nature research

Peer Review Information

Journal: Nature Structural and Molecular Biology
Manuscript Title: Mitochondrial complex I structure reveals ordered water molecules for catalysis and proton translocation
Corresponding author name(s): Prof. Judy Hirst

Reviewer Comments & Decisions:

Decision Letter, initial version:

13th May 2020

Dear Judy and Daniel,

Thank you again for submitting your manuscript "High-resolution cryo-EM structure of mitochondrial complex I reveals ordered water molecules for catalysis and proton translocation", and for your patience during this long process. I apologize for the delay in reaching this decision. We now have comments (below) from the 3 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that the reviewers are overall positive about the quality of the work and interest of the findings; they each have different questions for clarifications and suggestions to improve text/figures. You do have space to expand discussion as requested, and we do ask that you keep main text under 4,300 words (the current word count is ~4,100; the Results section could be streamlined a bit).

Please be sure to address/respond to all concerns of the referees in full in a point-by-point response. If you have comments that are intended for editors only, please include those in a separate cover letter.

I am attaching here 2 files:
- an edited word file with some comments and notes on formatting and organization; please follow those guidelines, as they would greatly simplify the next step.
- template for Table 1; please include EMDB and PDB IDs.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We expect to see your revised manuscript within 4 weeks. If you cannot send it within this time,

please contact us to discuss an extension; we would still consider your revision, provided that no similar work has been accepted for publication at NSMB or published elsewhere.

If there are additional or modified structures presented in the final revision, please submit the corresponding PDB validation reports.

Data availability: this journal strongly supports public availability of data. All data used in accepted papers should be available via a public data repository, or alternatively, as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found below:
https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data

We require deposition of coordinates into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication.

While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at http://www.nature.com/nsmb/authors/submit/index.html#costs

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Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,
Ines

Ines Chen, Ph.D.
Chief Editor

Nature Structural & Molecular Biology

ORCID 0000-0002-1405-9703

Referee expertise:

Referee #1: complex I

Referee #2: cryo-EM

Referee #3: complex I

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
This is a very well written manuscript that presents novel data linking the long-known active/deactive (A/D) conformational transition of the enzyme to mechanistic details of catalytic turnover. Using a 2.7A resolution structure of the yeast enzyme, the authors' determined important events that occurred in the membrane part of the enzyme during the A/D transition and also identified bound water molecules involved in proton translocation by complex I. This reviewer has no conceptual criticism, but only several comments.
1. When discussing the ubiquinone (UQ) binding site, recent biochemical data from the Miyoshi lab (Uno et al., 2019, 2020, JBC) should be mentioned. They showed accessibility of the UQ reduction site (in the mammalian enzyme, though) to various bulky quinone molecules that are much large than natural UQ. The structural data from the past and data presented in the manuscript suggested a long narrow cavity for UQ binding, which is highly unlikely to be traveled by such a large UQ molecule. It would be important to have a possible explanation of this discrepancy.
2. When discussing the models of complex I energy transfer (Page 2 last paragraph), references to a hypothesis of two-state stabilization change mechanism for complex I should be added.
3. The enzyme from yeast has lower activation energy for the A/D transition than the mammalian enzyme. What is the structural basis for this? Discussing this issue might significantly benefit the readers of Nature Structural Biology.
4. The NADH:Q reaction catalyzed by complex I is not strongly pH-dependent (within the range of 5.5-8.5). Therefore, protonation/deprotonation of amino acid residues affecting water molecules binding at the enzyme surface has very little functional effect in that pH range. How important are the ordered water molecules shown in the manuscript and what is the pK of amino acids involved in their binding?

Reviewer #2:
Remarks to the Author:
Mitochondrial complex I uses energy from NADH/ubiquinone oxidation/reduction to contribute to the proton gradient that powers ATP synthesis. To date there are several cryo-EM structures of complex I, which have defined different conformational states and have been central to our structural and mechanistic understanding of the complex. In the present study the authors present a new 2.7 Å-

resolution structure of complex I from the yeast Yarrowia lipolytica. This 2.7A map is currently the highest-resolution structure of complex I available. Importantly, this map contains details of structured water molecules, many of which are proposed to be vital for the enzyme's proton relays, energy transfer, and proton-pumping pathways. In addition, this structure is compared to other complex I structures from the literature, providing further insight into how different structural states relate to function.

The manuscript presented by Grba et al. is well written, and the figures are clear and easy to follow. The results presented here build on the current complex I knowledge. We recommend that this manuscript is published in nature structural and molecular biology with the following issues are addressed.

The authors extract 2 major populations of particles – those with ST1, and those without. The refinement leading to the map used for the majority of the analysis includes both populations. Presumably combining these populations allows for a higher resolution to be obtained which is essential for this study. However, combining these 'broken' (ST1 devoid) particles with the complete particles should be justified. Does the class without ST1 also have the alignment issues mentioned (Line 680)? If the classes are refined separately are the two maps similar (except for the ST1 region)? In particular, regions that are focused on in the analysis should be compared. Also, comparing these classes may provide details on how ST1 modulates complex I structure and function.

