nature portfolio

Peer Review File

The redox driven Na^+-pumping mechanism in *Vibrio cholerae* NADH-quinone oxidoreductase relies on dynamic conformational changes

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Na+ translocating NADH:ubiquinone oxidoreductase, Na+-NQR, is an important bioenergetic enzyme coupling NADH oxidation and quinone reduction with the translocation of Na+ ions across the membrane. The enzyme requires six structural subunits and six cofactors for this reaction. As this enzyme only occurs in bacteria, including some pathogens, it is a promising target for antibiotics. Although some structures of the enzyme have recently been elucidated, its mechanism is not yet understood. However, this is urgently needed in order to be able to use NQR as a potential target for future inhibitors. It is therefore an exciting and very topical subject that Ishikawa-Fukuda et al. are addressing here. Therefore, the scope of the work suits the journal 'Nature Communications' very well.

The authors describe novel cryo-EM structures of NQR in several states: NADH-reduced, NADH-reduced with an addition of two different inhibitors, NADH-reduced and in the absence of Na, the translocated substrate, as well as the oxidized and NADH-reduced preparation of two variants that lack distinct cofactors. The structures obtained from these studies are used by the authors as a basis for molecular dynamics (MD) calculations, leading to a reasonable reaction mechanism.

The manuscript is mainly well written and one can follow the train of thought. The text is concise and many data are provided. The quality of the structural data is excellent, therefore all the observed conformational changes described here can be considered as very reliable to justify the conclusions from this work. However, the data should be organized more easily as it is sometimes hard to follow through all the extended and supplemental data arranged in one large figure. In general, the data are interpreted in an appropriate manner in the light of the current literature. The manuscript contributes new and significant information about NQR, but there are a few points to be considered:

Extended data Fig. 1 should be divided in seven parts to make a more precise reference to structural data in the main text. The same holds true for Supplementary Fig.1.

Table 1: The assignment of the mutations to the strains is incorrect. PBD IDs 9UD3 and 9UD4 should be from variant NqrB-T236Y and not from NqrC-T225Y.

The B-factors of protein and ligand must be provided in Suppl. Tab. 1

Fig. 1: The meaning of the red lines around NqrF in Fig. 1A should be explained in the legend.

P. 3, l. 46/47: To me, it sounds a bit sophistical, but also misleading, to write in the introduction that the NQR only has one 2Fe2S cluster that is spectroscopically defined. It would be more understandable to write that there are two of these clusters, but only one of them has been described by EPR spectroscopy.

P. 7, l. 170: The FNR-like and ferredoxin-like domains are indeed structurally similar in the WT(ox) and the NqrF(up) state, but here it would be important to note that the distance between the cofactors, FAD(NqrF) and 2Fe2S(NqrF), has increased significantly as the cluster has rotated.

Fig. 3; p. 8, l. 208: The movement of NqrC is an important part of the manuscript. However, it is not clear to me, what is shown in Fig. 3b. It should be clearly described what the structural data is and how the rigid body fitting was placed on it. Furthermore, the meaning of the grey arrows and other signs in Fig. 3b are not explained.

P. 8, l. 211: Here, it is stated that there is a 27 A distance between the 2Fe2S cluster(NqrD/E) and FMN(NqrC). This raises questions about the mechanism of the enzyme, which is discussed later (p. 10, last paragraph and discussion: p. 12, l. 331). How can this distance be approximately halved to achieve a reasonably fast electron transfer from NADH to ubiquinone? This is also important for the mechanism, as Na is to be released into the periplasm in this electron transfer step (see Fig. 5).

P. 9, l. 243: How does the proposal that the redox reaction of 2Fe2S(NqrC) drives the inward/outward transition of the putative Na-pathway fits with the observation that in the Delta(FMN(NqrC)) sample always takes the 'outward open' form, regardless of whether the cluster is oxidized or reduced?

P. 9, l. 250: It is stated that electron transfer between the two 2Fe2S clusters is possible at a reasonable rate in the 'down' and 'middle' states of NqrF. This is true for the 'down' state, but electron transfer is not possible in the 'middle' state with a 26.3 A distance between the clusters.

In this and in some of the following paragraphs (e.g. p. 10, l. 263, ff; p. 14, l. 374) there seems to be a misunderstanding between cause and effect. NQR is a redox-driven Na+ pump. The flexibility of individual regions of the protein increases the probability of physiological electron transfer. This in turn induces the 'inward-outward' transition in NqrD/E. The flexibility of a protein alone cannot achieve this transition because flexibility is non-directional.

P. 11, l. 299: I am a little surprised that at least two waters should still be bound to the translocated Na during passage through the protein according to the MD simulations. Here it is very important to show the orientation of these waters and their position in relation to the calculated 'water channels' (Fig. 4a and b). If these waters form a continuous connection between periplasm and cytoplasm, a proton gradient would immediately collapse. This is the reason why ion channels bind the completely dehydrated ion at least at one point. As far as I know, the ATP synthase of Vibrio cholerae is a proton-driven enzyme that requires a proton gradient. If there is a proton gradient in Vibrio and if water bound to Na should form a channel across the membrane: Is there any evidence that the Na+ and H+ -gradients are spatially separated in Vibrio?

Fig. 5: I like the model proposed in Fig. 5 very much, but I generally see the problem that electron transfer between 2Fe2S cluster(NqrD/E) and FMN(NqrC) is not clarified in the NQF (and also not in the related RNF). One proposal to make this scheme clearer would be to distinguish between states and steps. The authors should describe 6 states that are converted into each other in 6 steps. The scheme should start with the protein in the oxidized state and the first step is to reduce it by NADH. This leads to the second state in which two electrons are donated to FAD(NqrF) leading to the reduction of 2Fe2S cluster(NqrF) by a single electron and the uptake of Na, and so on and so forth.

In my opinion, Suppl. Fig. 10 is too detailed and confusing. This should rather be placed in a more specialized journal.

Some minor points
P. 3, l. 64: Literature 25 is not relevant here.

P. 10, l. 273: There is no such thing as 'high homology'. Both enzymes are homologous to each other.

P. 13, l. 343: Please provide a literature for the UQ headgroup positioned approximately 20 A above the membrane surface.

P. 13, l.368: delete 'through'

P. 15, l. 391 and 392: Please provide citations

P. 15, l. 402: do not use square brackets

P. 15, l. 412: replace 'reduced' by 'reduce'

P. 16, l. 453: replace 'Molplobility' by 'MolProbity'

P. 17, l. 462: replace 'GalxyFill' by 'GalaxyFill' (this is spelled wrong on the homepage)

Legend to Fig. 4, l. 599: '.. are shown together with the position of the membrane surface.'

Same, l. 603: replace 'released' by 'release'

Extended Data Fig. 5: Why are three amino acid residues highlighted in yellow (and not mentioned in the legend)?

Reviewer #2

(Remarks to the Author)
The manuscript presents extensive cryo-EM study of Na-translocating NADH-quinone oxidoreductase (Na+-NQR) – a large

membrane protein complex with enigmatic mechanism. How the electron transfer between many cofactors of the complex is coupled to the translocation of sodium ions has been a major question since the discovery of the first structure of this bacterial enzyme. The current study resolves several conformations of NqrF and NqrC subunits, allowing temporary bridging of FeS and FMN cofactors, which are otherwise too far away from each other. Different redox conditions (presence of NADH) and mutants were studied, revealing that FeS cluster reduction in NqrDE subunits is linked to inward-to-outward-open transition of a putative Na+-translocating pathway. This is in contrast to previous ideas on the location of this pathway in NqrB subunit. The NQR mechanism proposed in Fig. 5 elegantly explains structural observations and appears to be quite feasible. This study is a major milestone in NQR studies and will be of interest to a wide audience of Nature Communications.

