nature portfolio

Peer Review File

The role of Tyr34 in proton coupled electron transfer and product inhibition of manganese superoxide dismutase

Corresponding Author: Dr Gloria Borgstahl

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)
In this manuscript, the authors present neutron crystal structures of the Tyr34Phe mutant of Mn SOD in the reduced, oxidized and H2O2-inhibited states. In addition, EXAFS and XANES-HERFD measurements, as well as DFT calculations are performed. The authors interpret the H2O2-inhibited state as Mn(II)-OOH–. Moreover, they provide protonation states of the nearby Gln143, His30 and Tyr34 residues. The results are compared to previous neutron structures of the wild-type enzyme and the Trp161Phe variant. A suggested reaction mechanism is given in Fig. 8 and the role of Tyr34 is discussed.. In principle, the study is of great interest, providing detailed information about the oxidation state and protonation state of MnSOD in different intermediates.
However, the main problem with this and previous studies is the low resolution of the crystallographic data and the large variation in the structural data. The authors often observe conspicuous differences in the structures of the A and B subunit of the enzyme. This is assigned to differences in the solvent accessibility. A more natural interpretation is that this reflects the uncertainty of the experimental data. The authors in two of the three current crystal structures choses to discard the observed structures in the B subunit and they likewise ignore previous findings for one of the subunits. Table S3 shows that the ignored structures involve one extra OH ligand (in subunit B in the Mn(II)-HO2- structure in the current study but in the A subunit in the reduced WT structure) or a missing OH ligand in the oxidized structure in this study). Still, the authors trust other (nearby) structures. Likewise, the orientation and exact location of the D atoms seems to vary in a random manner. It is unclear how a user could know if a part of the structure is reliable or not. This is a very serious problem that needs to be thoroughly discussed. The authors should also investigate what happens if the two subunits are enforced to have the same structure and evaluate what impression this gives regarding the precision of the structure and how this relates to other estimates of the precision and accuracy of the structure.

Detailed points
1. The Gln143 anion is observed only in the B subunit of reduced MnSOD. Likewise, Tyr34 is protonated only in the B subunit of reduced MnSOD. How should this difference in the two subunits be interpreted? Is the A subunit an artefact?
2. What are the pKa of the superoxide radical and H2O2?
3. The discussion of the product-inhibited complex at the end of p 4 is hard to follow and understand. In particular, what is “the inhibited complex”? Is it Mn3+-H2O2 or Mn2+-H2O2 or something else? Does H2O2 coordinate to the metal or not? It should also be clarified if this section applies to the WT enzyme or to any mutant.
4. Fig 1c: Does oxidized MnSOD really contain a doubly deprotonated His30?
5. On p. 7, it is said that reduced MnSOD is five-coordinate, but the authors neutron structure showed a six-coordinate Mn site in one subunit.
6. The article is about Tyr34, but the first sections of the result section are about the H2O2 inhibited state. This was the theme of the authors latest publication. Why is this study needed again? The authors need to motivate this section better, introduce it in the concept of their previous study and describe how the results differ and complement the previous study.
7. What was the oxidation state of MnSOD before adding superoxide or peroxide?
8. L254: In what way is the density in subunit B hard to interpret? Why not Mn(III)–OH–, as expected? If subunit B cannot be clearly interpreted, how could we trust the results for subunit A? Do the two OH ions have full occupancy? How could D atoms in subunit B be exchanged with H? Was H present in the buffer? Why does this not happen also in subunit A?
9. L260: “dioxo” should be replaced by O22– (dioxo sounds more like neutral O2).

