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Structure of clathrin coat with bound Hsc70 and auxilin: mechanism of Hsc70-facilitated disassembly

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 21 September 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the delay in getting back to you with a decision. Unfortunately, one of the referees was not able to get back to us with his/her report as quickly as initially expected.

Your manuscript has now finally been seen by three referees whose comments to the authors are shown below. As you will see while all three referees are generally positive and would support publication here after appropriate revision it also becomes clear that referees 1 and 2 express the concern that your conclusions are based on rather small effects; and referee 1 therefore feels strongly that some additional experimentation will be required to strengthen your evidence. We would therefore be able to consider a revised manuscript if you can address the referees' concerns in an adequate manner and if you can strengthen the experimental evidence for your conclusions along the lines put forward by referees 1 and 2 to their satisfaction.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Review of Xing et al.

This manuscript describes an electron microscopy single particle analysis of a complex of a clathrin assembly with the molecular chaperone Hsc70, and a segment of auxilin that is responsible for recruiting Hsc70 to clathrin.

The reconstruction is generated by a relatively small number of images (1500) at a resolution of 15Å, but is enhanced to 11Å by averaging using the internal symmetry of the clathrin D6-coat particles. From this reconstruction, the authors seek to define the location of the auxilin and Hsc70 segments included in the complex, using difference-density techniques. The authors make much of a 4% change in the axial ratios of the particle, and develop a discussion of the significance of this in the uncoating pathway of clathrin cages. Given the careful selection of a small (~10%) congruent set of images from the total dataset, this conformation may well be just one of many present in the total population of particles, and some other experimental approach will be needed if the claimed mechanistic significance of this conformation is to be established.

A significant weakness of the study is the reliance on difference-density features as the sole evidence for localizing the auxilin and the Hsc70 molecules in the complex. This is particularly an issue for the density attributed to Hsc70, which lies on the 3-fold axes at the centre of each clathrin triskelion. These symmetry axes were undoubtedly used in the n.c.s. symmetry averaging procedure used to enhance the signal-to-noise-ratio and improve apparent resolution. As noise inevitably builds up on symmetry axes during averaging procedures, there must be a serious concern that the difference density observed here may be an artifact of the procedure. This concern is reinforced by the author's own observation that the density has a three-fold symmetric shape. If it truly derives from averaging of a single molecule at each triskelion triad-axis, the resultant feature should have ~1/3 the electron density of the density for the surrounding clathrin molecules - is this the case ?

The authors need to provide objective experimental data that the small difference densities, which they attribute to auxilin (547-910) and to Hsc70 (1-554), do indeed arise from these molecules. This is particularly needed for the Hsc70. Labelling using Fabs, Ni-NTA gold-clusters targeted to an incorporated His-tag, or a reconstruction with Hsc70 and/or auxilin fusions with a large protein (eg MBP), would be sufficient and need only be done at modest resolution.

Referee #2 (Remarks to the Author):

Xing and colleagues present a novel cryoEM structure of a clathrin coat bound to Hsc70 and auxicillin. In their report, they use the 547-910 fragment of auxicilin that is known to bind to clathrin to recruit truncated Hsc70 (1-554)/ATP to the clathrin cage. They observed that ATP hydrolysis is necessary for tight binding of Hsc70 to clathrin. After determining the optimal molar ratio for all the proteins, they prepared them for cryoEM and solved the structure to 11.3Å. As with their previous study, they were able to achieve higher resolution by averaging the nine copies of clathrin found in the coat asymmetric unit. By fitting clathrin atomic structures in their EM density, they were able to determine that Hsc70 and auxicilin induce changes in the axial ratio of the coat compared to that of the undecorated cage. The authors suggest that their structure represents an intermediate where auxicilin and Hsc70 have induced strain in the coat structure, but the coats have not dissociated due to the low pH at which they performed their study. They suggest that low pH stabilizes interactions between adjacent clathrin molecules in the coat, but suggest that at neutral pH the accumulated strain from multiple auxicilin/Hsc70 proteins would lead to disassembly.