Some questions to be addressed in revision:
Overall strategy – it is not clear why the authors did not try to capture different conformational states during catalytic turnover, i.e. by freezing enzyme while showing activity in the presence of both NADH and Q. A similar approach, for example, revealed many key conformational states of Ca-ATPase from a single dataset, in addition to those previously revealed one-by-one in the presence of inhibitors, etc. For this approach to work it does not matter whether the enzymatic reaction is fast or slow, as the full distribution of different states populated during the catalytic turnover may be captured, and the observation of a particular state depends only on how populated it is overall (% of particles) during turnover. Additionally, this may reveal the location of Q binding site, currently disputed.

Fig. S3 – NADH density is badly visible in shown orientation, would be better to orient it lengthwise.

L. 165 “By focusing on the position of the ferredoxin-like domain, three distinct states of NqrF- up, middle, and down- were identified as separate classes …” There is no description of what exactly was done here – 3D classification (no align) with mask on this domain? – if so, it should be described in the main text and methods. If this was not done, it should have been, and it should be discussed why not. Overall, data processing methods description is currently very minimal and not sufficient to fully follow the workflow. Furthermore, the density for ferredoxin-like domain looks very fuzzy and it is not clear how well the density justifies the modelling of different states – to judge this, the detailed figures of density fit to the model for each discussed position of different domains should be added.

Fig. S2 – latent space distributions look quite homogenous without any features, suggesting absence of clearly defined different conformational states. There should be a discussion on how exactly the authors arrived at their proposed distribution of classes.

L. 198 “followed by focused refinement on the NqrC density.” – Again, not clear what was done here – there is no focused refinement step in ED Fig. 1e.

L. 204 “In the “stable” state (Fig. 3b), resolved at 2.93 Å,” – the resolutions of the two states seem to be mixed in the text.

L. 215 “that allows it to mediate electron transfer from 2Fe-2SNqrD/E to FMNNqrB” – How is that possible if the distance in shifted state is apparently still 27 A?

L. 236 – from this paragraph it is not clear if in all determined structures “inward-opened” state is always linked to “stable” state and “outward-opened” to “shifted” or not? This should be clarified.

Reviewer #3

(Remarks to the Author)
This paper describes an intensive investigation of redox-coupled Na+ ion translocation by NADH-quinone oxidoreductase (Na-NQR) using a combination of cryo-EM structural characterization of different states of the complex combined with molecular dynamics (MD) simulations designed to map those states onto proposed mechanism of translocation. The ingenious use of a combination of inhibited and mutated NQR states that slow or stop the reaction cycle at different points in the cycle combined with previous work by these authors and others in the field allow the authors to propose a potential mechanism for the Na+ translocation from cytosol to the periplasm.

Given that this reviewer is an expert in neither cryoEM nor MD, and assuming that other reviewers will be recruited to judge those technical aspects, I will settle for raising questions that an educated and interested reader might have.

1) Given that the Na-NQR complex is transmembrane, but no membrane components are present after purification, and detergent (DDM) concentrations are reduced after activity assays but prior to grid preparation, how comfortable are the authors that the cryo-EM movies accurately represent conformational changes that take place in the presence of the membrane? It is my experience that membrane-bound or membrane-associated proteins often take structural cues from their interaction with the membrane, and one notes that the MD simulations are performed in a POPC bilayer. Perhaps this is a question that frequently arises when looking at integral membrane structures by cryo-EM, but it probably would be a good thing to justify in more detail.

2) The "down" state of the NqrF, in which the FeS cluster of the ferredoxin domain is sufficiently close to that of the NqrD/E

domain for physiological electron transfer, is represented by only 5% of the particles observed (hence the lower resolution). Again, for those not expert in cryo-EM, this seems a small fraction but has large implications for the conclusions of the paper. How is this justified?

3) In Step 2 of the proposed mechanism (NADH binding), the authors claim that reduction of the FeS cluster in the ferredoxin domain of NqrF increases the flexibility of that domain, allowing it to approach the NqrD/E domain. In this reviewer's experience, reduction a 2Fe-2S cluster in a ferredoxin has the opposite effect, causing compaction of the protein near the cluster, slowing protein dynamics measured on a number of time scales, including H/D exchange and NMR relaxation times, with less motional freedom of surface features. This does not negate the proposed role of the domain, but in fact may make ferredoxin binding in the D/E pocket entropically more favorable. Analogous binding and electron transfer between ferredoxins and cytochromes show that the reduced ferredoxins bind much more tightly to their redox partners than the irrelevant oxidized forms. Once electron transfer has occurred, increased dynamics in the ferredoxin domain encourages dissociation (also preventing back e- transfer, since the rate of electron transfer between two centers with similar redox potentials is by Marcus theory expected to be quite fast). Rapid dissociation would also free up the pocket for Na ion uptake as proposed in step 3.

4) The possibility that a proline c/t isomerization is involved in in the D/E switch is intriguing and might be expanded upon. Is a particular proline indicated? If so, what happens if that proline is mutated to a Gly or Asn? We have ourselves found evidence that proline switches are much more common in enzymatic mechanisms and other conformational changes than is often appreciated.

Minor points: I assume NaPi is sodium phosphate buffer, but it should be listed in the abbreviations.

This reviewer is quite used to looking at protein structures, but cannot tell what the differences are between the up down and middle states of NqrF in Figure 1, part b. Perhaps using a rainbow coloring of the sequence would make those differences clearer.

Line 366, Prolines should not be capitalized.

Line 391, remove (REF)

Reviewer #4

(Remarks to the Author)
In the study "The Na+-pumping mechanism driven by redox reactions in the NADH- quinone oxidoreductase from Vibrio cholerae relies on dynamic conformational changes", Ishikawa-Fukuda et al. explores the molecular principles of redox-driven Na+ pumping in the NQR complex from Vibrio cholerae by combining cryo-EM experiments with MD simulations. The study provides an important contribution for the field, with mechanistic implications also for related redox-driven ion pumps. I find that the work deserves publication after some shortcomings have been clarified. I will limit here my comments (in no specific order) mainly to the computational aspects of the study:

1. It seems that all MD results are based on a single simulation replica. If this the case, the authors should perform at least a second MD replica for some of the key states to make sure that findings are statistically relevant.

2. The authors should report protonation states of titratable residues.

3. How were the redox states defined in the simulations? (see also comment 16)

4. The authors report an interesting connection between the redox state of the FeS center and the sodium binding. The authors should clarify if there are additional conformational / dynamic changes of the domains linked to the redox changes. If so, do these change the distance between redox cofactors, which could be important for the gating mechanism? Do the authors suggest that reduction of the 2Fe-2S cluster drives the inward/outward conformational change?

5. Many flavines are covalently bound to proteins. I cannot find a description of how this interaction was modelled. Please clarify.

6. How was the collective variable defined for the TMD simulations?

7. How was the gate size (Fig. 4c, Fig. S9) defined and calculated?

8. Table S3: how was a Na+ binding event defined?

9. The TMD simulations should be clarified in Table S2. Moreover, how does the resulting end state compare to unbiased MD simulations of the outward open state?

