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The mechanism of Vault opening from the high resolution structure of the N-terminal repeats of MVP

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Transaction Report:

(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 04 May 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Please excuse the slight delay in getting back to you with a decision - but as the three reviewers who have looked at your study were clearly very divided in their opinions and recommendations, I chose to additionally consult with an expert editorial advisor regarding the validity of some of the arguments raised. As you will see from the comments below, referees 2 and 3 bring up a number of important points (concerning technical as well as presentational aspects) but are generally supportive of publication given the interest of the topic and the importance of the structural improvements compared to previous work. On the other hand, referee 1 opposes publication, criticizing both limited amount of novel biological insights as well as major technical aspects of the work. Following discussions (especial about these technical criticisms) with our editorial advisor, we have come to conclude that these concerns should not preclude further consideration of your study further in line with the positive majority recommendation of the other two referees - given that also the expert advisor felt that data collection and data refinement appears to be done appropriately. With regard to the argument of limited direct biological insight, I realize that this would also apply to the recent structural work by Tanaka et al., and should therefore also not prevent publication, although a revision of the study would probably benefit from carefully reconsidering the discussions and proposals on functional implications.

In conclusion, I would therefore like to invite you to carefully consider the various comments and suggestions made by the referees, and to prepare a revised version of the manuscript in their spirit (a good starting point may be to heed referee 3's general suggestions for re-roganizing the manuscript; please also note that your manuscript is currently well below our length restrictions leaving space for various additions). Please be reminded that it is EMBO Journal policy to allow a single round of major revision only, and that we of course require PDB submission of all coordinates prior to acceptance. In any case, please do not hesitate to get back to us should you need feedback on any

issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to receiving your revision as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

What recent evidence indicates that the vault halves can dissociate in response to cellular signals (as stated in the abstract)? No evidence has been presented that vaults reversibly open at low pH, in fact, Goldsmith and colleagues (2007) state that the dissociation was irreversible (the manuscript says the data explains the reversibility).

The fact is that the human lung-resistance protein was found to be orthologous to the rat MVP linking vaults with multi-drug resistance.

The authors state that their data agrees with there being 39 copies of MVP that form half of the vault particle. How do 39-copies open into a structure with a central ring surrounded by 8-petals (Kedersha et al. 1991)? Perhaps there are really 40 copies of MVP/vault instead of the 39?

The manuscript is presented for review without coordinates, and indeed the document contains no statement that coordinates and structure factors have been or will be deposited anywhere in the wwPDB. Most journals require deposition prior to publication, and this manuscript specifically is suspicious without coordinates.

About prior MVP and vault structural work, and the persistence of errors: Tanaka, et al, and the present authors accepted as truth the Kozlov, et al.NMR model 1Y7X. Model 1Y7X looks to this reviewer(and to the various structure validation servers) like nobody looked at the model prior to publication (it has hydrophobic aspartates, for example). Similarly, the Tanaka et al model contains impossibilities that are obvious to the eye and to validation software, again as though nobody looked at the model. Querol-Audi, et al. do not mention Anderson, et al. (2007) Public Library of Science (PLoS) Biology, 5(11), 2661-2670, although the manuscript under consideration reads as though the authors read some of the supporting online materials for this PLoS article. That there have been previous structural efforts is easily discovered by searching the PDB for "vault" or searching www.ncbi.nlm.nih.gov/pubmed/ for terms such as "vault crystal structure" or "vault protein structure".

Starting with fundamentals, have the authors verified that they worked with MVP domains 1-7? In the p.9-10 sections about producing R1-R7, there is no mention of verifying the correct mass by mass spectrum analysis. If the R1-R7 construct was correctly made, shouldn't it have aggregated into barrel or ribbon shapes under the conditions of purification? The job of these domains is to aggregate. The crystallization condition seems aggressive, as though the second virial coefficient is strongly positive prior to crystallization. This indicates that the construct in the crystal has no tendency to form vault-like contacts. At the end of this analysis, the interactions shown in Fig. 3 do not look like crystal contacts.

The MAD and SAD phasing methods followed by ARP/wARP (p.10) should have produced at least a preliminary trace with side chains. That ARP/wARP could build 400 residues as poly-ala but not assigning side-chains indicates to me that the object in the crystal did not have the sequence input to ARP/wARP. P.10 middle, the authors incorporated the shape of the (wrong) 1Y7X model into the mask, automatically decreasing the credibility of any analysis that follows.