In figure 3a the authors display the model that highlights the protonation pathway. However, the authors only display the model (the results/interpretation of the data) and not the map (the data). The authors should also show map (or important parts of the map) in the figure, as they did in Figure 2c, to support their model.

Similar to figure 3, density should be shown for the models in figure 4.

Lines 280-285: The authors assertion about the level of deactivation is interesting, but may be too strong. This concern arises because they are comparing loops from maps at different resolutions. One would expect loops from a map at higher resolution to appear different than those from a map at lower resolution.

Lines 310-313: Similar to above, the authors should consider that the models they are comparing are developed from maps at different resolutions. We suggest directly comparing maps filtered to the same resolution. This analysis would help ensure that interesting differences between models are not due to the difference in resolution between their respective maps.

Line 345 – the authors should also consider that the structures being compared are from samples that were solubilized/exchanged in(to) different detergents. Detergents, like lipids, can affect structure and dynamics.

line 85 – a comma after 'transfer' would make this sentence more clear.

The 'E-channel' is cited throughout the text but not explicitly labelled in any of the figures. Adding this may help guide readers.

Reviewer #3:
Remarks to the Author:
Grba & Hirst presents here the structure of the mitochondrial complex I resolved from Yarrowia lipolytica at 2.7 Å resolution based on cryo-electron microscopy experiments. The work reveals experimentally for the first time water molecules that are likely to play an important functional role, and key conformational changes linked to complex I active/deactive transition. I find that the study is excellent quality, although the presentation is a bit too detailed in some sections. The study is of great importance for the complex I field, and of broad interest also for the readership of NSMB. However, a few points should be clarified, before I can recommend publication:

-p. 4/Fig. 3: the authors present a putative proton write leading to the Q site. Does the region / residues overlap with previous prediction by Jussupow et al. Sci Adv (2019) 5(3):eaav1850, particularly tunnel 1?

-Based on Fig. 3a, H95/Y144 do not seem to be in a conformation capable of binding Q. Please comment.

-p. 5/Fig. 2 and Fig 4: five water molecules are shown in ND2 in Figure 2, whereas eleven waters are shown in Fig 4. It would be good to show all resolved water molecules also in Fig. 2 to avoid confusion.

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-How well is the density of discussed carboxylate observed? Can the ion-pair conformations in ND2/4/5 or Glu side chains in ND1 be refined with certainty?

-p. 5-6/line 188-190: Does the number of observed water molecules correlate with local resolution of the cryoEM maps in ND2, ND4, ND5?

-p. 7: “the hydrated cavity in subunit ND1”-section. I find that this section would better fit after the “Q site hydration”- section, and it could be merged with the “ND1 cavity”-section on p. 8. I also find that the Figure 7 is a bit technical for a main text figure.

-Some lipids may have a functional role in complex I. Where are the 32 refined lipids observed?

-p.8 Cavity search: are the observed cavities sensitive to the employed probe radius?

-p. 10/lines 343-353: the list of observations is too technical for a general reader, and should be simplified.

- it has been proposed that the missing 42 kDa subunit in the Yarrowia complex I, could lead to its perturbed A/D transition (BBA 1859 (2018) 326–332). How does the findings compare to the resolved structure?

-The authors should clarify if they suggest that their resolved deactive-like state is an off-pathway form of complex I, or a part of its native reaction cycle?

-Minor comment: in the introduction, some key information is given in parenthesis. (e.g. in all that have it; 31 in mammals; for all but seven …; four protons are pumped for each NADH oxidised). Please write the sentence out if the information is relevant.

Author Rebuttal to Initial comments

Mitochondrial complex I structure reveals ordered water molecules for catalysis and proton translocation

Daniel N. Grba & Judy Hirst

Reponse to the comments of the reviewers

Reviewer 1

This is a very well written manuscript that presents novel data linking the long-known active/deactive (A/D) conformational transition of the enzyme to mechanistic details of catalytic turnover. Using a 2.7A resolution structure of the yeast enzyme, the authors' determined important events that occurred in the membrane part of the enzyme during the A/D transition and also identified bound water molecules involved in proton translocation by complex I. This reviewer has no conceptual criticism, but only several comments.

We thank the reviewer for this positive evaluation.

1. When discussing the ubiquinone (UQ) binding site, recent biochemical data from the Miyoshi lab (Uno et al., 2019, 2020, JBC) should be mentioned. They showed accessibility of the UQ reduction site (in the mammalian enzyme, though) to various bulky quinone molecules that are much large than natural UQ. The structural data from the past and data presented in the manuscript suggested a long narrow cavity for UQ binding, which is highly unlikely to be traveled by such a large UQ molecule. It would be important to have a possible explanation of this discrepancy.