10. L263: Table 2 contains 3 DFT calculations. The authors should clarify that they suggest that the Mn(II)-O2H– calculations agree with the experimental data. Some motivation to this interpretation (e.g. mean absolute or maximum deviation to neutron or EXAFS data) should also be given. Currently, the suggestions of the authors are vague and the reader has to guess what they really mean.
11. The omit density in Fig 3c seems to be in between Wat1 and Q143. The authors should provide difference density plots with either D on Wat1 and on Q143 (in SI) and perhaps also with D on both Wat1 and Q143..
12. L295: “anionic phenolate” is this deprotonated Tyr34 (if so, write it explicitly)?
13. What does “isolated” in L358 mean?
14. Why should we trust the protonation states of His30 and Tyr166 in the B subunit, when the structure around the Mn ion was deemed uninterpretable.
15. It should be clarified that Fig 6 shows the two subunits of the enzyme.
16. Fig 8 is said to show the reaction mechanism, but O2 is missing in a and b. It must be included. In addition, associating and dissociating molecules should be indicated. The full reaction of each step could be shown to the left, not only the change in Mn oxidation state.
17. What dielectric constant was used in the CPCM calculations? What atomic radii? What solvent radius?

Reviewer #2

(Remarks to the Author)

The authors present a detailed analysis of a tyrosine reside that is near the unoccupied 6th coordinate position of the manganese metal center in mitochondrial manganese superoxide dismutase. This region has a water situated between His 30 and Tyr34 directly above the metal but the water does not appear to become coordinated to the metal. While not mentioned in this article (perhaps it should be), there was (is) considerable debate about a 5-6-5 coordination change during the mechanism. When Try34 is mutated to Phe (this article), the water appears to be capable of metal coordination. Here, the evidence chosen shows that the metal remains in a distorted penta-coordination state throughout the steps of the mechanism. Interestingly, a second molecule in the unit cell appears to be hexa-coordinate. The authors chose not to discuss this since it contained partial occupancies but the water does become coordinated. Without providing the proper commentary, the reader is led to surmise that one of the primary roles of Tyr34 is to keep this particular water from coordinating?

The authors disregarded the guidelines for formatting with the main text being twice as long as suggested. There are also double the amount of references suggested. The authors should consider being more concise and moving significant portions to supplemental. Overall the manuscript describes the findings well and the data justifies the finding in this study. A few difficulties were noted. First, old and new information were, at times, not clearly delineated. For example, Table 1 is not from this study. These rate constants were determined from pulse radiolysis which are much faster than diffusion limits seen in enzymatic kinetic analysis. This represents the rates of rearrangement after the Michalis complex has formed. A short summary in the main text with the table in the supplementary information would be better.

The role of Try34 in the context of new information is a bit mysterious. The reader is required to gather bits of evidence and draw conclusions on their own with a reveal on lines 573 and 591. The ending paragraphs (lines 640 – 656) finally present the new findings. This needs to be presented near the beginning to afford the reader the opportunity to judge the findings when presented. A revised manuscript should make clear statements about the findings and the importance of the findings in each section, clearly separating literature information from new information. For example, the presentation promotes a distorted penta-coordinated metal as the active site. However, the mechanism (lines 48-49, 107 – 111) show the use of the uncoordinated site. Instead, an inner sphere coordinated water bound to the metal is exchanged during substrate processing but that must be inferred from the figures. Figures 7 and 8 appear very late in the manuscript but should be figures 1 (8) and 2 (7). This is the primary insight gained from this work. Also, Figures 7 and 8 use ideal octahedral configurations but the text implies a distorted penta-coordination. Perhaps these figures could be used to show the extent of the distortion.

The QM and spectroscopy studies have significant merit. The text contains a lot of technical information that belongs in the supplemental. A discussion on how the QM calculations handled the distorted Mn coordination should be included.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
This is a very interesting manuscript. It addresses the very important question of how manganese superoxide dismutase controls the levels of reactive oxygen species and it includes discussions of intricate microscopic details of proton coupled

electron transfer (PCET) processes in nature. The ambition of the study, namely to find a possible mechanisms for how a key catalytic residue Tyr34 modulates the activity of manganese superoxide dismutase by modulating the PCET catalysis, is very bold. And, I at least am not disappointed by the results. We learn a lot, we learn how, in the vicinity of the Mn ion of a metalloenzyme, ligands interplaty to donate and accept protons, how charge imbalances are facilitated and how unwanted pathways are inhibited.

