respectively, **Figure 1 C** and **F**). However, the volume fraction percentage of $CALM_{\Delta AH0}$ inserted in the headgroups-layer was found to be only 5±1% *v/v* (**Figure 1 F**), coherent with the presence of less amino acids in the membrane. This suggest that AH0 is inserted in the membrane.

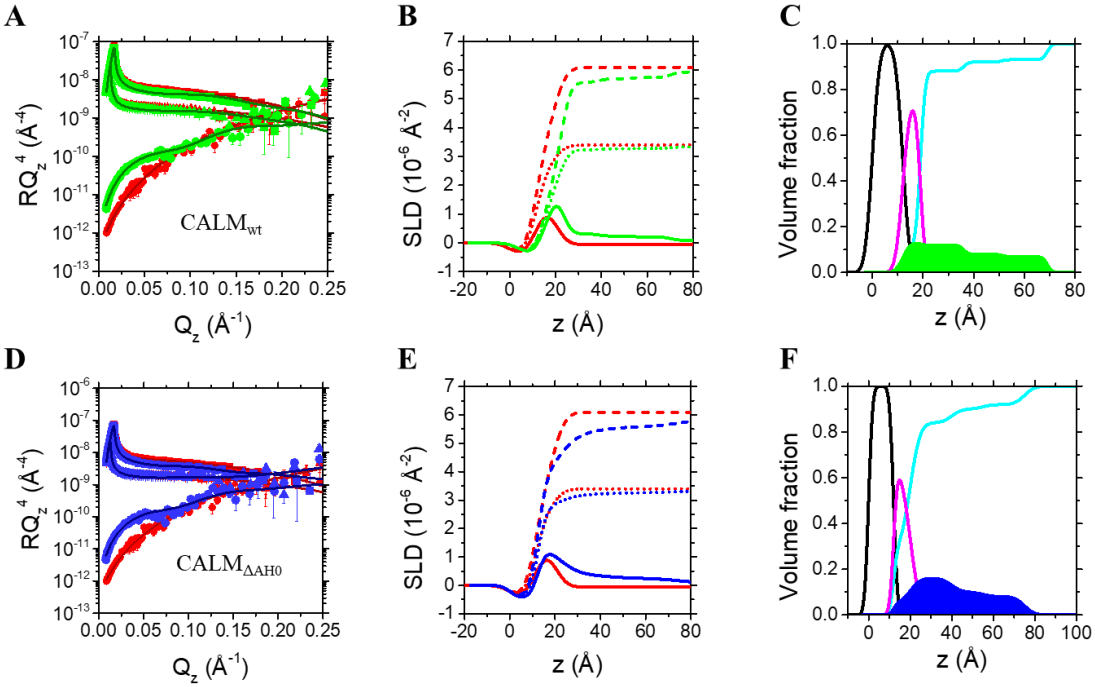

Figure 1 Experimental (symbols) and simulated (lines) NR profiles of lipid monolayers in the absence (red) and presence of **A** $CALM_{wt}$ (green) and **D** $CALM_{\Delta AH0}$ (blue). Data at three isotopic contrasts have been measured: D_2O (squares), 60% D_2O (triangles) and ACMW (circles). Figures are displayed on an RQ_z^4 scale to show the quality of the fits at high Q_z values. SLD profiles corresponding to fits are plotted in **B** and **E**. Continuous, short dotted, short dashed lines indicate the SLD profiles in ACMW, 60% D_2O and D_2O isotopic contrast, respectively. Red lines refer to purely monolayer profiles, green and blue lines refer to monolayer+$CALM_{wt}$ and monolayer+$CALM_{\Delta AH0}$ SLD profiles, respectively. Volume fraction profiles derived from the fit highlight the distribution of tails (black), heads (magenta), water (cyan) and **F** $CALM_{wt}$ (green), **I** $CALM_{\Delta AH0}$ (blue).

Small-angle neutron scattering on D11

SANS, on D11 instrument, was employed to investigate the binding of $CALM_{wt}$ with PIP_2-containing 100 nm sized liposomes, at a protein:lipid ratio of 1:25. This size was chosen since it is closer to the preferred size for CALM[4]. SANS profiles of lipids in two different contrast were measured: 100% D_2O and 42% D_2O *v/v*. The obtained intensity curves, plotted against the momentum transfer (Q), were modelled taking into account two different population: bilamellar vesicles of a total diameter of ~60 nm, which is compatible with the size of the membrane used for extrusion (100 nm), and nanodisks of lipids with a radius of ~18 nm (see **Figure 2 A** and **B**). When $CALM_{wt}$ was added to the liposomes solution, changes in the profile were observed, especially at low-Q and mid-Q. It is interesting to note the faster decay ($\sim Q^{-2}$) in the low-Q range (**Figure 2 C** and **D**), compared to profiles of pure lipid samples (Q^0), thus suggesting the presence of scattering objects bigger than 100 nm. We concluded that $CALM_{wt}$ has indeed an effect on liposome shape and size. However, we could not deduce whether the lipid+$CALM_{wt}$ sample contains larger vesicle, from fusion processes, or large tubules.

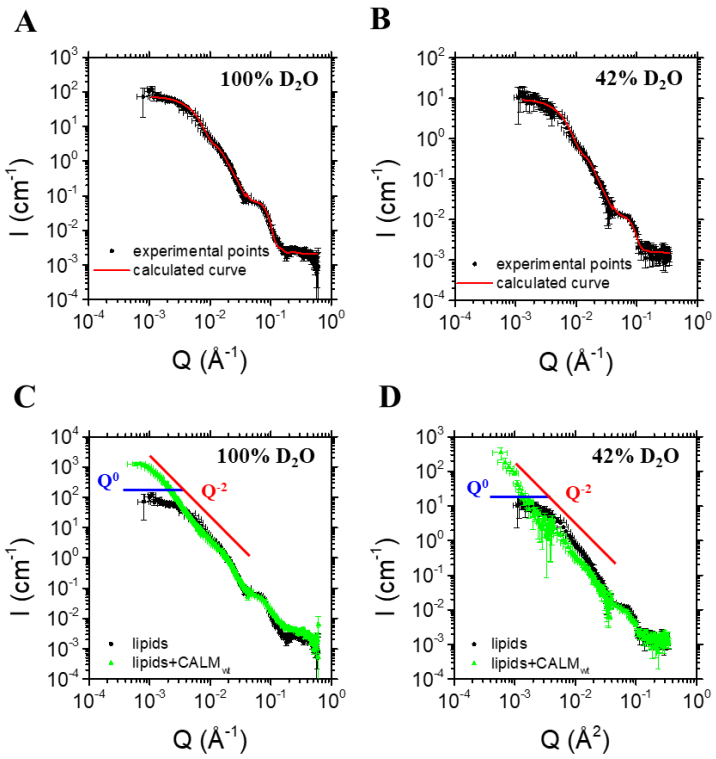

Figure 2 Panels showing the recorded SANS profiles: solely lipid samples in **A** 100% D_2O and **B** 42% D_2O *v/v*; comparisons of lipid sample and lipid+$CALM_{wt}$ sample profiles in **C** 100% D_2O and **D** 42% D_2O *v/v*. The experimental data points related to lipid and lipid+$CALM_{wt}$ samples are depicted as black circles and green triangles respectively. The simulated curve obtained from the model used are is shown as red lines.

References

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