About Table 1: The R-factors, especially for the P1 form seem qualitatively high. The R-factor indicates overlap between the model and crystal contents, but does not indicate correctness. The rms deviations from ideal geometry are meaningless because ideal geometry is a refinement target. Ramachandran-like analysis is not a refinement target and is an important independent check. 5% of 383 residues is 19 residues that attract too much attention. It might be useful to investigate why the gradient matrix, subject to restraints (as implemented in REFMAC), was unable to simultaneously fit side chains alongside a low-energy backbone. Does the sequence match the crystal contents?

The authors should use some widely available web tools to test the plausibility of the R1-R7 model. These analyses could be reported along with R-factors. Looking down the Google list, the PDB validation server does some checking of bond geometry, but does not check if the model is physically or biologically possible. Part of the JCSG structure validation software seems to be a re-invention of ERRAT, which is very sensitive to impossibilities, such as hydrophobic aspartates resulting from chain-trace errors. As an example, in SI Fig.3, Q82 appears too close to a very hydrophobic side chain, the sort of impossibility that is detected by ERRAT-like analysis, or by eye.

There are numerous annoying errors - for example:

p.5 the two monoclinic crystal forms and the ≈ resolution are reversed (either in the Table or in the text). The text states P21A (2.5≈) and P21B (3.0≈), the table
states P21A (3.0≈) and P21B (2.5≈).

p.6. Should refer to supplementary Table III not II.

Fig. 4. There are two part B's.

Referee #2 (Remarks to the Author):

One of the key points of this paper are a 2.1 A crystal structure of the R1-R7 fragment of the major vault protein (MVP) that is a major improvement over a recently published 3.5 A crystal structure by Tanaka et al, 2009 of the entire rat liver vault particle. The paper also describes two lower resolution structures of the entire vault particle at 8 A resolution.

The improvement of the quality of the R1-R7 fragment is an important contribution to the study of MVPs.

Comments:

1. it would be of interest to re-refine the structure by Tanaka et al, 2009 by molecular "replacement" of the R1-R7 fragment determined in this paper. This would produce a corrected structure of the entire vault particle at 3.5 A resolution, so at higher resolution than the 8 A structures presented here. I checked - the diffraction data for the structure by Tanaka et al. are available in the PDB.

2. a comparison of the entire (not just R1-R7) vault structure determined by Tanaka and the 8 A structures presented here should be performed.

3. The anomalous data sets appear to have relatively low phasing power perhaps explaining why density modification was required to obtain interpretable maps for "automatic" building of roughly half the molecule. I'm curious if the SeMet positions seen in (anomalous) difference Fourier maps actually match the location of the model's Met positions.

4. Supplementary figure 3 should be an SA-weighted omit map (or, better, annealed omit map).

Referee #3 (Remarks to the Author):

In their manuscript "The mechanism of Vault opening from the high resolution structure of the N-terminal repeats of MVP" Verdaguer and coworkers present the crystal structure of the seven N-terminal repeat domains of the major vault protein (MVP) together with the structure of the intact

vault particle at 8A resolution. Vaults are the largest known ribonucleoprotein complexes and are conserved among eukaryotes. Although their exact physiological role is still not known they have been implicated with molecular transport and multidrug-resistance. Vaults are composed of a highly symmetric shell formed by many copies of MVP, and two additional proteins and a small RNA, which are present in lower copy numbers. Very recently, Tanaka et al., published the first detailed structural model of the vault particle by X-ray crystallography at intermediate resolution and revealed the 39-fold dihedral symmetry of the shell composed of 78 MVP molecules, while earlier work suggested a 48-fold symmetry and the presence of 96 MVP molecules. In the current manuscript, Verdaguer et al. report an independent, crystallographically sound low resolution structure determination of the vault particle (in essentially the same crystal forms as reported by Tanaka and coworkers) by molecular replacement. To achieve this, Verdaguer crystallized the first 7 beta-sheet repeat domains of MVP in three different crystal forms and determined their structure at high-resolution. Remarkably, the crystal packing of this fragment resembles a flattened-out variant of their packing in the intact vault particle, reemphasizing the relevance of the observed conformation of this critical part of MVP. The R1-7 structure thus provides the first high resolution model of a central part of the vault particle. The tracing of the polypeptide chain in repeat domains 1 and 2 by Verdaguer et al. disagrees with the structure reported by Tanaka. The significantly higher resolution and quality of data as well as the independent observation in three different crystal forms clearly argue for the correctness of the model by Verdaguer; local deviations in the Tanaka model may result from the absence of sequence markers and a larger disorder in the affected domains. The improved structural model for R1-2 is highly relevant for the understanding of the dissociation of the vault particle into halves and their flowerlike opening - key dynamic features of vaults - and for the design of future experiments to study these processes. Due to the considerable interest in understanding the physiological role of vaults, these results are of general interest for the molecular biology community. The methods employed are described adequately and all conclusions are comprehensible and they are appropriately discussed with regard to previous literature. Detailed comments are provided below.