The authors present a very rigorous and exceptionally original analysis of data from a combination of nicely complementarey state-of-the-art methods (neutron crystallography for atomic structures, X-ray absorption spectroscopy for fine details of geometric and electronic structures of the vicinity of the Mn ion, quantum-chemical calculations for interpretation based on basics of molecular-orbital theory). The presentation is excellent. The manuscript is very written, with very clear and well chosen figures and very systematic and convincing arguments. The claims are very convincing, despite the fact that this is a very complex system.

The manuscript is full of convincing chains of arguments and many interesting relations to other systems and previously published results can be made. In terms of methods, this study could become a classic in how to best combine structural and electronic-structural insight to reveal the catalysis of metalloenzymes.

I cannot imagine how one could significantly improve this manuscirpt as I find it original, impactful, and very interesting as is. I can therefore with great certainty recommend publication in Nature Communications and irrespective of the outcome of this review process I want to congratulate the authors for their great achievement.

I have two minor comments and the authors may consider them in a potential revivision of their manuscript.

I thought that the introduction could be more concise. I understand that many aspects come in here for this complex system and complex catalysis. Still, maybe some of the results from previous investigations could be taken from the introduction and rather be placed into context with the results of the present study within the results and discussion section to shorten the introduction and enhance its accessibility.

In lines 181-183 I thought that citing references 57 and 77 to justify the claim that the measured pre-edge intensities indicate the presence of a distorted five-coordinate trigonal bipyramidal complex may be a bit short and hard to understand for a non-specialist readership. Adding 1 or 2 sentences about why that is the case may be helpful.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is the revised version of a study presenting neutron crystal structures, EXAFS and XANES measurements, as well as DFT calculations of the Tyr34Phe mutant of Mn SOD in the reduced, oxidized and H2O2-inhibited states. The article has been improved in many aspects following the suggestions by the reviewers and it can be published as it is now.

Reviewer #2

(Remarks to the Author)
A few minor comments/corrections:

PCET is misspelled a couple times as CPET (example: lines 113, 169, 174)

Figure 1: mentions that in order for solvent and substrate to enter the active site they must pass through gateway residues H30 and Y34. Proceed to compare the distances of nearby residues and water when Mn is oxidized and reduced. Does not mention or show the distances between H30 and Y34 under those two conditions. This is important as they are the gateway residues and seems to be a controlling factor of when things can come in or leave the active site. (closed or opened)

In figure 1d oxidized, is that water WAT2? Based on 1a it seems that it is, but a label there would be nice. Also, where does the water go in the 1d reduced?

Discussion of protonation state of Tyr34 in lines 70 and 71 need to be referenced to Fig. 1d

Line 114: Tyr34 protonates the dioxygen species twice to produce H2O2?

Line 122 is the first mention of Trp161 having any catalytic importance. Line 56 states Trp161 stabilizes the nearby hydrogen bonds. Comparing Trp161Phe to Tyr34Phe feels like apple to oranges. Also, what was the “similar kinetics” of the two.

Figure 2: overlay the two XANES regions of the superoxide and peroxide soaked since you are directly comparing them. (lines 206-207)

Line 286, 304, 309, 448, 453: use of the word “ablated” is not right in this context. Abrogated? Diminished?

Lines 293-294. Sentence about the anionic deprotonated Tyr34 is missing is not needed when talking about Tyr34Phe. (its mutated, we know it’s not there)

Lines 291-298: The structure was identical to wildtype but are still trying to use it in their favor by bringing in kinetics data

Line 299 states “The neutron structures of reduced and oxidized Tyr34Phe Mn2+SOD…. ” but the information is only from reduced compared to WT (not oxidized mutant).

Lines 331-361: Data from XANES in the HERFD mode should be presented here and not in the supplementary data. Since it is an improvement in energy resolution and sensitivity and presents the same results as the XANES data did earlier in the paper. Combine the two sections and reference then XANES data in the supplementary.

Lines 363-377: A casual reader is going to have a very hard time following and understanding this paragraph. Feels like a large step up from previous writing with limited explanation of the new complex topics.