10. cryoEM: How well could the redox cofactors be refined based on the cryoEM maps? Show example cryoEM maps and models in the SI. Are functional water molecules properly resolved? What about Na+ ions?

11. Fig. 2 is extremely dense and I have severe difficulties in seeing the main differences between the resolved structures. As this figure forms the basis for the proposal, the authors should improve the figure, highlighting also the detailed structure of the proposed Na+ binding site.

12. Fig. 4d show a dissociation of a Na+ ion. In which redox state does this take place? Can it be linked to the oxidation of the FeS cluster? Are there other conformational changes? (see comment 4)

13. "In the shifted state (Fig. 3b), while FMN^NqrC remains distant from 2Fe-2S^NqrD/E (~27°), the loop of Glu169-Gly177^NqrC, which includes Thr173 and Leu176 that stabilize the isoalloxazine ring of this FMN9, is embedded in the pocket formed by the NqrD/E subunits"

I cannot see the loop in the figure. Please clarify.

14. "The existence of these two distinct conformations shows that the periplasmic domain of NqrC, which houses FMN^NqrC, switches its position between NqrB and NqrD/E in a way that allows it to mediate electron transfer from 2Fe-2S^NqrD/E to FMNNqrB (Fig. 1a, 3)."

Report FMN^NqrC <-> 2Fe-2S^NqrD/E distances. I cannot find these in figures or text. Is this distance compatible with electron transfer?

15. "A notable example of this is the rearrangement of NqrF during electron transfer from FAD to 2Fe-2SNqrD/E (Fig. 1b,c). In the “down” and “middle” states, where 2Fe-2SNqrF is positioned close enough to 2Fe-251 2SNqrD/E to allow facile electron transfer, the ferredoxin-like domain of NqrF shows lower resolution, while the FNR-like domain containing FAD remains highly resolved (Supplementary Fig. 2a).

As far as I can see, in the middle state, the 2Fe-2S^NqrF <-> 2Fe-2S^NqrD/E distance to 26 Å. Electron transfer can thus only take place in the "down" state. Please clarify.

16. The mechanism requires clarification: how are the two electrons from the NADH oxidation are distributed during the pumping cycle? How is this considered in simulations? Also importantly: what is the proposed Na+ pumping stoichiometry based on the mechanistic model?

17. Previous studies on NQR have emphasized that NqrB is responsible for the Na+ pumping activity based on structural arguments, mutagenesis studies, but also based on the relation of the subunit to other transporters. It would be very useful if the authors further clarified what could be the reason(s) for the drastically different results and conclusions from previous NQR studies. It seems that the current study also supports the proposed model of the related RNF protein (of Ref. 27), suggesting that RNF and NQR employ the same mechanism. Please also clarify if the authors suggest that the mechanism is indeed the same as proposed for RNF or different in some way?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have taken all of the reviewers' objections into account and amended the revised version of the manuscript accordingly. Although the current version still contains minor typographical errors (e.g. line 289 “A. woodie” instead of “A. woodii”), it should be accepted in its current form.

Reviewer #2

(Remarks to the Author)
The manuscript has been carefully revised and can be published.

Reviewer #3

(Remarks to the Author)
The authors have answered my admittedly somewhat general questions quite adequately, thank you. Figures are much clearer, as well. I apologize for the lateness of this review. This paper is now ready for publication without further revision.

Reviewer #4

(Remarks to the Author)
All my comments have been adequately addressed and the authors made several important clarifications. I thus support publication of this work. The study will make an important contribution to the field. Congratulations!

Reply to Reviewer #1

Na^+ translocating NADH:ubiquinone oxidoreductase, Na^+-NQR, is an important bioenergetic enzyme coupling NADH oxidation and quinone reduction with the translocation of Na^+ ions across the membrane. The enzyme requires six structural subunits and six cofactors for this reaction. As this enzyme only occurs in bacteria, including some pathogens, it is a promising target for antibiotics. Although some structures of the enzyme have recently been elucidated, its mechanism is not yet understood. However, this is urgently needed in order to be able to use NQR as a potential target for future inhibitors. It is therefore an exciting and very topical subject that Ishikawa-Fukuda et al. are addressing here. Therefore, the scope of the work suits the journal 'Nature Communications' very well.

The authors describe novel cryo-EM structures of NQR in several states: NADH-reduced, NADH-reduced with an addition of two different inhibitors, NADH-reduced and in the absence of Na, the translocated substrate, as well as the oxidized and NADH-reduced preparation of two variants that lack distinct cofactors. The structures obtained from these studies are used by the authors as a basis for molecular dynamics (MD) calculations, leading to a reasonable reaction mechanism.

The manuscript is mainly well written, and one can follow the train of thought. The text is concise, and many data are provided. The quality of the structural data is excellent, therefore all the observed conformational changes described here can be considered as very reliable to justify the conclusions from this work. However, the data should be organized more easily as it is sometimes hard to follow through all the extended and supplemental data arranged in one large figure. In general, the data are interpreted in an appropriate manner in the light of the current literature. The manuscript contributes new and significant information about NQR, but there are a few points to be considered:

We thank the reviewer for the positive remarks and the valuable comments that have helped us to improve our manuscript.

1. *Extended data Fig. 1 should be divided in seven parts to make a more precise reference to structural data in the main text. The same holds true for Supplementary Fig.1.*
   We split Extend Data Fig. 1 and Supplementary Fig.1 into separate structural datasets. They are presented as Supplementary Fig. 1-9.

2. *Table 1: The assignment of the mutations to the strains is incorrect. PBD IDs 9UD3 and 9UD4 should be from variant NqrB-T236Y and not from NqrC-T225Y.*
   This is our careless mistake. We corrected mutant numbers.

3. *The B-factors of protein and ligand must be provided in Suppl. Tab. 1*
   We added B-factors of protein and ligand in Supplementary Table 1.

4. *Fig. 1: The meaning of the red lines around NqrF in Fig. 1A should be explained in the legend.*
   This symbol represents the flexibility of the corresponding region. We have modified Figure 1 and added the following explanation to the legend of Figure 1, as well as that of Figure 5: *Concentric partial circles in red* around NqrF indicate flexibility of this region.

5. *P. 3, l. 46/47: To me, it sounds a bit sophistical, but also misleading, to write in the introduction that the NQR only has one 2Fe2S cluster that is spectroscopically defined. It would be more understandable to write that there are two of these clusters, but only one of them has been described by EPR spectroscopy.*
   Thank you for your careful reading and suggestions. We amended the sentence as you suggested (p. 3)..

6. *P. 7, l. 170: The FNR-like and ferredoxin-like domains are indeed structurally similar in the WT(ox) and the NqrF(up) state, but here it would be important to note that the distance between the cofactors, FAD(NqrF) and 2Fe2S(NqrF), has increased significantly as the cluster has rotated.*
   As the reviewer pointed out, the distance between FAD^{NqrF} and 2Fe-$2S^{NqrF}$ has increased to 15.7Å in the "***up***" state as the ferredoxin-like domain has rotated, compared to the enzyme *as isolated* (**WT_{ox}**). We amended the sentence accordingly (p. 7).

7. *Fig. 3; p. 8, l. 208: The movement of NqrC is an important part of the manuscript. However, it is not clear to me, what is shown in Fig. 3b. It should be clearly described what the structural data is and how the*

rigid body fitting was placed on it. Furthermore, the meaning of the grey arrows and other signs in Fig. 3b are not explained.