Detailed comments:
1. Introduction:
The reference for (Esfandiary et al., 2008) should be updated.
2. Results and Discussion - Structure of the MVP N-terminal fragment R1-R7:
"was crystallized in three different space groups" should read "was crystallized in three different crystal forms"
3. Results and Discussion - The structure of R1-R7 provides ...:
Figure 3 is referenced before Fig. 2, the order of these figures should be adjusted. The different R1-R7 monomers should be highlighted in different colors, in particular for the insets. Some of the boxes indicating the position of insets appear to be misplaced.
4. Results and Discussion - The structure of R1-R7 provides ...:
"Intact vaults ... crystallize in different crystal forms": The authors should mention here that the crystal forms analyzed in their work are basically identical to the ones described by Tanaka et al.

5. Results and Discussion - Structural comparison of the seven ...:
The authors could additionally provide a more detailed analysis of the R1-R2 fragment as Supplementary Material. In particular, they could use crystallographic measures of quality, such as real space map correlation or temperature factor distribution to compare their model vs. the 3.5A model. In general, they could also compare the fit of their model derived from the high resolution R1-R7 structure against the deposited 3.5A data by Tanaka et al.

6. Results and Discussion - Hypothetical mechanism for vault opening ...:
In this section the authors should clearly focus on the mechanism suggested by their novel data. The detailed discussion on the comparison with the earlier model should be either moved to the previous paragraph or to the Supplementary Material. Also Fig. 4 should focus on a mechanism for vault opening. The comparison to the 3.5A structure should be moved to Supplementary Material, and more space should be given to adequately represent the interface between vault halves and to depict a mechanism of vault opening, possibly also in a schematic way more amenable for the general readership. In particular, the two aspects of vault opening, dissociation into halves and flower-like opening, should be discussed and represented in a figure and both interfaces, those between vaults halves and between MVP protomers within a half vault should be shown in the figure. A close-up inset to Fig.4b would be required to recognize the details indicated by arrows. The coloring levels

for electrostatic potential and the method of calculation should be provided. pKa calculations might be employed to analyse, which of the suggested residues is most likely protonated first, and thus triggers dissociation. In Fig. 4a the color choice (gold vs. orange) is not optimal.

7. Materials and Methods
General: Abbreviations should be spelled out.

8. Materials and Methods, Crystallization:
The authors have used a relatively low pH of 5.6 for crystallization, which might already lead to partial protonation of acidic side chains in particular environments. The authors could indicate whether the surrounding of acidic residues that are suggested to be involved in opening allows any conclusion on their protonation state.
Anisotropic scaling was applied to the raw data. Were scaled data used in refinement? Normally, the refinement software would take care of a model-based anisotropic scaling. If the data were severely anisotropic, this should be mentioned here and represented in a figure in the supplementary material.

9. Materials and Methods, Crystallization and X-ray analysis of the vault particle
The detailed comparison of the crystal packing modes of vaults could be moved to Supplementary Material because it is probably not of general interest. Supplementary Fig. 4 is of unacceptably low quality. The calculation should be repeated with more specialized computer programs, such as GLRF.

10. Materials and Methods, Molecular replacement and density modification
The implausibility of a fit of rings with more or less than 39 protomers could be represented in a figure. In addition to their current analysis, the authors could make use of the deposited data of Tanaka et al. to analyze the fit of the current model to these data.

11. Table 1.
Values for the highest resolution shell should be provided for Mean I/sigma, Rmerge and completeness. For P21Rm the I/sigma is strikingly lower than for any other data set. Overall Rcullis is very high, values for a limited resolution range could be provided.
12. SI Table 3.
Reasons for the low overall completeness should be provided in the Methods section. Have multiple crystals been used for data collection? Values for the highest resolution shell should be provided.
13. SI Figure 1c.
Coloring levels and method of electrostatic potential calculation should be provided.
14. SI Figure 3.
The authors might consider to show an unbiased omit map instead of a 2Fo-Fc map.