Put bond distances on Fig 5

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

REVIEWER COMMENTS

We thank the reviewers for their positive remarks and helpful feedback to further strengthen the manuscript! The reviewers also found the work to be of high impact saying - “In terms of methods, this study could become a classic in how to best combine structural and electronic-structural insight to reveal the catalysis of metalloenzymes”

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors present neutron crystal structures of the Tyr34Phe mutant of Mn SOD in the reduced, oxidized and H2O2-inhibited states. In addition, EXAFS and XANES-HERFD measurements, as well as DFT calculations are performed. The authors interpret the H2O2-inhibited state as Mn(II)-OOH–. Moreover, they provide protonation states of the nearby Gln143, His30 and Tyr34 residues. The results are compared to previous neutron structures of the wild-type enzyme and the Trp161Phe variant. A suggested reaction mechanism is given in Fig. 8 and the role of Tyr34 is discussed. In principle, the study is of great interest, providing detailed information about the oxidation state and protonation state of MnSOD in different intermediates.

We thank the reviewer for their enthusiasm for the detailed information this study provides.

However, the main problem with this and previous studies is the low resolution of the crystallographic data and the large variation in the structural data. The authors often observe conspicuous differences in the structures of the A and B subunit of the enzyme. This is assigned to differences in the solvent accessibility. A more natural interpretation is that this reflects the uncertainty of the experimental data. The authors in two of the three current crystal structures choses to discard the observed structures in the B subunit and they likewise ignore previous findings for one of the subunits. Table S3 shows that the ignored structures involve one extra OH ligand (in subunit B in the Mn(II)-HO2- structure in the current study but in the A subunit in the reduced WT structure) or a missing OH ligand in the oxidized structure in this study). Still, the authors trust other (nearby) structures.

Yes, in crystal structures, there are differences between the individual subunits that make up the dimer in the asymmetric unit. For this crystallographic form of MnSOD, in some of our structures the chains are similar and in others they have different ligands bound to the active site metal. This is not a data quality issue as even in higher resolution (better than 1.8 Å) X-ray structures of MnSOD showed differences between the active sites (PDB 3K9S[1], 1.55 Å; PDB 5T30[2], 1.77 Å). We added a comment on this page 5 lines 139-141. The solvent accessibility differences between the chains of the asymmetric unit were illustrated in Supplementary Figure 4 in our 2021 publication[4].

Our interpretation of the structural data for mechanistic insight was guided by the matching X-ray spectroscopy data. These data indicate five-coordinate complexes rather than the six-coordinate complexes seen in the B subunit because our X-ray spectroscopy data indicate homogenous five-coordinate complexes – from two different methods (HERFD-XANES and EXAFS Fig. 2). These items are noted in Table S3. In fact, one of the reasons we invested large efforts into pursuing X-ray spectroscopy was to delineate the coordination of the Mn ion. In these solution studies, the subunits are identical.

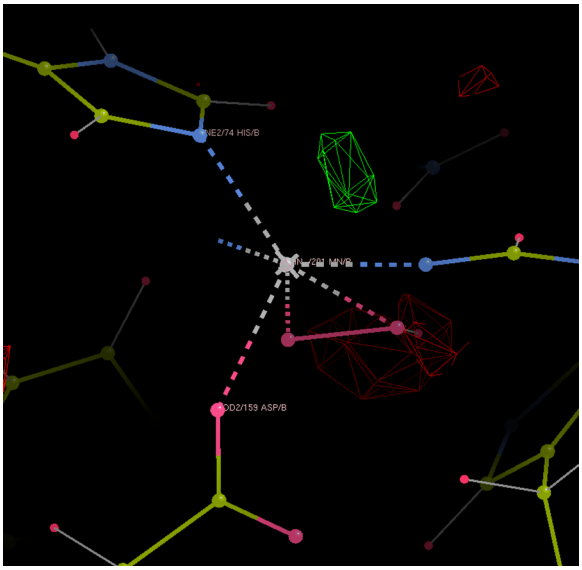
It should also be noted that the Chain A structures for the peroxide-soaked and reduced counterparts are like those seen in Trp161Phe MnSOD (Fig. 4 from our previous publication)[3]. This is thoroughly discussed on pages10-14 in the supplementary information.

Likewise, the orientation and exact location of the D atoms seems to vary in a random manner. It is unclear how a user could know if a part of the structure is reliable or not. This is a very serious problem that needs to be thoroughly discussed.