The manuscript was well written, and the hypotheses are fairly well supported, but there are a few concerns about the study.

1) Major concern - when performing the n.c.s symmetrization, how were the molecular contours selected for the Hsc70 density? Was Hsc70 symmetrized during the n.c.s averaging? If so, then perhaps more information could be extracted on Hsc70 structure if less averaging was performed.

2) More details are need in the Hsc70:ATP and Hsc70:ADP titration experiments as shown in figure 2A. The methods section seems to only describe the experiment shown in Fig. 2B.

3) In the 3D reconstruction, the authors describe collecting 14,000 particles but only using the 1500 best. Throwing away such a large number of particles is fairly unusual. How were the good particles selected? What would the reconstruction look like with all particles included? Is it possible that the other particles represent different conformations?

4) How do the authors define the molecular contours for the comparison of axial ratios between different reconstructions?

5) Though the fits of the atomic structures look convincing, the authors make their conclusions about the strain induced by auxicilin and Hsc70 based on their observations of small shifts in the positions of the atomic structures. The primary structures are never shown. The report would be more convincing if both the EM maps and the atomic structures were shown.

6) The middle of the structure looks surprisingly clean. Was an inner mask applied during the reconstruction?

7) In Fig. 4C, it is too hard to see the superposition of the two structures because the colors are too hard to distinguish from one another.

8) An image of an electron micrograph should be included to show the quality of their data.

Referee #3 (Remarks to the Author):

This paper provides new structural insight into the mechanism of hsc70-mediated disassembly of clathrin coats. The work is a very nice extension of their previous structure of the clathrin coat with bound auxilin.

1st Revision - authors' response 26 October 2009

Point-by-point response to reviewers

Reviewers 1 and 2 raised two major points. The first concerned the density ascribed to Hsc70, for which they requested further assurance of its significance. The second concerned the selection of particles, as only about 10% of the total number of initial images were retained in the final reconstructions. We have modified the MS to address these two points as follows.

Point 1. Both reviewers assumed that the difference map showing the Hsc70 location reflected n.c.s. threefold averaging, and naturally wondered whether pile-up of features near a symmetry axis could contribute. The difference density was NOT n.c.s. averaged, and we have added sentences to the second paragraph of the section entitled "Auxilin and Hsc70" to make this point clear. The difference maps are local differences between unaveraged, low-pass filtered maps, as described in Methods. To assess the difference density objectively, we give an analysis of its significance (in a new section of Methods), based on standard-deviation estimates from the independent, paired maps used to determine FSC. We also show the same feature in a lower-resolution (non-difference) map (Fig. S2: not n.c.s.-averaged), added to the supplementary information in response to reviewers' point 2. The standard-deviation estimates and the new, lower-resolution map show directly that the density surrounding the C-terminal extensions of the heavy chain is the principal new feature produced by the addition of Hsc70 to the coats and that this density is significant at the level of 4σ.

We note further that a mechanistically meaningful Hsc70 site must be in the region of the difference density, because we know from detailed mutagenesis studies (Rapoport et al, 2008) that a polypeptide chain segment "dangling" from the tripod is essential for Hsc70:ATP-dependent uncoating.

One reviewer suggested attempting to add density (e.g., NiNTA gold labels) to Hsc70, in order to identify the molecule directly. We have carried out a set of experiments of this kind, using NiNTA-gold, but the non-specific Hsc70 binding that inevitably accompanies specific binding (see Fig. 2) and the relatively high background level of non-specific binding of NiNTA-gold to the coat (independent of His-tagged-Hsc70, even in the presence of reasonable concentrations of imidazole (up to nearly 0.1 M) turns out to make this approach unacceptably ambiguous. Attaching additional tags to the N-terminus of Hsc70 would not be likely to strengthen the feature significantly, as the ATPase domain itself is probably "smeared out" with respect to the substrate binding domain, and attaching large additional tags to the C-terminus would prevent the substrate-binding domain from reaching its target in the first place. We have therefore chosen to demonstrate the significance of the Hsc70 density, using the computational and analytical approaches described above.

