Crystal structure of yeast V\textsubscript{1}-ATPase in the autoinhibited state

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Transaction Report:

(No\te: 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 February 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies again for the very extended duration of the review process in this case. Your study has now been seen by three referees whose comments are shown below.

As you will see from the reports, the referees all express great interest in the findings reported in your manuscript and they would consequently support publication following adequate revision. They are not requesting much in terms of new experiments but they do raise a number of points with data description and presentation that would need to be addressed. On a more general note, I would also encourage you to think about writing the manuscript in a way that makes the findings more directly accessible to the non-specialist reader.

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

REFeree COMMENT

Referee #1:

Summary: Oot et al. presents a structure of the dissociated V\textsubscript{1}-ATPase complex of the yeast V-ATPase in its auto-inhibited state. The manuscript describes significant conformational changes in the V\textsubscript{1} region compared to intact V-ATPase, which explain how the catalytic activity of the V\textsubscript{1} region is blocked.
upon dissociation of the intact enzyme. In particular, the C-terminal region of the H subunit undergoes a surprisingly large rotation when the enzyme transitions from the assembled (active) state to the disassembled (inactive). This manuscript describes exciting results that answer a long-standing question regarding the structural basis for V1 auto-inhibition in V-ATPase regulation, and as such the manuscript which will have a significant impact on the V-ATPase field. Technically, the crystallization of this large and complicated molecular motor protein complex is an impressive feat.

The manuscript is well written and there are no major concerns regarding the study. We strongly recommend publication of this exciting study in the EMBO Journal after addressing the minor concerns described below and considering the suggestions given below that may improve the manuscript.

Minor concerns:
- In several places throughout the manuscript the authors state that the C subunit is released upon V-ATPase dissociation and "little, if any, C subunit" is bound to V1 purified from yeast cytoplasm and other sources. However, Hildenbrand et al (2010, PLoS One 5, e12588) show a significant amount of bound C subunit in affinity purified V1-ATPase from yeast cytoplasm. This discrepancy should be explained or the blanket statement that dissociated V1 lacks subunit C should be softened.

- page 2, paragraph 2: Defects in V-ATPase are described as being associated with osteoporosis and cancer. The references given describe targeting V-ATPase to treat osteoporosis and V-ATPase activity at the plasma membrane being involved in cancer invasion and metastasis. These activities cannot really be described as defects. To my knowledge V-ATPase defects are associated only with osteopetrosis, not osteoporosis.

- page 2, paragraph 3: "membrane integral proton channel, VO (ac_8c'c'de)". How do the authors know the stoichiometry of the c-ring (subunits c,c',c'')? No reference is provided and their statement of c8c'c'' is different from what most of the literature states.

- page 4, paragraph 1-2: The authors first describe the 7 A model, which did not resolve subunit F, middle of D, and N termini of EG2/3. The authors then describe the 6 A model, which did allow the modeling of the above subunits. The authors suggest possible disorder in the crystal lattice as the reason for being able to model the subunits in one case, but not the other. Considering that subunit H can be resolved in both cases and it supposedly binds the middle of D, it's not clear why the D subunit (and F) are disordered in one crystal versus the other. Furthermore, for a non-specialist reader, it may seem a bit odd that <1 A difference in the resolution would allow the subunits to be resolved in one structure versus the other. Either remove details about the 7 A model (which is not important for the manuscript) or add more detail to clarify.

- page 4, paragraph 3: "The three peripheral stators are bound via their C-terminal domains to the N-termini and along the face of each B subunit". The meaning of this sentence is unclear.

- page 5, paragraph 3: "the structure of the ScV1 catalytic core is overall very similar to its bacterial counterpart...rationalizing the success with using the EhA3B3 as MR search model". How does the authors define "success" in this case? Using an MR model will always result in model bias. Perhaps the authors mean "rationalizing the use of EhA3B3 as a MR search model"?

-Figure 4. Panel a: The alignment of the A subunits by their beta-barrel region seem to indicate that there are significant conformational changes in the N-terminal regions between the A subunits, which would be different from what has been found previously for the intact yeast V-ATPase. Is this a novel finding for the V1 subcomplex? If so, the authors should discuss this in more detail. However, these differences could also be due to noise because beta-sheets are not well defined at 6 A resolution, so aligning by the beta-barrel region may not be optimal. Furthermore, the two "closed" conformations appear different in this figure. If they are actually different then this should be discussed; or is it a result of alignment error? The authors may consider aligning the A subunits by the entire N-terminal region for a more robust comparison.

-Supplementary Figure 1. Some of the densities shown is not modeled or labeled. What are they? From the perspective that is presented, the unmodeled densities appear to contact H_CT. Is this real?
The authors should comment on possible contributions from non-specific protein-protein contacts due to crystallization.

Suggestions that may help make the manuscript more clear:
-page 4, paragraph 3: "three EG heterodimers that serve as peripheral stators". A reference to (Fig. 3a and 3b, EG1-3) may be helpful.
-page 5, paragraph 1: "peripheral stalk". This terminology is inconsistent with previous use of "peripheral stator", it may help readers to stay with one name.
-page 5, paragraph 1: "bulge' region in subunit G". It may help the reader if this is indicated in a figure.
-page 5, paragraph 3: "N- and C-termini found distal and proximal to the membrane, respectively (Fig. 3a-c)". Indicate the membrane in Figure 3.
-page 5, paragraph 3: "beta structure along the top of the molecule". Indicate the beta structure in Figure 4.
-page 6, paragraph 1: "the open catalytic site is (AB)1 and the two closed ones (AB)2 and (AB)3". Indicate this in a figure of the V1 complex.
-page 6, paragraph 3: The authors begin the paragraph by saying that comparison of the "catalytic cores reveals considerable differences regarding the position of the D subunit". The rest of the paragraph then refers to differences regarding the position of the catalytic cores (Fig. 4c), which were aligned based on the D subunit. The change in perspective is not initially obvious and it would help to state explicitly that the catalytic cores were aligned to subunit D in the main text.
-page 6, paragraph 3: "central rotor is bent...whereas in ScV1, the base of the central rotor appears to be more straight". A reference to Figure 7b,c would be helpful.
-page 6, paragraph 4: "Subunit H is a two domain polypeptide...(Fig. 5)". The boundary between H_NT and H_CT is not clear. An additional reference to Supplementary Figure 2 may be helpful.
-page 6, paragraph 4: "density modified MR map showed clear electron density for H_NT and H_CT (Fig. 2)". It's not clear where the densities for H_NT and H_CT are in Fig. 2.
-page 8, paragraph 2: "very C-terminal alpha helix". The term "very" is a bit confusing in its use here. Perhaps "most" would be better.
-page 8, paragraph 2: "H_NT and N-terminal domain of EG1 move outwards towards the periphery of the V1". It would be helpful to reference Movie 1.
-page 8, paragraph 2: last sentence. A reference to figure 5c may be appropriate.
-page 9, paragraph 2: "F_O-F_C density". This is technical jargon that may confuse readers unless defined or explained.
-page 10, paragraph 1: "dual and opposing functions of these binding sites on H_CT". This sentence is a bit misleading because it suggests that each binding site performs two opposing functions, but actually each binding site has a distinct and different function.
-page 10-11, paragraph 2: model of V1 dissociation. It may help to have a diagram or movie showing this process and reference it in the main text.
-Figure 1. Label "V/A or A-ArPase" and "V-ArPase" in the figure. Panel c is not consistent with Movie 1 (attachment of subunit C).
-Figure 2. Panel a: indicate which part of the peak was collected and pooled for the gel shown. Panel b: It is unclear which densities correspond to subunits D, E, G, and H. Label MR model as EhV1.
-Figure 3. Use of the colors "orchid" and "cornflower" is confusing. It may help the reader if the authors use the same color scheme defined by Walker in the mid-1990s (red for non-catalytic subunits and yellow for catalytic subunits), which has also been used in previous structural studies of the yeast V-ArPase. Panel b: indicate bulge. Panel c: label "Catalytic core". Panel d: label "Peripheral stators" and draw a box around E_NT and G_NT to indicate location of inset on right.
-Figure 4. Panel a: indicate beta-barrel region and which of the A subunits (1-3) is depicted. Panel c: the reference of the alignment is unclear. Consider just a slice through the V1 region to show the two alpha helices in the central rotor. Scale bar?
-Figure 5. Panel b: Indicate sites of contact and residue numbers. Scale bar?
-Figure 6. Panel b: Label the growth conditions of each plate. Panel e: Label size of ladder.
-Figure 6, caption: "insect, frog, mouse, bovine, chimpanzee, and human". Reference these organisms with the labels used in the figure.
-Figure 6, caption: "chromosomally encoded H subunit (H_ch)". H_ch is not in panel b/c.
-Figure 7. Label which subunits are from V1 and which are from V1VO. Scale bar?
-Supplementary Figure 2, caption: "ScH_CT". This is inconsistent with what is used in the figure. Scale bar?
Referee #2:

This is an exciting manuscript explaining the molecular mechanisms of V-type ATPase inhibition by subunit H. Subunit H is unique to eukaryotic V-type ATPases where it plays important regulatory roles that are relatively well understood on a biochemical, but not on a structural level. The crystal structure of subunit H in isolation has been known for a number of years and the cryo-EM structure of the intact V1Vo proton pump has recently been published to 11 Å resolution showing the enzyme with subunit H in an ATP hydrolyzing conformation.

This work presents the 6.2 Å crystal structure of the 15 subunit, ~600 kDa soluble V1 sector in an inhibited form. It shows major structural changes in subunit H catching it in action as a molecular brake and explaining formerly puzzling biochemical features of inhibition. Although the resolution is low for a crystal structure, the electron density for the main chain is clear and unambiguous and the methods for solving the structure are sound and convincing. I unreservedly recommend the manuscript for publication in EMBO Journal.

Below are a few minor suggestions for improvement.

- What was the incentive behind the subunit C deletion? Is V1 silenced with sub C present? What are the differences?

- What was the rationale for selecting the wavelengths for data collection? In particular collecting the Sr containing data at 0.6279 Å rather than at the Sr absorption edge, which might have helped phasing and/or identification of potential Ca binding sites?

- Figure 2 might be better suited for supplementary information, whereas supplementary Figure 2 would be useful to show in the main text.

- Supp Figure 4: "The 2mFo-DFc electron density map (contoured at 1.2σ) is shown as a blue mesh and the mFo-DFc map (contoured at 3σ) is showing positive density in green mesh." Mesh?