Unlike with other methods (for example, cryoEM), crystal structures have the same resolution throughout. The exterior of the enzyme has more motion, but the interior is very stable, with low B values, in the MnSOD structures. The locations of the D atoms in the active site were placed very carefully using Fo-Fc omit density after the rest of the structure had been solved. This is described in the Crystallographic Data Processing and

Refinement methods section and summarized on Page 10 lines 239-240. In neutron crystallography, the protons can be seen when the resolution of the data is better than 2.5 Å.

The authors should also investigate what happens if the two subunits are enforced to have the same structure and evaluate what impression this gives regarding the precision of the structure and how this relates to other estimates of the precision and accuracy of the structure.



Good idea! We put the peroxide from chain A into chain B by super position, deleted the water ligands in chain B and refined the structure with both subunits having identical ligand structures. The Fo-Fc map is shown here. There is green density indicating that OL (the ligand opposite of Asp 159; see supplementary Figure 1b) should be modeled. Also, there is red density, showing that it is inappropriate to model a peroxide in the Wat2 position. This refinement supports our interpretation that the two subunits in the crystal structure do not have the same ligand structure. We put a sentence about this on page 26-27 lines 622-625 in the Crystallographic Data Processing and Refinement methods section.

Detailed points

1. The Gln143 anion is observed only in the B subunit of reduced MnSOD. Likewise, Tyr34 is protonated only in the B subunit of reduced MnSOD. How should this difference in the two subunits be interpreted? Is the A subunit an artefact?

We observed these differences in our recent publication[3] about a different MnSOD variant W161F (see supplementary Figure 2 in that publication). We observed the same sort of differences in Y34F. As described above, this seems to be the nature of MnSOD crystals not an artefact.

2. What are the pKa of the superoxide radical and H2O2?

The pKa for Superoxide is 4.89, and for Peroxide, it is 11.6. So, at physiological pH, superoxide exists primarily in its anionic form ($O_2^{\bullet -}$) and hydrogen peroxide exists in its neutral form (H_2O_2) when free in solution.

3. The discussion of the product-inhibited complex at the end of p 4 is hard to follow and understand. In particular, what is “the inhibited complex”? Is it Mn3+-H2O2 or Mn2+-H2O2 or something else? Does H2O2 coordinate to the metal or not? It should also be clarified if this section applies to the WT enzyme or to any mutant.

All these questions were unknown before this study and the previous study on W161F. Some mutants (Y34F and W161F) predominantly use the product-inhibited pathway. Whether or not these variants have the same product-inhibited structure as each other is part of this study. Our X-ray spectroscopy studies suggest that the three forms of MnSOD are very similar (Supplementary Fig. 3d). It is probable that the variants have a similar product-inhibited structure with wild-type but the neutron structure for wild-type is future work. We have rewritten this section for clarity (page 4 line 98). We summarize the similarities between Y34F peroxide and W161F peroxide on page 15 lines 352-358. The technical discussion of these data are included in the supplementary Information pages10-14 where the spectroscopic similarities are discussed and structural comparison to W is made.

4. Fig 1c: Does oxidized MnSOD really contain a doubly deprotonated His30?

Yes. We have observed a doubly deprotonated His30 in wildtype MnSOD (See Fig. 5d in our previous publication[4]). His 30 is in an interesting proton shuttle involving Tyr166 and His30 and Wat2. Protons are shared between these residues. We have not observed this doubly deprotonated His30 in either variant.

5. On p. 7, it is said that reduced MnSOD is five-coordinate, but the authors neutron structure showed a six-coordinate Mn site in one subunit.

There is a bit of confusion here. Let me try to clarify. In this paper, chain A and B were both solved with a five-coordinate active site for the reduced MnSOD (see Supplementary Fig 1cd). It was chain B of the peroxide-soak that was six-coordinate (see Supplementary Fig 1b). Again, we attribute this difference in chains to the nature of the MnSOD asymmetric unit in this crystal form. It does not match the in-solution X-ray absorption spectroscopy (XAS) data. So, we reported this chain in the supplement to avoid distracting from the paper's main points. In fact, in solution X-ray absorption spectroscopy (XAS), a five-coordinate active site for all states of MnSOD was measured (oxidized, reduced, peroxide-treated).

