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A virus capsid-like nanocompartment that protects bacteria from oxidative stress

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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.)

Editor: Andrea Leibfried

1st Editorial Decision 07 April 2014

Thank you for submitting your manuscript entitled 'A virus capsid-like nanocompartment that protects bacteria from oxidative stress' to The EMBO Journal. I have now received all reports from the referees, which are enclosed below.

As you will see, all referees find your study well performed. However, they propose some amendments and additional experiments to better support your data and claims, all of which are clearly outlined in the reports. Referee #1 furthermore indicates that the study would be better suited in a more specialized journal. This is particularly expressed in the general evaluation form provided by the referee.

Given the constructive comments provided by the referees, we can offer to consider a revised version should you be able to amend your manuscript along the lines suggested by the referees. We would like to strongly encourage the inclusion of your cryo-EM data as requested by referee #2. We think that this would enhance the general interest of the paper and would thus probably positively contribute to referee #1's opinion on your manuscript.

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

REFEREE REPORTS

Referee #1:

McHugh et al. characterize the structure and partially the possible functions of a nanocompartment contained in Myxococcus xanthus. By using various methods, cryoelectron microscopy, energy-dispersive X-ray spectroscopy, coupled plasma mass spectrometry, SDS gel electrophoresis, genome mining, and modelling they arrive at a 180 units shell that encapsulates 3 proteins of which two are similar to rubrerythrin/ferritin and one to peroxidases. The shells contain an accumulation of iron and phosphorus in a 4:1 ratio. The amount of the shell-forming protein, designated encapsulin, is five-fold increased during starvation but absent in the subsequently formed spores. Mutants lacking the encapsulin show an increased sensitivity to hydrogen peroxide, suggesting lack of protection by iron.
Similar structures, assemblies and ferritin /peroxidase functions were published by Sutter et al. 2008 who characterized a particle from Thermotoga maritima. These authors determined in addition the crystal structure of encapsulin and found it similar to gp5 of phage HK97. They did not determine the Fe and P content and protection against hydrogen peroxide.
Comments
1. The authors should calculate the average number of iron ions per particle and compare it with ferritin related to the volume.
2. They should comment on the difference of 180 protein subunits as compared to 60 monomers in the Sutter paper forming particles of similar size.
3. They should state whether the ferrooxidase center is the most highly conserved domain suggesting that it functions in iron oxidation.
4. Lack of particles in spores may not only be caused by lack of assembly but most likely by protein degradation.

Referee #2:

This manuscript reports the analysis and structure of a bacterial iron-containing nano-compartment that resembles virus capsids with HK97 fold. The findings are exciting, the work is very well conducted and the manuscript is nicely written. The combination of different methods is excellent. I have only a few minor points and suggestions regarding the technical electron microscopy aspects of the paper.

Minor points:

More detail on some of the image processing methods would help others to follow the methods and apply similar approach using Bsoft on other structures. Especially:

Lines 407-421: It is not clear if two halves of the datasets were kept fully independent from the beginning. While this is not required for the C200 data set, it would be required for the Polara dataset. I believe this has been done (as 0.143 FSC threshold has been used in this case), but it is not clear from the text. This part needs to be clarified.

Line 418: Were the particles selected manually or using a template or something else? How much was the initial reference filtered to start the refinement? These details are important to exclude any doubt on reference bias in structure refinement (although I don't suspect that is the case here)

Line 451: How were the two maps normalised for difference imaging?

Data deposition: It seems that the structure containing the internal components has not been submitted in the EMDB. This would be required so that others can verify the difference imaging

Non-essential suggestions:

It would be nice if the structural cryo-EM analysis of the iron-containing capsids was included in this paper. That would strengthen the paper even more, as it could be compared to the two maps presented in the paper.

Referee #3:

From the abstract, it is hard to understand how the authors conclude the nanocompartments under study contain encapsulin or iron. The idea of iron storage/concentration and oxidant protection inside a protein nanocomparments has been attributed to members of the ferritin family of nanoproteins, which occur in Archea, bacteria and eukaryotes. Here an unrelated protein with the same function is described. It has an apparently independent origin and structure from the ferritin family.