Indeed, the aim of this work was to probe whether the addition of bulky features to the substrate would prevent it entering the proposed narrow tunnel, as would be required for turnover. Uno et al. showed that short bulky substrates (OS-UQ1–3) could be reduced by submitochondrial particles (SMPs) but not by purified complex I reconstituted in proteoliposomes. They interpreted their SMP results to mean that the narrow channel is not a viable quinone binding channel – and that the purified complex must have been functionally altered. However, we disagree. We contend that the observation of sustained NADH oxidation by SMPs upon the addition of the soluble OS-UQ is due to redox equilibration of OS-UQ with the Q10 present in the membranes. The redox equilibration of different Q pools is a well-known phenomenon. In this case, complex I reduces Q10 to Q10H2, and the large pool of OS-UQ equilibrates with the Q10/Q10H2,

reoxidising the Q10H2 to Q10 – and enabling NADH oxidation to continue. In the proteoliposomes no Q10 is present, so the reaction does not occur. We note that complex I in proteoliposomes was able to reduce OS-UQ8 – a molecule long enough to access the active site with its bulky group remaining outside the narrow binding tunnel. In regard of the labelling experiments reported, the 12-hour incubation involved in the alkynylation assay will inevitably result in conversion to the deactive state, in which the ubiquinone site is disordered and solvent accessibility is known to be increased. We therefore contend that the results of Uno et al. have obvious alternative interpretations from those presented, and that they do not, in fact, challenge the long, narrow binding channel. We therefore do not include reference to this work in our manuscript, which allows for only a brief introduction to the mechanism of the complex.

2. When discussing the models of complex I energy transfer (Page 2 last paragraph), references to a hypothesis of two-state stabilization change mechanism for complex I should be added.

Thank you for pointing out this omission. We have added a statement and reference to the two-state stabilization change mechanism on page 2 accordingly:

"Proton pumping may be initiated by a conserved aspartate linked by hydrogen bonding to the ubiquinone redox centre[22], or the ubiquinone may switch between two subsites in a two-state stabilization change mechanism[23]."

3. The enzyme from yeast has lower activation energy for the A/D transition than the mammalian enzyme. What is the structural basis for this? Discussing this issue might significantly benefit the readers of Nature Structural Biology.

The yeast enzyme presented here lacks the mammalian NDUFA10 subunit. As noted by Agip et al (2018), the NDUFA10 subunit on the membrane domain interacts with the NDUFA5 subunit on the hydrophilic domain, forming a second interface between the two domains and thereby stabilising their relative arrangement (which changes in the active/deactive transition). Computational analyses of complex I dynamics were subsequently presented by Di Luca and Kaila (2018), and indicated that the same two subunits have a major impact on dyanmics and conformational transitions in the mammalian enzyme.

We have now expanded our text on page 10 as follows:

"The relaxation occurs at different rates and to different depths in different species (Figure 7). It occurs more slowly and to greater depth in the mammalian enzyme than in *Y. lipolytica*, perhaps due to the extra NDUFA10–NDUFA5 interface that must adjust in the mammalian enzyme[20]. NDUFA10 is absent from *Y. lipolytica*, and the NDUFA10–NDUFA5 interaction was identified in simulations to have a major impact on the global enzyme dynamics[50]."

4. The NADH:Q reaction catalyzed by complex I is not strongly pH-dependent (within the range of 5.5-8.5). Therefore, protonation/deprotonation of amino acid residues affecting water molecules binding at the enzyme surface has very little functional effect in that pH range. How important are the ordered water molecules shown in the manuscript and what is the pK of amino acids involved in their binding?

First, we disagree that the NADH:Q reaction catalyzed by complex I is not strongly pH dependent – in our hands, the rate shows a well-defined bell-shaped curve with a maximum at around pH 7.5, and very little activity at higher (pH 10) or lower (pH 5) values. Second, although (as expected) many of the water molecules we have modelled here are associated with the outside of the protein (bound on its surface), those that form the basis for the majority of our work are buried in the protein interior, separated from the bulk solvent. It is correct that surface-bound waters are subject to the bulk pH (provided that the pKs of the residues involved are in range), but here we discuss the surface waters only where they accumulate, perhaps around patches of polar residues, and may indicate potential hydration/proton-transfer pathways. Importantly, many of the buried waters are associated with key residues that are crucial for energy transduction / proton transfer and defining them is a key requirement for understanding the mechanism of catalysis. Of course the pK values of these key catalytic buried residues are central to the mechanism, but our contribution to defining them is to provide high resolution structural data as the basis for advanced computational work to evaluate their values; the computational work itself is clearly outside the scope of this current manuscript.