Referee #3:

Oot et al., present the structure of the isolated V1 part of the V-type ATPase of Saccharomyces cerevisiae in the autoinhibited state. The structure was determined to a resolution of 6.2-6.5 Å by X-ray crystallography and densities for α-helices are clearly visible. The novelty of the structure lies in the location of the C-terminal part of subunit H, which is responsible for preventing wasteful hydrolysis of ATP by the disassembled enzyme. Through mutation studies, the authors were able to pinpoint the inhibitory mechanism to a non-conserved loop region in the C-terminal region of the yeast's subunit H. These findings represent a significant advancement in the understanding of how V-type ATPases are regulated. Although the manuscript is well written, the figures fail to adequately portray the novel features of the structure or support some of the issues discussed in the text.

Comments

i) Page 5: The authors refer to a bulge region on the peripheral stalk EG3, however it is not clear what the bulge is. What is the bulge? Please indicate the location of the bulge region in Figure 3 and show the corresponding densities to clarify the statement "the density is patchy and flattened" (line 11, page 5)
ii) The authors also discuss how EG are attached to the A3B3 hexamer. These connections and the corresponding density should be presented in the figure.
iii) Page 5: The authors state that EG3 bind to subunit A and therefore may hold it in closed conformation adding regulation. This is a main point in the discussion and should be clearly shown in a figure. In addition, a figure comparing the difference between the binding of EG1/2 and EG3 with the A3B3 hexamer should be presented.

iv) Figure 3c: What is the purpose of figure 3c? What does this figures show that is not shown in figure 3a or 3b? Also the significance or purpose of 3d is not clear.

v) What criterion was used to assign the peripheral stalks as EG1, EG2 and EG3.

vi) In the section "catalytic hexamer and DF rotary shaft" (page 6), the text states that there is little difference in the position of subunits A or B between the bacterial and eukaryotic enzyme. However this is not clear in figure 4. An overlay of the bacterial and eukaryotic enzyme is required.

vii) The text describing the structure of the catalytic hexamer also states that "subunit DCT protrudes from the top of the hexamer by ~10Å, a feature unique to the eukaryotic enzyme". This novel feature needs to be illustrated.

viii) Figure 4c would be clearer if the (A3B3) hexamer was overlaid rather than subunit D. This would then demonstrate how the central stalk is stalled in relation to the different catalytic sites of the (A3B3) hexamer and that subunit A and B in the bacterial and eukaryotic enzyme are in the same position and conformation as stated in the text.

ix) The significance of figure 4a and b in relation to novel findings of the presented structure is unclear. Are these states not seen in previously published structures or are they the standard states? Is it necessary to show this figures in the manuscript?

x) Section "subunit H inhibitory interactions" is the most significant part of the manuscript and could benefit with being expanded. Of particular interest is the connection of subunit H to EG1 and the (A3B3) hexamer in relation to the catalytic state. E.g. could subunit H bind to the closed states or can it only bind to the open state?

xi) Figure 5a and b are very informative, however an additional image showing the connection of EG1 to subunit H from a different orientation would be beneficial. In addition the loop region on which the mutational experiences are based need to be clearly indicated in figure 5b e.g. with a different colour.

xii) A table or schematic figure summarising the different mutations of Subunit H constructs tested would greatly assist the understanding of figure 6.

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xiv) Figure 6e. The MW markers are not the same scale in the two gels. Please either add MW markers to the right gel or rescale the gels so the MW markers on the left gel correspond to the markers on the right gel.

xv) Figure 6. Labeling nomenclature for WT subunit H expressed from a plasmid is inconsistent between figure 6d, 6e and legend.

xvi) What is the significance of the C-subunits being co-purified with the isolated V1 for each mutant as shown in figure 6e? how does this affect the interpretation? This needs to be discussed in the text of the manuscript.

xvii) Why was only one point mutation tested? Is this sufficient to conclude that a "single point mutation in the loop region is not sufficient to negate function"?

xviii) Figure 7 requires an overview of the overlay between the holoenzyme and the auto inhibited V1 structure to make it clear that these two structures are being compared. The features described in the text should be indicated on the overview with a second image dedicated to these features. These include: a slice through the (A3B3) hexamer showing the similarity in position of subunit A and B, and close ups of HNT & EG1, HCT and EG3.

xix) Discussion, page 9, first paragraph, line 13. Please change may to "maybe" in the sentence starting "Photochemical crosslinking on the other hand...

xx) Is it really possible to assign ADP to the density shown in supplementary Fig 4 when the resolution is 6.2-6.7Å? It is probably more accurate to say that the density indicates that a "nucleotide" is bound to the closed catalytic site and that the nucleotide is likely to be ADP because of....
Referee #1:

Summary:
Oot et al. presents a structure of the dissociated V1-ATPase complex of the yeast V-ATPase in its autoinhibited state. The manuscript describes significant conformational changes in the V1 region compared to intact V-ATPase, which explain how the catalytic activity of the V1 region is blocked upon dissociation of the intact enzyme. In particular, the C-terminal region of the H subunit undergoes a surprisingly large rotation when the enzyme transitions from the assembled (active) state to the disassembled (inactive). This manuscript describes exciting results that answer a long-standing question regarding the structural basis for V1 autoinhibition in V-ATPase regulation, and as such the manuscript which will have a significant impact on the VATPase field. Technically, the crystallization of this large and complicated molecular motor protein complex is an impressive feat.

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Minor concerns:
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Authors’ response 1.1:
The reviewer makes a good point. While conventionally purified V1 from yeast or insect contains no C subunit as judged by Coomassie stained SDS-PAGE (Graf et al, 1996; Parra et al, 2000), yeast V1 purified via affinity capture contains varying amounts of C, with traces seen in refs. Zhang et al, 2003; Diab et al., 2009 and a more significant amount in the study pointed out by the reviewer (Hildenbrand et al, 2010). The reason for this discrepancy is not clear but it is possible that the more gentle treatment of the complex during affinity purification allows for more subunit C to remain bound. We have previously shown that the C subunit head domain binds with nanomolar affinity to one EG heterodimer (Oot & Wilkens, 2010), an interaction that must be broken for release of subunit C during regulated disassembly (Kane, 1995). It is possible that the breaking of this interaction is not complete or that some re-binding of C occurs during cell lysis and subsequent purification of V1.

Please find the text amended to include a statement about this in the section “Crystallographic Investigations of the autoinhibited ScV1” (page 3):

“While subunit C is released into the cytoplasm during reversible enzyme disassembly (Kane, 1995) (Fig 1C), variable but typically substoichiometric levels of C have been seen to co-purify with ScV1 (Diab et al, 2009; Hildenbrand et al, 2010; Zhang et al, 2003) (see also Fig 5E below). To ensure a homogeneous preparation for crystalllogenesis, ScV1 was therefore purified from a yeast strain deleted for the C subunit (Fig EV1A).”

Please also find the statement “very little, if any at all” to be amended to (page 11):

“Indeed, V1 purified from yeast cytoplasm or insect midgut has variable levels of C subunit bound, ranging from nondetectable (Graf et al, 1996; Parra et al, 2000) to more significant amounts (Diab et al, 2009; Hildenbrand et al, 2010; Zhang et al, 2003), consistent with EG3 adopting a conformation unfavorable for Chead binding.”

- page 2, paragraph 2: Defects in V-ATPase are described as being associated with osteoporosis and cancer. The references given describe targeting V-ATPase to treat osteoporosis and V-ATPase
activity at the plasma 3 membrane being involved in cancer invasion and metastasis. These activities cannot really be described as defects. To my knowledge V-ATPase defects are associated only with osteopetrosis, not osteoporosis.

Authors' response 1.2:
The reviewer is of course correct. The sentence has been changed to (page 2):

“While complete loss of V-ATPase function in animals is embryonic lethal (Inoue et al, 1999), partial loss or hyper activity of the enzyme has been associated with a wide spectrum of human diseases including osteoporosis...”

Authors’ response 1.3:
The Vo subunit stoichiometry (including the c subunit ring) was given for the yeast enzyme, with the stoichiometry of ten proteolipids as seen in the recent yeast cryo EM map (Zhao et al, 2015), and each one c’ and c” as determined earlier by Tom Stevens’ group (Powell et al, 2000). The text has been changed to:

“In the V-ATPase from S. cerevisiae, a well characterized model system for the enzyme from higher organisms, energy coupling requires the concerted action of fourteen different polypeptides that are organized into the ~640 kDa membrane extrinsic V1ATPase (A3B3CDE3FGH) (Kitagawa et al, 2008) and the ~330 kDa membrane integral Vo proton channel (ac8c’c’de) (Powell et al, 2000; Zhao et al, 2015).”

Authors’ response 1.4:
When we referred to the “middle of (the) D (subunit)”, we meant the middle segment of the polypeptide (amino acids ~50-150), in some studies referred to as the “base” of the D subunit as it is in contact with the membrane sector in holo V1Vo. Unlike the N and C-terminal alpha helices that form the coiled coil of subunit D (which were resolved in both of our higher and lower resolution models), the “base” of the subunit is composed of mostly random coil and some beta structure. Interestingly, while this part of the D subunit has also not been resolved in the much higher resolution (2.17 – 2.68 Å) X-ray structures of the bacterial V1-ATPase from E. hirae (3vr4, 3vr6; Arai et al, 2013), it was resolved and could be modeled in the lower resolution (3.9 Å) structure of the T. thermophilus A/V-type ATPase sector (3w3a; Nagamatsu et al, 2013). The base of the D subunit may be resolved or unresolved due to protein interactions or crystal packing differences rather than resolution being the major dictating factor. It is important to point out that the base of the D subunit is not involved in the interaction with the H subunit, which as the reviewer pointed out was resolved in both higher and lower resolution models. Instead, as mentioned above, this region of D subunit would be bound to the membrane sector in the assembled enzyme.

In describing the procedure of how the 7 Å model was solved, our intention is to convince the reader that we exercised caution with trying to avoid model bias by only placing subunit structures, or regions thereof, for which there was convincing electron density in the maps before the model was extended. We think that this is important to point out, in particular since we are dealing with low-resolution data. However, this stepwise procedure was not necessary for solving the higher resolution structure as we already had the 7 Å model deposited when the higher resolution data were collected. In addition to the higher resolution, this dataset had overall somewhat better statistics,
which likely explains why it allowed modeling of parts of the structure that were poorly resolved in the 7 Å structure. Furthermore, as the 7 Å structure was used in solving the higher resolution structure, we needed to discuss the lower resolution model to accurately describe how the 6.2-6.5 Å structure was solved and modeled.

