Peer Review File

Manuscript Title: Hydrogen-dependent CO2 reductase filaments establish nanowire-like structures to maximize enzymatic activity

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

Dietrich et al. report a CryoEM structure of the filament-forming enzyme hydrogen-dependent CO2 reductase from the thermophilic acetogenic bacterium Thermoanaerobacter kivui. This enzyme catalyzes the reversible hydrogenation of CO2 by hydrogen to formic acid, a process of outstanding interest for biotechnology, ecology, and hydrogen-power research. They validate their results and in particular the role of filament formation and electron transfer using in vitro activity assays. Experiments are generally conclusive and relevant. Further characterization of an iron sulfur cluster deletion variant central to this paper is required, confirmation of filament formation in cells would strengthen the manuscript. Throughout the manuscript the authors use experiments with low number of replicates starting at N=2 but use descriptive statistics for representation in contradiction to Nature guidelines. Cryo EM data are of adequate quality to support all claims, but no validation reports for the structural work have been provided for review. The potential impact of radiation damage to metal centers could be addressed in extended data. For detailed comments, see below.

Detailed comments:

Line 99: Could the authors determine the average filament length observed on the grid? Evidence for the presence of extended filaments in this preparation should be provided because the authors use illustrations of longer filaments throughout.
Line 102: Large dataset: please specify number of movies (33853). Replace “high-resolution” by 3.4A resolution.
Line 106: Check cross-references: Fig S2 = Ext Data Figure 2? (for whole document)
Line 108: Density map appears to not be sufficient in resolution for individual atom-based cluster modelling. What types of additional information have been used in modelling or refinement? Restraints should be deposited with models. Have the authors considered possible effects of radiation damage on metal centers?
Line 143: 0.961A: reduce number of significant digits.
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Line 159: His-tagged protein had the same…Comparative data for wild type natively purified enzyme should be show in the same graphs and gels.
Line 673 … Ext. Data Figures: Experiments use descriptive statistics and bar graph/error bar representation for replicate number starting at N=2 violating Nature statistics guidelines. The relevance and interpretability of error bars as in Ext. Data Fig. 5b (N=4 left bar, N02 right bar) is doubtworthy. Please follow Nature guidelines.
Line 214: It is critically important to show data on the intact assembly of filaments for the variant with deleted iron sulfur cluster, using the same methods applied for the HycB3 and HycB4 deletions

(EM, Size Exclusion, Pull down).
Line 223: Could the authors determine an average filament length (in vitro and in vivo; see other comments), so that the dependence of enhanced catalysis by electron transfer on filament length could be further studied e.g. in simulations?

Line 232: Demonstrating filament formation in cells would strengthen the relevance of the findings of this paper. One established method would be immunogold labelling EM of sections of fixed cells based on the His-tagged variant and commercial anti-His antibodies.
Line 435: Electron dose is listed as 51 in text and 52 in table 1.
Line 522: Hydrogen production measurements: see comment on number of replicates above.
Note 1: No validation reports were provided.
Note 2: The manuscript quotes supplementary data and figures, while no supplementary data were provided. Some information is apparently lost, e.g. the list of primer (line 489), please carefully check cross-references to all Ext. Data items.

Referee #2 (Remarks to the Author):

The manuscript by Dietrich and coworkers reports on the cryo-EM structure of the HDCR enzyme from Thermoanaerobacter kivui, confirming the previously reported filamentous nature of the enzyme. It also reprts studies of truncated versions of HDCR that are purported to support the main conclusion that the function of the filaments is to maximise activity. While the manuscript reports a significant amount of work, I found the novelty to be rather limited and the evidence for the function of filamentation not sufficiently robust. In terms of the structure, the subunit composition of HDCR was already known, which includes proteins for which there are several structures available. In addition, it was obvious from the HDCR composition that the FeS proteins would make to electric connection between the two catalytic subunits. The filamentous nature of the enzyme was also previously reported. So the novelty here is only the structural basis for filamentation, but alas this is not really clear from the manuscript. The studies with variants yielded results that are totally expectable from the structure, and do not provide solid evidence for why the filaments are formed or that it is for maximising activity.

More specific remarks:
1.The authors mention that HDCR is the most active biological or chemical catalyst for direct reduction of CO_2 by H_2 to produce formate. However, they should make it more clear that it is in fact the ONLY biological catalyst known to perform this reaction, as the formate-hydrogen lyase of E.coli operates mostly in formate oxidation. Also, previous reports show that HDCR activity is limited by the activity of its formate dehydrogenase and this is in fact not higher than the activity of other isolated W-containing Fdh enzymes. So, the advantage of HDCR is to combine in a single protein the hydrogenase and formate dehydrogenase enzymes, but its catalytic activity is not that outstanding in biological terms.

2. The stoichiometry of the enzyme is not clear. Previous reports suggested that the enzyme is a heterotetramer formed by HydA, HycB4, HycB3 and FdhF in a 1:1:1:1 arrangement, but this

manuscript seems to indicate that there are 2 HydA/HycB4 dimers per FdhF/HycB3 unit. The subunit stoichiometry should be determined.

3. Line 102: what is a minimal HDCR filament? How many hexamers does it comprise?

4. Lines 125-126: The authors do not seem aware that the catalytic mechanism of Fdhs is still under strong debate, namely regarding metal coordination of the substrate during turnover, the role of the metal sulfido ligand and whether the Cys/Sec ligand remains bound to the metal during turnover (see recent reviews on the topic). The present structure offers no insight into this topic, so it is not correct to say that the mechanism of FdhF is “in accordance with previously described enzymes” (lines 125-126).

5. An important point is that the interactions allowing formation of the “nanowire” are not clear in the manuscript. From Fig. 1 and Fig.3 it appears that HycB4(1) and HycB4(2) form the core of the wire, with the HycB3/FdhF dimer protruding from it. However, only the interactions between HycB4(1) and HycB4(2) and between HycB4 (which?) and HycB3 are described, but not those allowing the repeating nature of the filament. We are missing the interaction site between one hexamer and the following one. If indeed HycB4 forms the core of the filament, as suggested by Figs. 1, 3a and 4, then this contradicts the conclusions on lines 196-198 that HycB3 is responsible for linking the hexamers together, whilst HycB4 is responsible for integrity of the hexameric complex.

6. Several variants produced do not really provide additional evidence from that already available from the structure. It is obvious from the structure that if HycB4 is deleted then HydA will not be associated with the other proteins, or that if HycB3 is deleted then FdhF will not be associated. I do not really see the point of these experiments.

7. Figure 3 and lines 192-195 – How do the authors know what is the composition of the proteins from the HDCR_ΔHelix HycB3 and HDCR_ΔHelix HycB4 variants? No evidence is provided and it seems they are guessing from the molecular mass, which is certainly not reliable. How can they be certain that HDCR_ΔHelix HycB4 is composed of a pair of HydA2-HycB4 dimers and HDCR_ΔHelix HycB3 of a hexamer? Furthermore, how do they know if the protein integrity of the subunits present is still OK? The deletion of these helices may have unexpected impacts on the structure of the subunits in several ways. Without determining the structure of these variants we are looking at a black box, and it is not possible to make solid interpretations of their activity!! Do the numbers in Fig. 3c represent the molecular mass? They are missing units. If they are the molecular mass then the mass of HDCR_ΔHelix HycB3 is much higher than expected for a hexamer. The activity for CO2 reduction from H2 should also be shown for these variants.

8. Lines 214-216: When the cluster [4Fe4S] VI is deleted how can the authors be certain that electron transfer to the closest HydA subunit is retained? From Fig. 4a and 4b, it seems to me that this cluster is essential for electron transfer to both HydA subunits, so it is little surprising that activity is reduced by 95%.

9. Lines 217-222: The comments in these lines are supposed to be the core of the manuscript finding (that activity increases with filamentation), but the evidence is certainly not sufficiently robust to

conclude this, as no evidence is provided for the composition and integrity of the proteins present in the various variants, and the activity alone does not tell us much. Why do the authors say that the protein obtained in the HDCR_HycB4Δ[4Fe4S] VI variant has only four subunits, and that HDCR_ΔHelix HycB3 has more connected subunits? I do not see evidence for this. Also comparing HDCR_HycB4Δ[4Fe4S] VI and HDCR_ΔHelix HycB3 is misleading since in one the electron transfer is interrupted, while presumably not in the other. The authors are forcing a narrative for which there is not sufficient evidence.