We included the text to clarify how rigid-body fitting was performed to model the "***shifted***" state of NqrC in the Methods section (p. 18). In addition, Figure 3b has been updated to more clearly illustrate the rearrangement of key structural elements in NqrC, including the TMH, hydrophilic domain, and FMN. The figure legend has also been amended to explain the meaning of each symbol.

8. *P. 8, l. 211: Here, it is stated that there is a 27 A distance between the 2Fe2S cluster(NqrD/E) and FMN(NqrC). This raises questions about the mechanism of the enzyme, which is discussed later (p. 10, last paragraph and discussion: p. 12, l. 331). How can this distance be approximately halved to achieve a reasonably fast electron transfer from NADH to ubiquinone? This is also important for the mechanism, as Na is to be released into the periplasm in this electron transfer step (see Fig. 5).*

As pointed out, the distance between 2Fe-2S$^{NqrD/E}$ and FMN^{NqrC} in the "***shifted***" state (~27 Å) would indeed need to be reduced by approximately half to enable physiological electron transfer. This implies the existence of an additional conformational intermediate in which the hydrophilic domain of NqrC approaches NqrD/E more closely. Such a state may correspond to the structure of the FMN^{NqrC}-lacking mutant enzyme. We mentioned this point in the revised manuscript (p. 9).

As already described in the MD section (p. 11), the redox-dependent *inward-to-outward* transition of the TMH^{NqrCDEF} bundle was not observed in our MD simulations. The "***stable***" state appears to be energetically much more favorable than the "***shifted***" state. The stability of the "***stable***" (*inward-open*) state and the relative instability of the "***shifted***" (*outward-open*) state might be essential for the Na^+ translocation against the sodium motive force, that is, to prevent Na^+ from moving back into cytoplasm.

9. *P. 9, l. 243: How does the proposal that the redox reaction of 2Fe2S(NqrC) drives the inward/outward transition of the putative Na-pathway fits with the observation that in the Delta(FMN(NqrC)) sample always takes the 'outward open' form, regardless of whether the cluster is oxidized or reduced?*

As the reviewer noted, the electron transfer pathway in FMN-lacking mutants differs from that in the wild type, so these structures alone cannot establish that the redox state of 2Fe-2S$^{NqrD/E}$ drives the conformational change. Instead, they show that the position of the NqrC hydrophilic domain correlates with the *inward/outward* transition. The *outward-open* conformation consistently observed in the ΔFMN^{NqrC} enzyme may be stabilized by the absence of FMN^{NqrC}.

The conclusion can be drawn from the structures of wild-type enzyme. In the wild type, when 2Fe-2S^{NqrE} is reduced and its ferredoxin-like domain approaches NqrD/E, the enzyme remains in the inward-open state. By process of elimination, this suggests that the reduction of 2Fe-2S$^{NqrD/E}$ drives the inward-to-outward transition. We have clarified this logical flow in the revised manuscript (p. 9-10).

10. *P. 9, l. 250: It is stated that electron transfer between the two 2Fe2S clusters is possible at a reasonable rate in the 'down' and 'middle' states of NqrF. This is true for the 'down' state, but electron transfer is not possible in the 'middle' state with a 26.3 A distance between the clusters.*
In this and in some of the following paragraphs (e.g. p. 10, l. 263, ff; p. 14, l. 374) there seems to be a misunderstanding between cause and effect. NQR is a redox-driven Na^+ pump. The flexibility of individual regions of the protein increases the probability of physiological electron transfer. This in turn induces the 'inward-outward' transition in NqrD/E. The flexibility of a protein alone cannot achieve this transition because flexibility is non-directional.

For the former point, we have deleted the word "middle" state from the corresponding sentence.

For the latter point, we appreciate the reviewer's careful reading and agree with this important point. In the revised manuscript (p. 10 and 15), we have rephrased the sentence to state that "the flexibility increases the probability of physiological electron transfer" instead of "the flexibility enables physiological electron transfer."

11. *P. 11, l. 299: I am a little surprised that at least two waters should still be bound to the translocated Na during passage through the protein according to the MD simulations. Here it is very important to show the orientation of these waters and their position in relation to the calculated 'water channels' (Fig. 4a and b). If these waters form a continuous connection between periplasm and cytoplasm, a proton gradient*

would immediately collapse. This is the reason why ion channels bind the completely dehydrated ion at least at one point. As far as I know, the ATP synthase of Vibrio cholerae is a proton-driven enzyme that requires a proton gradient. If there is a proton gradient in Vibrio and if water bound to Na should form a channel across the membrane: Is there any evidence that the Na+ and H+ -gradients are spatially separated in Vibrio?

Thank you for this insightful comment. As mentioned, a continuous water connection between the two sides of the membrane would allow proton translocation via the Grotthuss mechanism. We checked the MD trajectory and confirmed that such a water connection was not observed. Instead, we observed a spatial gap in water in both the cytoplasmic-open and the periplasmic-open state, as shown in the Figure below. Thus, proton translocation (or leak) across the membrane is unlikely. We have added this point in the revised manuscript (p. 12), and the figure below has also been included as Supplementary Fig. 23.

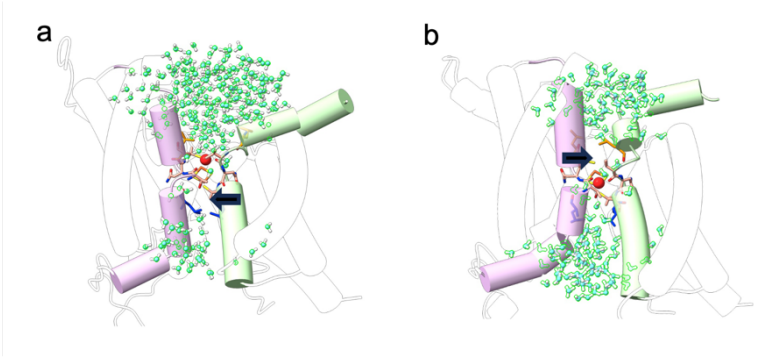

Figure. *Water orientation in the cytoplasmic-open (a) and in the periplasmic-open state (b). The oxygen and hydrogen atoms of water are shown in cyan and white, respectively. The arrow points to a spatial gap in water.*

The function of NQR should not interfere with the proton gradient, as in most cells, proton and sodium gradients coexist on the same lipid bilayer membrane and are independent, except that they share the same electrical potential.

12. *Fig. 5: I like the model proposed in Fig. 5 very much, but I generally see the problem that electron transfer between 2Fe2S cluster (NqrD/E) and FMN(NqrC) is not clarified in the NQR (and also not in the related RNF). One proposal to make this scheme clearer would be to distinguish between states and steps. The authors should describe 6 states that are converted into each other in 6 steps. The scheme should start with the protein in the oxidized state and the first step is to reduce it by NADH. This leads to the second state in which two electrons are donated to FAD(NqrF) leading to the reduction of 2Fe2S cluster(NqrF) by a single electron and the uptake of Na, and so on and so forth.*

In response to the reviewer's suggestion, we have distinguished between "states" and "steps" to clarify the scheme described in Figure 5 (p. 13-14). We have also slightly modified Figure 5 and its legend to clearly indicate which models represent states and which symbols represent steps. We greatly appreciate the reviewer's constructive proposal.

13. *In my opinion, Suppl. Fig. 10 is too detailed and confusing. This should rather be placed in a more specialized journal.*

We have removed Supplementary Fig. 10b-10e from the revised manuscript, as we agree that the level of detail may be excessive for the present report. In addition, we have improved Fig. S10a (Supplementary Fig. 24 in the revised manuscript) to illustrate how the two electrons donated from NADH are distributed during the catalytic cycle and presented this as an independent figure to complement Fig. 5. This model is consistent with our simulations, in which the one-electron reduction of 2Fe-2S$^{NqrD/E}$ is coupled to the translocation of one Na^+ ion.