We understand that with so many crystal structures and the variation we sometimes see in the ASU due to the crystal lattice, it can be confusing, and we have tried to clarify this throughout the manuscript and label our figures carefully.

6. The article is about Tyr34, but the first sections of the result section are about the H2O2 inhibited state. This was the theme of the authors latest publication. Why is this study needed again? The authors need to motivate this section better, introduce it in the concept of their previous study and describe how the results differ and complement the previous study.

We realized from this comment that this section was not written logically, and we rewrote the subtitle for this section on page 7, line 167, and revised the section. We also rewrote the title of the manuscript to better reflect the topics this study answered. In our previous study[4], we found that Tyr34 was structurally part of the product-inhibited state. The Tyr34Phe mutation disrupts the binding of peroxide near Tyr34 but does not prevent binding at the Mn. By describing the XAS results first, it clarifies our interpretation of the neutron structures that come in the next section. Tyr34 is an important residue involved in the CPET enzymatic mechanism as well as product-inhibition.

7. What was the oxidation state of MnSOD before adding superoxide or peroxide?

Trivalent. This information is on page 25 lines 581-582 in the methods section. These sentences were rewritten for clarity.

8. L254: In what way is the density in subunit B hard to interpret? Why not Mn(III)–OH–, as expected? If subunit B cannot be clearly interpreted, how could we trust the results for subunit A? Do the two OH ions have full occupancy? How could D atoms in subunit B be exchanged with H? Was H present in the buffer? Why does this not happen also in subunit A?

This sentence could be misinterpreted from these comments, so we rewrote it. What we meant was that it was difficult to interpret in the context of XAS data and was a result of the special nature of the Asymmetric Unit and the crystal lattice in this crystal form. We did not mean that the nuclear density was difficult to interpret. We rewrote it on page 10 line 252-254.

9. L260: “dioxo” should be replaced by O22– (dioxo sounds more like neutral O2).

Sure! Fixed this.

10. L263: Table 2 contains 3 DFT calculations. The authors should clarify that they suggest that the Mn(II)-O2H– calculations agree with the experimental data. Some motivation to this interpretation (e.g. mean absolute or maximum deviation to neutron or EXAFS data) should also be given. Currently, the suggestions of the authors are vague and the reader has to guess what they really mean.

We agree. We rewrote this part and put the absolute value of the difference between the neutron structure bond lengths and the DFT calculations in parenthesis in Table 2 (now Table 1 when renumbered). See page lines 261-265.

11. The omit density in Fig 3c seems to be in between Wat1 and Q143. The authors should provide difference density plots with either D on Wat1 and on Q143 (in SI) and perhaps also with D on both Wat1 and Q143.

We believe the omit density in Fig 3c looks like that in part because of the figure orientation. Also, the reduced Y34FMnSOD neutron diffraction data is of lower resolution (2.5 Å) compared to the other two neutron datasets

(2.3 Å). This was the best we could do with the beamtime we had in 2020 during the pandemic. We had to ship the crystal, rather than hand carry on an airplane and it did not diffract as well as usual. In the refined structure for chain A there is no residual Fo-Fc density that would suggest the determined structure in Fig 3c is incorrect. This is the best interpretation we have for this data. Neutron beamtime is in high demand and we won't be able to remeasure these data. We put a note in the figure legend for Figure 3 and Supplementary Figure 1 pointing out that this structure had the lowest resolution of the set of neutron structures.

We can't do the last thing the reviewer asks us to do because there isn't enough space for a D on both Wat1 and Q143.

12. L295: "anionic phenolate" is this deprotonated Tyr34 (if so, write it explicitly)?

Done

13. What does "isolated" in L358 mean?

We meant cryotrapped. Fixed this.

14. Why should we trust the protonation states of His30 and Tyr166 in the B subunit, when the structure around the Mn ion was deemed uninterpretable.

Wildtype enzyme has similar protonation states.

