Uncoating Characteristics of Clathrin Cages

Lahari de Alwis

THE UNIVERSITY OF WARWICK

Introduction

Investigating the uncoating characteristics of auxilin-bound clathrin cages in the presence of hsc70 and ATP.

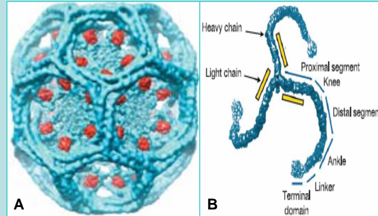
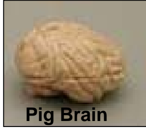


Figure 1: *A) Auxilin-bound clathrin D6 barrel at 12Å resolution. The auxilin is in red. Taken from reference 1. B) Clathrin triskelions. Taken from reference 2.*

Clathrin is a vesicle protein, triskelions of which combine to form cages which are stabilized by auxilin, as shown in Figure 1.

The hsc70 protein initiates the uncoating and is powered by the hydrolysis of ATP.

Purification of Clathrin



Wash and immerse in buffer (pH7).

Add **AEBSF** protease inhibitor.

Homogenise the brains until there are no large lumps remaining.

Prepare the resulting mixture for a series of varying spins.

The centrifuging speeds and times are designed to **isolate coated vesicles** with as few contaminants as possible.

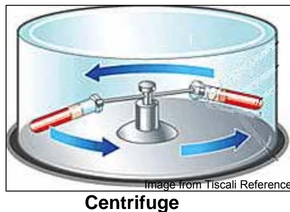
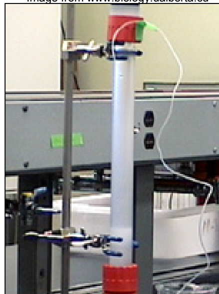


Figure 2: *Centrifuging separates molecules according to weight, with the larger forming a pellet at the bottom.*

Centrifuge

Add 2M Tris buffer and 0.1% β-mercaptoethanol and leave for 1 hour to **strip** the protein coat off the vesicles.

Spin for 20 minutes at 50k to isolate proteins.



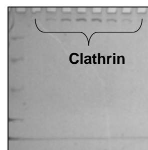
Gel Filtration

Gel filter the supernatant using Tris running buffer.

Running at 1ml/min, clathrin comes off after approximately the first 600ml.

Run a gel of the appropriate fractions.

Pool those containing clathrin.



Locating Clathrin

Precipitate the protein by adding an equal volume of saturated **ammoniumsulphate**.

Spin and resuspend pellet.

Dialyse into '**Depolymerisation**' buffer.

Spin to remove unfolded aggregates.

Dialyse into '**Polymerisation**' buffer.

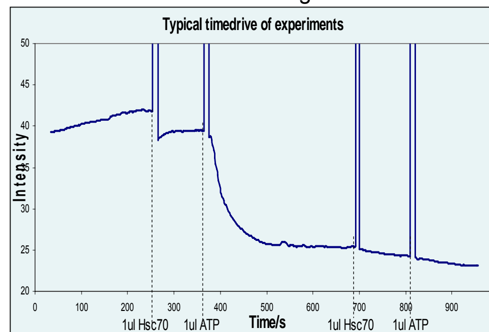
Spin, dialyse supernatant into column buffer and filtrate in superdex 200 column. Locate clathrin and dialyse in 'depolymerisation' buffer and then 'polymerisation' buffer.

Spin and resuspend pelleted cages in **Barouch** buffer.

Experiments & Results

The experiments involved observing changes in scattering intensity.

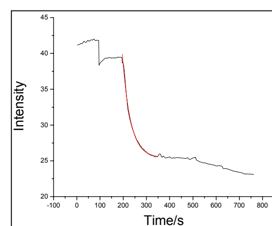
Hsc70, and then ATP were added to varying concentrations of clathrin cages and auxilin.



The drop in intensity signifies the disintegration of the cages into triskelions.

Graph Fitting

The decay curve was analysed using Origin® Data Analysis Software.



The majority of curves fitted a 2nd order exponential decay:

$$I = I_0 + A_1 e^{-\left(t/T_1\right)} + A_2 e^{-\left(t/T_2\right)}$$

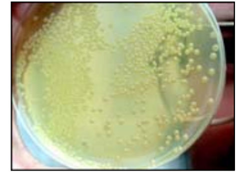
This suggests two separate reactions in the system.

Conclusions

- The value of T_1 was consistently between 29 s and 59 s, implying that the time constant of the decay is of the order 10.
- T_2 was much larger, ranging from 130 s to 1500 s. There was no noticeable pattern to these values.
- As the auxilin:clathrin ratio decreased, T_1 increased drastically at 1:40, giving a value of about 150 s. This implies that the lack of auxilin slows the uncoating due to the terminal domain being less accessible for hsc70 binding.
- For ratios less than 1:40, the decay began before the addition of ATP, suggesting that the stability of the cages was reduced by the absence of auxilin and thus the ATP-powered hsc70 cycle was unnecessary.

Production of Auxilin

Express the GST–tagged auxilin, residues 401 – 910, in a suitable E. coli strain.



Protein Expression

Add AEBSF protease inhibitor.

Lyse cells by sonication.

Centrifuge at 20k for 30 minutes.

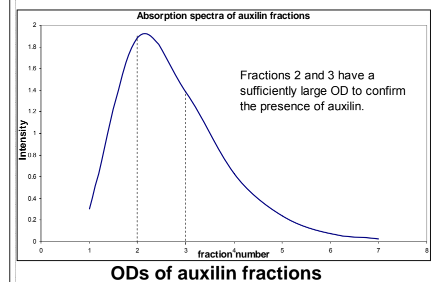
Add the supernatant to a GST-sepharose bead column and allow the GST tag on the auxilin to attach to the beads.

Sepharose filtration

Wash out other molecules.

To elute auxilin, wash through with glutathione buffer. The GST–tagged auxilin will detach from the beads.

Measure the optical density of the fractions collected to locate auxilin.



ODs of auxilin fractions

Pool auxilin-containing fractions.

Dialyze in Barouch buffer.

Microfuge to remove any contaminants.

Add AEBSF, as auxilin degrades easily.

Always store at 4°C.

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References

1. Fotin, A., Cheng, Y., Grigorieff, N., Walz, T., Harrison, S. & Kirchhausen, T. Structure of an Auxilin-bound clathrin coat and its implications for the mechanism of uncoating. *Nature* **432** (2004), 649
2. Fotin, A., Cheng, Y., Sliz, P., Grigorieff, N., Harrison, S., Kirchhausen, T & Walz, T. Molecular Model for a complete clathrin lattice from electron cryomicroscopy. *Nature* **432** (2004), 573