Reviewer #2

Mitochondrial complex I uses energy from NADH/ubiquinone oxidation/reduction to contribute to the proton gradient that powers ATP synthesis. To date there are several cryo-EM structures of complex I, which have defined different conformational states and have been central to our structural and mechanistic understanding of the complex. In the present study the authors present a new 2.7 Å-resolution structure of complex I from the yeast Yarrowia lipolytica. This 2.7A map is currently the highest-resolution structure of complex I available. Importantly, this map contains details of structured water molecules, many of which are proposed to be vital for the enzyme's proton relays, energy transfer, and proton-pumping pathways. In addition, this structure is compared to other complex I structures from the literature, providing further insight into how different structural states relate to function.

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We thank the reviewer for this positive evaluation.

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The reviewer is correct.

However, combining these 'broken' (ST1 devoid) particles with the complete particles should be justified. Does the class without ST1 also have the alignment issues mentioned (Line 680)? If the classes are refined separately are the two maps similar (except for the ST1 region)? In particular, regions that are focused on in the analysis should be compared. Also, comparing these classes may provide details on how ST1 modulates complex I structure and function.

i) We apologise for the lack of clarity on line 680. The alignment issue mentioned is only the result of focusing the alignment on the flexible ST1 subunit. As the relative arrangement of complex I and ST1 varies, focussing the alignment on either one causes loss of resolution in the other – an effect that is particularly marked on the large complex I when the alignment is focussed on the small ST1 subunit (as is evident in Supplementary Figure 2b, particularly on the peripheries of the complex, far from ST1). We have now clarified our text accordingly (page 22):

"The ST1 subunit was built independently from the rest of the complex using its focused refinement map. The rest of this map (for the main complex) was not used for building because the flexible interface between ST1 and the rest of the complex results in poor alignment outside of the ST1 mask, and therefore it is of low resolution."

ii) Separate refinement (prior to focussed refinement) of the ST1-containing particles and the ST1-free particles gives very similar results: 3.0 Å resolution (~25.5K particles) compared to 3.1 Å resolution (~24K particles), respectively. The two maps are essentially indistinguishable, except for the presence/absence of ST1. Consistent with this, the map produced from all the particles together shows no loss of local resolution in complex I at the ST1 contact point, and no adjustment to the structures of the complex I subunits that form the interface with ST1 can be identified by comparing the maps. As Supplementary Figure 3 shows, the interface comprises mainly loop regions, consistent with a weak and flexible interface. As a result, we do not expect the presence of ST1 to modulate the core catalytic function of complex I. We have now extended our description of the ST1 map and model on page 4 as follows:

"However, targeted classification (Extended Data Figure 1) revealed that only 25.5K (51%) of these particles contain the sub-stoichiometric, *Y. lipolytica*-specific sulfur-transferase 1 (ST1) subunit[37,38]. Focused refinement maps assisted in modelling this 43rd subunit, which is resolved to 3.5–5.7 Å, revealing its weak interactions with the hydrophilic domain through NDUFS3, NDUFA7, NDUFA12 and NDUFA13 (Extended Data Figures 2 and 3). As no differences in any of these contact subunits could be identified between the ST1-containing and ST1-free complexes, both sets of particles were included in the final reconstruction of the 2.7 Å resolution map."

In figure 3a the authors display the model that highlights the protonation pathway. However, the authors only display the model (the results/interpretation of the data) and not the map (the data). The authors should also show map (or important parts of the map) in the figure, as they did in Figure 2c, to support their model. Similar to figure 3, density should be shown for the models in figure 4.

We thank the reviewer for this helpful suggestion. Originally, we did not include the map in these figures to try to keep them as simple and clear to visualise as possible, but of course we recognise the importance of validating the model with the map presentation. We attempted to add the map to Figure 3a accordingly, but this made the figure unclear and hard to interpret, so instead we have added the additional panel c, in which we now display the densities for the water molecules modelled in each of ND2, ND4 and ND5 separately. In Figure 4 it was possible to add the water densities directly. We hope the reviewer will find these additions satisfactory.

Lines 280-285: The authors assertion about the level of deactivation is interesting, but may be too strong. This concern arises because they are comparing loops from maps at different resolutions. One would expect loops from a map at higher resolution to appear different than those from a map at lower resolution.

We thank the reviewer for raising this important point. In Line 280–285 we compared our *Y. lipolytica* map (2.7 Å) with the published map of deactive mouse (3.9 Å, Agip et al. 2018) and we understand the reviewer's concern about their different resolutions. In the deactive mouse map, densities for the ND1 TMH5–6, ND3 TMH1–2 and NDUFS2 catalytic loops cannot be identified. However, our *Y. lipolytica* map filtered to the same resolution still exhibits clearly visible continuous densities for both the ND1 and NDUFS2 loops; densities for the short regions of the ND3 loop that we modelled without side chains lose continuity, but the majority of the loop (much more than for deactive mouse) remains visible. We have amended our text (page 8) accordingly:

"We clearly establish our *Y. lipolytica* structure as in a deactive state (see Extended Data Table 4), but note that its deactive status is less severe than observed in mammalian enzymes[10,20,21] because the loops described above are poorly resolved, not lost from the map completely, even when filtered to the same resolution as the highest resolution available map for the mammalian deactive state (3.9 Å)[20]."