15. It should be clarified that Fig 6 shows the two subunits of the enzyme.

Sure! We modified the figure title (now Fig. 4) to be "Protonation states of Tyr166 and His30 in Tyr34Phe MnSOD between adjacent subunits across the dimeric protein-protein interface".

16. Fig 8 is said to show the reaction mechanism, but O2 is missing in a and b. It must be included. In addition, associating and dissociating molecules should be indicated. The full reaction of each step could be shown to the left, not only the change in Mn oxidation state.

We do not have structural data for the binding of superoxide yet, so we do not want to bias the mechanism and just indicated that superoxide binding gives electrons to the active site metal in part a with a thick arrow. This is how we handled this complication in our 2021 publication[4] (please see Fig 7b in that publication). We added a comment on this in the figure legend. We put the full reaction for each step as requested. Good idea! We think that this indicates the associating and dissociating molecules very well. We don't want to add them to the right side of the figure as it would further clutter it. We put a description in the figure legend that the associating and dissociating molecules are indicated by the reaction on the left.

17. What dielectric constant was used in the CPCM calculations? What atomic radii? What solvent radius?

The dielectric constant was 80.4. The atomic radii was 2.04 Å for Carbon, 1.86 Å for Nitrogen, 1.32 Å for Hydrogen, 1.82 Å for Oxygen, and 2.40 Å for Manganese. The solvent probe radius was 1.3 Å. We added this list of parameters to the methods section page 28 lines 660-662.

Reviewer #2 (Remarks to the Author):

The authors present a detailed analysis of a tyrosine reside that is near the unoccupied 6th coordinate position of the manganese metal center in mitochondrial manganese superoxide dismutase. This region has a water situated between His 30 and Tyr34 directly above the metal but the water does not appear to become coordinated to the metal. While not mentioned in this article (perhaps it should be), there was (is) considerable debate about a 5-6-5 coordination change during the mechanism. When Try34 is mutated to Phe (this article), the water appears to be capable of metal coordination. Here, the evidence chosen shows that the metal remains in a distorted penta-coordination state throughout the steps of the mechanism. Interestingly, a second molecule in the unit cell appears to be hexa-coordinate. The authors chose not to discuss this since it contained partial occupancies but the water does become coordinated. Without providing the proper commentary, the reader is led to surmise that one of the primary roles of Tyr34 is to keep this particular water from coordinating?

We are well aware of the 5-6-5 debate in the field. In this study, the XAS data showed five-coordinate active sites for all samples. This indicates that the partially occupied 6-coordinate site in chain B is merely a result of the challenges of this crystal form and crystal contact effects on chain B. Perhaps our future studies with superoxide ligand will provide resolution of the 5-6-5 debate.

The authors disregarded the guidelines for formatting with the main text being twice as long as suggested. There are also double the amount of references suggested. The authors should consider being more concise and moving significant portions to supplemental. Overall the manuscript describes the findings well and the data justifies the finding in this study. A few difficulties were noted. First, old and new information were, at times, not clearly delineated. For example, Table 1 is not from this study. These rate constants were determined from pulse radiolysis which are much faster than diffusion limits seen in enzymatic kinetic analysis. This represents the rates of rearrangement after the Michalis complex has formed. A short summary in the main text with the table in the supplementary information would be better.

We have done our best to shorten the text and to clarify it. This is a complex system and set of experiments. We have moved Table 1 to the supplement as suggested. We have referenced the papers we pulled the data from. We have also dramatically shortened the manuscript by moving two sections and two figures to the supplement and only include a short summary in the main text. We feel this leads to a more easily understood manuscript. Thank you for these suggestions. We have trimmed the number of references cited as much as we could (reduced from 119 to 106), but this research stands on the shoulders of a wealth of research and we felt this previous work should be properly referenced.