We have modified the text in the section “Crystallographic investigations of the autoinhibited ScV1” (page 3-4) to streamline the description of solving the structure and to clarify some of the points summarized above.

-Page 4, paragraph 3: "The three peripheral stators are bound via their C-terminal domains to the N-termini and along the face of each B subunit”. The meaning of this sentence is unclear.

Authors’ response 1.5:
The sentence has been changed to (page 5):

“Each of the three peripheral stalks is bound via its C-terminal domain (E\textsuperscript{CT}G\textsuperscript{CT}) to the N-terminal β barrel domain of the corresponding B subunit and crosses a non-catalytic AB interface on its way towards the base of the hexamer (Fig 2B).”

Please also note that a new panel B in Fig 2 illustrates the interactions discussed in this sentence, showing peripheral stalk (EG3) interacting with the A3B3 hexamer.

-Page 5, paragraph 3: "the structure of the ScV1 catalytic core is overall very similar to its bacterial counterpart...rationalizing the use of EhA3B3 as a MR search model”. How does the authors define "success” in this case? Using an MR model will always result in model bias. Perhaps the authors mean “rationalizing the use of EhA3B3 as a MR search model”?

Authors’ response 1.6:
Reviewer #1 is of course correct that solving a structure by MR will carry the risk of model bias, especially at low resolution, which is why we described the stepwise procedure to solve the structure starting with the 7 Å dataset. We considered the molecular replacement to be “successful” since (i) the phases provided by the MR search model revealed interpretable electron density beyond the search model and (ii) since the final model could be refined to R/Rfree values reasonable for this resolution. This indicates that the search model is appropriate and that the model was placed correctly in the asymmetric unit. In what is now Figure EV1 (previously Fig 2), we show the NCS averaged and density modified map of the MR solution, which shows clear density beyond that of the search model, corresponding to subunits H and D and parts of EG1, subunits not present in the structure of the bacterial enzyme used as MR search model. To clarify the matter, we have modified the text on page 3 in the section “Crystallographic investigations of the autoinhibited ScV1”:

“A 7 Å resolution dataset collected from one ScV\textsubscript{1} crystal was used to start the structure determination by molecular replacement (MR) (Table 1). Since there is no crystal structure available for the eukaryotic V\textsubscript{1}-ATPase, we employed the structure of the nucleotide free A\textsubscript{3}B\textsubscript{3} catalytic hexamer from the E. hirae sodium pumping V-type ATPase (EhA\textsubscript{3}B\textsubscript{3}) (Arai et al, 2013) for MR. The primary structures of E. hirae and yeast A and B subunits are highly similar (48 and 54% identity, respectively), indicating that the tertiary structures of the bacterial and eukaryotic catalytic subunits are conserved, and that the bacterial A\textsubscript{3}B\textsubscript{3} catalytic hexamer represents a suitable MR search model for solving the structure of the eukaryotic V\textsubscript{1}..”

And page 5, section “Catalytic hexamer and rotary shaft”

“As mentioned above, the catalytic core (A\textsubscript{3}B\textsubscript{3}:DF) of V-type rotary ATPases is highly conserved, with primary sequence identities between bacterial, yeast and mammalian A and B subunits of ~50-80%, respectively (Muench et al, 2011). Not surprisingly therefore, the structure of the ScV\textsubscript{1} catalytic core is overall very similar to its bacterial counterpart from E. hirae (EhV\textsubscript{1}) with an rmsd of 2.3 Å.”

-Figure 4. Panel a: The alignment of the A subunits by their beta-barrel region seem to indicate that there are significant conformational changes in the N-terminal regions between the A subunits, which would be different from what has been found previously for the intact yeast V-ATPase. Is this
a novel finding for the V1 subcomplex? If so, the authors should discuss this in more detail. However, these differences could also be due to noise because beta-sheets are not well defined at 6 Å resolution, so aligning by the beta-barrel region may not be optimal. Furthermore, the two "closed" conformations appear different in this figure. If they are actually different then this should be discussed; or is it a result of alignment error? The authors may consider aligning the A subunits by the entire N-terminal region for a more robust comparison.

Authors’ response 1.7:
Alignment of the N-terminal beta-barrels (residues 1-90) of the A subunits was chosen based upon the work of Arai et al, (2013) that reported the crystal structure of the bacterial V1-ATPase from E. hirae. In that study, the authors aligned by this region due to the rigid nature of the contiguous beta-barrel structure formed by the N-termini of the A3B3 complex, thereby allowing visualization of conformational variability in the nucleotide binding and C-terminal domains. This seems a fair rationalization because the alignment is intended to show conformational differences between the catalytic subunits from the context of the hexameric arrangement. Indeed, the density for beta strand regions of the structure is less well resolved at this low resolution, but larger tertiary beta structures such as the beta barrels that we used for alignment are resolved in our density map. In the revised Figure 3D, we now show the density for some of the beta barrels and a portion of electron density depicting the longer C-terminus of the central rotor (with respect to that of the bacterial enzyme as per reviewer #3’s suggestions). The differences seen in the N-terminus of the catalytic A subunits, primarily for example in the non-homologous region, are not large scale and are in fact also seen upon alignment of the catalytic A subunits from the recent cryo-EM structure of the intact yeast V-ATPase solved at a similar resolution (Zhao et al., 2015). As these small differences in conformation at the N-terminus are seen in the intact V-ATPase, the A/V-type and the V1 structure presented here, we do not feel that this merits additional discussion in the manuscript. Below, please find the alignment of the A subunits from “state 2” of the intact yeast V-ATPase (3j9u), to illustrate the similarity (to the alignment shown in revised Fig 3B) in N-terminal conformational changes.

As reviewer #1 points out, the two closed conformations seen in the alignment (revised Fig 3B) are indeed different, with one site appearing more closed than the other. Indeed, both the A/V-type and intact V-ATPase structures discussed above are in similar conformations showing two closed states,
with one more closed than the other in both cases. In the publication for the A/V-type V1 from E. hirae (Arai et al, 2013), which compared the structures of the A3B3 hexamer with A3B3DF (bacterial V1 complex) both solved in different nucleotide bound states, it was concluded that in the presence of the central rotor subunits, DF, the hexamer always has two closed and one open site, regardless of nucleotide content. Further, when comparing the A3B3DF structures, they observed a “bound” form (less closed) and “tight” form (more closed).

Of the two closed states in our structure of autoinhibited eukaryotic V1, the more closed “tight” AB pair, (AB)2 seems to contain tightly bound inhibitory nucleotide (likely ADP) as indicated by positive difference density (Appendix Fig S6). Our biochemical experiments measuring bound endogenous nucleotide indicate that slightly more than 1 mol of ADP is bound per mol of V1. It is possible that the less closed AB pair, (AB)3 in our study, contains nucleotide in some copies of V1, supporting the possibility of a lower affinity site for nucleotide in the autoinhibited ScV1.

The text has been amended to point out the two non-equivalently closed sites as well as an interpretation of what this might mean in terms of nucleotide content (section “Catalytic Hexamer and rotary Shaft”, page 6):

“Note that of the two closed catalytic sites, (AB)2 appears more closed than (AB)3 (Fig 3B and C). The presence of two non-equivalently closed sites has been also observed in the EhV1 (Arai et al, 2013), and in the cryo EM maps of holo eukaryotic V-ATPase (Zhao et al, 2015) (see “Discussion” section below for more detail).”

And in the “Discussion” (page 10):

“Interestingly, while ScV1 purified using ammonium sulfate precipitation and ion exchange chromatography contained no bound nucleotide (Purra et al, 2000), the ScV1 as purified here by affinity chromatography contains ~1.3 mol/mol ADP. ScV1ΔH on the other hand retained only ~0.4 mol/mol of the nucleotide, indicating that subunit H, by stabilizing an open catalytic site near EG1, leads to increased affinity for inhibitory ADP at another site, which in turn results in activity silencing. Since there are two non-equivalently closed catalytic sites in ScV1 (Fig 3B and C), it is likely that one of these sites is completely filled and the other partially occupied with nucleotide.”

---Supplementary Figure 1. Some of the densities shown is not modeled or labeled. What are they? From the perspective that is presented, the unmodeled densities appear to contact H_CT. Is this real? The authors should comment on possible contributions from non-specific protein-protein contacts due to crystallization.

Authors’ response 1.8:
The unlabeled densities do indeed belong to symmetry related copies of the complex, which is now pointed out in the figure and explained in the legend (now Appendix Fig S2). Specifically, there is a crystal contact involving HCT and the N-termini from a NCS related EG1 peripheral stalk. While we can not rule out that this nonspecific crystal contact influences the position of HCT, our mutagenesis data and the available published literature support the binding position of HCT at the bottom of the V1 as seen in the structure.

Please find the caption to Appendix Fig S2 to now include the text:

“The unmodeled electron density near H_CT and D belongs to a NCS related copy of ScV1. While it cannot be ruled out that non-specific crystal contacts have some influence on the structure, the model is consistent with both the available literature and the mutagenesis studies presented in the current study (see Fig 5 of the main text).”

Suggestions that may help make the manuscript more clear:

-Page 4, paragraph 3: "three EG heterodimers that serve as peripheral stators". A reference to (Fig. 3a and 3b, EG1-3) may be helpful.

Authors’ response 1.9:
Done. Please see reference to Revised Fig 2A (page 4).
-page 5, paragraph 1: "peripheral stalk". This terminology is inconsistent with previous use of "peripheral stator", it may help readers to stay with one name.

Authors’ response 1.10:
Agreed. We settled on “peripheral stalk”.

-page 5, paragraph 1: "bulge’ region in subunit G”. It may help the reader if this is indicated in a figure.

Authors’ response 1.11:
Please find the bulge region now indicated on Figure 2D (previously Figure 3). In addition, a new Supplemental Figure (Appendix Figure S3) has been added which highlights the density for the bulge region in subunit G as well as a figure showing the 2.9 Å crystal structure (4dl0; Oot et al, 2012), previously solved by our group, of the EG heterodimer to illustrate the bulge region as seen at higher resolution for readers less familiar with the field. The text on page 5 was modified to:

“In that study, we provided evidence that the EG heterodimers contain two hinges and a partially disordered “bulge” region in subunit G that provide flexibility and that we speculated would play an important role in the mechanism of reversible disassembly (Appendix Fig S3B). In the ScV1 structure presented here, this region of the G subunit is involved in the interacting surface between EG and the B subunit. In peripheral stalks EG1 and EG2, the segment of G is resolved as continuous tubular density, consistent with α helical structure. In EG3, however, the density is patchy and flattened, indicating presence of the “bulge” structure as seen in isolated EGCGhead (Fig 2D and Appendix Fig S3C).”