10. Furthermore, in a previous study on the relevance of the filamentous nature of the first reported HDCR (Ref. 30) experiments are described where it is shown that by depolymerizing the enzyme only about 30% of activity is lost versus a similarly treated control. This finding also does not support the present proposition that the role of filamentation is to increase activity.

11. In conclusion, while this is a nice structure, allowing nice figures due to its repeating nature, I do not see sufficient evidence to conclude that the role of filamentation is to increase activity. There could be a number of other reasons for why filamentation occurs. It is also not clear whether filamentation has physiological relevance and occurs in vivo.

Author Rebuttals to Initial Comments:

Dietrich et al. Response to Reviewer Comments

We warmly thank the reviewers for their constructive feedback, which we feel has significantly improved our manuscript. Before responding to the **reviewer comments** below, we would like to briefly outline the major additions and changes to our revised manuscript.

We included a detailed functional comparison of wild-type HDCR with the overproduced HDCR_His (**Extended Data Figs. 6, 7A-D**) and extended characterization of the HDCR variants to include filamentation of HDCR_HycB4Δ[4Fe4S] IV, filament subunit composition and enzymatic activity of filament variants (**Fig. 3B-D**). Furthermore, we repeated all enzymatic measurements to provide statistically robust data, with each experiment conducted in three biological replications, each with three or two (pH and temperature optimum) technical repetitions (**Figs. 2D, 3D; Extended Data Figs. 6C-D, 7**). In addition, we remade most of the figures to improve the representation of the enzyme structure, subunit interactions, and electron connectivity through the [4Fe4S]-cluster network.

Most prominently, our study now includes convincing structural and functional evidence for the role of HDCR filaments *in vivo*. Cryo-electron tomography (cryo-ET) revealed unexpected HDCR superstructures inside native cells: we observed large ring-shaped bundles of HDCR filaments bound to the cell's membrane (**Fig. 5**). Determination of a native filament structure (**Fig. 5G-I, Extended Data Fig. 9**) and comparison to the Δ*hdcr* mutant (**Extended Data Fig. 8**) confirmed that these filament bundles are indeed HDCR, and they are required for robust cell growth (**Extended Data Fig. 6A**). These novel HDCR superstructures may enable an anaerobic hydrogen- and carbon-concentrating mechanism, driving HDCR's unsurpassed catalytic activity and enabling metabolism in energy-limited environments. By integrating precise functional dissection with structural analysis that spans scales from atoms to the cells, our study presents one of the most detailed descriptions of enzyme filamentation to date.

Referee #1 (Remarks to the Author):

Dietrich et al. report a CryoEM structure of the filament-forming enzyme hydrogen-dependent CO2 reductase from the thermophilic acetogenic bacterium Thermoanaerobacter kivui. This enzyme catalyzes the reversible hydrogenation of CO2 by hydrogen to formic acid, a process of outstanding interest for biotechnology, ecology, and hydrogen-power research. They validate their results and in particular the

role of filament formation and electron transfer using in vitro activity assays. Experiments are generally conclusive and relevant. Further characterization of an iron sulfur cluster deletion variant central to this paper is required, confirmation of filament formation in cells would strengthen the manuscript. Throughout the manuscript the authors use experiments with low number of replicates starting at N=2 but use descriptive statistics for representation in contradiction to Nature guidelines. Cryo EM data are of adequate quality to support all claims, but no validation reports for the structural work have been provided for review. The potential impact of radiation damage to metal centers could be addressed in extended data. For detailed comments, see below.

We thank the reviewer for this positive assessment of our structural biology, and helpful suggestions that we have incorporated into our revised manuscript. We increased the number of replicates for all assays in our study (N = 3 biological replicates, each with 3 or 2 [pH and temperature optimum] technical replicates), and although our main conclusions are not altered, our data are now more robust. As requested, we were able to show that the HDCR_HycB4Δ[4Fe4S] IV variant is not structurally corrupted and still forms filaments (**Fig. 3B**). Furthermore, this variant can complement the knock-out, indicating structural integrity of the enzyme (**Extended Data Fig. 6A**). On the other hand, this variant shows the physiological importance of intact electron transmission through the HDCR nanowire, as growth is much slower than wild-type cells (**Extended Data Fig. 6A**). We have also rewritten our manuscript to describe how we carefully modeled the cofactors (homology models and restrained refinement) because of the limited resolution. We clarify that we do not draw conclusions from observations that we cannot see (such as radiation damage), and that all our observations are experimentally validated. The validation report for our cryo-EM structure of the enzyme is included in the revised manuscript. Finally, to support the relevance of this structural work *in vivo*, we used cryo-ET to directly resolve HDCR filament inside native cells and discovered that they bundle to form ring-shaped superstructures attached to the cell membrane (**Fig. 5, Extended Data Figs. 8-9, Movie 3**).

1. Line 99: Could the authors determine the average filament length observed on the grid? Evidence for the presence of extended filaments in this preparation should be provided because the authors use illustrations of longer filaments throughout.

Thank you for the suggestion. We now elaborate that the short filaments are 3-4 repeating units and longer filaments do exist in the isolated HDCR preparation (examples provided in

Extended Data Fig. 2), although they are quite rare (about 1 every 30 micrographs). We tried to process these longer filaments, however they are multilayered bundles that prevent single particle analysis. Please note that these isolated filament bundles are consistent with the bundled HDCR superstructures we observe inside cells (**Fig. 5**).

We modified the text accordingly: “The micrographs were dominated by individual bent short filaments (**Extended Data Fig. 1**). Reference-free 2D class averages revealed that they consist of 3-4 repeating units. Longer filaments, similar to those previously described[32], were only rarely observed (**Extended Data Fig. 2**), likely due to fragmentation caused by mechanical stress during purification and blotting onto EM grids. These longer filaments formed bundles that prevented structural analysis (**Extended Data Fig. 2**).”

2. Line 102: Large dataset: please specify number of movies (33853). Replace ”high-resolution” by 3.4A resolution.

Thank you, we corrected these passages in the manuscript.

3. Line 106: Check cross-references: Fig S2 = Ext Data Figure 2? (for whole document)

Thank you for pointing this out. We changed the labeling of all figures and tables according to the Nature layout.

4. Line 108: Density map appears to not be sufficient in resolution for individual atom-based cluster modelling. What types of additional information have been used in modelling or refinement? Restraints should be deposited with models. Have the authors considered possible effects of radiation damage on metal centers?

Thank you for raising these concerns. This question also occupied us for a very long time, and we took great care not to use resolution regimes in our analysis that are not warranted by the data. Due to the flexibility of the short filaments and the associated subunits, the local resolution is high in the core regions and low in the edge regions. The highest resolution we describe is at the residue level (2.7 Å). As rightly pointed out by the reviewer, this prevents an atom-based cluster modeling of the complex cofactors. Thus, we used a .cif file generated from the PDB (ligand ID 402) to constrain the co-factor coordinates in the PHENIX

real_space_refine. The .cif files are available upon request. We added the generation of the .cif file to the Methods section.

We tested additional particle polishing routines that could mitigate the effects of radiation damage, but processing with RELION only results in lower resolution reconstructions (>4.5 Å). CryoSPARC Non-Uniform Refinement with integrated global and local CTF correction seems to cope better with this particular protein. We would like to point out that published work analyzing radiation damage to non-protein cofactors is in the 2.0 Å resolution range[1,2], well beyond the attained resolution for HDCR. We note that a co-first author on this study of radiation damage (Dr. Righetto) is now also a co-first author of our HDCR manuscript.

Because of the inhomogeneous resolution and potential radiation damage, we have also thoroughly experimentally validated all of our conclusions. To illustrate this, we have modified the following passages:

1) "We acquired 33,853 cryo-EM images and determined the single-particle structure of a short HDCR filament with a global resolution of 3.4 Å (**Fig. 1, Movies 1-2**). In the core of the molecule, a local resolution of 2.7 Å was achieved, enabling reliable modelling at the single-residue level (**Extended Data Figs. 1, 3**). However, the periphery only reached lower resolutions (> 5 Å) due to flexibility of the filament and associated enzymes, so we modeled these regions with the aid of AlphaFold predictions[33] for the protein backbone and homology models for cofactor positioning (**Extended Data Figs. 1, 3, 4, 5**)."