The abstract lacks specific data on the properties of encapsulin subunits, especially in relationship to the ferritins. Numerical and rudimentary structural information in the abstract are unusually sparse. Such information in the abstract should include the succinct/concise answers to the following questions
1.What re the dimensions of the protein and the iron-containing cavity?
2. How many iron atoms are sequestered? Range? specific number
3.Is the phosphate content high as in bacterial ferritins (1:1)? Or low as in eukaroytic (P:Fe ~ 1:8)ferritins?
5. How do the authors know the function is solely oxidant protection and not iron storage for later utilization/nutrition functions as well?
6. What is the predicted (or known) secondary structure of the subunits? What is the subunit size?
7. Does Myxococcus also have ferritin genes (many bacteria have multiple ferritin-like or ferritin protein nanocage genes, which are expressed under different environmental conditions.

RESULTS:
The description of the results lacks information on the reproducibility of the data. How many times were each of the analyses in the results repeated (multiple cultures? multiple cryoEM preps and analyses? What is the type and magnitude of experimental variations observed?

In general, the results appear to be very significant and of interest to a wide segment of EMBO readers. However, from the information provided, evaluation of the data reproducibility is difficult. Moreover, the full significance of the information will be obscure from the abstract in is current form.

1st Revision - authors' response 09 May 2014

Referee #1:

McHugh et al. characterize the structure and partially the possible functions of a nanocompartment contained in Myxococcus xanthus. By using various methods, cryoelectron microscopy, energy-dispersive X-ray spectroscopy, coupled plasma mass spectrometry, SDS gel electrophoresis, genome mining, and modeling they arrive at a 180 units shell that encapsulates 3 proteins of which two are similar to rubrerythrin/ferritin and one to peroxidases. The shells contain an accumulation of iron and phosphorus in a 4:1 ratio. The amount of the shell-forming protein, designated encapsulin, is five-fold increased during starvation but absent in the subsequently formed spores. Mutants lacking the encapsulin show an increased sensitivity to hydrogen peroxide, suggesting lack of protection by iron.
Similar structures, assemblies and ferritin /peroxidase functions were published by Sutter et al. 2008 who characterized a particle from Thermotoga maritima. These authors determined in addition the crystal structure of encapsulin and found it similar to gp5 of phage HK97. They did not determine the Fe and P content and protection against hydrogen peroxide.
Comments

1. The authors should calculate the average number of iron ions per particle and compare it with ferritin related to the volume.
   This is now done. To do so Figures 4 and 6, which were formerly in an accompanying manuscript, have been transferred into this (revised) paper. Figure 6 reports the masses of many individual nanocompartments as determined by STEM microscopy.

Subtracting the masses of the icosahedral protein shell and the internal proteins, we obtain the masses of iron (and phosphorus), and hence the number of iron atoms. This number is not fixed. We are able to estimate the range from the spread of the STEM-derived masses. Further to this point, Figure 4 shows by cryo-electron tomography that the encapsulin cores are made up of a variable number (11 – 19) of dense (iron-rich) granules, each 5-6nm in diameter and we are able to estimate the number(s) of iron atoms per granule See lines 36/37, 167-176 and 208-226.

2. They should comment on the difference of 180 protein subunits as compared to 60 monomers in the Sutter paper forming particles of similar size.
Triangulation geometries of encapsulin shells and those of other HK97-like proteins compared in the new Figure 8. The 180-subunit T=3 architecture is shared by the M. xanthus encapsulin and that of P. furiosus, whereas that of T. maritima has 60 subunits (T=1; Sutter et al). In fact, HK97-like folds appear in all or almost all tailed bacteriophage capsids and they exhibit a wide range of T-numbers – up to at least 27. Returning to the M. xanthus system, the 15% of smaller particles assembled upon expressing EncA in E. coli have T=1 geometry. This issue is discussed in lines 309-319, and the small T=1 capsid – which we have reconstructed - is referred to as "unpublished results" as we are max'ed out with 9 Figures.