-page 5, paragraph 3: "N- and C-termini found distal and proximal to the membrane, respectively (Fig. 3a-c)”. Indicate the membrane in Figure 3.

Authors’ response 1.12:
The position of the membrane with respect to the N- and C-termini of the catalytic subunits is now indicated in the revised Figure 3A.

-page 5, paragraph 3: "beta structure along the top of the molecule”. Indicate the beta structure in Figure 4.

Authors’ response 1.13:
Please find Figure 3A, B (previously Figure 4) amended with the beta barrels indicated with labels.

-page 6, paragraph 1: "the open catalytic site is (AB)1 and the two closed ones (AB)2 and (AB)3”.
Indicate this in a figure of the V1 complex.

Authors’ response 1.14:
Please find that revised Figure 2E and F now include the labels of the AB pair designations on a slice through the V1 complex.

-page 6, paragraph 3: The authors begin the paragraph by saying that comparison of the "catalytic cores reveals considerable differences regarding the position of the D subunit". The rest of the paragraph then refers to differences regarding the position of the catalytic cores (Fig. 4c), which were aligned based on the D subunit. The change in perspective is not initially obvious and it would help to state explicitly that the catalytic cores were aligned to subunit D in the main text.

Authors’ response 1.15:
This is an excellent point that was also raised by Reviewer #3. We have included a side view of an alignment of the A3B3 in panel D of the revised Figure 3 (Previously Figure 4), which shows a difference in position of the central rotor subunit D when aligning by the catalytic cores. In addition, we have modified the text, which now reads:

“Comparing the crystal structures of yeast (ScV1) and bacterial (EhV1) catalytic cores reveals considerable differences regarding the position of the D subunit (Fig 3D). Aligning the structure of
autoinhibited ScV1 to available structures of nucleotide-free and AMP-PNP bound EhV1 (Arai et al, 2013) using the D subunit as reference illustrates that the eukaryotic V1 is halted in a different rotational position (Fig 3E)."

"central rotor is bent...whereas in ScV1, the base of the central rotor appears to be more straight". A reference to Figure 7b,c would be helpful.

Authors’ response 1.16:
Please find a reference to newly added panels D and F in the revised Figure 3 (depicting the differences in central rotor conformation) in the text on page 6.

"Subunit H is a two domain polypeptide...(Fig. 5)". The boundary between H_NT and H_CT is not clear. An additional reference to Supplementary Figure 2 may be helpful.

Authors’ response 1.17:
Please find a revised Figure 4 (previously Fig 5), which now has labels pointing out HNT, HCT and the flexible linker between the domains on the figure. The previous Supplementary Figure 2 is now a new panel F in the main text Figure 6 (previously Fig 7).

"density modified MR map showed clear electron density for H_NT and H_CT (Fig. 2)". It's not clear where the densities for H_NT and H_CT are in Fig. 2.

Authors’ response 1.18:
This Figure is now Fig EV1 as per Reviewer #2’s suggestion. Please find the figure modified to include labels on the densities for HNT and HCT. See also, response 1.26 in reference to the comment on Figure 2 Panels A and B below.

"very C-terminal alpha helix". The term "very" is a bit confusing in its use here. Perhaps "most" would be better.

Authors’ response 1.19:
Please find the text in the following sections amended:

In the results section “Comparison of autoinhibited ScV1 to holo ScV1Vo (page 9)”

“The most dramatic change is seen in the position of H_CT, rotating ~150º from a position in V1Vo where its C-terminal α helix is in contact with a_NT to its position at the bottom of the catalytic hexamer in free ScV1 as described above (Fig 6C and E).”

See also in the “Discussion” section “Comparison of ScV1 and ScV1Vo and implications for the mechanism of reversible dissociation” (page 11):

“For example, it has been shown that ScV1 does not bind a_NT in vitro (Diab et al, 2009), a result that can be explained by the fact that the most C-terminal α helix on H_CT that binds a_NT in V1Vo is hidden in V1 due to the 150º rotation of H_CT during disassembly.”

Also please see the caption to Fig 6E:

“In ScV1Vo, the most C-terminal α helix (dark green) of H_CT (green) binds to a_NT (light gray), with the inhibitory loop (green spheres) rotated away and inaccessible for binding the B subunit and the central stalk (transparent red).”

And in the same caption:

“Note that the H_CT loop (spheres) is rotated away from the central stalk in ScV1Vo whereas in ScV1, the C-terminal α helix (orange) involved in binding a_NT is rotated away and unavailable for rebinding to the membrane.”

"H_NT and N-terminal domain of EG1 move outwards towards the periphery of the V1". It would be helpful to reference Movie 1.
Authors’ response 1.20:
The Figure associated with this text (now Fig 6, was previously Fig 7) has now been expanded to show a zoomed view of EG1 and HNT in V1 versus intact V-ATPase (Fig 6B) as per Reviewer #3’s suggestion. As a result of this expansion of the Figure, the text in that section has been reorganized with discussion of the movements of the peripheral stalks first, followed by a discussion of the interactions involving HCT. In the initial submission, the information about HCT was discussed first in this section. In addition, this Figure now includes a new panel (Fig 6F), which shows the alignment between the isolated H subunit crystal structure (1ho8; Sagermann et al, 2001), H subunit from V1Vo (3j9u; Zhao et al, 2015) and H subunit from autoinhibited V1. This figure was previously Supplemental Figure S2. We have referenced Movie EV3 (previously Movie 1) in the revised text.

-page 8, paragraph 2: last sentence. A reference to figure 5c may be appropriate.

Authors’ response 1.21:
The revised text (page 9) now references the relevant revised figures:

“In addition, a striking change is seen in peripheral stalk EG3 that is bound to the C subunit in the assembled enzyme but binds to the C-terminal domain of an A subunit of closed catalytic site (AB)2 in ScV1 (Fig 6C and D; see also Fig 2DF).”

-page 9, paragraph 2: "Fo-Fc density". This is technical jargon that may confuse readers unless defined or explained.

Authors’ response 1.22:
“Fo-Fc density” has been changed to “difference density” (page 10).

-page 10, paragraph 1: "dual and opposing functions of these binding sites on H_CT". This sentence is a bit misleading because it suggests that each binding site performs two opposing functions, but actually each binding site has a distinct and different function.

Authors’ response 1.23:
Please find the text amended to now read (page 11):

“Besides the different and opposing function mediated by each of these two distinct binding sites on H_CT, …”

-page 10-11, paragraph 2: model of V1 dissociation. It may help to have a diagram or movie showing this process and reference it in the main text.

Authors’ response 1.24:
Thank you for this suggestion. We have now added a movie (Expanded View Movie EV4; referred to on page 11) that highlights the conformational changes at the V1-Vo interface as a result of enzyme disassembly. However, as pointed out in the legend to the movie, the exact order of the structural changes is currently unknown. See also response to the next comment.

-Figure 1. Label "V/A or A-ATPase" and "V-ATPase" in the figure. Panel c is not consistent with Movie 1 (attachment of subunit C).

Authors’ response 1.25:
Please find Figure 1 amended to include the suggested labels. Furthermore, Movie EV3 (previously Movie 1) has been modified so that subunit C is now faded out prior to detachment of V1 from Vo. In the initial submission, we were apprehensive about the treatment of subunit C in the movie because while it is generally accepted that C subunit is released during regulated disassembly, the details of the process and the timeline for the events surrounding the release of C in disassembly of V1 and Vo are not understood. Your comment made us reflect on this, and we concluded that it is more correct to show C subunit disappearing during dissociation than having it remain on Vo.

-Figure 2. Panel a: indicate which part of the peak was collected and pooled for the gel shown.
Panel b: It is unclear which densities correspond to subunits D, E, G, and H. Label MR model as EhV1.

Authors’ response 1.26:
This figure has been moved to the Expanded View Figure EV1 as per Reviewer #2’s suggestion. Please find the figure annotated according to your suggestions. The chromatogram is boxed to indicate the fractions that were pooled for use in running the gel and crystallization of V1. In addition, the density modified MR map is now labeled with the search model (EhA3B3) and the densities corresponding to subunits E, G, D and HNT and HCT.

-Figure 3. Use of the colors "orchid" and "cornflower" is confusing. It may help the reader if the authors use the same color scheme defined by Walker in the mid-1990s (red for non-catalytic subunits and yellow for catalytic subunits), which has also been used in previous structural studies of the yeast V-ATPase. Panel b: indicate bulge. Panel c: label "Catalytic core". Panel d: label "Peripheral stalks" and draw a box around E_NT and G_NT to indicate location of inset on right.

Authors’ response 1.27:
The names of the colors such as “orchid” and “cornflower” have been simplified to “pink” and “blue.” The coloring scheme defined by Walker in the mid 1990s indeed has been also used in the coloring of the recent models from the intact V-ATPase from yeast (Zhao et al., 2015), however, the color scheme Zhao et al. used for other subunits in V1 does not provide sufficient contrast to highlight the subunits not found in F-ATPase. As suggested by the reviewer, we tried to use Zhao et al.’s color scheme but the yellow catalytic A subunits and the orange H are difficult to distinguish in some of the views in our figures so we decided to keep the coloring scheme as in our original manuscript. Furthermore, please find revised Figure 2 (previously Figure 3) with the bulge region in subunit G now indicated on the figure panel D. The previous panel C has been moved to revised Fig 3A and panel D can now be found in Appendix Fig S3A (as per Reviewer #3’s suggestion). Please find Fig 3A labeled with “Catalytic core” and Appendix Fig S3A with the EG_NT/G_NT boxed and the label “Peripheral stalks.” In the new Appendix Fig S3, please also find additional panels related to the G subunit bulge, according to reviewer #3’s suggestion.

-Figure 4. Panel a: indicate beta-barrel region and which of the A subunits (1-3) is depicted. Panel c: the reference of the alignment is unclear. Consider just a slice through the V1 region to show the two alpha helices in the central rotor. Scale bar?