2) "HydA2 is composed of two domains, which adopt the mushroom-shaped structure of a classical [FeFe] hydrogenase (**Extended Data Figs. 3A, 4A**). The stem domain contains two [4Fe4S]-clusters that directly route electrons to the active site in the two-lobed cap domain (**Fig. 2A**), which harbors the enzyme's catalytic H-cluster (HC). A structural superposition with the closely related CpI from *Clostridium pasteurianum* (PDB 3C8Y) (**Extended Data Fig. 4**) shows that all residues necessary for the active site are conserved, including Cys 387 (bridging [2Fe]-subcluster and [4Fe4S]-cluster of the HC), Cys183 (proton reduction), Met237 and Met381 (H-cluster coordination), Pro115 (substrate regulation), and the cysteines responsible for [4Fe4S]-cluster coordination[34] (**Extended Data Fig. 4D**)."

3) In the Methods Section: "For refining the H-clusters in the complex, a geometry file containing the coordinates of the H-cluster (RCSB ligand ID - 402) was used to generate a restraint CIF file using the electronic Ligand Builder and Optimization Workbench (eLBOW) tool[62] integrated in the PHENIX work suite. This CIF file was then used as an input to constrain the

real space refinement. The statistical quality of the final protein model was assessed using MOLPROBITY[63]. Figures were prepared using PyMOL[64] or UCSF ChimeraX[65]."

4) In the Methods Section: "Additional refinement approaches including density subtraction, local refinement and per-frame reconstructions to compensate for electron damage did not lead to noteworthy improvements despite multiple attempts."

5. Line 143: 0.961A: reduce number of significant digits.,

Thank you for pointing out this error. We have reduced the number of digits in the manuscript.

6. Line 158: Where are data shown for the HDCR activity increase to 68.7U/mg?

Thank you for mentioning this. We included a corresponding graph in the Extended Data section (**Extended Data Fig. 5D**). However, after repeating the experiment several more times, we have now updated the enzyme activities to 5 U/mg (wild type) and 98 U/mg (HDCR_His), as can be seen in the figure.

7. Line 159: His-tagged protein had the same; Comparative data for wild type natively purified enzyme should be show in the same graphs and gels.

In the revised manuscript, we included the data for both enzymes in the same graphs and gels in **Extended Data Fig. 5** (growth, substrate turnover, protein subunit composition) and **Extended Data Fig. 6 A-D** (pH and temperature optima).

8. Line 673; Ext. Data Figures: Experiments use descriptive statistics and bar graph/error bar representation for replicate number starting at N=2 violating Nature statistics guidelines. The relevance and interpretability of error bars as in Ext. Data Fig. 5b (N=4 left bar, N02 right bar) is doubtworthy. Please follow Nature guidelines.

Thank you for asking that we increase the statistical rigor of our study. All enzymatic measurements have been repeated, so every experiment has now been performed in 3 biological replicates, each with 3 (minimum 2) technical repetitions (N=9 or N=6).

9. Line 214: It is critically important to show data on the intact assembly of filaments for the variant with deleted iron sulfur cluster, using the same methods applied for the HycB3 and HycB4 deletions (EM, Size Exclusion, Pull down).

Thank you for the helpful comment. We have now included the size exclusion chromatogram of the HDCR_HycB4Δ[4Fe4S] IV protein showing filamentation in **Fig. 3B**.

10. Line 223: Could the authors determine an average filament length (in vitro and in vivo; see other comments), so that the dependence of enhanced catalysis by electron transfer on filament length could be further studied e.g. in simulations?

See our response to Comment #1 for in *vitro* filaments. For *In vivo* HDCR, we performed cryo-ET of FIB-milled cells and made an exciting discovery. Around 100 HDCR filaments bundle together to form membrane-bound ring-shaped superstructures that are about 200 nm in diameter. Each filament can have dozens of repeating units. We thank the referee for this idea— we are cooperating with an expert computational biologist Prof. Ville Kaila on other topics and plan to carry out simulations of HDCR filament bundles in future studies.

11. Line 232: Demonstrating filament formation in cells would strengthen the relevance of the findings of this paper. One established method would be immunogold labelling EM of sections of fixed cells based on the His-tagged variant and commercial anti-His antibodies.

Thank you for mentioning this. We completely agree that showing filament formation in cells would strengthen the relevance of our paper. Therefore, we included comprehensive structural analysis of HDCR filaments inside native cells by cryo-electron tomography (cryo-ET). **Fig. 5, Movie 3** and **Extended Data Figs. 8-9** show the corresponding results in the new manuscript. Not only does this cryo-ET work definitively demonstrate filament formation within cells, but it also yielded the surprising discovery that these HDCR filaments bundle to form membrane-attached superstructures (See also Referee #2 Comment 11c).

12. Line 435: Electron dose is listed as 51 in text and 52 in table 1.

The electron dose is 51.9, so the rounding error has now been corrected to 52 in the text.

13. Line 522: Hydrogen production measurements: see comment on number of replicates above.

Thank you. As mentioned in Comment #8, we have increased the number of measurements to N=9.

Note 1: No validation reports were provided.

In the initial submission, we sent the validation reports to the editor, as there was an error in the data upload. In our resubmission, we have added new reports, as we updated the model of FdhF using an AlphaFold prediction instead of homology modelling using PHYRE2.

Note 2: The manuscript quotes supplementary data and figures, while no supplementary data were provided. Some information is apparently lost, e.g. the list of primer (line 489), please carefully check cross-references to all Ext. Data items.

We are sorry for this misunderstanding, “supplementary data and figures” were meant to be Extended Data figures and tables. The primer list was removed from the initial manuscript due to limits on the number of figures and tables, but has been included in the revised manuscript.

Referee #2 (Remarks to the Author):

The manuscript by Dietrich and coworkers reports on the cryo-EM structure of the HDCR enzyme from Thermoanaerobacter kivui, confirming the previously reported filamentous nature of the enzyme. It also reports studies of truncated versions of HDCR that are purported to support the main conclusion that the function of the filaments is to maximize activity. While the manuscript reports a significant amount of work, I found the novelty to be rather limited and the evidence for the function of filamentation not sufficiently robust. In terms of the structure, the subunit composition of HDCR was already known, which includes proteins for which there are several structures available. In addition, it was obvious from the HDCR composition that the FeS proteins would make to electric connection between the two catalytic subunits. The filamentous nature of the enzyme was also previously reported. So the novelty here is only the structural basis for filamentation, but alas this is not really

clear from the manuscript. The studies with variants yielded results that are totally expectable from the structure, and do not provide solid evidence for why the filaments are formed or that it is for maximizing activity.

We are grateful for a critical review of our work, which has highlighted the main areas to improve our study. We have therefore revised our manuscript to show that HDCR filaments do dramatically increase enzymatic activity (**Fig. 3D**), and that *in vivo* they not only form the expected filament structures but bundle together into novel ring-shaped superstructures attached to the cell membrane (**Fig. 5, Extended Data Figs. 8, 9, Movie 3**). These *in vivo* HDCR filament bundles are required for efficient cell growth. Furthermore, we show that a mutant that is unable to transfer electrons through the filament has a severely delayed growth phenotype, emphasizing the biological relevance of filamentation for maximizing HDCR activity (**Extended Data Fig. 6A**). We hope that this thorough combination of structural biology, enzymatic analysis and cellular tomography, all showing that HDCR filament formation is crucial for its function *in vitro* and *in vivo*, will now convince the reviewers of the novelty of our work.

1.The authors mention that HDCR is the most active biological or chemical catalyst for direct reduction of CO2 by H2 to produce formate. However, they should make it more clear that it is in fact the ONLY biological catalyst known to perform this reaction, as the formate-hydrogen lyase of E.coli operates mostly in formate oxidation.

Thank you for your comment, we rephrased the corresponding text in the introduction.

1.b Also, previous reports show that HDCR activity is limited by the activity of its formate dehydrogenase and this is in fact not higher than the activity of other isolated W-containing Fdh enzymes. So, the advantage of HDCR is to combine in a single protein the hydrogenase and formate dehydrogenase enzymes, but its catalytic activity is not that outstanding in biological terms.

We disagree with the reviewer's opinion that the activity of HDCR is not outstanding in biological terms. It is true that FdhF and HydA2 activities alone (with methyl viologen) are in the range of previously described similar enzymes. However, there is no other enzyme reported that converts CO_2 to formate with a turnover rate even close to HDCR. Therefore, the activity of the formate dehydrogenase FdhF (reducing CO_2 with H_2 as electron donor via HydA2) **is** by all means outstanding.