3. They should state whether the ferrooxidase center is the most highly conserved domain suggesting that it functions in iron oxidation.
Two supplementary figures (S2 & S3) present bioinformatic analysis of EncB & EncC. It shows that in both proteins the rubrerythrin/ferritin-like domain, including one of the predicted iron binding motifs in EncB and the predicted one in EncC, is highly conserved. Lines 102-112.

4. Lack of particles in spores may not only be caused by lack of assembly but most likely by protein degradation.
Agreed. This eventuality is now mentioned in lines 242-243.

Referee #2:

This manuscript reports the analysis and structure of a bacterial iron-containing nano-compartment that resembles virus capsids with HK97 fold. The findings are exciting, the work is very well conducted and the manuscript is nicely written. The combination of different methods is excellent. I have only a few minor points and suggestions regarding the technical electron microscopy aspects of the paper.

We thank the reviewer for his/her positive and encouraging remarks. We have now revised the manuscript to address the specific points raised, as detailed below.

Minor points:

More detail on some of the image processing methods would help others to follow the methods and apply similar approach using Bsoft on other structures. Especially:

Lines 407-421: It is not clear if two halves of the datasets were kept fully independent from the beginning. While this is not required for the C200 data set, it would be required for the Polara dataset. I believe this has been done (as 0.143 FSC threshold has been used in this case), but it is not clear from the text. This part needs to be clarified.
The two half data sets of images coming from the Polara were kept fully independent from the beginning. This is now described in lines 504-508.

Line 418: Were the particles selected manually or using a template or something else? How much was the initial reference filtered to start the refinement? These details are important to exclude any doubt on reference bias in structure refinement (although I don't suspect that is the case here)
Particles were manually selected. The initial reference used for the Polara data set was the CM200

EncA map band-limited to 15 Å. Now described in lines 497 & 505.

Line 451: How were the two maps normalised for difference imaging?
A complete account of the map normalization is given by Heymann et al., 2003, and is outlined in lines 513-518. In brief, the relative scaling of the reconstructions were determined using the Bsoft programs, bint to resize a map, and bop to determine the fit between two maps (one resized) using a linear least squares fitting algorithm.

Data deposition: It seems that the structure containing the internal components has not been submitted in the EMDB. This would be required so that others can verify the difference imaging
We have started the deposition of 3D reconstruction of the core-lacking native nanocompartments.
EMDB code: EMD-5953*This is now mentioned in the Data deposition section (line 401).*

Non-essential suggestions:

It would be nice if the structural cryo-EM analysis of the iron-containing capsids was included in this paper. That would strengthen the paper even more, as it could be compared to the two maps presented in the paper.
Taking into account the editorial encouragement to do so, this material is now included in the revised paper, both as a cryo-EM analysis showing the averaged 3D structure from 700 images (the new Figure 3), as cryo-ET analysis showing the 3D structures of individual nanocompartments (the new Figure 4), and STEM mass measurements (the new Figure 6), showing 2D mass maps of numerous individual particles. See accompanying text in revised manuscript.

Referee #3:

From the abstract, it is hard to understand how the authors conclude the nanocompartments under study contain encapsulin or iron. The idea of iron storage/concentration and oxidant protection inside a protein nanocomparments has been attributed to members of the ferritin fmaily of nanoproteins, which occur in Archea, bacteria and eukaryotes. Here an unrelated protein with the same function is described. It has an apparently independent origin and structure from the ferritin family.

The abstract lacks specific data on the properties of encapsulin subunits, especially in relationship to the ferritins. Numerical and rudimentary structural information in the abstract are unusually sparse.
We have rewritten the abstract to cover as many as possible of the points listed by the reviewer, given the word limit of 175 words for an EMBO J abstract. The other points are more prominently featured on the text so that the relevant information is readily accessed by a reader. The principal differences between encapsulin nanocompartments and ferritin cages are summarized in lines 271-275.