Authors’ response 1.28:
The beta barrel region used for alignment as well as the identity of each A subunit corresponding to AB pairs 1-3 is now indicated in panel B of the revised Figure 3 (previously Figure 4). A new panel D showing a side view of the alignment by the A3B3 has been added to illustrate the difference in position of the D subunit between EhV1 and ScV1. The original alignment by the D subunit is now shown in panel E of the revised Figure 3 and a new panel F has been added to highlight the different conformations between ScV1 and EhV1. Furthermore, a new Appendix figure has been added (Appendix Fig S4) showing the alignment between the A3B3 cores of ScV1 and T. thermophilus A3B3DF (3w3a; Nagamatsu et al, 2013). Text has been added on page 6 to point out that ADP bound TtA3B3DF is more similar to ScV1 in terms of the subunit D conformation:

“Interestingly, a more straight central rotor is also seen in the ADP bound catalytic core from the T. thermophilus A/VATPase (TtV1; Appendix Fig S4) (Nagamatsu et al, 2013), suggesting that the conformation of the DF subcomplex may be influenced by the nucleotide occupancy of the two closed catalytic sites of the complex.”

Regarding the suggested addition of scale bars, please find scale bars included in Fig 2C and D and in other figures, the ribbon representation of alpha helices provides an internal size reference.

-Figure 5. Panel b: Indicate sites of contact and residue numbers. Scale bar?

Authors’ response 1.29:
Please find the amended Fig 4C (previously Fig 5b) showing the regions involved in the contact between HCT, BCT and DNT with different coloring and labeled with residue ranges. The residue
ranges are also listed in the main text as follows (page 7):

“The contacts between H_CT and the catalytic core are mediated by a loop in H_CT (residues 408-414) and two short segments in the C-terminal domain of the B subunit of the open catalytic site (AB)1 (residues around Ile427 and Glu471) together with two α helical turns in D_NT (residues 38-45) (Fig 4C)”

-Figure 6. Panel b: Label the growth conditions of each plate. Panel e: Label size of ladder.

Authors’ response 1.30:
Please find revised Figure 5 (formerly Figure 6) amended with the growth conditions of the plates and the molecular masses of the ladder labeled on the Western blot.

-Figure 6, caption: "insect, frog, mouse, bovine, chimpanzee, and human”. Reference these organisms with the labels used in the figure.

Authors’ response 1.31:
Please find the caption for Fig 5A (previously Fig 6) amended according to these suggestions.

-Figure 6, caption: "chromosomally encoded H subunit (H_ch)". H_ch is not in panel b/c.

Authors’ response 1.32:
Please find the caption for Fig 5C (previously Fig 6 panels b/c) corrected.

-Figure 7. Label which subunits are from V1 and which are from VIVO. Scale bar?

Authors’ response 1.33:
Please find revised Figure 6 (previously Fig 7), with the subunits labeled as belonging to either V1 or V1Vo.

-Supplementary Figure 2, caption: "ScH_CT". This is inconsistent with what is used in the figure. Scale bar?

Authors’ response 1.34:
The original Supplemental Figure S2 has now been incorporated into revised Fig 6F and the caption has been corrected.

-Supplementary Figure 4, caption: “the nucleotide superimposes to the mF_O-DF_C density”. There are other green densities that the nucleotide does not superimpose with.

Authors’ response 1.35:
With Appendix Fig S6 (formerly S4), we are merely pointing out that there is green (positive difference) density near the P-loop of closed catalytic site AB2, which suggests that it is AB2 that has inhibitory ADP bound. The difference density is displayed at the default 3 sigma contour level. At 4 sigma, only the green density near the P-loop is visible (though a lot smaller), highlighting its significance. The origin of the positive difference densities aside from the one near the P-loop in AB2 is unclear. Some may be due to unmodeled density, some may be due to noise. It is acknowledged that at this resolution, we can only suggest based on the difference density near the P-loop that the ADP we know is present in the V1 preparation (from measuring the nucleotide content) is bound in AB2.

The legend to Appendix Fig S6, panel A, has been amended to:

“A ScV1 shown with the model sliced through the top of the hexamer to the level of the phosphate binding (P) loops. The electron density map (2mFo-DFo; contoured at 1.2 σ) is shown as a blue mesh and the difference map (mFo-DFo; contoured at 3 σ) is showing positive density as a dense green mesh. The boxed area highlights the strong positive density present in the P loop region of (AB)2, which is shown enlarged in B. We have quantified the nucleotide content of ScV1 to be ~1.3 mol/mol ADP.”
Referee #2:

This is an exciting manuscript explaining the molecular mechanisms of V-type ATPase inhibition by subunit H. Subunit H is unique to eukaryotic V-type ATPases where it plays important regulatory roles that are relatively well understood on a biochemical, but not on a structural level. The crystal structure of subunit H in isolation has been known for a number of years and the cryo-EM structure of the intact V1V0 proton pump has recently been published to 11 Å resolution showing the enzyme with subunit H in an ATP hydrolyzing conformation.

This work presents the 6.2 Å crystal structure of the 15 subunit, ~600 kDa soluble V1 sector in an inhibited form. It shows major structural changes in subunit H catching it in action as a molecular brake and explaining formerly puzzling biochemical features of inhibition. Although the resolution is low for a crystal structure, the electron density for the main chain is clear and unambiguous and the methods for solving the structure are sound and convincing. I unreservedly recommend the manuscript for publication in EMBO Journal.

Below are a few minor suggestions for improvement.

- What was the incentive behind the subunit C deletion? Is V1 silenced with sub C present? What are the differences?

Authors’ response 2.1:

The incentive behind deletion of subunit C was that while typically either non-detectable or substoichiometric amounts of C subunit have been observed to co-purify with V1, the levels are variable and can be present in more significant amounts (Graf et al, 1996; Zhang et al, 2003; Kitagawa et al, 2008; Hildenbrand et al, 2010). As it has been shown that the C subunit is released from the enzyme upon dissociation (Kane, 1995), we deleted the subunit to ensure a homogenous preparation for crystallization. The reasons for variable levels of C subunit co-purifying with V1 are not clear at this point, but as we have shown previously that the “head” domain of subunit C binds one peripheral stalk with nanomolar affinity (Oot & Wilkens, 2010) it may be that either the breaking of this interaction is not efficient in some cases or perhaps some C re-binds V1 during the purification procedure. Please see also our response 1.1 to reviewer #1 for additional details about the levels of C subunit in V1 purifications. Please also find the text amended in the section “Crystallographic investigations of the Autoinhibited ScV1” (page 3):

“While subunit C is released into the cytoplasm during reversible enzyme disassembly (Kane, 1995) (Fig 1C), variable but typically substoichiometric levels of C have been seen to co-purify with ScV1 (Diab et al, 2009; Hildenbrand et al, 2010; Zhang et al, 2003) (see also Fig 5E below). To ensure a homogeneous preparation for crystallogenesis, ScV1 was therefore purified from a yeast strain deleted for the C subunit (Fig EV1A).”

As to the question whether V1 is silenced with subunit C present we can say that from the published literature and our experience, MgATPase activity (or better, lack thereof) is the same for V1 affinity purified from ‘wild type’ cells containing (substoichiometric amounts of) C subunit and cells deleted for subunit C (Parra et al, 2000; Zhang et al, 2003). In other words, whether or not subunit C is present, V1 purified from ‘wild type’ or C deleted cells has no measurable MgATPase activity. See also response 3.15 to reviewer #3, point (xvi) below for more details.

- What was the rationale for selecting the wavelengths for data collection? In particular collecting the Sr containing data at 0.6279 Å rather than at the Sr absorption edge, which might have helped phasing and/or identification of potential Ca binding sites?

Authors’ response 2.2:

MacCHESS recently upgraded their beam lines to improve photon flux, but this upgrade required increasing the X-ray energy to the specified (fixed) wavelength due to technical reasons. It should be noted that information transfer should actually be improved when going to higher energy (Fourme et al, "Reduction of radiation damage and other benefits of short wavelengths for macromolecular crystallography data collection", J. Appl. Cryst. 45, 652, 2012). We did collect
some data at the tuneable APS beam line 17-ID-B but due to the limited resolution of the data, we
did not attempt to collect data at the Sr edge (0.77 Å).

- Figure 2 might be better suited for supplementary information, whereas supplementary Figure 2
would be useful to show in the main text.

Authors’ response 2.3:
Thank you for this suggestion. We agree with Reviewer #2 about moving Figure 2 out of the main
text figures and moving original Supplemental Figure S2 to the main text. The previous
Supplemental Figure 2 is now panel F in Fig 6. The previous Figure 2 is now Expanded View Fig
EV1 and revised to include annotations suggested by Reviewer #1. With the promotion of the
original Supplemental Fig 2 to the main text (now Fig 6F), we have amended the text on page 9:

“The apparent flexibility of H_CT can be further illustrated upon comparison of the available structure
of the isolated H subunit to that in ScV1_V_o and in ScV1 (Fig 6F). Based on this comparison, H_CT
undergoes an 180° rotation from its position in isolated H to that in the autoinhibitory conformation
in ScV1. The alignment further suggests that H_CT can adopt multiple low energy conformations and
that its flexibility is required for the inhibitory function in ScV1 and for coupling ATP hydrolysis to
proton transport in ScV1_V_o.”

- Supp Figure 4: "The 2mFo-DFc electron density map (contoured at 1.2 σ) is shown as a blue mesh
and the mFo-DFc map (contoured at 3 σ) is showing positive density in green mesh." Mesh?

Authors’ response 2.4:
It is a rather fine or dense mesh and perhaps would be better described as a “dense green mesh”.
Please find the caption to Appendix Fig S6, previously Fig S4) amended to include this description.

Referee #3:
Oot et al., present the structure of the isolated V1 part of the V-type ATPase of Saccharomyces
cerevisiae in the autoinhibited state. The structure was determined to a resolution of 6.2-6.5 Å by X-
ray crystallography and densities for α-helices are clearly visible. The novelty of the structure lies in
the location of the C-terminal part of subunit H, which is responsible for preventing wasteful
hydrolysis of ATP by the disassembled enzyme. Through mutation studies, the authors were able to
pinpoint the inhibitory mechanism to a non-conserved loop region in the C-terminal region of the
yeast's subunit H. These findings represent a significant advancement in the understanding of how
V-type ATPases are regulated. Although the manuscript is well written, the figures fail to adequately
portray the novel features of the structure or support some of the issues discussed in the text.