2. The stoichiometry of the enzyme is not clear. Previous reports suggested that the enzyme is a heterotetramer formed by HydA, HycB4, HycB3 and FdhF in a 1:1:1:1 arrangement, but this manuscript seems to indicate that there are 2 HydA/HycB4 dimers per FdhF/HycB3 unit. The subunit stoichiometry should be determined.

In previous studies, a 1:1:1:1 stoichiometry was assumed due to the genetic organization of the *hdcr* genes and the seemingly similar protein staining in an SDS gel (no signal quantification was performed). The structural analysis that we show here is a much more accurate method than quantification from an SDS gel and clearly shows that the intact HDCR complex has a stoichiometry of 1:1:2:2 (FdhF:HycB3:HycB4:HydA2). Therefore, we do not see the point of further studies on the stoichiometry, since it has already been precisely determined by our cryo-EM structure.

3. Line 102: what is a minimal HDCR filament? How many hexamers does it comprise?

We now elaborate that the short filaments are 3-4 repeating units, and longer bundled filaments were also observed in the *in vitro* preparations, although they are quite rare (about 1 every 30 micrographs; see **Extended Data Fig. 2**). *In vivo*, we observed that filaments have dozens of repeating subunits and bundle together to form large ring-shaped superstructures (**Fig. 5**). We have modified the text accordingly. Please also see comments to Referee #1 Point 1.

4. Lines 125-126: The authors do not seem aware that the catalytic mechanism of Fdhs is still under strong debate, namely regarding metal coordination of the substrate during turnover, the role of the metal sulfido ligand and whether the Cys/Sec ligand remains bound to the metal during turnover (see recent reviews on the topic). The present structure offers no insight into this topic, so it is not correct to say that the mechanism of FdhF is "in accordance with previously described enzymes" (lines 125-126).

Thank you for pointing out this omission. We added the sentence *"The precise catalytic mechanism of molybdenum- and tungsten-dependent formate dehydrogenases is still under debate and is widely discussed in the field*[35-39]*."* in the corresponding paragraph to make the reader aware of the ongoing and much-discussed research in this field. Additionally, we rephrased the text passage mentioned by the reviewer to "*the catalytic mechanism for FdhF*

seems to be in accordance with previously-described enzymes of this family[40]" in order to describe it in a more restrained way.

5. An important point is that the interactions allowing formation of the "nanowire" are not clear in the manuscript. From Fig. 1 and Fig.3 it appears that HycB4(1) and HycB4(2) form the core of the wire, with the HycB3/FdhF dimer protruding from it. However, only the interactions between HycB4(1) and HycB4(2) and between HycB4 (which?) and HycB3 are described, but not those allowing the repeating nature of the filament. We are missing the interaction site between one hexamer and the following one. If indeed HycB4 forms the core of the filament, as suggested by Figs. 1, 3a and 4, then this contradicts the conclusions on lines 196-198 that HycB3 is responsible for linking the hexamers together, whilst HycB4 is responsible for integrity of the hexameric complex.

Thank you for asking us to describe the interactions more clearly. We rephrased the corresponding paragraph describing the interactions of the Hyc-subunits as follows:

"HDCR oligomerization is mediated by long C-terminal α-helices in HycB3 (residues 160-184) and HycB4 (residues 190-210) (**Figs. 2B, 3A**). These helices are nested in a binding groove formed by the β3 and β4 anti-parallel β-sheets of the respective neighboring HycB subunit. This binding interface is maintained by hydrophobic interactions and H-bonds to the peptide backbone. HycB4 has an additional interaction surface (**Fig. 3A**), a loop insertion between β9 and β10 (residues 135-160) that latches onto the sheets of the second ferredoxin-like domain of the following HycB4 molecule in the filament. The corresponding loop in HycB3 is not involved in the oligomerization interface, instead binding exclusively to FdhF. These differences in the binding interfaces cause an uneven stoichiometry of HycB3-HycB4$_1$-HycB4$_2$ proteins that form the repeating core of the HDCR filament."

6. Several variants produced do not really provide additional evidence from that already available from the structure. It is obvious from the structure that if HycB4 is deleted then HydA will not be associated with the other proteins, or that if HycB3 is deleted then FdhF will not be associated. I do not really see the point of these experiments.

We decided to leave the corresponding figure panels (**Fig. 2C, D**) in the manuscript, as we feel it adds experimental rigor to further verify our structural model. However, we shortened the description in the result section as suggested to: "…we confirmed the interactions in the

minimal HDCR protomer by complementation experiments and pull-downs (**Fig. 2C**, detailed report in Extended Data File)."

7a) How do the authors know what is the composition of the proteins from the HDCR_ΔHelix HycB3 and HDCR_ΔHelix HycB4 variants? No evidence is provided and it seems they are guessing from the molecular mass, which is certainly not reliable. How can they be certain that HDCR_ΔHelix HycB4 is composed of a pair of HydA2-HycB4 dimers and HDCR_ΔHelix HycB3 of a hexamer?

Thank you for your comment. In the revised manuscript, we analyzed the elution fractions of all variants after size exclusion chromatography for their subunit composition. As the reader can now see in the new **Fig. 3B** in combination with the updated **Fig. 3C**, the elution fractions of HDCR_His, HDCR_HycB4Δ[4Fe4S] IV and HDCR_HycB3ΔC contain all four subunits of the enzyme, whereas HDCR_HycB4ΔC only contains HydA2 and HycB4 proteins. Additionally, we added a corresponding sentence with a description to **Fig. 3B** in the revised manuscript. The size of the respective protein complexes from **Fig. 3B** was calculated after calibration of the Superose 6 column using commercially available calibration proteins (Thyroglobulin, Ferritin, Aldolase, Conalbumin and Ovalbumin).

7b) Furthermore, how do they know if the protein integrity of the subunits present is still OK? The deletion of these helices may have unexpected impacts on the structure of the subunits in several ways. Without determining the structure of these variants we are looking at a black box, and it is not possible to make solid interpretations of their activity!!

We thank the referee for their concern that the reduced activity is not caused by the disruption of filamentation, but rather by partially disrupting the electron transfer within HDCR's repeating hexameric unit, e.g. caused by incompletely assembled proteins. We do not have enough protein to carry out structure determination of all the mutants, which would extend beyond the scope of this revision. However, there is solid evidence from our experiments that the proteins are intact and functional. First, our wild-type HDCR structure clearly shows that the C-terminal helices of HydB3 and HydB4 stick out alone from the core of the proteins, and thus their deletion should not affect [4Fe4S] cluster integrity (easy to see in **Fig. 2B**). Second, the HDCR mutants migrate at their predicted size with size-exclusion chromatography (**Fig. 3B**). HDCR_HydB3ΔC migrates as a minimal repeating hexamer unit (i.e., protomer) (**Fig. 3B** – purple), whereas HDCR_HydB4ΔC disrupts the protomer and forms a tetramer

(HydA2HydB4) (**Fig. 3B** – pink). The tetramer is inactive, as we disrupt the electron transfer between enzymatic partners HydA2 and FdhF (**Figs. 2D, 3D**). The minimal hexamer has this connection intact, and thus shows ~20% activity compared to the wild-type HDCR filament (**Fig. 3D**). If the proteins were disrupted, this activity would also be lost. Instead, our activity assay shows that detached protomers of HDCR have reduced activity compared to the intact filament. This conclusion is further supported by the reduction of activity when deleting [4Fe4S] cluster IV of HycB4 (**Fig. 3D**). Filamentation of HDCR_HydB4Δ[4Fe4S] IV is not disrupted, as indicated by size exclusion chromatography (**Fig. 3B**). Rather, activity is reduced to ~10% of wild-type HDCR when electron transfer between repeating units of the filament is blocked. This level of activity is similar to the ~20% activity we measured for the HDCR_HydB3ΔC single protomer unit.

7C. Do the numbers in Fig. 3c represent the molecular mass? They are missing units. If they are the molecular mass then the mass of HDCR_ΔHelix HycB3 is much higher than expected for a hexamer.

Thank you for pointing this out. The unit was indeed missing, and the labelling of the elution maxima was shifted. We made sure that the revised **Fig. 3B** now has the correct formatting.

7D. The activity for CO2 reduction from H2 should also be shown for these variants.

Thank you for pointing this out. We have now included the corresponding experiments in **Fig. 3D**.