Lines 310-313: Similar to above, the authors should consider that the models they are comparing are developed from maps at different resolutions. We suggest directly comparing maps filtered to the same resolution. This analysis would help ensure that interesting differences between models are not due to the difference in resolution between their respective maps.

In Line 310-313 we compared our *Y. lipolytica* map (2.7 Å) with a previously published *Y. lipolytica* map with ubiquinone bound (3.2 Å). We note *increased* order in the ubiquinone-bound structure, which is of lower overall resolution, confirming that the different resolutions of the two maps do not underpin the comparison. We have adjusted our text (page 9) accordingly:

"Strikingly, in the ubiquinone-bound enzyme, ND1-TMH4 is straight and ND6-π1 present (matching deactive mouse) but the ND3-TMH1–2 and ND1-TMH5–6 loops are ordered and block access to the matrix (matching active mouse, Figure 7b). Differences in resolution cannot account for the loop ordering, as the ubiquinone-bound map with the ordered loops is of lower resolution (3.2 Å) than that described here (2.7 Å). These hallmarks (see Extended Data Table 4) place the ubiquinone-bound enzyme in a hybrid state."

Line 345 – the authors should also consider that the structures being compared are from samples that were solubilized/exchanged in(to) different detergents. Detergents, like lipids, can affect structure and dynamics.

We fully agree that the lipid and detergent environment can greatly influence the structure and dynamics of the protein, and that some of the differences observed between the different *Y. lipolytica* structures may result from this. On page 9 we explore this aspect in more detail, and consider that the DDM we see intercalated into our structure may be absent (and replaced by ubiquinone) in the structure of Parey and coworkers because they used LMNG instead, a detergent likely too large to fit in the cavity. We further considered the possibility that the DDM binding may contribute to inducing the greater disorder we observe. These sections underpin the discussion referred to.

line 85 – a comma after 'transfer' would make this sentence more clear.

Thank you for this suggestion, we have edited our text to read: "…structured water molecules that are vital for the enzyme's proton relays, energy transfer mechanisms, and proton-pumping pathways."

The 'E-channel' is cited throughout the text but not explicitly labelled in any of the figures. Adding this may help guide readers.

Thank you for this suggestion, we have added a label to Figure 2d accordingly.

Reviewer 3

Grba & Hirst presents here the structure of the mitochondrial complex I resolved from Yarrowia lipolytica at 2.7 Å resolution based on cryo-electron microscopy experiments. The work reveals experimentally for the first time water molecules that are likely to play an important functional role, and key conformational changes linked to complex I active/deactive transition. I find that the study is excellent quality, although the presentation is a bit too detailed in some sections. The study is of great importance for the complex I field, and of broad interest also for the readership of NSMB.

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However, a few points should be clarified, before I can recommend publication:

-p. 4/Fig. 3: the authors present a putative proton write leading to the Q site. Does the region / residues overlap with previous prediction by Jussupow et al. Sci Adv (2019) 5(3):eaav1850, particularly tunnel 1?

No, the tunnel 1 predicted by Jussupow et al. is at the interface of the NDUFS2, NDUFS7 and ND3 subunits. None of the residues involved overlap with the proton channel proposed here, which leads through the interior of NDUFS2.

-Based on Fig. 3a, H95/Y144 do not seem to be in a conformation capable of binding Q. Please comment.

H95 is on the NDUFS2 loop that is known to be poorly ordered in the deactive state (as highlighted and referenced extensively throughout our manuscript), and this likely arises from its increased flexibility. Considering this, the modelled H95 is positioned in its most favourable orientation (based on protein geometry requirements and the cryo-EM map) but this may not be the optimal quinone-binding orientation. Local reordering of this dynamic region of the deactive state may be induced by the presence of quinone or by transition to the active state, to create the quinone-binding conformation. The deactive state is a non-turnover state. To make this observation clear in relation to the catalytic involvement of H95 we amended the legend to Fig. 3 to read:

"Glu34, conserved in the hydrogenases, is replaced by the His95 ubiquinone ligand in complex I. The flexible NDUFS2 loop in the non-turnover state depicted suggests the resulting His95 conformation is not optimal for coordinating ubiquinone."

-p. 5/Fig. 2 and Fig 4: five water molecules are shown in ND2 in Figure 2, whereas eleven waters are shown in Fig 4. It would be good to show all resolved water molecules also in Fig. 2 to avoid confusion.

Figure 2d focusses on the waters associated with the residues on the hydrophilic axis (as stated in the figure legend), aiming to display how connections are formed between these residues. (All the waters are shown in 2a and 2b). Adding all the other water molecules to 2d adds complexity to the figure, makes it hard to interpret, and detracts from the focus. On the other hand, Figure 4 is the basis for a detailed description of the water molecules present in just ND2, ND4 and ND5 - both along the hydrophilic axis *and* forming potential pathways of entry for waters and/or protons to these subunits, so all the water molecules are included.