Comments
i) Page 5: The authors refer to a bulge region on the peripheral stalk EG3, however it is not clear
what the bulge is. What is the bulge? Please indicate the location of the bulge region in Figure 3
and show the corresponding densities to clarify the statement "the density is patchy and flattened”
(line 11, page 5)

Authors’ response 3.1:
The G subunit bulge is now pointed out in the revised Figure 2D and illustrated in more detail in a
new Appendix Figure S3B showing the crystal structure of the EG heterodimer in isolation (pdb
code 4dl0; Oot et al, 2012). This structure, and its accompanying paper was the first description of
this “bulge” feature in the G subunit of the peripheral stalks from the yeast V-ATPase. Appendix
Figure S3C also shows the density corresponding to the bulge region in the current ScV1 structure,
as requested.

The text in the section “Overall structure of ScV1” has been modified to indicate the potential
contribution of the “bulge” region of subunit G in providing flexibility to that peripheral stalk (page
5):

“In that study, we provided evidence that the EG heterodimers contain two hinges and a partially
disordered “bulge” region in subunit G that provide flexibility and that we speculated would play an
important role in the mechanism of reversible disassembly (Appendix Fig S3B).”
ii) The authors also discuss how EG are attached to the A3B3 hexamer. These connections and the corresponding density should be presented in the figure. Please see response 3.2, to point iii below.

iii) Page 5: The authors state that EG3 bind to subunit A and therefore may hold it in closed conformation adding regulation. This is a main point in the discussion and should be clearly shown in a figure. In addition, a figure comparing the difference between the binding of EG1/2 and EG3 with the A3B3 hexamer should be presented.

Authors’ response 3.2
In response to points (ii) and (iii) please find revised Figure 2 (panels C-F) amended to include density for the peripheral stalks binding to the A3B3 including the contact between EG3 and the A subunit C-terminal domain of (AB)2. We have also included an Expanded View Movie (EV1) to show sectioning through the model and density map that illustrates these connections for all three stalks along the periphery of the entire V1.

iv) Figure 3c - What is the purpose of figure 3c? What does this figure show that is not shown in figure 3a or 3b? Also the significance or purpose of 3d is not clear.

Authors’ response 3.3:
The original purpose of Figure 3c was to illustrate and point out that the V-ATPase A3B3DF catalytic core is conserved within the family of rotary ATPases. The rationale was that this figure would drive home the point that bacterial V1s are active ATP hydrolases, whereas the eukaryotic V1 is autoinhibited. In addition, we discuss several points in the text focused around the catalytic core subunits specifically, for example, in the comparison of the ScV1 to the bacterial enzyme. Further, there are comments in the text about the central rotor’s positioning with respect to the phosphate binding P loop, and the central rotor protruding out of the top of the hexamer. We believe that showing the catalytic core of ScV1, with new annotations, provides an important visual tool accompanying the text descriptions, particularly for those less familiar with the architecture of rotary ATPases. We agree that as it was initially presented, this panel did not contribute much information to the rest of the figure. Please find revised Fig 3A, with new annotations to accompany the text found in the “Catalytic Hexamer” section. The rationale behind Fig 3d was simply to show that the three peripheral stalks are in different conformations at their N-terminal helices, but in the same conformation in the (A3B3-binding) C-termini. However, we agree with the reviewer that this panel does not provide information essential for the main text and we have therefore moved this panel to Appendix Figure S3A.

v) What criterion was used to assign the peripheral stalks as EG1, EG2 and EG3.

Authors’ response 3.4:
This naming scheme was initially introduced in Diepholz et al. (Structure 16, 1789 (2008)) and has since been used, most recently in the cryo-EM reconstructions of the yeast holo enzyme. Since the A/V-type ATPases from bacteria and archaea contain two peripheral stalks (EG1 and EG2) that both connect to the Vo subunit a N-terminal domain (aNT), the peripheral stalk that is only found in the eukaryotic enzyme has been referred to as EG3.

vi) In the section "catalytic hexamer and DF rotary shaft" (page 6), the text states that there is little difference in the position of subunits A or B between the bacterial and eukaryotic enzyme. However this is not clear in figure 4. An overlay of the bacterial and eukaryotic enzyme is required.

Authors’ response 3.5:
An overlay of the bacterial and eukaryotic enzyme is now shown in panel D of the revised Figure 3 (formerly Figure 4).

vii) The text describing the structure of the catalytic hexamer also states that "subunit DCT protrudes from the top of the hexamer by ~10Å, a feature unique to the eukaryotic enzyme". This novel feature needs to be illustrated.
Authors’ response 3.6:
Please find the model and density map now shown as a zoomed view in the revised Figure 3D.

viii) Figure 4c would be clearer if the (A3B3) hexamer was overlaid rather than subunit D. This would then demonstrate how the central stalk is stalled in relation to the different catalytic sites of the (A3B3) hexamer and that subunit A and B in the bacterial and eukaryotic enzyme are in the same position and conformation as stated in the text.

Authors’ response 3.7:
We absolutely agree that the change in perspective between the discussion in the text and how this was illustrated in the Figure was not as clear as it should be. In fact, Reviewer #1 brought up the same point. To address this, we have now added an alignment of the A3B3 hexamers (see panel D in the revised Figure 3). See also response 1.15 to reviewer #1 above.

ix) The significance of figure 4a and b in relation to novel findings of the presented structure is unclear. Are these states not seen in previously published structures or are they the standard states?

Authors’ response 3.8:
Initially, we had put an emphasis on these open and closed states because it is a main point in the context of the inhibitory binding of HCT to the open state and that this in turn stabilizes adjacent closed sites, leading to ADP inhibition. Indeed, these states have been seen in previously published structures, including the bacterial A/V-type enzyme (Arai et al, 2013; Nagamatsu et al, 2013) and the intact V-ATPase (Zhao et al, 2015), and so can be regarded as somewhat standard states. It should be noted that while these can be considered to be standard states, these states and their positioning with respect to other subunits in the context of the autoinhibited V1 are of mechanistic interest. However, while we agree that these features are not entirely novel they do help us to explain a mechanism for autoinhibition. Further, we feel that because some emphasis is put on the presence of the two closed and one open catalytic site, these panels would serve an important purpose for those less familiar with the field. We have retained both of these panels (revised Fig 3) to point out the different catalytic subunit conformations, as these states are important to other figures and the discussion. For example, in Figure 4D (previously Figure 5, and discussed in more detail in point (x) below), we point out that the inhibitory HCT interacts with the open state and discuss implications of this in terms of ADP inhibition. In addition, our endogenous nucleotide measurements and ATPase activity data combined with the structure containing two closed and one open site, help to decipher a mechanism of silencing.

x) Section "subunit H inhibitory interactions" is the most significant part of the manuscript and could benefit with being expanded. Of particular interest is the connection of subunit H to EG1 and the (A3B3) hexamer in relation to the catalytic state. E.g. could subunit H bind to the closed states or can it only bind to the open state?

Authors’ response 3.9:
This is a good point. Please find the text of that section expanded to now read (page 7):

“During catalysis, conformational changes in the catalytic hexamer (Movie EV2) drive rotation of the central DF rotor. As the D subunit forms part of the rotor, the site on DNT for the HCT interaction would only be available for binding in this rotational position (Fig 4D). Further, the site on the B subunit for the HCT interaction is only exposed and available for binding in the open conformation of the (AB)1 pair (Movie EV2). From its inhibitory interactions, it appears that part of the mechanism of HCT inhibition is to stabilize the open conformation of the (AB)1 pair specifically, which would have the additional effect of stabilizing the adjacent closed conformations of the (AB)2 and (AB)3 catalytic sites due to the highly cooperative nature of the catalytic sites in rotary ATPases. As we are able to resolve this interaction in the crystal structure, this must be a specific stopping point associated with inhibition of the enzyme.”

Please also note that we have included a reference to a Movie (EV2), which shows the interpolation between the three states observed recently for the intact yeast V-ATPase and our structure of V1. This movie shows rotation of the central rotor and the conformational changes in the A3B3 in
relation to where HCT is binding.

xi) Figure 5a and b are very informative, however an additional image showing the connection of EG1 to subunit H from a different orientation would be beneficial. In addition the loop region on which the mutational experiences are based need to be clearly indicated in figure 5b e.g. with a different colour.

Authors’ response 3.10:
We thank Reviewer #3 for the excellent suggestions. We agree that the EG1-H interaction is an important aspect of the inhibitory function of subunit H and provides a clearer picture of the breadth of these interactions. Please find revised Figure 4 (previously Figure 5), which now includes a panel (B) showing density and the interactions of EG1 with subunit H N-terminal domain from a different orientation. In addition, the regions of interaction between the loop in HCT and subunits D and BCT are now highlighted in panel C of the revised Fig. 4. More detail regarding the mutational manipulations in this region of HCT are now shown in a new panel (B) in the revised Figure 5 (previously Figure 6).

xii) Figure 5c needs to be discussed in the text. E.g. does DCT penetrate into the open state between A and B? Can DCT interact with subunits A and B in a closed state? A side view of figure 5c maybe beneficial.

Authors’ response 3.11:
Fig 4D (previously Fig 5C) is now discussed towards the end of the revised section “Subunit H inhibitory interactions” on page 7 (please see above response 3.9 to point (x)). Furthermore, a side view is now included in the revised Fig. 4A to help orient the reader.

xiii) A table or schematic figure summarising the different mutations of Subunit H constructs tested would greatly assist the understanding of figure 6.

Authors’ response 3.12:
Please find a new panel B in the revised Figure 5 (previously Figure 6) to include a schematic representation of the mutations tested along with a structural perspective of the mutants. Along with the Loop region of the autoinhibited V1, the structural perspective includes homology models (generated using the Phyre2 server) of the human H subunit loop region and that of the yeast mutant referred to in the text as H(Loop). The H(Loop) mutant was designed to mimic the length and overall shape of the human H subunit, while keeping the yeast Loop sequence largely in place.

xiv) Figure 6e. The MW markers are not the same scale in the two gels. Please either add MW markers to the right gel or rescale the gels so the MW markers on the left gel correspond to the markers on the right gel.

Authors’ response 3.13:
Please find the molecular masses labeled on the blot in the revised panel E of the new Figure 5 (previously Figure 6).

xv) Figure 6. Labeling nomenclature for WT subunit H expressed from a plasmid is inconsistent between figure 6d, 6e and legend.

Authors’ response 3.14:
Please find the legend for revised Figure 5D,E (previously Fig 6D,E) amended accordingly.

xvi) What is the significance of the C-subunits being co-purified with the isolated V1 for each mutant as shown in figure 6e? how does this affect the interpretation? This needs to be discussed in the text of the manuscript.