8. Lines 214-216: When the cluster [4Fe4S] IV is deleted how can the authors be certain that electron transfer to the closest HydA subunit is retained? From Fig. 4a and 4b, it seems to me that this cluster is essential for electron transfer to both HydA subunits, so it is little surprising that activity is reduced by 95%.

We agree with the reviewer that the coloring of [4Fe4S] clusters in $HycB4_1$ in **Fig. 4A-B** was indeed difficult to recognize. Therefore, we recolored in this figure to make [4Fe4S] center localization and electron transfer routes easier to understand. In addition, we added a more detailed description of the electron transfer routes and the consequences of HycB4-[4Fe4S] IV deletion.

The purpose of **Fig. 4B** is to show that HycB4-[4Fe4S] IV is needed for electron transfer within the filament. In the original manuscript, the second HydA2 domain ($HydA2_1$) was oriented away to the background, so the remaining functional electron transfer route between FdhF and $HydA2_1$ was not visible in this view. In the revised manuscript, we added a second view to this panel with a 120° rotation of the filament.

9a. Lines 217-222: The comments in these lines are supposed to be the core of the manuscript finding (that activity increases with filamentation), but the evidence is certainly not sufficiently robust to conclude this, as no evidence is provided for the composition and integrity of the proteins present in the various variants, and the activity alone does not tell us much.

Thank you for your comment, which motivated us to improve our analysis. We hope the reviewer agrees that the revised **Fig. 3** now provides strong evidence for how electron transfer through the HDCR filament greatly enhances activity, while the new **Fig. 5** shows the relevance of HDCR filaments and bundled superstructures *in vivo*. As a control, we confirmed the protein composition of the HDCR variants by SDS-PAGE after gel filtration, as now shown in **Fig. 3C**. Furthermore, by assessing the activity of the individual HydA2 and FdhF enzymes using artificial electron acceptors, we showed that the enzymes of all purified HDCR complexes are active at wild-type levels (**Extended Data Fig. 7E,F**). Thus, all complexes used for the activity assays with the physiological partners have confirmed composition, show expected behavior in size exclusion chromatography, and have confirmed activity of the individual enzymes. These controls support the significance of our findings that the hexameric minimal repeating unit (HDCR_HydB3ΔC) has ~20% activity compared to wild-type filamentous HDCR, while blocking electron transport through the intact filament (HDCR_HydB4Δ[4Fe4S] IV) drops the activity to ~10% (**Fig. 3D**). We therefore conclude that filamentation and electric conductivity of HDCR are important to maximize its activity.

9b. Why do the authors say that the protein obtained in the HDCR_HycB4Δ[4Fe4S] IV variant has only four subunits, and that HDCR_ΔHelix HycB3 has more connected subunits? I do not see evidence for this.

It seems that there might be a misunderstanding. In HDCR_HycB4Δ[4Fe4S] IV, there are not only 4 subunits (filamentation is intact, as can be seen in **Fig. 3B**), but the [4Fe4S] cluster essential for electron transfer between neighboring HDCR protomers is disrupted. In contrast, HDCR_HycB3ΔC is a complex of 6 protein subunits including all 4 different HDCR proteins

(as can be seen in **Fig. 3C**) and a molecular mass consistent with our expectations of a hexamer (**Fig. 3B**). This hexamer is the minimal protomer unit of HDCR. The connections between the subunits within the HDCR_HycB3ΔC protomer are not modified. See also the response to Comment 7b, above.

9c. Also comparing HDCR_HycB4Δ[4Fe4S] IV and HDCR_ΔHelix HycB3 is misleading since in one the electron transfer is interrupted, while presumably not in the other. The authors are forcing a narrative for which there is not sufficient evidence.

Electron transfer is interrupted in both, but in different ways. In HDCR_HycB4Δ[4Fe4S] IV, filamentation is still intact, but electron transport between HDCR protomers is interrupted by deletion of an essential [4Fe4S] cluster. In HDCR_HycB3ΔC, electron transfer is interrupted by disruption of filamentation (hexameric HDCR protomers are still intact). See also the response to Comment 7b, above.

10. Furthermore, in a previous study on the relevance of the filamentous nature of the first reported HDCR (Ref. 30) experiments are described where it is shown that by depolymerizing the enzyme only about 30% of activity is lost versus a similarly treated control. This finding also does not support the present proposition that the role of filamentation is to increase activity.

We disagree with this statement, since the mentioned treatment of the *A. woodii* HDCR with $MgSO_4$ in Schuchmann *et al*[3] did indeed yield shortened filaments but did not result in *depolymerization* of HDCR. To clarify this contradiction, we wrote the following paragraph, which we present in the supplement due to space constraints:

“To date, there are two organisms identified to produce an HDCR, *T. kivui* and the closely related *Acetobacterium woodii*, and characterization of these enzymes showed that both HDCRs form large filaments[2,3]. In previous studies, filamentation of *A. woodii* HDCR was reported to be dependent on the presence of divalent cations, and in accordance with our findings, the filamentous form was the most active state of the enzyme[32]. While similar experiments with the *T. kivui* HDCR did not show a divalent cation dependency on filamentation, it must be noted that the shortened protomers reported in the study of Schuchmann *et al*.[32] showed an elution profile corresponding to a molecular mass of about 3500 kDa. This indicates that the reported enzyme still forms roughly a 14-mer (assuming the *A. woodii* HDCR forms hexameric protomers, as in *T. kivui*) or a 20-mer (tetrameric form of

the HDCR) and makes comparison of the depolymerized states of the two enzymes difficult. However, *A. woodii* HDCR in the 3500 kDa state showed a ~30% reduction in activity compared to a similarly treated control of the filamentous form (>5000 kDa), and therefore, is consistent with our findings of a stepwise reduction in HDCR activity, depending on the number of connected active centers."

11a. In conclusion, while this is a nice structure, allowing nice figures due to its repeating nature, I do not see sufficient evidence to conclude that the role of filamentation is to increase activity.

We respectfully disagree with the reviewer's opinion that the evidence is not convincing for the role of filamentation in maximizing HDCR enzymatic activity. In our revised manuscript, we show with detailed controls that both protein connectivity (see response to Comments 7b and 9a) and electric coupling (see response to Comments 9b,c) along the filament are important to maximize enzyme activity. In HDCR_HycB4Δ[4Fe4S] IV, filamentation is still intact (**Fig. 3B**), but electron transport between HDCR repeating units is interrupted by deletion of a required [4Fe4S] cluster. This is further supported by a phenotypic growth defect in the HDCR_HycB4Δ[4Fe4S] IV mutant compared to the wild type (**Extended Data Fig. 6A**). In summary, electron transport between repeating protomers of the HDCR filament greatly enhances enzymatic activity *in vitro*, and this enhanced activity of HDCR filaments supports robust cell growth *in vivo*.

11b. There could be a number of other reasons for why filamentation occurs.

We do not claim that filamentation of HDCR is only relevant for maximizing its catalytic activity, and therefore we agree it is valid to speculate about other possible functions of filamentation, especially in light of the supramolecular organization of HDCR filaments we observed with our new cryo-ET experiments. Therefore, we extended the discussion in the revised manuscript, integrating new results regarding the *in vivo* superstructure of the HDCR:

"The functional consequences of HDCR bundling and membrane connection remain areas for future investigation. This could serve a structural role, helping to stabilize filaments and perhaps facilitate the nucleation of new filaments. As the HDCR structure described in our study shows no clear membrane-binding domains, the mechanism of HDCR membrane anchoring is yet to be discovered. It will be fascinating to explore whether there is energetic coupling between HDCR electron transport and the cell's membrane potential, which could

modulate the function of HDCR as well as proteins embedded in the plasma membrane. It will also be important to investigate whether additional membrane-bound or soluble factors are enriched around the ring-shaped HDCR superstructures to form specialized metabolic subcompartments within the cell. Many acetogenic bacteria live in extreme environments that demand efficient capture of rare gaseous substrates such as H_2 and CO_2[50,51]. Perhaps the molecular connectivity and supramolecular architecture of HDCR help coordinate a hydrogen- and carbon-concentrating mechanism, enabling metabolism at the thermodynamic limit of life[50]."