1st Revision - authors' response 28 July 2009

Please find enclosed the revised version of our manuscript entitled: "The mechanism of vault opening from the high resolution structure of the N-terminal repeats of MVP" addressing most reviewers criticisms. In particular, the manuscript is reorganized following the indications of referee 3. The last section of discussion was modified and extended. It is now focused on the mechanism of vault opening suggested by our new data. We also added a new figure (Figure 4C) that schematically shows the mechanism of vault opening.

The answers to referees questions and comments follow :

Reviewer 1:

What recent evidence indicates that the vault halves can dissociate in response to cellular signals (as stated in the abstract)?

The sentence in the abstract has been changed in the modified version of the manuscript

No evidence has been presented that vaults reversibly open at low pH, in fact, Goldsmith and colleagues (2007) state that the dissociation was irreversible (the manuscript says the data explains the reversibility).

We agree that the reversibility of the vault opening has not yet been explicitly demonstrated. However, as indicated in the text, vault-like particles (VLPs), similar to purified endogenous vaults, are observed when rat MVP is expressed in insect cells indicating that MVP is sufficient to direct the assembly of VLPís (Stephen et al., 2001). In turn, Goldsmith et al., (2007) showed that vault particles dissociated into half-vaults at low pH. The phenomenon was visually confirmed by transmission electron microscopy. The same authors pointed that identification of conditions for reversible vault disassembly and reassembly could enable application of these particles in drug delivery. Herlevsen et al., 2007 found that MVP knockdown disrupted the lysosomal compartment and suggested that the acidic nature of the lysosomes may serve as an excellent microenvironment with which to trigger vault dissociation. Finally, Poderycki and colleagues in 2006 managed to incorporate vault-associated proteins into preformed MVP-only recombinant vaults proving is not a rigid, impenetrable box but more a fluctuating dynamic structure presenting substantial flexibility. Our structural results, showing important charge complementarity at the interface between the vault halves, led us to propose the pH dependent interaction mechanism presented in the manuscript. At low pH vault disassembly would be facilitated by charge repulsion due to the protonation of the acidic residues at the contacting interface, while stability would be favoured at higher pHs. In this context, we think it is reasonable to refer to the proposed dissociation mechanism as reversible.

The authors state that their data agrees with there being 39 copies of MVP that form half of the vault particle. How do 39-copies open into a structure with a central ring surrounded by 8-petals (Kedersha et al. 1991)? Perhaps there are really 40 copies of MVP/vault instead of the 39?

As we explained in the methods section: "Molecular replacement and density modification" (Page 12): The models with rotational symmetries of 40 or higher result in unacceptable steric clashes between neighbour vaults in the crystal. A rotational symmetry of 39 allows interaction between neighbour vault particles without introducing steric problems. The implausibility of a fit of rings of 40 protomers is now presented as supplementary material (New supplementary Figure 2, referenced in pages 6 and 12).

A possible explanation of how the vault particle may open like a flower that seems to be composed of eight petals, surrounding a central ring is now provided in the last section of Results and discussion (page 9).

The manuscript is presented for review without coordinates, and indeed the document contains no statement that coordinates and structure factors have been or will be deposited anywhere in the PDB. Most journals require deposition prior to publication, and this manuscript specifically is suspicious without coordinates.

Coordinates and structure factors are deposited in the PDB with codes: 3GNF, 3GNG and 3GF5. The deposition dates were February 26, and March 17th of 2009. This information has been included in the revised version of the manuscript (Methods section; page 12).

About prior MVP and vault structural work, and the persistence of errors: Tanaka, et al, and the present authors accepted as truth the Kozlov, et al.NMR model 1Y7X. Model 1Y7X looks to this reviewer (and to the various structure validation servers) like nobody looked at the model prior to publication (it has hydrophobic aspartates, for example). Similarly, the Tanaka et al model contains impossibilities that are obvious to the eye and to validation software, again as though nobody looked at the model. Querol-Audi, et al. do not mention Anderson, et al. (2007) Public Library of Science (PLoS) Biology, 5(11), 2661-2670, although the manuscript under consideration reads as though the authors read some of the supporting online materials for this PLoS article. That there have been previous structural efforts is easily discovered by searching the PDB for "vault" or searching www.ncbi.nlm.nih.gov/pubmed/ for terms such as "vault crystal structure" or "vault protein structure".