Authors’ response 3.15:
The levels of C subunit co-purified with the mutants, and indeed non-mutant V1, are highly variable (see also our response 1.1 in reference to the first comment by reviewer #1). In fact, the substoichiometric and variable levels of C found in V1 preparations was the impetus for our deletion of the subunit for crystallization. The C subunit is known to dissociate from the V1Vo complex.
during regulated disassembly and its departure is thought to trigger the disassembly process. However, while in the literature some preparations contain negligible amounts of C (Kitagawa et al, 2008), the subunit C content in some purifications of V1 is more significant (Hildenbrand et al, 2010). The reasons for the highly variable levels of C co-purifying with V1 are not clear at this point. We have previously quantified the affinity of the C subunit (and its individual domains) for the peripheral stalks and found that one domain binds with nanomolar affinity to the EG heterodimer (Oot & Willems, 2010). While this high affinity interaction occurs in the intact V-ATPase, it is broken for release of subunit C during regulated enzyme disassembly. It seems that in some cases, where subunit C co-purifies with V1, the breaking of this interaction is either not complete or some C subunit re-binds at some point during the purification procedure. The inefficiency of re-forming this interaction is displayed in variable amounts of subunit C found in V1 preparations. The positioning of EG3 seen in the autoinhibited V1 may help to occlude a binding interface for subunit C on that peripheral stalk, as proposed in the Discussion of the manuscript.

In terms of how the co-purification of subunit C affects interpretation of the activity data, a previous study from our laboratory tested functional properties of a V1 with wild type (and stoichiometric) C subunit and found the enzyme to lack any Mg-ATPase activity (as in our autoinhibited V1 with C subunit deleted), suggesting that the variable levels of subunit C do not directly affect the MgATPase activity of V1 (Zhang et al, 2003). In another study, it was found that overexpression of subunit C in yeast allowed for increased levels to be copurified with ScV1 but no activation of MgATPase activity was observed (Keenan Curtis & Kane, 2002). Consistent with this, there does not appear to be an effect from the amount of C subunit present in the preparations on the level of ATPase activity in the current study. For example, in the V1 deleted for subunit C used for crystallization, there is no detectable MgATPase activity at all, whereas the mutants with the most C subunit co-purified contain the least activity. Taken together with the available literature, we conclude that the level of subunit C co-purified with V1 does not affect the MgATPase activity in a measurable way.

Please find the caption for Fig 5E amended to include the text:

“SDS-PAGE and Western Blot analysis of ScV1 preparations. Left panel, 10% SDS PAGE of ScV1 purified from the subunit H mutant and WT strains. Note that H WT,ch is purified from a yeast strain deleted for the C subunit (used for crystallization). The remaining strains contain subunit C, which is co-purified with ScV1 in varying levels. Note that while H WT,ch ScV1, which is expressed from a strain deleted for subunit C, displays no detectable MgATPase activity, the H subunit mutants co-purified with the most C subunit display the least activity, suggesting that absence or presence of subunit C has no effect on MgATPase activity. Right panel, immunoblot probed with an antibody directed against the N-terminal Myc tagged mutant H subunits expressed from a plasmid. Note that the levels of mutant H expression does not appear to vary from strain to strain.”

xvii) Why was only one point mutation tested? Is this sufficient to conclude that a "single point mutation in the loop region is not sufficient to negate function"?

Authors’ response 3.16:
The single point mutation was tested because it is the only conserved residue in the HCT Loop region. We had seen from the Chimeric H subunit studies that the entire Human H subunit C-terminal domain was unable to inhibit ATPase activity, and we therefore sought to examine the importance of the only conserved residue in this region of the yeast HCT Loop. As the human subunit could not silence and the point mutant could, we then examined the importance of the length of the Loop and surrounding alpha helices, as these are predicted to be shorter in the human enzyme. These studies stress the importance of the shape and accessibility of this region to silencing, and that the conservation of that residue does not reflect an obvious importance to inhibitory function. We agree that the wording of the text was too strong, as only one point mutation was examined. Please find the text amended to read (page 9):

“…mutagenesis of the only conserved residue in this region is not sufficient to negate function…”

xviii) Figure 7 requires an overview of the overlay between the holoenzyme and the auto inhibited V1 structure to make it clear that these two structures are being compared. The features described in the text should be indicated on the overview with a second image dedicated to these features.
These include: a slice through the (A3B3) hexamer showing the similarity in position of subunit A and B, and close ups of HNT & EG1, HCT and EG3.

Authors’ response 3.17:
Thank you for this suggestion. Please find Figure 6 (previously Figure 7) amended to include overlays of the autoinhibited V1 structure and the intact V-ATPase “state 2” (3j9u; Zhao et al, 2015)). The overlays are aligned by the A3B3s and shown in two different views with zoomed images shown for EG1-HNT, EG3, HCT. These views include parts of the A3B3 hexamer, showing the similarity of the positions of the A3B3 subunits. Please find an enlarged view of the peripheral stalk EG1-HNT showing the change in position between V1 and intact V1Vo, as discussed in the text. In another enlarged view, peripheral stalk EG3 is highlighted to point out the loss of its binding partner (C subunit) as well as its conformational change to the position at the base of the catalytic hexamer, as also is discussed in the text. We kept the original panel from this figure that highlighted the interactions involving HCT but added annotation and included more of the central rotor domain. This figure now also includes a panel indicating the flexibility of the HCT by aligning with the isolated H subunit crystal structure (1ho8; Sagermann et al, 2001) as well as with the structure from the V1Vo holoenzyme (3j9u; Zhao et al, 2015). This panel was previously part of the Supplement (Fig S2) and as per Reviewer #2’s suggestion has been moved into a main text figure.

As a result of the expansion of this figure, the text in the section “Comparison of autoinhibited ScV1 to holo ScV1Vo” has been reorganized to flow with the figure. Specifically, in the initial manuscript submission we had discussed the HCT conformational changes first, followed by discussion of the conformational changes of the peripheral stalks. This order is reversed, with the peripheral stalk conformations coming first and HCT afterwards in the text of this section. Further, as we have added an additional panel that was previously in the Supplement (as per Reviewer #2’s suggestion), we have amended the text in this section (page 9) to read:

“The apparent flexibility of H_{CT} can be further illustrated upon comparison of the available structure of the isolated H subunit to that in ScV1\textsubscript{V0} and in ScV1_{1} (Fig 6F). Based on this comparison, H_{CT} undergoes an 180° rotation from its position in isolated H to that in the autoinhibitory conformation in ScV1_{1}. The alignment further suggests that H_{CT} can adopt multiple low energy conformations and that its flexibility is required for the inhibitory function in ScV1 and for coupling ATP hydrolysis to proton transport in ScV1_{V0}.”

xix) Discussion, page 9, first paragraph, line 13. Please change may to "maybe" in the sentence starting "Photochemical crosslinking on the other hand...”

Authors’ response 3.18:
Done

xx) Is it really possible to assign ADP to the density shown in supplementary Fig 4 when the resolution is 6.2-6.7Å? It is probably more accurate to say that the density indicates that a "nucleotide" is bound to the closed catalytic site and that the nucleotide is likely to be ADP because of....

Authors’ response 3.19:
The reviewer is of course correct that we cannot assign the nucleotide bound to the V1 based upon the structural data at this resolution. We have changed the text to (page 10):

“The presence of strong positive difference density near the P-loop in (AB)2 indicates that the inhibitory nucleotide is bound in the closed catalytic site near EG2 and while we can not assign the identity of the nucleotide at this resolution, our biochemical measurements indicate that it is likely to be ADP (Appendix Fig S6).”

Additional changes to the manuscript:
In addition to the changes in response to the reviewers’ comments, we made the following changes to the text and/or figures:

(1) We moved the details of the experimental methods section to the Appendix, leaving an
abbreviated version of the methods in the main text.

(2) We included new appendix figures to illustrate (i) the contents of the asymmetric unit (Appendix Fig S1) and (ii) the alignment of the catalytic cores of yeast and Thermus thermophilus V1.

(3) We added a new Appendix Table S2 that lists the residues modeled for each of the subunits in the two copies in the asymmetric unit.

(4) During the revision process, we realized that the ATPase activity for the D410A mutant was measured from only one preparation and plotted as the average of four technical replicates. We have therefore repeated this measurement from an additional preparation of this mutant and the averaged activity value is now shown in the revised figure (Fig 5D). Furthermore, in the original submission, activity data for each mutant was plotted as the average of all technical replicates combined. In the revised figure, we have first averaged the technical replicates for each preparation and then plotted the mean of these two values with error bars showing the standard error of the mean. Please note that the conclusions drawn from the activity data remain the same as in the original submission.

References


Thank you for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by two of the original referees whose comments are shown below.

As you will see, the referees both find that all major criticisms have been sufficiently addressed and recommend the manuscript for publication, pending very minor revision. Before we can go on to officially accept the manuscript for publication I would therefore ask you to address the few remaining suggestions from ref #3 in a final revised version.

In particular, I would ask you to comment on the conformation of EG1 and EG2 relative to EG3 - and include additional depictions if you find this informative for the reader. I would also ask you to comment on/indicate the position of the hinge regions discussed in figure 2D and on the bar for H WT-CH in figure 5D. As for figures 2E, and 2F it is fine from our side to leave them as they are now.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to receiving your final revision.

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Referee #1:  
My compliments to the authors on a thoughtful and thorough revision. This manuscript should be in the EMBO Journal without delay.

Referee #3:  
The authors have satisfied the majority of my concerns however a few points remain outstanding.
Main concerns:

Page 5, Overall structure of ScV1: The main points of this section are still not clearly represented in figures 2. Line 11 of this section states: "Each of the three peripheral stalks is bound via its C-terminal domain (ECTGCT) to the N-terminal β barrel domain of the corresponding B Subunit and crosses a non-catalytic A/B interface on its way towards the base of the hexamer (Fig2B)." Figure 2B shows very nicely the path of EG3 however, I thought EG3 was very different to EG1 and EG2. Therefore the interaction of EG1 and EG2 should also be shown in similar style figures.

Having the bulge region indicated in figure 2D is greatly appreciated. However, in the text, hinge regions are discussed. Where are these? are the same place as the bulge region? Do the different conformations of the peripheral stalk in the ScV1 structure confirm the presence of the hinges?

The information contribution of fig2E and 2F is not obvious. Are these figures necessary?

Figure 5D: The bar for H WTCH (chromosomal encoded H subunit) in panel 5D appears to be missing despite it being discussed in the figure legend

Minor comment:
In both the printed out and on screen version, the color of subunit A appears purple not pink.