11c. It is also not clear whether filamentation has physiological relevance and occurs in vivo. (Line 232)

Thank you for raising this concern, which motivated our new cryo-ET investigation (**Fig. 5**, **Movie 3**, **Extended Data Figs. 8, 9**). In the revised manuscript, we included extensive cryo-ET imaging and structural analysis of wild-type HDCR-producing *T. kivui* cells, as well as Δ*hdcr* mutant cells. By directly visualizing the native cellular environment with molecular resolution, we discovered that HDCR not only forms filaments *in vivo* but also bundles into huge ring-shaped superstructures composed of hundreds of HDCR filaments, and thus, thousands of [4Fe4S] clusters. We discuss the possible functional implications of these membrane-bound superstructures in the revised manuscript. In addition, the revised manuscript includes growth studies of wild-type, HDCR_His and HDCR_HycB4Δ[4Fe4S] IV strains, the latter of which produces filaments that cannot transfer electrons between repeating units (**Extended Data Fig. 6A**). The HDCR_HycB4Δ[4Fe4S] IV variant displays a strong growth defect, demonstrating the physiological relevance of electron transfer along the HDCR filaments *in vivo*.

1 Righetto, R. D. *et al.* High-resolution cryo-EM structure of urease from the pathogen Yersinia enterocolitica. *Nat. Commun.* **11,** 5101 (2020).

2 Kato, K. *et al.* High-resolution cryo-EM structure of photosystem II reveals damage from high-dose electron beams. *Commun. Biol.* **4,** 382 (2021).

3 Schuchmann, K., Vonck, J. & Müller, V. A bacterial hydrogen-dependent CO_2 reductase forms filamentous structures. *FEBS J.* **283,** 1311-1322 (2016).

Reviewer Reports on the First Revision:

Referees' comments:

Referee #2 (Remarks to the Author):

The authors have significantly improved the manuscript and addressed several of the points highlighted by the reviewers. The inclusion of the in-vivo imaging of HDCR filaments by cryo-ET is a very important addition that solves the question of whether filamentation is physiological and occurs in vivo. The new data raises a number of interesting questions for future studies, as pointed out in the manuscript.

However, despite the addition of new relevant information, I still have doubts about the experimental support for the theory that filamentation greatly increases activity. This is based essentially on two variants, the HDCR_HycB3ΔC and HDCR_HycB4Δ[4Fe4S] IV.

The deletion of a C-terminal helix in HycB3 (variant HDCR_HycB3ΔC), which is involved in the interaction with HycB4, was successful in preventing filamentation. However, whether an intact hexamer is present is still not fully demonstrated. In fact, the gel in Fig. 3c suggests that there is less FdhF relative to HydA compared to the WT enzyme, as the FdhF band is weaker than the HydA one, whereas the opposite should occur given their masses. The results shown in Extended Fig. 7e and f are not helpful since these data were obtained with cytoplasmic fractions where all enzymes are present, regardless of whether they are interacting or not. This activity data should be determined for the purified variants.
Since it was observed in vivo that the FdhF and HydA enzymes can detach from the filament, the apparently lower amount of FdhF raises the questions of whether the lower activity may come not from the presence of an hexamer but from other, less functional, forms of the enzyme. Also, although deletion of this helix may in theory not interfere with the electron transfer path, it can still disturb it due to conformational changes provoked by the deletion and/or absence of other subunits. This cannot be discarded with the present data. What is the effect of this deletion on growth? This is only shown for the HDCR_HycB4Δ[4Fe4S] IV variant but would also be interesting in this case.

For the HDCR_HycB4Δ[4Fe4S] IV variant high molecular mass filaments are still present as shown by gel filtration, but these are different from the WT enzyme, as the two elution profiles are clearly different (Fig. 3b), but again it is not clear what is the reason for this. As I stated previously, this deletion may also cause local structural variations that may impact electron transfer between HydA and FdhF, and so the assumption that electron transfer between these two proteins is not affected, but only that along the filament, is not well supported.

The loss of activity of HDCR_ HycB4ΔC is not conclusive since this mutation completely disrupts the subunits interaction and only HydA/HycB4 are present with no FdhF/HycB3.

In conclusion, I think the evidence with these variants is not conclusive in terms of saying that filamentation is essential for high activity. Having the structures of the HDCR_HycB3ΔC and

HDCR_HycB4Δ[4Fe4S] IV variants would help to clear these doubts. In alternative, the authors may consider to separate filaments of different sizes and measure their activity. This would have the advantage of using native forms of the enzyme, and thus preventing side effects that are difficult to avoid when deleting parts of the enzyme.

A final point regarding the effect of filamentation on activity is what do the authors think could be the molecular basis for the effect they are proposing? A reasonable explanation is not given. Storing electrons and temporally separating both reactions may play a role physiologically but does not explain why the activity would increase in vitro upon filamentation.

Additional point:
The discussion in lines 174-182 and Fig. 2c is still not clear. In the HycB4 deletion interaction with FdhF/HycB3 is lost and so only HydA is recovered if a tag is put on it. When a tag is put also on FdhF of course both HydA and fdhF are both purified but the two proteins will not be interacting!! This has to be clearly stated.

Referee #3 (Remarks to the Author):

I was asked to step in instead of Referee #1, and thus tried to critically assess the work from the perspective of the previous comments, most of which I agree with. All the comments have been adequately addressed. Although my background is structural biology of ribosomes and bioenergetics, I enjoyed reading this study very much. Not only that it aims the big question of CO2 capture and reduction, but also the data is explained in an intelligent way that is accessible for non-experts, yet without compromising the depth of the analysis. The descriptions of the complex results are concise and straight to the point, the illustrations are stunning, and the writing is engaging and generally inspiring.

1) In conclusions, maybe also add a sentence or two of how the study relates back to climate change mitigation, carbon capture? It would bring the ideas together that you discuss at the start.
2) In movies, labels for proteins would help for orientation.
3) Page 10, Line 243 “attached to the plasma membrane”.

Author Rebuttals to First Revision:

Reviewer #2 (comments to the authors):

The authors have significantly improved the manuscript and addressed several of the points highlighted by the reviewers. The inclusion of the in-vivo imaging of HDCR filaments by cryo-ET is a very important addition that solves the question of whether filamentation is physiological and occurs in vivo. The new data raises a number of interesting questions for future studies, as pointed out in the manuscript.

We thank the referee for recognizing our efforts and progress regarding the main criticisms in our initial submission.

However, despite the addition of new relevant information, I still have doubts about the experimental support for the theory that filamentation greatly increases activity. This is based essentially on two variants, the HDCR_HycB3ΔC and HDCR_HycB4Δ[4Fe4S] IV.

1. The deletion of a C-terminal helix in HycB3 (variant HDCR_HycB3ΔC), which is involved in the interaction with HycB4, was successful in preventing filamentation. However, whether an intact hexamer is present is still not fully demonstrated. In fact, the gel in Fig. 3c suggests that there is less FdhF relative to HydA compared to the WT enzyme, as the FdhF band is weaker than the HydA one, whereas the opposite should occur given their masses.

It is true that the protein ratio in filamentous HDCR variants (HDCR_His and HDCR_HycB4Δ[4Fe4S] IV) differs from those where filamentation is disrupted (HDCR_HycB3ΔC and HDCR_HycB4ΔC). However, it is not surprising that the FdhF protein staining is weaker than HydA2 in HDCR_HycB3ΔC, considering that this variant is unable to form filaments, so the sample contains the isolated hexameric HDCR complex with two HydA2 subunits and only one FdhF subunit. Therefore, a more intense staining of HydA2 is the logical consequence of this 2:1 stoichiometry.

In contrast to that, as we described in the manuscript and as the referee emphasizes in their comment #3, our cryo-ET observations suggest that the occupancy of the catalytic subunits on longer bundled HDCR filaments could be variable and might not follow the strict 2 HydA2 : 1 FdhF ratio discovered in our structure of a small filament fragment. Consistent with this idea, the protein ratio of FdhF and HydA2 in variants HDCR_His and HDCR_HycB4Δ[4Fe4S] IV is closer to 1:1, differing from the non-filamentous variants. We thank the referee for their helpful comment and have added this hypothesis to our revised text:

"while the central HycB3-HycB4 electron wire fits well into the density of the cryo-ET average (Fig. 5H), the peripheral densities corresponding to HydA2 and FdhF were present but not as well resolved (Fig. 5I). This could be due to variable pitch between different bundled filaments, which would blur peripheral densities in the average, or alternatively, it could indicate non-stoichiometric occupancy of HydA2 and FdhF along the filaments. The latter idea is consistent with the stoichiometries of HydA2:FdhF in high-molecular mass fractions of filamentous

HDCR (Fig. 3C-D), which differ from the 2:1 ratio seen in our cryo-EM structure of a completely occupied filament fragment."