14. *Some minor points*
    a. *P. 3, l. 64: Literature 25 is not relevant here.*

We have removed the Literature 25 as a reference for this sentence.

b. P. 10, l. 273: There is no such thing as 'high homology'. Both enzymes are homologous to each other.

We have corrected the sentence accordingly (p. 11). We thank the reviewer for the careful reading.

c. P. 13, l. 343: Please provide a literature for the UQ headgroup positioned approximately 20 A above the membrane surface.
We have added Ito et al. (2017) as a reference for this sentence.

d. P. 13, l.368: delete 'through'
This is our mistake. We corrected the sentence accordingly.

e. P. 15, l. 391 and 392: Please provide citations
We have added two references, Kishikawa et al. (2022) and Barquera et al. (2004), to this sentence.

f. P. 15, l. 402: do not use square brackets
We corrected the sentence accordingly.

g. P. 15, l. 412: replace 'reduced' by 'reduce'
We have corrected the sentence accordingly. We thank the reviewer for careful reading.

h. P. 16, l. 453: replace 'Molplobility' by 'MolProbity'
We have corrected the sentence accordingly.

i.P. 17, l. 462: replace 'GalxyFill' by 'GalaxyFill' (this is spelled wrong on the homepage)
We have corrected the sentence accordingly.

j. Legend to Fig. 4, l. 599: '.. are shown together with the position of the membrane surface.'
We have corrected the sentence accordingly.

k. Same, l. 603: replace 'released' by 'release
We have corrected the sentence accordingly.*'*

l. Extended Data Fig. 5: Why are three amino acid residues highlighted in yellow (and not mentioned in the legend)?
We have modified Extended Data Fig. 5 (Supplementary Fig. 22 in the revised manuscript).

Reply to Reviewer #2

The manuscript presents extensive cryo-EM study of Na-translocating NADH-quinone oxidoreductase (Na^+-NQR) – a large membrane protein complex with enigmatic mechanism. How the electron transfer between many cofactors of the complex is coupled to the translocation of sodium ions has been a major question since the discovery of the first structure of this bacterial enzyme. The current study resolves several conformations of NqrF and NqrC subunits, allowing temporary bridging of FeS and FMN cofactors, which are otherwise too far away from each other. Different redox conditions (presence of NADH) and mutants were studied, revealing that FeS cluster reduction in NqrDE subunits is linked to inward-to-outward-open transition of a putative Na^+-translocating pathway. This is in contrast to previous ideas on the location of this pathway in NqrB subunit. The NQR mechanism proposed in Fig. 5 elegantly explains structural observations and appears to be quite feasible. This study is a major milestone in NQR studies and will be of interest to a wide audience of Nature Communications.

We thank the reviewer for the positive remarks and the valuable comments that have helped us to improve our manuscript.

1. *Some questions to be addressed in revision:*
   Overall strategy – it is not clear why the authors did not try to capture different conformational states during catalytic turnover, i.e. by freezing enzyme while showing activity in the presence of both NADH and Q. A similar approach, for example, revealed many key conformational states of Ca-ATPase from a single dataset, in addition to those previously revealed one-by-one in the presence of inhibitors, etc. For this approach to work it does not matter whether the enzymatic reaction is fast or slow, as the full distribution of different states populated during the catalytic turnover may be captured, and the observation of a particular state depends only on how populated it is overall (% of particles) during turnover. Additionally, this may reveal the location of Q binding site, currently disputed.
   As an initial attempt, we also tried to capture different conformational states during catalytic turnover by incubating Na^+-NQR in the presence of NADH, UQ_1 (or UQ_2), and AOX (alternative quinol oxidase from *Trypanosoma brucei*). However, under these conditions, no conformations other than one identical to the wild-type as isolated (**WT_{ox}**) structure were obtained. Therefore, we decided to examine nine different experimental conditions listed in Table 1. We thank the reviewer for this important comment.

2. *Fig. S3 – NADH density is badly visible in shown orientation, would be better to orient it lengthwise.*
   We appreciate the reviewer's suggestion. We have updated Fig. S3 (Supplementary Fig. 12 in the revised manuscript) by reorienting NADH lengthwise to better illustrate how it occupies the RNF domain of NqrF.

3. *L. 165 "By focusing on the position of the ferredoxin-like domain, three distinct states of NqrF- up, middle, and down- were identified as separate classes …" There is no description of what exactly was done here – 3D classification (no align) with mask on this domain? – if so, it should be described in the main text and methods. If this was not done, it should have been, and it should be discussed why not. Overall, data processing methods description is currently very minimal and not sufficient to fully follow the workflow. Furthermore, the density for ferredoxin-like domain looks very fuzzy and it is not clear how well the density justifies the modelling of different states – to judge this, the detailed figures of density fit to the model for each discussed position of different domains should be added.*

   We thank the reviewer for this helpful comment. In the revised manuscript, we have added a detailed description of the data processing workflow in both the main text (p. 7) to clarify how the three classes were obtained. Moreover, the workflow in Extended Data Fig. 1b (Supplementary Fig. 2 in the revised manuscript)has been improved to better illustrate this process. In addition, Fig. 1b has been updated with a density-fit view to clearly show how the ferredoxin-like domain was fitted into the corresponding EM density.

4. *Fig. S2 – latent space distributions look quite homogenous without any features, suggesting absence of clearly defined different conformational states. There should be a discussion on how exactly the authors arrived at their proposed distribution of classes.*
   As the reviewer pointed out, the latent space distribution shown in Fig. S2 (Supplementary Fig. 10 in the revised manuscript), generated by **3D Flex Analysis**, appears homogeneous with no clearly defined clusters. This is a common and expected output for this algorithm in CryoSPARC. The classification of different conformational states was performed using **Heterogeneous Refinement** or **3D Variability**

Analysis, not 3D Flex Analysis. The 3D Flex Analysis was utilized solely to improve the density map by accounting for volume bending, not for separating distinct conformational states. To avoid misunderstanding, we have also revised Extended data Figure 1 (Supplementary Fig. 1-9 in the revised manuscript).

5. *L. 198 "followed by focused refinement on the NqrC density." – Again, not clear what was done here – there is no focused refinement step in ED Fig. 1e.*
   This is our careless mistake. The processing was performed against the entire image of Na+-NQR. We did not perform focused refinement of the NqrC density. We have amended the sentence in the revised manuscript (p. 8).

6. *L. 204 "In the "stable" state (Fig. 3b), resolved at 2.93 Å," – the resolutions of the two states seem to be mixed in the text.*
   This is our careless mistake. We amended the sentence in the revised manuscript (p. 8). We thank for reviewer's careful reading.

7. *L. 215 "that allows it to mediate electron transfer from 2Fe-2SNqrD/E to FMNNqrB" – How is that possible if the distance in shifted state is apparently still 27 A?*
   As pointed out, the distance between 2Fe-2S$^{NqrD/E}$ and FMN^{NqrB} in the "shifted" state (~27 Å) would need to be reduced by approximately half to allow physiological electron transfer. This suggests the existence of an additional conformational snapshot in which the hydrophilic domain of NqrC approaches NqrD/E more closely. Such a state may correspond to the structure of the FMN^{NqrC}-lacking mutant enzyme. We have clarified this point in the revised manuscript (p. 9). We appreciate the reviewer's careful reading.