Authors’ response:
Our intention in showing Figure 2B is to present a representative view of the interaction between the EG C-termini and the catalytic hexamer and the crossing of a non-cat interface by the stalks on their way towards the base of the hexamer (interactions which are overall similar for EG1, EG2 and EG3). As reviewer #3 correctly points out, EG3 differs from the other peripheral stalks, a difference that lies in the conformation of its N-terminus. We began our discussion of the peripheral stalks in the “Overall structure” section by pointing out the similar binding modes and conformations for each peripheral stalk in their C-terminus, specifically in the text that reviewer #3 quotes above. In the following sentences we move on to describe that while the stalks are the same as each other in their C-termini, they are each different in the conformation of their N-terminal domains. As panel 2B was included to show the binding of the stalks to the hexamer, an interaction that is the same in each stalk, we chose to show only one of the stalks’ interactions in this figure. We do not think that also showing Fig 2B style depictions of EG1 and EG2 would add essential information. The crossing of a noncatalytic A/B interface by each peripheral stalk is also depicted in panel 2E-F and Movie 2. One benefit of showing this from the perspective illustrated in panels E and F is that the similarity in binding and crossing the non catalytic interfaces can be shown for all three stalks, along with the change in conformation of the N-terminus, as evidenced by the different proximity of the EG N-termini to an adjacent catalytic A subunit.
The text referred to by the reviewer has been amended, adding a qualifier to make more clear the point that while the three peripheral stalks are binding to the hexamer via the EG C-terminus in a very similar manner to each other, the conformations of their N-terminal domains is different (page 5):

“Each of the three peripheral stalks is bound via its C-terminal domain (ECTGCT) to the N-terminal β barrel domain of the corresponding B subunit and crosses a non-catalytic A/B interface on its way towards the base of the hexamer (Fig 2B). However, while the stalks’ C-terminal domains are largely invariant, their N-termini (ENTGNT) are in different conformations (Fig 2C and D; Appendix Fig S3A).”

Further, we have modified the legend to Figure 2B to clarify this point:

“Interaction of the peripheral stalks with the catalytic hexamer. Each peripheral stalk crosses a non-catalytic A/B interface on its way from the top of the V1 to the base of the catalytic core. Representative view of the interaction is shown for EG3.”

Having the bulge region indicated in figure 2D is greatly appreciated. However, in the text, hinge regions are discussed. Where are these? are the same place as the bulge region? Do the different conformations of the peripheral stalk in the ScV1 structure confirm the presence of the hinges?

Authors’ response:
Please find the hinge regions labeled on the previously solved structure of the peripheral stalk (4dl0) in Appendix Figure S3B. In addition, we have labeled and indicated these residues on the alignment of the three peripheral stalks shown in Appendix Figure S3A. Indeed, these hinges are flanking the “bulge” in subunit G. It can be seen on the alignment that the hinge at subunit E Glu54 is in the area at which the N-termini of the three stalks diverge in conformation, suggesting that the structure of V1 confirms the presence of at least one of the previously identified hinges. The other hinge (at subunit E Met84) may allow some flexibility during catalysis that is not obvious in our autoinhibited structure.

Please find a new reference to these panels included in the revised text (page 5):

“In that study, we provided evidence that the EG heterodimers contain two hinges and a partially disordered “bulge” region in subunit G that provide flexibility and that we speculated would play an important role in the mechanism of reversible disassembly (Appendix Fig S3A and B).”

Further, as a result of these changes to the Figure, please find the Appendix Figure S3 legend to panels A and B amended:

“A Conformational differences in the three peripheral stalks. The three peripheral stalks are shown EG1 (E dark blue, G orange red), EG2 (E light blue, G gold) and EG3 (E blue, G orange). EG1-3 were aligned by their invariant C-termini illustrating the different conformations adopted by their N-termini (boxed and enlarged at right). The residues on subunit E (EGLU54 and EMET84) that were previously shown to be part of two flexible hinge regions in the peripheral stalk are labeled (Oot et al, 2012).

B The available structure of the yeast peripheral stalk at 2.9Å resolution (4dl0) showing the “bulge” in subunit G as well as the residues on subunit E (EGLU54 and EMET84) that have been identified previously as flexible hinges (Oot et al, 2012).”

The information contribution of fig2E and 2F is not obvious. Are these figures necessary?

Authors’ response:
As mentioned above, in response to the comments on Figure 2B, these panels were presented (along with a Movie) to depict the crossing of the non-catalytic interfaces of the hexamer by each of the three peripheral stalks as well as to show the change in conformation at the N-terminus (particularly of EG3) and it’s proximity to the catalytic A subunit from an adjacent A/B pair. We wished to include a panel that would highlight these features globally, from a different perspective and in the
corresponding electron density.

Figure 5D: The bar for H WT\textsubscript{CH} (chromosomal encoded H subunit) in panel 5D appears to be missing despite it being discussed in the figure legend

Authors’ response:
This is a good point and we can see that the Figure legend may have been somewhat unclear as it was originally presented. When we mentioned chromosomally encoded H subunit, we should not have used the abbreviation “H WT\textsubscript{CH}” because that annotation suggests that a bar would be shown on the Figure. As the V1 with chromosomally encoded H subunit has no detectable Mg\textsuperscript{2+}-ATPase activity, we cannot present a bar indicating this. However, this property of the V1 is widely accepted and well documented so we have now included references to this in the legend. The purpose of mentioning this property in the Figure legend was for comparison to the V1 with plasmid-borne WT H subunit, which has low levels of activity likely due to expression from the low copy plasmid.

We have amended the Figure legend to clarify this point:

“The ScV\textsubscript{1} sector containing chromosomally encoded H subunit shows no detectable Mg\textsuperscript{2+}-ATPase activity (Parra et al, 2000; Zhang et al, 2003), whereas ScV\textsubscript{1} containing plasmid-borne wild type H (H WT\textsubscript{pl}) exhibits low levels of activity likely due to reduced expression from the low copy plasmid.”

Minor comment:
In both the printed out and on screen version, the color of subunit A appears purple not pink.

Authors’ response:
In the initial submission, the coloring of the A subunits was described as “orchid”, which is between pink and purple but reviewer #1 felt that this color description was ambiguous so we changed it to “pink”. Since there is no other color used in the context of V1 that is similar to purple or pink, “pink” should be an unequivocal identifier for subunit A.
A. Figures
1. Data
The data shown in figures should satisfy the following conditions:
- The data were obtained and processed according to the study's design and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- Figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- Graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n ≥ 2, the individual data points from each experiment should be plotted and any statistical test employed should be justified.
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- A specification of the experimental system investigated (e.g., cell line, species name).
- The assay(s) and method(s) used to carry out the reported observations and measurements.
- An explicit mention of the biological and chemical entity(ies) that are being measured.
- An explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- The exact sample size (n) for each experimental group/condition, given as a number, not a range.
- A statement of how many times the experiment was independently replicated in the laboratory.
- Definitions of statistical methods and measures:
  - A common test, such as t-test (please specify whether paired or unpaired); simple p-test (Welch and Mann-Whitney tests, etc.), can be unambiguously identified by name only, but more complex techniques should be described in the methods section.
  - Are tests one-sided or two-sided?
  - Are there adjustments for multiple comparisons?
  - Exact statistical test results, e.g., F-value = x but P-value < y.
  - Definition of center values: mean or median; s.d. or s.e.m.; N/A.
  - Definition of error bars: s.d. or s.e.m.; N/A.

Any descriptions too long for the figure legends should be included in the methods section and/or with the source data.

Please ensure that answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write N/A (non-applicable).

B. Statistics and general methods
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?
Activity measurements were taken from two independent purifications of each mutant. Activity measurements from 3-8 technical replicates for each proportion were averaged and the mean of the two values was plotted with error bars indicating the standard error of the mean (Fig 5D, caption and Appendix Supplementary Methods).

B.6. For animal studies, include a statement about randomization even if no statistical methods were used.

B.8. Describe inclusions/exclusions criteria if samples or animals were included from the analysis. Were criteria pre-established?

B.9. Were any steps taken to minimize the effects of subjective bias when assigning samples to treatment (e.g. randomization procedure)? If yes, please describe.

B.9. For animal studies, include a statement about randomization even if no randomization was used.

B.9. Were any steps taken to minimize the effects of subjective bias during group allocation or when assessing results (e.g. blinding of the investigator)? If yes, please describe.

B.9. For animal studies, include a statement about blinding even if no blinding was done.

C. Reagents

C.9. Are the variance similar between the groups that are being statistically compared?

Please fill out these boxes. (Do not worry if you cannot see all your text once you press return.)
6. To show that antibodies were profiled for use in the system under study (assy and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. E.g., anti-CD45 (Proteintech; see link at top right).
5. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma.

D- Animal Models
4. For experiments involving in vivo or in vitro (e.g., cell culture, bioreactors), include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.
3. We recommend consulting the ARRIVE guidelines: (see link at top right) to ensure that all relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these recommendations. Please confirm compliance.
2. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

E- Human Subjects
1. Identify the committee(s) approving the study protocol.

F- Data Accessibility
1. Provide accession codes for deposited data. See author guidelines, under ‘Data Deposition’.
   - Data deposition in a public repository is mandatory for:
     a. Protein, DNA and RNA sequences
     b. Microscopic images
     c. Crystallographic data for small molecules
     d. Functional genomics data
     e. Proteomics and molecular interactions
2. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal’s data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under ‘Expanded View’ or in unstructured repositories such as Dryad (see link at top right) or Figshare (see link at top right).
3. Access to human clinical and genetic datasets should be provided with active instructions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access controlled repositories such as dbGaP (see link at top right) or EGA (see link at top right).
4. If for any possible, primary and referenced data should be formally stored in a Data Availability section. Please state whether you have included this section.
   - Examples:
   c. AF-MS analysis of human histone deacetylase interactions in CEM-T cells (2012). PROTEOMICS 1200208
5. Functional models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized formats (BMRB, CATH) should be used instead of scripts (e.g., MATLAB). Authors are strongly encouraged to follow the MIBML guidelines (see link at top right) and deposit their model in a public database such as BioModels (see link at top right) or PubMedcentral (see link at top right). If a final source code is provided with the paper, it should be deposited in a public repository of included in supplementary information.

G- Dual use research concern
2. Could your study fall under dual use research restrictions? Please check biosecurity documents.

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