Furthermore, the integrity of HDCR_HycB3ΔC as an intact hexamer is demonstrated in various experiments. The purified complex includes all four HDCR subunits (revised Fig. 3D). Within this complex, all catalytic sites show regular enzymatic activity separately (Extended Data Fig. 7E-F), whilst the coupling of both reactions is functional but impaired significantly compared to the WT enzyme (revised Fig. 3E). The complex has the apparent size of a hexamer (revised Fig. 3C), with one copy of FdhF and two copies of HydA2 explaining the uneven amounts of these proteins (revised Fig. 3D, discussed above). Therefore, all data indicate the presence of the intact hexameric HDCR repeating unit.

2. The results shown in Extended Fig. 7e and f are not helpful since these data were obtained with cytoplasmic fractions where all enzymes are present, regardless of whether they are interacting or not. This activity data should be determined for the purified variants.

We can understand the referee´s concerns regarding our choice to use cytoplasmic fractions for these measurements, since indeed all proteins of the complex are present in the reaction mixture. However, since the reaction monitors the direct electron transfer from H_2 to the artificial electron acceptor methylviologen (HydA2) or from formate to methylviologen (FdhF), the presence or absence of the other subunits does not affect these separate reactions (except FdhF needs HycB3 for its activity, as can be seen in Extended Data Fig. 7F). The aim of this experiment was to show that for all HDCR variants used in this study, HydA2 and FdhF proteins incorporate functional active centers regardless of interacting subunits, except if the active center itself was destroyed or missing by design. Therefore, there is no impact on the experiment´s outcome whether the reaction mixture includes cytoplasmic fractions or the purified proteins.

3. Since it was observed in vivo that the FdhF and HydA enzymes can detach from the filament, the apparently lower amount of FdhF raises the questions of whether the lower activity may come not from the presence of an hexamer but from other, less functional, forms of the enzyme.

The question of how bacteria regulate attachment or detachment of HydA2 and FdhF to and from HDCR filaments *in vivo* is indeed very fascinating and will be interesting to investigate in future studies.

We do agree with the reviewer that disruption of filamentation in HDCR may not only lead to interruption of the electron network but also to conformational changes that could decrease enzymatic activity. Therefore, in the revised manuscript we moderated our interpretations on the importance of the electron network and followed the reviewer´s suggestion to strengthen our focus more on the impact of filament length and putative protein-protein interactions for enzymatic activity (see our detailed reply to reviewer comment #4).

Nevertheless, the non-filamentous variant HDCR_HycB3ΔC mentioned by the reviewer provides important data, since it shows the catalytic properties of HDCR when filamentation is completely disabled. Whether the decreased activity is caused by disruption of the electron nanowire or from structural changes is indeed not to be answered with this mutant alone.

However, we still believe that disruption of the electron nanowire has a significant impact on enzymatic activity, since activity was severely impaired not only in the hexameric variant HDCR_HycB3ΔC but also in the filamentous HDCR_HycB4Δ[4Fe4S] IV, where the electrical connection of the HycB3-HycB4 nanowire was disrupted (revised Figs 3C-E, 4A-B). In our revised manuscript, we added *in vivo* data to confirm that the impaired but not abolished activity of both mutants result in partial rescue of the HDCR deletion mutant, with slower growth rates (revised Fig. 3B).

To provide a more well-rounded interpretation, in our revised manuscript we state that the reduced activity is most likely caused by a combination of structural changes and inhibition of rapid electron transport across the nanowire.

4. Also, although deletion of this helix may in theory not interfere with the electron transfer path, it can still disturb it due to conformational changes provoked by the deletion and/or absence of other subunits. This cannot be discarded with the present data.

We share the reviewer's beliefs that HDCR_HycB3ΔC differs from the wild type HDCR in terms of iron sulfur cluster orientation for the rapid transfer of electrons, however, we previously restrained from speculating on this. Our aim was to show that filamentation is important for the connectivity and hence activity of the complex. In the revised manuscript, we included a new discussion paragraph (see point #9 below) and updated figures that explain a potential mechanism for how filamentation increases activity of the complex. Our main proposal is that filamentation not only enables electronic conductivity along the filament, but also increases the stability of the protein-protein interactions. In the filament, protein interactions are tight, whereas the minimal active heterohexamer is likely flexible and less stable. Filament formation thus rigidifies the HycB backbone and locks the proteins in a conformation that is favorable for electron transport, therefore reducing the reorganization energy needed for electron transfer and enhancing the electronic coupling between [4Fe4S] clusters. In other words, distances between the [4Fe4S] clusters in the electron wire are kept constant, which allows for rapid and favorable electron flow that leads to coherent electron transfer. Our cryo-EM structure elucidated that these [4Fe4S] clusters are located at an average distance of ~10 Å (revised Fig. 2A), which provides rapid microsecond transfer rates[1]. As product formation mainly depends on the electron flow, higher rates increase the activity of the complex. Thus, rigidification by filamentation would provide a molecular basis for why filamentation greatly increase enzymatic activity.

This explanation elaborates our initial statement that filamentation has a stimulating effect on enzymatic activity of HDCR. When HDCR forms the natural filament, the enzyme enters its

most active state due to several reasons: rapid electron shuttling, alternate active sites, and possibly even building a scaffold for microcompartments of WLP metabolism that would "pull" the reaction by subsequent conversion of the HDCR reaction product. We hope the referee agrees that we have made these ideas clearer in the revised manuscript.

5. What is the effect of this deletion on growth? This is only shown for the HDCR_HycB4Δ[4Fe4S] IV variant but would also be interesting in this case.

We thank the reviewer for this helpful suggestion. The revised manuscript now also includes the growth phenotype of the HDCR_HycB3ΔC variant. We moved the analysis of growth comparison between HDCR variant strains differing in filament formation to the main figures (revised Fig. 3B) to emphasize the functional consequence of filamentation *in vivo* in the native cellular context without potentially harsh isolation procedures. As we describe in the text:

"Impairing filament formation and electron transfer not only reduced HDCR activity in vitro but also in vivo. The HDCR_HycB3ΔC and HDCR_HycB4Δ[4Fe4S] IV mutants partially rescued the Δhdcr strain phenotype but showed greatly impaired growth (doubling time 4.4 h) compared to wild-type (2.4 h) and HDCR_His complementation strains (2.6 h), even with glucose as growth substrate and electron donor for HDCR (Fig. 3B)."

6. For the HDCR_HycB4Δ[4Fe4S] IV variant high molecular mass filaments are still present as shown by gel filtration, but these are different from the WT enzyme, as the two elution profiles are clearly different (Fig. 3b), but again it is not clear what is the reason for this. As I stated previously, this deletion may also cause local structural variations that may impact electron transfer between HydA and FdhF, and so the assumption that electron transfer between these two proteins is not affected, but only that along the filament, is not well supported.

We recognize that filaments of WT and HDCR_HycB4Δ[4Fe4S] IV do not show the exact same elution profile by gel filtration, however it can be clearly seen that HDCR_HycB4Δ[4Fe4S] IV does form high molecular mass filaments, as the referee pointed out. Despite the differential abundance of large filaments between these two variants, the aim of this experiment was to show that, in HDCR, filamentation alone (and therefore a structural change of enzymatic conformation) does not lead to an increase of enzymatic activity. This sets HDCR apart from several published filamentous enzymes. Thus, there must be an additional reason for the increase in HDCR activity.

In fact, the generation of variant HDCR_HycB4Δ[4Fe4S] IV was intended to show that an impact in electron transfer between HydA2 and FdhF does have severe consequences for HDCR activity, more precisely in taking away the second HydA2 reaction partner for every single FdhF subunit in the filament, in addition to the disruption of electron transfer through the filament. We apologize for the misunderstanding, and we have clarified this by more clearly indicating the position of the mutation in Fig. 4A-B.

However, it is true that with the current data we cannot exclude (even if it is unlikely) an unforeseen change of the position of the other [4Fe4S] clusters in HycB4 that may also affect electron transfer between FdhF and HydA2$_1$. Therefore, we rephrased our message to focus on the statement that filamentation increases HDCR activity. To emphasize this hypothesis and avoid potential side effects that could arise from mutations, we followed the suggestion of the referee to analyze differently sized filaments of the complete HDCR. For a detailed description, please see our answer to reviewer comment #8, below.