Point 2. The relatively stringent particle selection follows a procedure (based on phase residuals) analyzed in some detail by Fotin et al, J Struc Biol, 2006 -- a reference that was unfortunately missing from the previous version. As we now write in the section on "Electron cryomicroscopy of D6 coats with bound auxilin and Hsc70", clathrin coats are less rigid and less uniform than icosahedral virus particles or ribosomes, and elimination of particles with high phase residuals selects for minimally distorted coats. To verify that stringent selection of undistorted particles did not affect the molecular interpretation, we compared the model based on our final map with one of the intermediate maps we obtained in the course of refinement -- a reconstruction at 21Å resolution derived from about 7000 particles. All the features described and analyzed in our paper can be seen in this lower resolution map (which was not subjected to ncs averaging): the density due to Hsc70 beneath each vertex, the overall change in axial ratio of the coat, and the outward movement of the most distal portions of the triskelion legs (see the new Fig. S2 and the new panel in Fig. 6). To validate directly directly that discarded particles are distorted in some way, we carried out a multi-reference alignment of the complete data set, yielding six classes. The largest class contained about 44% of the particles. The remaining five classes showed clear evidence of distortion or damage (for details, see the new section in Methods entitled "Multi-reference alignment").

Reviewer 2 had some additional points (his/her points 1 and 3 correspond to points 1 and 2 above).

(Reviewer's point 2). Details of the Hsc70:ATP titration experiments. Several sentences from an earlier draft were indeed missing from the methods sections. These have been added.

(Reviewer's point 4). Axial ratio determination. The reviewer's questions have been answered by adding details to the caption of Fig. 6.

(Reviewer's point 5). The requested maps and atomic structures are shown in the new panel C of Fig. 6.

(Reviewers point 6). Masking of the center of the structure. Yes, the center was masked, to remove disordered density from AP2 molecules. This removal prevents density from the substoichiometric AP2 from pushing refinement of individual image orientations into false local minima. We have added a remark in the Methods section. See also Fotin et al, 2004a and Fotin et al, 2006, in which the procedure is explained in more detail.

(Reviewers point 7). Fig. 4C. Colors have been changed as requested.

(Reviewer's point 8). We have added a panel to Fig. S1 showing an image of an electron micrograph, as requested.

2nd Editorial Decision 23 November 2009

Thank you for sending us your revised manuscript. Our original referees 1 and 2 have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Before this will happen, however, I was wondering whether you would like to consider addressing the minor issues suggested by referee 2 (see below). Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2 (Remarks to the Author):

The revised manuscript is significantly improved and adequately addresses the criticisms raised from the original manuscript. A couple of minor concerns still remain, and these are listed below.

Minor points

More details are still required for the Hsc70:ATP and Hsc70:ADP titration experiments. One can infer that the centrifugation was performed to separate free proteins from assembled cages, but that should be explicitly stated in both the results and the methods. The figure legend and the text should also state what samples are run on Fig2, the pellet or the supernatant.

In the model for the uncoating mechanism shown in Fig 7 a yellow sphere is shown but nothing in the text describes it. In figure 1A) yellow is used to describe the nucleotide binding region of Hsc70 and green is used to show the substrate binding region. Is the yellow sphere supposed to indicate the nucleotide binding region?

2nd Revision - authors' response 25 November 2009

Here is a revision of the text of our MS, incorporating in the captions to Figs. 2 and 7 the clarifications requested by reviewer 2.

The Methods was already clear on the point of the gel showing a resuspended pellet, but the addition to the caption will now make it unambiguous.