8. *L. 236 – from this paragraph it is not clear if in all determined structures "inward-opened" state is always linked to "stable" state and "outward-opened" to "shifted" or not? This should be clarified.*
   We appreciate this suggestion. We have amended the revised manuscript (p. 9-10) to clarify that all determined structures in the "*inward-open*" state are always associated with the "***stable***" state of NqrC, whereas those in the "*outward-open*" state are associated with the "***shifted***" state.

Reply to Reviewer #3

This paper describes an intensive investigation of redox-coupled Na^+ ion translocation by NADH-quinone oxidoreductase (Na^+-NQR) using a combination of cryo-EM structural characterization of different states of the complex combined with molecular dynamics (MD) simulations designed to map those states onto proposed mechanism of translocation. The ingenious use of a combination of inhibited and mutated NQR states that slow or stop the reaction cycle at different points in the cycle combined with previous work by these authors and others in the field allow the authors to propose a potential mechanism for the Na^+ translocation from cytosol to the periplasm.

Given that this reviewer is an expert in neither cryoEM nor MD, and assuming that other reviewers will be recruited to judge those technical aspects, I will settle for raising questions that an educated and interested reader might have.

We thank the reviewer for the positive remarks and the valuable comments that have helped us to improve our manuscript.

1) Given that the Na-NQR complex is transmembrane, but no membrane components are present after purification, and detergent (DDM) concentrations are reduced after activity assays but prior to grid preparation, how comfortable are the authors that the cryo-EM movies accurately represent conformational changes that take place in the presence of the membrane? It is my experience that membrane-bound or membrane-associated proteins often take structural cues from their interaction with the membrane, and one notes that the MD simulations are performed in a POPC bilayer. Perhaps this is a question that frequently arises when looking at integral membrane structures by cryo-EM, but it probably would be a good thing to justify in more detail.

As is sometimes the case with membrane-bound proteins, the key question is: to what extent do detergent-solubilized proteins maintain all properties of their membrane-bound forms? We confirmed that both enzyme activity and inhibitor sensitivity were well maintained during purification process from the bacterial membrane, indicating that the overall structural integrity of Na^+-NQR was preserved. In addition, the detergent (DDM) concentration was reduced prior to cryo-EM grid preparation, this step did not cause any structural alterations such as aggregation. This suggests that sufficient amounts of detergent and phospholipids remained to stabilize the enzyme.

Nevertheless, as the reviewer rightly pointed out, we cannot completely rule out the possibility that interactions with surrounding phospholipids affect the structure and function of membrane proteins. We are currently working on determining the structure of Na^+-NQR reconstituted into liposomes or nano-discs to assess its conformation in a more native membrane environment. We greatly appreciate the reviewer's valuable comment.

2) The "down" state of the NqrF, in which the FeS cluster of the ferredoxin domain is sufficiently close to that of the NqrD/E domain for physiological electron transfer, is represented by only 5% of the particles observed (hence the lower resolution). Again, for those not expert in cryo-EM, this seems a small fraction but has large implications for the conclusions of the paper. How is this justified?

We thank the reviewer for this valuable comment. It is not unexpected that the three structural states of NqrF are not equally populated, as this likely reflects a low steady-state population of the "down" conformation due to the rapid electron transfer from FAD to 2Fe-2S^{NqrF} via 2Fe-2S$^{NqrD/E}$. Moreover, even though the "down" state represents a minor population, the 77k particles used for reconstruction (Extended Data Fig. 1b, Supplementary Fig. 2 in the revised manuscript) were sufficient to obtain a well-resolved EM density map that provides direct structural insight into the electron transfer mechanism.

Similar examples have been reported in another cryo-EM studies. For instance, Sanghai *et al.* (***Nature* 556**, 126–129, 2018) identified three conformational states (States 1–3) of the nucleolar pre-60S ribosomal subunit composed of 126k, 201k, and 31k particles, respectively. State 3, determined from less than 10% of the total particles, still provided a high-quality map (*Extended Data Fig. 2 therein*). This demonstrates that a relatively small particle population can still yield a reliable EM density if the data quality is high.

3) In Step 2 of the proposed mechanism (NADH binding), the authors claim that reduction of the FeS cluster in the ferredoxin domain of NqrF increases the flexibility of that domain, allowing it to approach the NqrD/E domain. In this reviewer's experience, reduction a 2Fe-2S cluster in a ferredoxin has the opposite effect,

causing compaction of the protein near the cluster, slowing protein dynamics measured on a number of time scales, including H/D exchange and NMR relaxation times, with less motional freedom of surface features. This does not negate the proposed role of the domain, but in fact may make ferredoxin binding in the D/E pocket entropically more favorable. Analogous binding and electron transfer between ferredoxins and cytochromes show that the reduced ferredoxins bind much more tightly to their redox partners than the irrelevant oxidized forms. Once electron transfer has occurred, increased dynamics in the ferredoxin domain encourages dissociation (also preventing back e- transfer, since the rate of electron transfer between two centers with similar redox potentials is by Marcus theory expected to be quite fast). Rapid dissociation would also free up the pocket for Na ion uptake as proposed in step 3.

Regarding the motion of the ferredoxin-like domain of NqrF, we have carefully described this aspect in the Results section ("Shift in local resolution map reflects redox-driven conformational changes of Na^+-NQR"). Based on this observation, we confidently propose that reduction of the 2Fe-2S cluster in NqrF increases the flexibility of the ferredoxin-like domain, thereby enhancing the probability of physiological electron transfer to $2Fe\text{-}2S^{NqrD/E}$.
However, as the reviewer pointed out, it is also possible that binding of the ferredoxin-like domain to the NqrD/E pocket becomes entropically more favorable when $2Fe\text{-}2S^{NqrF}$ is reduced. We have added this possibility to the revised manuscript (p. 10). We thank the reviewer for this intriguing and valuable comment.

4) The possibility that a proline c/t isomerization is involved in in the D/E switch is intriguing and might be expanded upon. Is a particular proline indicated? If so, what happens if that proline is mutated to a Gly or Asn? We have ourselves found evidence that proline switches are much more common in enzymatic mechanisms and other conformational changes than is often appreciated.
We thank the reviewer for this constructive comment. Among the conserved proline residues in the NqrD/E subunits, the proline at position 114 in NqrE appears to play a key role in amplifying the conformational switching of the D/E subunits. We have clarified this point in the revised manuscript (p.15) and illustrated the local relationships surrounding this residue during the *inward/outward* transition (Supplementary Fig. 19 in the revised manuscript). We are also interested in examining how mutation of this proline, or nearby hydrophobic residues, may affect the conformational dynamics. We plan to carry out site-directed mutagenesis experiments to assess the role of this proline in the switching mechanism.

Minor points: I assume NaPi is sodium phosphate buffer, but it should be listed in the abbreviations.
We added NaPi (sodium phosphate) in the abbreviation list.

This reviewer is quite used to looking at protein structures, but cannot tell what the differences are between the up down and middle states of NqrF in Figure 1, part b. Perhaps using a rainbow coloring of the sequence would make those differences clearer.
We updated Figure 1b to clarify the difference between three structures.

Line 366, Prolines should not be capitalized.
We amended the sentence in the revised manuscript (p. 15). We thank for reviewer's careful reading.

Line 391, remove (REF)
This is our careless mistake. We amended the sentence in the revised manuscript (p. 15). We thank for reviewer's careful reading.