7. The loss of activity of HDCR_ HycB4ΔC is not conclusive since this mutation completely disrupts the subunits interaction and only HydA/HycB4 are present with no FdhF/HycB3.

As the referee has rightly pointed out, and as we describe in the manuscript, the aim of the HDCR_HycB4ΔC variant was to show that the C-terminal helix of HycB4 is responsible for interaction of the protein pairs FdhF-HycB3 and HydA2-HycB4. Therefore, deletion of this helix completely disrupts the integrity of the HDCR complex. However, since we did not want to restrict our analysis concerning this finding on the structural analysis or optical assays such as the SDS gel (revised Fig. 3D), we included the activity assays for the sake of completeness. Considering that it underlines our statement on the importance of this helical peptide, we would keep the experiment in our manuscript.

8. In conclusion, I think the evidence with these variants is not conclusive in terms of saying that filamentation is essential for high activity. Having the structures of the HDCR_HycB3ΔC and HDCR_HycB4Δ[4Fe4S] IV variants would help to clear these doubts. In alternative, the authors may consider to separate filaments of different sizes and measure their activity. This would have the advantage of using native forms of the enzyme, and thus preventing side effects that are difficult to avoid when deleting parts of the enzyme.

Thank you for suggesting a complementary analysis of native HDCR to display the importance of filamentation for increased HDCR activity. Despite the already published findings that the level of HDCR activity is dependent on filamentation in *Acetobacterium woodii*[2], we followed the advice of the referee and performed analysis of the enzymatic activity of the complete HDCR from *T. kivui* after separating different filament sizes, from ~5000 kDa (void volume of the size exclusion) to ~800 kDa (three repeats of the hexameric HDCR complex). Here, no putatively destabilizing mutations are affecting the catalytic properties, which allows a reliable comparison of activity. In the revised manuscript, we clearly show that enzymatic activity is coupled to filament length, and that activity continuously decreases with shorter filaments (revised Fig. 3F, revised Extended Data Fig. 2E-F). As we described in the text:

"Furthermore, wild-type HDCR shows variations in filament size[32], enabling us to check the filamentation-activity relationship without mutagenesis. When we separated HDCR_His by gel

filtration, there was a clear correlation between filament size and activity, which decreased from 100 % to 50 % and 32 % (Fig. 3F).”

9. A final point regarding the effect of filamentation on activity is what do the authors think could be the molecular basis for the effect they are proposing? A reasonable explanation is not given. Storing electrons and temporally separating both reactions may play a role physiologically but does not explain why the activity would increase in vitro upon filamentation.

As we described in our answer to reviewer comment #4, our proposed molecular basis is the increase of stability and rigidity of the enzymes in the filament, in combination with the tight HycB filament core providing constantly close distances between [4Fe4S]-clusters and therefore increasing electron tunneling for rapid product formation. We have elaborated on this in our revised discussion:

“Besides the improved connectivity of the filament, the additional protein-protein interactions in the filament likely stabilize attachment of the peripherally-associated enzymes. In particular, the HycB3-FdhF subcomplex would have a very exposed position in the minimal repeating unit. Additionally, filament formation likely rigidifies the HycB backbone, locking this central nanowire in a conformation that is favorable for efficient electron transport, with constant distances between [4Fe4S] clusters. This may allow electrons to be transported over long distances to reduce a CO_2 molecule far from the H_2 oxidation site (Fig. 4C-D). The HDCR nanowire might store electrons, as previously described for non-enzymatic multicytochrome and multiheme proteins[44-46], allowing a spatial and temporal separation of the two reactions that helps maximize enzymatic activity. To the best of our knowledge, HDCR is the first example of multiple enzymes connected by an electron nanowire, a molecular architecture that has great potential for biotechnology applications.”

10. Additional point: The discussion in lines 174-182 and Fig. 2c is still not clear. In the HycB4 deletion interaction with FdhF/HycB3 is lost and so only HydA is recovered if a tag is put on it. When a tag is put also on FdhF of course both HydA and fdhF are both purified but the two proteins will not be interacting!! This has to be clearly stated.

We thank the referee for pointing out this misleading description. To provide an unambiguous description of the enzymatic activity of HDCR variants, we re-phrased the mentioned paragraph as follows:

“We next tested whether the integrity of the central filament affected enzymatic activity by producing variants devoid of either HycB3, HycB4 or HydA2 (Fig. 2C, Extended Data File). Proteins purified with HydA2-His_6 or His_6-FdhF were unable to produce formic acid from H_2 + CO_2 as well as H_2 from formic acid (Fig. 2D) but retained H_2:methylviologen and formate:methylviologen oxidoreductase activity, respectively (Extended Data Fig. 7E). Hydrogenase activity was independent of HycB3 and HycB4. When HydA2 was omitted from overexpression (HDCR_ΔHydA2) or the H-cluster coordinating Cys387 was substituted with

alanine (HDCR_HydA2 C387A), there was no hydrogenase activity. Formate dehydrogenase activity was marginally affected by removal of HycB4 and HydA2, but HycB3 was required for FdhF activity (Extended Data Fig. 7F).”

Furthermore, to clarify the interactions between co-purified proteins, we re-worded the corresponding paragraph in the Extended Data file as follows:

“Genetic complementation of the Δhdcr strain was used to probe the connectivity of the HDCR complex. When HycB4 was omitted from the overexpression (HDCR_ΔHycB4), only HydA2 was found in the eluate after purification with HydA2-His_6 (Fig. 2C). However, addition of a second His_6-tag to the HDCR_ΔHycB4 construct (HydA2-His_6 & His_6-FdhF) led to copurification of monomeric FdhF and the protein pair HycB3-HydA2, although there was no functional interaction between FdhF and HycB3-HydA2. This verifies HycB4 as the connecting subunit between HydA2 and the protein pair of FdhF and HycB3. When HycB3 was omitted from the overexpression (HDCRΔHycB3), HydA2-His_6 purification yielded a complex containing HydA2 and HycB4, but not FdhF. This proves that HycB3 is the subunit linking HycB4 to FdhF, revealing the chain of protein interactions to be FdhF-HycB3-HycB4-HydA2. This conclusion is in complete accordance with the cryo-EM structure (Fig. 1) and is also supported by the genetic organization of the hdcr operon, where the genes for the enzyme complex are arranged in the same order[88]*.”*

Reviewer #3 (comments to the authors):

I was asked to step in instead of Referee #1, and thus tried to critically assess the work from the perspective of the previous comments, most of which I agree with. All the comments have been adequately addressed. Although my background is structural biology of ribosomes and bioenergetics, I enjoyed reading this study very much. Not only that it aims the big question of CO2 capture and reduction, but also the data is explained in an intelligent way that is accessible for non-experts, yet without compromising the depth of the analysis. The descriptions of the complex results are concise and straight to the point, the illustrations are stunning, and the writing is engaging and generally inspiring.

We thank the referee for their encouraging comments on both the impact and accessible presentation of our study.

1) In conclusions, maybe also add a sentence or two of how the study relates back to climate change mitigation, carbon capture? It would bring the ideas together that you discuss at the start.

Great idea. We have now added two concluding sentences that bring these ideas together at the end of our discussion section:

"The unsurpassed catalytic activity of HDCR makes it a promising tool for H_2 storage and carbon capture[5,23,52-54], reactions that underly the production of renewable fuels and potentially even the development of negative emission technologies to combat climate change. Our study reveals the exquisite connectivity of HDCR filaments and bundles, providing a molecular blueprint for future bioengineering applications."

2) In movies, labels for proteins would help for orientation.

Thank you for the suggestion. We have added labels to the movies. In addition, we merged Movies 1 and 2 in a single, more informative movie (revised Movie 1). Movie 3 (now revised Movie 2) has also been remade to provide an even more compelling 3D tour of the HDCR filament bundles inside the cell.

3) Page 10, Line 243 "attached to the plasma membrane".

Fixed, thank you.

References:

1 Page, C. C., Moser, C. C., Chen, X. & Dutton, P. L. Natural engineering principles of electron tunnelling in biological oxidation–reduction. *Nature* **402**, 47-52 (1999).

2 Schuchmann, K., Vonck, J. & Müller, V. A bacterial hydrogen-dependent CO_2 reductase forms filamentous structures. *FEBS J.* **283**, 1311-1322 (2016).

Reviewer Reports on the Second Revision:

Referees' comments:

Referee #2 (Remarks to the Author):

I am happy with the changes made to the manuscript, which has much improved since the initial submission.