We agree that the NMR model of repeats R4R5 contains some errors, but even so the quality was good enough as to provide a partial molecular replacement solution of the R1R7 structure when using the NMR coordinates of repeat R5. In turn, the model published by Anderson et al., modelled in an averaged electron density map (at 9 ≈ resolution) that was obtained assuming an erroneous vault symmetry (of D48 instead of D39), did not allow us any molecular replacement solution, neither for the the entier vault particle nor for the R1R7 structures. Finally, considering only the R1R7 structures, superimpositions of each of the seven repeats from our crystal structures with those reported by Anderson et al. obtained by computational methods show large discrepancies specially regarding the length and shape of the -strands in each module. Therefore, we would prefer to skip considerations about the Anderson model that, in our opinion, do not provide any relevant information for the present work.

Starting with fundamentals, have the authors verified that they worked with MVP domains 1-7? In the p.9-10 sections about producing R1-R7, there is no mention of verifying the correct mass by mass spectrum analysis. If the R1-R7 construct was correctly made, shouldn't it have aggregated into barrel or ribbon shapes under the conditions of purification? The job of these domains is to aggregate. The crystallization condition seems aggressive, as though the second virial coefficient is strongly positive prior to crystallization. This indicates that the construct in the crystal has no tendency to form vault-like contacts. At the end of this analysis, the interactions shown in Fig. 3 do not look like crystal contacts.

The DNA sequence of the construct was carefully verified prior to the overexpression and purification of the R1-R7 construct. Moreover, the X-ray maps of the three structures allowed to identify with confidence most residues and side chains of the R1R7 recombinant fragment (triclinic P1 structure: 2.1 ≈ resolution, residues determined: from Glu5 to Ile380; P21A structure: 3.0 ≈ resolution, residues determined: from Ala2 to Ile380 and P21B 2.5 ≈ resolution, residues determined: from Met1 to Ile380, plus the four extra amino acids at the N-terminus, corresponding to the linker between R1R7 and the cleaved GST-Tag). Taking into account the quality of the X-ray data we consider that mass spectrometry analysis is not necessary.

The fact that we obtained the different crystal forms, diffracting at high resolution, demonstrates that the recombinant R1R7 domain was in good shape and aggregates in an ordered form

Crystallization conditions are totally standard (Crystal Screen INDEX, Hampton Research; with small variations around the condition D10).

The MAD and SAD phasing methods followed by ARP/wARP (p.10) should have produced at least a preliminary trace with side chains. That ARP/wARP could build 400 residues as poly-ala but not assigning side-chains indicates to me that the object in the crystal did not have the sequence input to ARP/wARP. P.10 middle, the authors incorporated the shape of the (wrong) 1Y7X model into the mask, automatically decreasing the credibility of any analysis that follows.
About Table 1: The R-factors, especially for the P1 form seem qualitatively high. The R-factor indicates overlap between the model and crystal contents, but does not indicate correctness. The rms deviations from ideal geometry are meaningless because ideal geometry is a refinement target. Ramachandran-like analysis is not a refinement target and is an important independent check. 5% of 383 residues is 19 residues that attract too much attention. It might be useful to investigate why the gradient matrix, subject to restraints (as implemented in REFMAC), was unable to simultaneously fit side chains alongside a low-energy backbone. Does the sequence match the crystal contents?

The authors should use some widely available web tools to test the plausibility of the R1-R7 model. These analyses could be reported along with R-factors. Looking down the Google list, the PDB validation server does some checking of bond geometry, but does not check if the model is physically or biologically possible. Part of the JCSG structure validation software seems to be a re-invention of ERRAT, which is very sensitive to impossibilities, such as hydrophobic aspartates resulting from chain-trace errors. As an example, in SI Fig.3, Q82 appears too close to a very hydrophobic side chain, the sort of impossibility that is detected by ERRAT-like analysis, or by eye.

The anomalous data for the SeMet derivative showed relatively low phasing power (Table I). This fact would explain why we needed additional information, as a partial molecular replacement

solution and two fold averaging density modification, to obtain more interpretable maps.
R-factors in the three space groups as well as the geometry of the models, with all residues in the allowed regions of the Ramachandran plot, are correct.