Reply to Reviewer #4

In the study "The Na+-pumping mechanism driven by redox reactions in the NADH- quinone oxidoreductase from Vibrio cholerae relies on dynamic conformational changes", Ishikawa-Fukuda et al. explores the molecular principles of redox-driven Na+ pumping in the NQR complex from Vibrio cholerae by combining cryo-EM experiments with MD simulations. The study provides an important contribution for the field, with mechanistic implications also for related redox-driven ion pumps.
I find that the work deserves publication after some shortcomings have been clarified. I will limit here my comments (in no specific order) mainly to the computational aspects of the study:

We thank the reviewer for the positive feedback and the valuable comments, which have significantly helped us improve our manuscript.

1. It seems that all MD results are based on a single simulation replica. If this the case, the authors should perform at least a second MD replica for some of the key states to make sure that findings are statistically relevant.

As noted by the reviewer, only one trajectory is displayed in Fig. 4 of the main text. However, we conducted five independent trajectories following the targeted molecular dynamics (MD) simulation, and Na+ translocation was observed in two of these trajectories. This is illustrated in the bottom panels of Supplementary Fig. 9 (now labeled as Supplementary Fig. 21 in the revised manuscript). This result is mentioned in the main text on page 11: "Na+ translocation was observed in two additional independent trajectories following the targeted MD simulation (bottom panels in Supplementary Fig. 21)." These findings demonstrate that our results are reproducible.

2. The authors should report protonation states of titratable residues.

The following sentence was added in Methods section (p. 18)*: "The pKa of titratable residues was evaluated using PROPKA, and the protonation state was determined at pH 8, which is the same pH at which the experiments were conducted."*

3. How were the redox states defined in the simulations? (see also comment 16)

We thank the reviewer for this useful comment. We added the following sentences (p.18).
"The redox states of the cofactors were defined as the previous study [SI in ref. 27]. The oxidized and reduced 2Fe–2S were modeled as Fe[III]-Fe[III] and Fe[II]-Fe[III], respectively. The oxidized FAD was modeled as a neutral flavin ring. The oxidized and reduced FMN were modeled as a neutral flavin ring and $1e^-$ radical flavin ring, respectively. RBF is modeled as $1H^+/1e^-$ neutral radical flavin ring."

4. The authors report an interesting connection between the redox state of the FeS center and the sodium binding. The authors should clarify if there are additional conformational / dynamic changes of the domains linked to the redox changes. If so, do these change the distance between redox cofactors, which could be important for the gating mechanism? Do the authors suggest that reduction of the 2Fe-2S cluster drives the inward/outward conformational change?

We appreciate the reviewer's comment. In addition to the Na^+ binding and unbinding associated with the redox changes, we observed a shift in the distance between the cofactors 2Fe-2S NqrD/E and the FMN in NqrC, as illustrated in Extended Data Fig. 4 (Supplementary Fig. 20 in the revised manuscript). This shift resulted from a rearrangement of the NqrC subunit, which was triggered by the conformational change of NqrD/E to the outward-open state. However, this rearrangement was not directly linked to the redox change. We did not mention this observation in the main text because it was only noted in a single trajectory and not in others, possibly due to the timescale of the process.

Concerning the driving force behind the *inward/outward* conformational change, we believe that Na^+ binding, prompted by the reduction of the 2Fe-2S cluster, induces this conformational change. The binding of Na^+ helps to neutralize the negative charge introduced by the reduction of the 2Fe-2S cluster, leading to a stable occluded conformation and facilitating the transition to the *outward-open* state.

We added the following sentence in the main text (p.11): *"The negative charge introduced by the reduction of 2Fe-2S$^{NqrD/E}$ should be neutralized by Na$^+$ to achieve a stable occluded conformation and further allow the conformational change to the outward-open state."*

5. Many flavines are covalently bound to proteins. I cannot find a description of how this interaction was modelled. Please clarify.

We appreciate the reviewer for highlighting this important point. We have added the following sentence to the Methods section (p. 18): *"FAD^{NqrF} is not covalently attached to NqrF. 2Fe-2S^{NqrF} is covalently attached to NqrF-Cys70, NqrF-Cys76, NqrF-Cys79, and NqrF-Cys111. The 2Fe-2S$^{NqrD/E}$ is covalently attached to NqrD-Cys29, NqrD-Cys112, NqrE-Cys26, and NqrE-Cys120. FMN^{NqrC} is covalently attached to NqrC-Thr225, while FMN^{NqrB} is attached to NqrB-Thr236. RBF^{NqrB} is not covalently attached to NqrB."*

6. How was the collective variable defined for the TMD simulations?

We added the following sentence in Methods (p.19): *"The collective variable of the TMD simulations is a root-mean-square deviation (RMSD) to the reference structure using all Ca atoms of NqrD/E."*

7. How was the gate size (Fig. 4c, Fig. S9) defined and calculated?

The gate size was defined by using a closest heavy-atom distance. We clarified this in the captions of Fig. 4c, Fig. S9 (Supplementary Fig. 21 in the revised manuscript), and Extended Data Fig. 4 (Supplementary Fig. 20 in the revised manuscript).

8. Table S3: how was a Na+ binding event defined?

The Na$^+$ binding was defined if a distance is less than 3 Å. It was explained in the caption of Table S3.

9. The TMD simulations should be clarified in Table S2. Moreover, how does the resulting end state compare to unbiased MD simulations of the outward open state?

Details of the TMD simulations are listed in Table S2. In Extended Data Fig. 4 (Supplementary Fig. 20 in the revised manuscript), the TMD trajectory is compared to the unbiased MD in the outward-open state. The end conformation of the TMD simulation overlaps with the unbiased MD trajectory.

10. cryoEM: How well could the redox cofactors be refined based on the cryoEM maps? Show example cryoEM maps and models in the SI. Are functional water molecules properly resolved? What about Na+ ions?

We thank the reviewer for this important comment. We assume that this question specifically refers to the key cofactors involved in electron transfer from 2Fe-2S^{NqrF} to FMN^{NqrC} via 2Fe-2S$^{NqrD/E}$. As the EM density for the ferredoxin-like domain of NqrF containing 2Fe-2S^{NqrF} is somewhat fuzzy, this domain was modeled by rigid-body fitting. Therefore, it is impractical to refine the redox cofactor within this region at higher resolution. Nevertheless, this approach is commonly used for modeling flexible protein regions, as in the structural determination of the oxidized state of Na$^+$–NQR (*ref. 7*).

Accordingly, we confidently show that the ferredoxin-like domain containing 2Fe-2S^{NqrF} approaches the NqrD/E subunit to reduce 2Fe-2S$^{NqrD/E}$, based on the three structural states: *up*, *middle*, and *down*. Figure 1b has been updated with a density-fit view to clearly illustrate how the ferredoxin-like domain coordinating 2Fe-2S^{NqrF} was fitted into the corresponding EM density. Following the same strategy, we modeled the hydrophilic domain of NqrC in the "*shifted*" state, which supports the repositioning of FMN^{NqrC} to accept an electron from 2Fe-2S$^{NqrD/E}$. The position of 2Fe-2S$^{NqrD/E}$ was stable and well refined (See Fig. 3 in *ref.7*).

With respect to water molecules and Na$^+$ ions, although we observed densities resembling Na$^+$ or water near the 2Fe-2S$^{NqrD/E}$, we did not assign them as Na$^+$ or water because they cannot be unambiguously identified from the EM map alone. The discussion of Na$^+$ and water binding sites is instead based on MD simulations.