-p.5/line 178: I do not see how dissociation of the Lys-Glu bridge is contradicting the findings of higher hydration.

Computational simulations on the hydration of the ND2/4/5 subunits proposed that the association of the TMH5-Glu and TMH7-Lys ion pair should correlate with the level of hydration from the matrix (ref. 31). However, in our models the Lys–Glu bridge is in the same dissociated state in all cases, despite that the hydration of subunit ND2 is substantially higher than for ND4 and ND5. Therefore, our findings are not consistent with the prediction. We have rewritten this section of our text (page 5) to improve the clarity:

"Notably, simulations on the membrane domain of *T. thermophilus* complex I suggested that association/dissociation of the 'ion pair' formed by the TMH5-Glu and the TMH7-Lys should correlate with hydration from the matrix and the protonation state of the central TMH8-Lys residue[31]. However, we observe ND2 to be more hydrated from the matrix than ND4/5, but the Lys–Glu salt bridges in all three subunits appear similarly dissociated."

-How well is the density of discussed carboxylate observed? Can the ion-pair conformations in ND2/4/5 or Glu side chains in ND1 be refined with certainty?

This is an interesting question; as the reviewer is obviously aware, negatively charged side chains are often poorly resolved in cryo-EM density maps. It has been suggested that the negative charge interferes with imaging (Hryc et al., *Proc. Natl. Acad. Sci. USA* **114**, 3103–3108 (2017)), and this has resulted in the idea that carboxylate sidechains with clear map features are likely protonated (neutral). Computational work to explore the possibility of interpreting the protonation states is ongoing in some groups, and we are excited about the future possibility of being able to interpret our maps in this way. In the meantime, although the carboxylate groups in our model show varying map definition, map features for at least the C-beta atoms are usually clearly visible to denote the initial orientation of the sidechain, and often extend out towards the carboxylate. This provides a good idea of the location (if not the rotation) of the carboxylate headgroup, that we support, particularly for the longer Glu residues, by structure refinement, which considers the protein/residue geometry and minimises steric clashes with the (well-resolved) surrounding residues. As is standard practice, we therefore present the most likely location for cases in which the complete side chain is not visualised – and in the few cases in which the density is too weak for us to be confident, the sidechains have been clipped from our model (see Extended Data Table 1). Specifically, for the glutamates of the ion pairs in ND2/4/5 and the E-channel residues in ND1 discussed above, we have sufficiently clear density to assign the side chain rotamers, but the oxygen atoms of the carboxylates are typically not defined.

-p. 5-6/line 188-190: Does the number of observed water molecules correlate with local resolution of the cryoEM maps in ND2, ND4, ND5?

Although the resolution decreases towards the peripheries of the domains, substantial loss in resolution does not occur in the membrane arm until the supernumerary subunits around the toe of ND5. The local resolutions for subunits ND2 and ND4 are very similar whereas ND5, as expected, is marginally worse (local resolution over the 10 TMH bundles: 2.69 Å (ND2); 2.73 Å (ND4); 2.88 Å (ND5)). However, the decrease is small, and filtering the map to consider the ND2 waters at 2.88 Å resolution did not decrease the number it is possible to visualise. Therefore, the higher number of water molecules around ND2 relative to ND5 is not the result of decreasing resolution.

Related to this point, a very recent paper (Djurabekov et al, 2020, just published) has explored the simulated hydration of just the ND5 subunit. They explore differences in ND5 hydration relative to ND4 and ND2,

and attribute them to the specific architecture of ND5. Although they predict greater internal hydration than we are able to observe, the water channels they predict as proton-transfer pathways agree with the locations of our water molecules at the surface of ND5, at ND5-TMH7b ("entry") and at the Asp397/CastP predicted cavity ("exit"). We have therefore updated our manuscript with the following statement at (page 6):

"Recently, simulations of the hydration of ND5[43] have, consistent with our observations, suggested that water molecules are positioned in the CastP-predicted cavity around Asp397, that water influx from the matrix side occurs at TMH7b, and that the observed internal hydration of ND5 differs from that predicted for ND4 and ND2."

-p. 7: "the hydrated cavity in subunit ND1"-section. I find that this section would better fit after the "Q site hydration"- section, and it could be merged with the "ND1 cavity"-section on p. 8. I also find that the Figure 7 is a bit technical for a main text figure.

We ordered the sections to start by describing the ordered water molecules (the focus of this paper) as the reader is guided along a logical catalytic reaction sequence – starting from ubiquinone reduction and ending with proton transfer in the ND subunits. We then return to consider the protein architecture and specific structural elements (such as the pi-bulges), and only in the later sections do we add comparisons to published models. We considered and tested several different constructions for the order and flow of our text, and found that the one adopted allowed the most clear and concise presentation.