The side chain residue 82 of SI. Figure 3, mentioned by the referee, is located apart of L74 (the shortest distance between the polar atoms of Arg82 and the Leu side chain mentioned by the referee is 4.5 ≈). In the revised version of the manuscript, the old SI. Figure 3 corresponds to the SI. Figure 5 and the 2Fo-Fc map has been changed by an annealed omit map.

p.5 the two monoclinic crystal forms and the resolution are reversed (either in the Table or in the text). The text states P21A (2.5 A) and P21B (3.0 A), the table states P21A (3.0 A) and P21B (2.5A).

Text is modified in the revised version of the manuscript in order to be coherent with Table I.

p.6. Should refer to supplementary Table III not II.

There are only two supplementary tables. The statistics of the X-ray data of the complete vaults (referred in page 6) are summarised in Supplementary Table II.

Fig. 4. There are two part B's.

Figure 4 is modified in the revised version and the labeling has been corrected.

Reviewer 2:

1. it would be of interest to re-refine the structure by Tanaka et al, 2009 by molecular "replacement" of the R1-R7 fragment determined in this paper. This would produce a corrected structure of the entire vault particle at 3.5 A resolution, so at higher resolution than the 8 A structures presented here. I checked - the diffraction data for the structure by Tanaka et al. are available in the PDB.

We think this is a very important suggestion.

In the structure of the vault particle reported by Tanaka et al., the 39 copies of the MVP molecule were independently refined with individual atomic b factors (at 3.5 A resolution and with an averaged temperature factor of 121.4), using the CNS protocols (PDB ids. 2ZUO, 2ZV4 and 2ZV5). We produced a hybrid model containing the R1R7 structure, at the N-terminus (residues form Glu4 to Val380), and the coordinates corresponding to monomer A of the structure of Tanaka et al., 2009 (2ZV4), at the C-terminus (residues from P381 to G814). This hybrid model fitted reasonably well in the averaged electron density map calculated with DM (referred in page 8 in the manuscript) with the deposited diffraction data of Tanaka et al. Using also this Tanaka et al. diffraction data the fitted hybrid model was then subjected to rigid body refinement with CNS, treating each of the twelve domains defined by Tanaka et al., 2009 as independent bodies and using the 39-fold non-crystallographic symmetry as a constraint. The resulting structure was then further refined using the minimize protocol of CNS also maintaining strict 39-fold non-crystallographic symmetry. Convergence was reached with an R-factor of 37 % and an overall temperature factor of 78 ≈. The model was used to re-analyse the interactions between the two vault halves (essentially the same to those described in the previous version of the manuscript) and to produce the new figure 4.
At this point we feel better stopping our report of the vault refinement (based on the Tanaka et al. diffraction data), as completion of the work requires a revision of the coordinates also outside the R1-R7 region, where no new high resolution experimental information is added in the present work.

2. a comparison of the entire (not just R1-R7) vault structure determined by Tanaka and the 8 A structures presented here should be performed.

A comparison of the entire vault structures is now provided in pages 6-7 and the new supplementary Figure 4.

3. The anomalous data sets appear to have relatively low phasing power perhaps explaining why density modification was required to obtain interpretable maps for "automatic" building of roughly half the molecule. I'm curious if the SeMet positions seen in (anomalous) difference Fourier maps actually match the location of the model's Met positions.

Yes, the SeMet positions seen in the anomalous maps are coincident with the locations of the Met positions. The main problem seemed to be related with the anisotropy of the data. A new supplementary Figure 8 was added to illustrate this point.

4. Supplementary figure 3 should be an SA-weighted omit map (or, better, annealed omit map).

The new figure is a stereo view of an annealed omit map. In the revised version, this new figure is Supplementary Figure 6

Reviewer 3:

1. Introduction: The reference for (Esfandiary et al., 2008) should be updated.

The revised version contains the updated reference.

2. Results and Discussion - Structure of the MVP N-terminal fragment R1-R7:
"was crystallized in three different space groups" should read "was crystallized in three different crystal forms"

The text of page 5 has been modified.

3. Results and Discussion - The structure of R1-R7 provides ...:
Figure 3 is referenced before Fig. 2, the order of these figures should be adjusted. The different R1-R7 monomers should be highlighted in different colors, in particular for the insets. Some of the boxes indicating the position of insets appear to be misplaced.