11. Fig. 2 is extremely dense, and I have severe difficulties in seeing the main differences between the resolved structures. As this figure forms the basis for the proposal, the authors should improve the figure, highlighting also the detailed structure of the proposed Na+ binding site.

We thank the reviewer for this helpful comment. We have revised Fig. 2 to enhance its clarity, helping readers better understand the correlation between this panel and the proposed Na^+-pumping mechanism illustrated in Figs. 4f and 5d.

12. Fig. 4d show a dissociation of a Na+ ion. In which redox state does this take place? Can it be linked to the oxidation of the FeS cluster? Are there other conformational changes? (see comment 4)

Indeed, the dissociation of Na^+ took place in the oxidized state of 2Fe-2S$^{NqrD/E}$. As described in our response to the comment 4, we also observed a change in distance of the cofactors 2Fe-2S$^{NqrD/E}$ and FMN^{NqrC}, although this was not observed in additional independent trajectories.

13. "In the shifted state (Fig. 3b), while FMN^NqrC remains distant from 2Fe-2S^NqrD/E (~27°), the loop of Glu169-Gly177^NqrC, which includes Thr173 and Leu176 that stabilize the isoalloxazine ring of this FMN9, is embedded in the pocket formed by the NqrD/E subunits"
I cannot see the loop in the figure. Please clarify.

We update Fig. 3b to clarify the position of the region Thr169-Leu177. We appreciate the reviewer for careful reading.

14. "The existence of these two distinct conformations shows that the periplasmic domain of NqrC, which houses FMN^NqrC, switches its position between NqrB and NqrD/E in a way that allows it to mediate electron transfer from 2Fe-2S^NqrD/E to FMNNqrB (Fig. 1a, 3)."
Report FMN^NqrC <-> 2Fe-2S^NqrD/E distances. I cannot find these in figures or text. Is this distance compatible with electron transfer?

We thank the reviewer for this careful comment. The distance between FMN^{NqrC} and 2Fe-2S$^{NqrD/E}$ in the "*shifted*" state is indicated in the text (p. 9). As pointed out, the distance between 2Fe-2S$^{NqrD/E}$ and FMN^{NqrC} in the "shifted" state (~27 Å) would need to be reduced by approximately half to allow physiological electron transfer. This implies the existence of an additional conformational snapshot in which the hydrophilic domain of NqrC moves closer to NqrD/E, possibly corresponding to the structure of the FMN^{NqrC}-lacking mutant enzyme. We have clarified this point in the revised manuscript (p. 9).

15. "A notable example of this is the rearrangement of NqrF during electron transfer from FAD to 2Fe-2SNqrD/E (Fig. 1b,c). In the "down" and "middle" states, where 2Fe-2SNqrF is positioned close enough to 2Fe-251 2SNqrD/E to allow facile electron transfer, the ferredoxin-like domain of NqrF shows lower resolution, while the FNR-like domain containing FAD remains highly resolved (Supplementary Fig. 2a).
As far as I can see, in the middle state, the 2Fe-2S^NqrF <-> 2Fe-2S^NqrD/E distance to 26 Å. Electron transfer can thus only take place in the "down" state. Please clarify.

We thank the reviewer for this careful observation. Indeed, only the "*down*" state is compatible with electron transfer between 2Fe-2S^{NqrF} and 2Fe-2S$^{NqrD/E}$. We have clarified this point in the revised manuscript (p. 8).

16. The mechanism requires clarification: how are the two electrons from the NADH oxidation are distributed during the pumping cycle? How is this considered in simulations? Also importantly: what is the proposed Na+ pumping stoichiometry based on the mechanistic model?

We thank the reviewer for this important comment. We have improved Fig. S10a to better illustrate the distribution of the two electrons donated by NADH during the catalytic cycle. This is now shown as a standalone figure (Supplementary Fig. 24) to complement Fig. 5. Our model aligns with our simulations, indicating that the one-electron reduction of 2Fe-2SNqrD/E is coupled to the translocation of one Na^+ ion. Since NADH donates two electrons, this reaction cycle occurs twice. Based on previous studies (see Ref. 2) as well as our current work, we propose a Na^+ pumping stoichiometry of 2 Na^+ ions per 2 electrons (2 Na^+/2 e^-).

17. Previous studies on NQR have emphasized that NqrB is responsible for the Na+ pumping activity based on structural arguments, mutagenesis studies, but also based on the relation of the subunit to other transporters. It would be very useful if the authors further clarified what could be the reason(s) for the drastically different results and conclusions from previous NQR studies.

We thank the reviewer for this important point. In this study, we demonstrate for the first time that the transition of the NqrD/E subunits from inward to outward is driven by redox reactions within the enzyme. This was confirmed by identifying key reaction intermediates through cryo-electron microscopy (cryo-EM) analysis. Additionally, our molecular dynamics (MD) simulations, based on these structures, clearly indicate that the translocation of Na^+ from the cytoplasm to the periplasm is tightly coupled to this redox-driven structural transition. These findings lead to conclusions that differ significantly from those of previous studies (Hau et al., 2023, Nat. Struct. Mol. Biol.). We have included this clarification in the revised manuscript (p.14).

It seems that the current study also supports the proposed model of the related RNF protein (of Ref. 27), suggesting that RNF and NQR employ the same mechanism. Please also clarify if the authors suggest that the mechanism is indeed the same as proposed for RNF or different in some way?

We thank the reviewer for this insightful comment. Given the structural similarity and evolutionary relationship between RNF and Na^+-NQR, it is almost certain that, in broad mechanistic terms, these enzymes operate via the same general principle. This is particularly evident for the Na^+-pumping machinery governed by the RnfA/E subunits, which correspond to the NqrD/E subunits in Na^+-NQR (for the mechanism of the RNF complex, see *ref. 27*).

However, there are clear differences in both structure and redox chemistry between RNF and Na^+-NQR, indicating that their detailed electron-transfer mechanisms cannot be identical. Functionally, RNF transfers electrons from a one-electron donor (ferredoxin) to a two-electron acceptor (NAD^+), whereas Na^+-NQR transfers electrons from a two-electron donor (NADH) to a two-electron acceptor (ubiquinone). In both systems, however, the Na^+-pumping step itself appears to be associated with a one-electron process.

These functional differences are reflected in the RnfB and NqrF subunits, which each donate one electron to the FeS in RnfA/E and NqrD/E to pump one Na^+, respectively. RnfB accepts a single electron from ferredoxin and passes it through a series of Fe-S clusters, whereas NqrF receives two electrons from NADH. The two-electron nature of NADH oxidation by NqrF may serve as a kind of "electron gating" mechanism that regulates Na^+ translocation. Such functional divergence between RnfB and NqrF, which may differently affect conformational changes to donate electrons to the Na^+-translocating RnfA/E and NqrD/E subunits, could determine the directionality of Na^+ transport: Na^+-NQR catalyzes Na^+ extrusion only, whereas RNF can operate in the reverse direction, coupling NADH oxidation to Na^+ uptake. We added this point in the revised manuscript (p.15).

Reply to Reviewer #1

The authors have taken all of the reviewers' objections into account and amended the revised version of the manuscript accordingly. Although the current version still contains minor typographical errors (e.g. line 289 "A. woodie" instead of "A. woodii"), it should be accepted in its current form.

We corrected the typo. We appreciate the reviewer's positive remarks and valuable comments, which have helped improve our manuscript.