Such information in the abstract should include the succinct/concise answers to the following questions
1.What re the dimensions of the protein and the iron-containing cavity? *See abstract lines 32-33.*

2. How many iron atoms are sequestered? Range? specific number *See abstract, line 37 for mean, and Results, lines 208-226 for range and further discussion.*

3.Is the phosphate content high as in bacterial ferritins (1:1)? Or low as in eukaroytic (P:Fe ~ 1:8)ferritins?
Our ICP-MS data make it lower at about 1 : 4. See Table 1 and lines 122-123 & 218-226.

4. How do the authors know the function is solely oxidant protection and not iron storage for later utilization/nutrition functions as well?
We agree encapsulin nanocompartments may also have a nutrition function. This is discussed in lines 276-285.

5. What is the predicted (or known) secondary structure of the subunits? What is the subunit size?

EncB and EncC are predicted to be substantially a-helical, See lines 345-350 and Supplementary Figures S2 & S3.

6. Does Myxococcus also have ferritin genes (many bacteria have multiple ferritin-like or ferritin protein nanocage genes, which are expressed under different environmental conditions.
M. xanthus does possess ferritin genes. Now discussed in lines 276-285.

RESULTS:
The description of the results lacks information on the reproducibility of the data. How many times were each of the analyses in the results repeated (multiple cultures? multiple cryoEM preps and analyses? What is the type and magnitude of experimental variations observed?
A summary of this information is now included in tabular form in the Supplemental Material (and see below).

In general, the results appear to be very significant and of interest to a wide segment of EMBO readers. However, from the information provided, evaluation of the data reproducibility is difficult. Moreover, the full significance of the information will be obscure from the abstract in is current form.

We thank the reviewer for the positive assessment. We have altered the title and the abstract accordingly.

- *Supplemental Table 1: Statistical Summary of Experiments Performed.*
-
- *Total number of purifications:*
    - *At least 27 independent isolations of the native nanocompartments were performed. Found to be consistent by negative staining EM and SDS-PAGE.*
    - *2 independent isolations of EncA shells from E coli were performed. SDS-PAGE and Western-blot confirmed that they were composed of >95% EncA.*
    -
- *The proteins forming the encapsulin nanocompartments were identified by two approaches from two independent isolations: MALDI/TOF mass spectrometry and Edman degradation analysis. During optimization of the purification protocol, the same proteins were found by MALDI/TOF, resulting in at least 4 identifications of the encapsulin nanocompartment components.*
-
- *EDX elemental analysis was performed on 6 encapsulin particles. Iron was consistently found at high levels in the core of the encapsulin particles and it was not present in the proteinaceous rim of the particles (corresponding to the EncA shell) nor in the grid background.*
-
- *ICP-MS: measurements consisted of 5 independent isolations done in triplicate.*
-
- *EM:*
    - *The same preparation of EncA shells was used (on separate occasions) to collect first the CM200 data, and then the Polara data, which gave a reconstruction to 4.6Å. The FSC curve shown in Figure S6 attests to the internal consistency of these data.*
    - *Two independent preparations of native nanocompartments were studied by cryo-EM/cryo-ET and by STEM. They were consistent except that the first preparation contained ~6% of the core-lacking particles while the second contained < 1%.*
- *To quantify yields of dense-cored nanocompartments, equal samples of the respective isolates were applied for 15 s to carbon-coated 400 mesh copper grids and the numbers of particles per area counted. At least three independent fields were analyzed in each experiment for two independent isolations for each condition.*
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- *Peroxide stress assays were repeated three times with independent cultures. Each*

experiment included three replicates for each sample tested. Further, the disruption and deletion strains used in these experiments were generated completely independently.

2nd Editorial Decision 27 May 2014

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Referee #2:

The Authors have adequately addressed all of the minor issues I had indicated. I recommend the article for publication.

Referee #3:

The demonstration that encapsulation of iron particles in bacterial viruses as part of antioxidant protection is an important complement to other mechanisms of antioxidant protection found in the ferritin superfamily of mineral-synthesizing protein nanocages.