Figure 2 is referenced in the first paragraph of page 6, together with supplementary table I. Figure 3 is referenced in the second paragraph, Title:
"The structure of R1R7 providesÖ" after Figure 2.

The new Figure 3 shows the symmetry related monomers in a different colour and the boxes indicating the position of the insets are now placed in the correct position.

4. Results and Discussion - The structure of R1-R7 provides ...:
"Intact vaults ... crystallize in different crystal forms": The authors should mention here that the crystal forms analyzed in their work are basically identical to the ones described by Tanaka et al.

The paragraph is modified in the revision version, mentioning that the crystal forms analysed here are basically identical to those described in Tanaka et al.

5. Results and Discussion - Structural comparison of the seven ...:
The authors could additionally provide a more detailed analysis of the R1-R2 fragment as Supplementary Material. In particular, they could use crystallographic measures of quality, such as real space map correlation or temperature factor distribution to compare their model vs. the 3.5A model. In general, they could also compare the fit of their model derived from the high resolution R1-R7 structure against the deposited 3.5A data by Tanaka et al.

Density correlations are calculated for the R1R7 structures in the P1 crystal and for the 3.5 A vault structure. A new figure (Figure 5) is provided as Supplementary material and the results obtained are discussed in page 7.

6. Results and Discussion - Hypothetical mechanism for vault opening ...:
In this section the authors should clearly focus on the mechanism suggested by their novel data. The detailed discussion on the comparison with the earlier model should be either moved to the previous

paragraph or to the Supplementary Material.
Also Fig. 4 should focus on a mechanism for vault opening. The comparison to the 3.5A structure should be moved to Supplementary Material, and more space should be given to adequately represent the interface between vault halves and to depict a mechanism of vault opening, possibly also in a schematic way more amenable for the general readership. In particular, the two aspects of vault opening, dissociation into halves and flower-like opening should be discussed and represented in a figure and both interfaces, those between vaults halves and between MVP protomers within a half vault should be shown in the figure. A close-up inset to Fig.4b would be required to recognize the details indicated by arrows. The colouring levels for electrostatic potential and the method of calculation should be provided. pKa calculations might be employed to analyse, which of the suggested residues is most likely protonated first, and thus triggers dissociation. In Fig. 4a the colour choice (gold vs. orange) is not optimal.

In the revised version, this section is extended and totally focused to the vault opening mechanism. The comparison of the Tanaka model is moved to the previous paragraph.

Figure 4 is also modified to adequately represent the interface between vault halves as the reviewer suggested. Close-up insets showing both, half vault and lateral contacting surfaces are added. Electrostatic potential was calculated and rendered with pymol (colouring levels -66.7 66.7) This information is now provided in the figure legend

A new panel was also created (Figure 4C) that shows a schematic diagram illustrating the mechanism of vault opening. The two aspects of vault opening:
The dissociation into halves and flower-like opening are represented in the figure and discussed in the main text (pages 8-9).

Colours in Figure 4A are changed.

9. Materials and Methods, Crystallization and X-ray analysis of the vault particle.
The detailed comparison of the crystal packing modes of vaults could be moved to Supplementary Material because it is probably not of general interest. Supplementary Fig. 4 is of unacceptably low quality. The calculation should be repeated with more specialized computer programs, such as GLRF.

The comparison of the crystal packing modes of vaults is moved to supplementary material, as suggested by the referee. A new supplementary figure of the self rotation function with better quality is provided in the revised version (SI Figure 9).

10. Materials and Methods, Molecular replacement and density modification
The implausibility of a fit of rings with more or less than 39 protomers could be represented in a figure. In addition to their current analysis, the authors could make use of the deposited data of Tanaka et al. to analyze the fit of the current model to these data.

A new supplementary figure (SI. Figure 2) is provided, showing the implausibility of a fit of rings with more or less than 39 protomers.

11. Table 1.
Values for the highest resolution shell should be provided for Mean I/sigma, Rmerge and completeness. For P21Rm the I/sigma is strikingly lower than for any other data set. Overall Rcullis is very high, values for a limited resolution range could be provided.

The values (Rmerge, completeness and I/sigma) for the highest resolution shell are provided in the revised Table 1.
Old Table I contained some errors in the values of phasing power and Rcullis. These errors are corrected in the modified Table.