Likewise, we spent considerable time and effort to optimise the presentation of Figure 7. It is very difficult to represent and compare three-dimensional empty spaces (i.e. Figure 7) and we have done our best to produce a simple, clear figure that conveys the core messages. Accompanying movies have been used throughout to assist the reader in appreciating the structural information we describe (and of course, the model will be released upon publication for individual reads to investigate and navigate for themselves). Figure 7 describes the formation and hydration of the ND1 cavity and how it is linked to the quinone-binding channel and A/D. It thus presents information that is central to our manuscript and is an important image to retain in the main text.

-Some lipids may have a functional role in complex I. Where are the 32 refined lipids observed?

Lipids are indeed known to be important for complex I activity, and we have shown (Figure 5a and Supplementary Figure 6) some of the most interesting ones we identified. However, we did not identify any new bound lipids in this work, and for the sake of maintaining the focus of the paper and controlling the length to be within journal limits, we did not include any extensive description or discussion of them.

-p.8 Cavity search: are the observed cavities sensitive to the employed probe radius?

The CASTp cavity-searching tool allows for the manipulation of the probe size (default 1.4 Å, allowed range of 0 to 10 Å). We used a 1.4 Å probe as the accepted probe size for evaluating water molecule accessibility/exclusion to protein pockets and cavities. The probe size is defined in the Methods. As expected, adjusting the size of the probe alters the extent and shape of the cavities detected (larger probe = smaller cavity).

-p. 10/lines 343-353: the list of observations is too technical for a general reader, and should be simplified.

We agree that this list is dense with information. However, the list simply puts a series of observations arising from information presented earlier into order and we expect that most readers will be able to appreciate this (especially with the assistance of Extended Data Table 4, which we included for this purpose).

- it has been proposed that the missing 42 kDa subunit in the Yarrowia complex I, could lead to its perturbed A/D transition (BBA 1859 (2018) 326–332). How does the findings compare to the resolved structure?

As we discussed in comparisons of A/D transitions in mouse and Yarrowia, the mammalian A/D transition does involve transitions of the interface between the 42 kDa subunit (absent from Yarrowia) and the B13 subunit. The reference highlighted by the reviewer is a good addition to the discussion and has now been included (page 10) as follows:

"The relaxation occurs at different rates and to different depths in different species (Figure 7). It occurs more slowly and to greater depth in the mammalian enzyme than in *Y. lipolytica*, perhaps due to the extra NDUFA10–NDUFA5 interface that must adjust in the mammalian enzyme[20]. NDUFA10 is absent from *Y. lipolytica*, and the NDUFA10–NDUFA5 interaction was identified in simulations to have a major impact on the global enzyme dynamics[50]."

-The authors should clarify if they suggest that their resolved deactive-like state is an off-pathway form of complex I, or a part of its native reaction cycle?

This is an area of on-going debate in the community. In our opinion, our structure is in a deactive-like, off-pathway state because we believe that the loss of key ordered structural elements and substantial increase in hydration we observe is unlikely during the turnover of such an efficient and highly coupled enzyme. We have added the following statement to our manuscript (page 11):

"We propose our deactive-like structure represents an off-pathway state because the disordering of key structural elements and substantial hydration we observe is unlikely during turnover of such an efficient and highly coupled enzyme."

-Minor comment: in the introduction, some key information is given in parenthesis. (e.g. in all that have it; 31 in mammals; for all but seven …; four protons are pumped for each NADH oxidised). Please write the sentence out if the information is relevant.

Thank you for this suggestion – we have made the requested changes accordingly.

Decision Letter, first revision:

11th Jun 2020

Dear Judy, Daniel,

Thank you again for submitting your manuscript "Mitochondrial complex I structure reveals ordered water molecules for catalysis and proton translocation". We consulted one of the original reviewers,

who was satisfied with the revision and response. We are thus happy to accept your paper, in principle, for publication as an Article in Nature Structural & Molecular Biology, on the condition that you revise your manuscript in response to our editorial requirements.

The text and figures require revisions. Note that, within a few days, we will send you detailed instructions for the final revision, along with information on editorial and formatting requirements. We recommend that you do not start revising the manuscript until you receive this additional information, likely early next week.

Data availability: this journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found below:
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We hope that you will support this initiative and supply the required information. Should you have any query or comments, please do not hesitate to contact me.

If you have any questions, please do not hesitate to contact me directly.

Sincerely,
Ines

Ines Chen, Ph.D.
Chief Editor
Nature Structural & Molecular Biology

ORCID 0000-0002-1405-9703

Reviewer #1 (Remarks to the Author):

In the revised version of this paper, the authors addressed all the comments of this reviewer.