12. SI Table 3.
Reasons for the low overall completeness should be provided in the Methods section. Have multiple crystals been used for data collection? Values for the highest resolution shell should be provided.

We used multiple crystals for data collection of the complete vault particle.
However, most of the analysed crystals were non isomorphous.
An explanation is now provided in the methods section (page12).
Values for the highest resolution shell are included in the revised SI:Table II.

13. SI Figure 1c.
Colouring levels and method of electrostatic potential calculation should be provided.

Electrostatic potential was calculated displayed with Pymol (colouring levels from ñ56.8 to 56.8).
This information is now provided in the figure legend.

14. SI Figure 3.
The authors might consider to show an unbiased omit map instead of a 2Fo-Fc map.

The new figure is a stereo view of an annealed omit map. In the revised version, this new figure is Supplementary Figure 6

Preliminary decision letter 12 August 2009

Thank you for submitting your revised manuscript for our consideration. We have now received the comments of the original referee 3 (attached below), and I am pleased to inform you that s/he considers the study satisfactorily improved and retains no principle reservations towards publication. We shall therefore be happy to proceed shortly with acceptance of your manuscript for publication in The EMBO Journal.

Nevertheless, there remain, as you will see below, a few minor issues of mostly editorial nature to be dealt with. With the paper by Tanaka et al already published, my personal preference would be to get your study into print as soon as possible, and I would thus disfavor having an additional round of revision to effect these changes. Given that the 'transparent editorial process' initiative implemented at The EMBO Journal since January 2009 actually includes online publication of anonymous referee comments and authors' responses with all accepted manuscript, I find that this may indeed suffice here to let the readers see both the final comments of the referee, as well as your description of the refinement of the Tanaka et al model that the referee wants included in the manuscript. If you agree to proceeding in this manner, we will swiftly move on to formal acceptance and transfer to our publisher as soon as we will have heard back from you.

Yours sincerely,

Editor
The EMBO Journal

Referee 3 (comments to authors):

In their letter of response and in the revised manuscript, the authors have addressed all issues - most of them convincingly - raised by the reviewers regarding the original manuscript and they have improved the discussion of their findings in the general context of vault biology.
In particular, they have modified statements regarding the reversibility of vault dissociation and have answered important questions regarding the quality of their structural models. They provide evidence -beyond typical levels for structure determination at this resolution - for the agreement between the sequence

assignment in the structure and the sequence of the overexpression plasmid by providing an all-residue real space density correlation plot and by the confirmation of all SeMet positions from anomalous difference density peaks.
They also provide the requested additional visualization for the choice of 39-fold symmetry, which is further substantiated by their successful molecular replacement/NCS averaging procedure, based on their high-resolution models of R1-R7. Further analysis of the rotational symmetry of the particle would be possible. However, such analysis has already been published by Tanaka et al. (2008) for a basically identical crystal form (resulting in the later structure determination by Tanaka et al.). Still, the authors could considerably improve SIFig 9 by re-calculation with a more suited program, using sharpening and careful adjustment of contour levels.
The authors hesitate to explicitly discuss the "draft structure" of the vault published by Anderson et al. 2007, which appears plausible, because this 9≈ resolution structure obtained under the assumption of 48-fold symmetry in the vault particle does not match common standards of crystallographic structure determination.
Regarding the relatively high R-factors obtained in refinement and unsuccessful attempts of initial automated model building the authors now provide additional documentation for the observed anisotropy of the diffraction data. Their use of anisotropically scaled data in initial steps of structure refinement is certainly justified, however, it is less common for final rounds of model refinement. The authors should additionally supply information about the results of refinement against the original isotropically scaled data using the internal anisotropic scaling of typical refinement programs.
The re-refinement of an updated vault model against the deposited data of Tanaka et al. could certainly be improved but this is probably in the responsibility of Tanaka et al., as the authors indicate in their letter of response. Still, the use of the Tanaka et al. data for the generation of the model shown in the new Fig. 4 has to be described in the manuscript (e.g. in the supplemental methods section of SI), similarly to how this is done in the rebuttal letter.
The authors should re-check Table I to confirm some the data. Is Mean() equivalent to Mn(I/sd)in the SCALA output? If so, the authors should comment on the more unusual numbers, including the Rmerge and Mn(I/sd) in the highest shells for some of the P21 crystal forms. They should state whether these data were obtained before or after anisotropic scaling.
The manuscript still requires careful technical editing.