17th Jun 2020

Dear Judy,

Thank you for your patience as we’ve prepared the guidelines for final submission of your Nature Structural & Molecular Biology manuscript, "Mitochondrial complex I structure reveals ordered water molecules for catalysis and proton translocation" (NSMB-A43134A).

Please follow the instructions provided here and in the attached files (sent separately), as the formal acceptance of your manuscript will be delayed if these issues are not addressed.

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2. DATA DEPOSITION: We require deposition of coordinates (and structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). EM maps must be deposited in the EMDB. Accession codes must be provided in your final submission for acceptance, and entries must be accessible or HPUB at the galley proof stage.

3. Nature Research is taking an active approach to improving our transparency standards and increasing the reproducibility of all of our published results. Detailed information on experimental design and reagents is now collected on our Life Sciences Reporting Summary, which will be published alongside your paper. Please provide an updated version of the Reporting Summary (which will be published with the paper) with your final files.
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GENERAL FORMATTING:

4. The main text, title and abstract are all fine.

5. The Online methods section is currently 1442 words; that's fine as well.

6. References: the current manuscript has 52 references in main text and 14 in methods. That's fine too. It is not possible to accommodate a reference list for Extended Data Figures; it seems several of those papers were already cited in main text or methods, in which case you may simply use the same number; for "new" citations, please provide the reference in line. If the same procedure is not feasible for Supplementary Tables, we can have a separate table at the end of that PDF.

7. References: the reference list should contain papers that have been published or accepted by a named publication or recognized preprint server. Published conference abstracts, numbered patents and research datasets that have been assigned a digital object identifier may also be included in the reference list.

8. Our style for PDB codes is "PDB XXXX" (no hyphens or ID).

FIGURES AND TABLES:
9. There are currently 7 Figures and 1 Tables in main article. Some figures are on the small side, and you may want to consider merging those cited close together (e.g., Figs 6-7), which would simplify layout.

10. Please make sure all figures and tables, including Extended Data Figures, are cited in the text in numerical order.

11. SUPPLEMENTARY INFORMATION: you currently have 7 Extended Data Figures, 4 Supplementary Videos (not Extended Data Movies) and 4 Supplementary Tables (not Extended Data Tables); sorry if our previous instructions were confusing.

All Supplementary Information must be submitted in accordance with the instructions in the attached Inventory of Supporting Information, and should fit into one of three categories:

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All Extended Data Figure must be called out in order as Extended Data Fig. 1, Extended Data Fig 2, etc.

2. SUPPLEMENTARY INFORMATION: these would be
- Supplementary Tables 1–4 (in single PDF document; in this case, if unavoidable, a reference list at the end of the PDF is feasible), listed in section 2A of inventory.
- Supplementary Videos 1–4, listed in section 2B of inventory.

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Supplementary items (such as Supplementary Tables, Videos, Notes, and additional Supplementary Figures if permitted), should be numbered and called out in main article, as Supplementary Figure 1 (not SI1) and so on.

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Best regards,
Ines

Ines Chen, Ph.D.
Chief Editor
Nature Structural & Molecular Biology

ORCID 0000-0002-1405-9703

Reviewer #1:
Remarks to the Author:
In the revised version of this paper, the authors addressed all the comments of this reviewer.

Author Rebuttal, first revision:

Dear Ines,

We are pleased to see that the reviewers were happy with our responses to their questions.

We have made the formatting adjustments and uploaded all required and relevant files for the next stage. For the uploaded figures, we have included versions ten-times the size of the minimum column value to aid in any adjustments. All figures are intended to be single-column width except Figure 2. We have decided to keep all figures separate and not merge any. The extended data figures are all sized so they should accommodate a single page with their respective figure legends when initially opened. If you find any problems with the files or if there is something missing, please let us know and we can amend it straight away.

As indicated in the uploaded form also, we wish to participate in transparent peer review, and are happy for the reviewers' comments and our rebuttals to be included in the process.

I (Dan) could not find a way to link my ORCID (0000-0003-2915-951X) within the manuscript tracking system, but I have messaged the helpdesk suggested.

We look forward to hearing from you further,
Best wishes,
Daniel Grba and Judy Hirst

Final Decision Letter:

Dear Judy and Daniel,

We are now happy to accept your revised paper "Mitochondrial complex I structure reveals ordered water molecules for catalysis and proton translocation" for publication as a Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Before the manuscript is sent to the printers, we shall make any detailed changes in the text that may be necessary either to make it conform with house style or to make it intelligible to a wider readership. If the changes are extensive, we will ask for your approval before the manuscript is laid out for production. Once your manuscript is typeset you will receive a link to your electronic proof via email within 20 working days, with a request to make any corrections within 48 hours. Please read proofs with great care to make sure that the sense has not been altered. If you have queries at any point during the production process then please contact the production team at rjsproduction@springernature.com. Once your paper has been scheduled for online publication, the Nature press office will be in touch to confirm the details.

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Ines

Ines Chen, Ph.D.
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ORCID 0000-0002-1405-9703