The role of Try34 in the context of new information is a bit mysterious. The reader is required to gather bits of evidence and draw conclusions on their own with a reveal on lines 573 and 591. The ending paragraphs (lines 640 – 656) finally present the new findings. This needs to be presented near the beginning to afford the reader the opportunity to judge the findings when presented. A revised manuscript should make clear statements about the findings and the importance of the findings in each section, clearly separating literature information from new information. For example, the presentation promotes a distorted penta-coordinated metal as the active site. However, the mechanism (lines 48-49, 107 – 111) show the use of the uncoordinated site. Instead, an inner sphere coordinated water bound to the metal is exchanged during substrate processing but that must be inferred from the figures. Figures 7 and 8 appear very late in the manuscript but should be figures 1 (8) and 2 (7). This is the primary insight gained from this work. Also, Figures 7 and 8 use ideal octahedral configurations but the text implies a distorted penta-coordination. Perhaps these figures could be used to show the extent of the distortion.

These initial mechanistic equations do not have structural evidence. We feel that it is inappropriate to assume that the uncoordinated site being used just from looking at the equations. Whether or not the 6th site is used comes from the structures not the equations. These equations came from previous publications that are cited.

Figures 7 and 8 (now Figures 5 and 6) come at the end because they are the summary of our results. We build our case for the summary of our results before introducing the mechanism. Also, Reviewer 4 thought the manuscript was clear and appreciated our flow of logic. We are trying to be unbiased in deciphering the role of Tyr34. So, we would like to keep the figures in their current order. Also, the configuration in the figures is not meant to be taken literally and were made this way for clarity and the needed space for labeling.

As an alternative, at end of introduction (page 5 lines 145-148), we put a brief summary of our main findings in this study and their biological importance.

The QM and spectroscopy studies have significant merit. The text contains a lot of technical information that belongs in the supplemental. A discussion on how the QM calculations handled the distorted Mn coordination should be included.

We have moved two sections of technical analysis to the supplementary information (see pages 10-19 in the supplement). How the QM calculations were performed is described in the methods section lines 653-662 and are similar to our previous publication.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4 (Remarks to the Author):

This is a very interesting manuscript. It addresses the very important question of how manganese superoxide dismutase controls the levels of reactive oxygen species and it includes discussions of intricate microscopic details of proton coupled electron transfer (PCET) processes in nature. The ambition of the study, namely to find a possible mechanisms for how a key catalytic residue Tyr34 modulates the activity of manganese superoxide dismutase by modulating the PCET catalysis, is very bold. And, I at least am not disappointed by the results. We learn a lot, we learn how, in the vicinity of the Mn ion of a metalloenzyme, ligands interplay to donate and accept protons, how charge imbalances are facilitated and how unwanted pathways are inhibited.

Thank you for your enthusiastic appreciation of the work!

The authors present a very rigorous and exceptionally original analysis of data from a combination of nicely complementary state-of-the-art methods (neutron crystallography for atomic structures, X-ray absorption spectroscopy for fine details of geometric and electronic structures of the vicinity of the Mn ion, quantum-chemical calculations for interpretation based on basics of molecular-orbital theory). The presentation is excellent. The manuscript is very written, with very clear and well chosen figures and very systematic and convincing arguments. The claims are very convincing, despite the fact that this is a very complex system.

The manuscript is full of convincing chains of arguments and many interesting relations to other systems and previously published results can be made. In terms of methods, this study could become a classic in how to best combine structural and electronic-structural insight to reveal the catalysis of metalloenzymes.

Thank you for your appreciation of the presentation and the logic of our arguments!

I cannot imagine how one could significantly improve this manuscirpt as I find it original, impactful, and very interesting as is. I can therefore with great certainty recommend publication in Nature Communications and irrespective of the outcome of this review process I want to congratulate the authors for their great achievement.

I have two minor comments and the authors may consider them in a potential revision of their manuscript.

I thought that the introduction could be more concise. I understand that many aspects come in here for this complex system and complex catalysis. Still, maybe some of the results from previous investigations could be taken from the introduction and rather be placed into context with the results of the present study within the results and discussion section to shorten the introduction and enhance its accessibility.

We appreciate this idea and moved some of the text as suggested. We also worked to make the introduction more concise.

In lines 181-183 I thought that citing references 57 and 77 to justify the claim that the measured pre-edge intensities indicate the presence of a distorted five-coordinate trigonal bipyramidal complex may be a bit short and hard to understand for a non-specialist readership. Adding 1 or 2 sentences about why that is the case may be helpful.

We have added the suggested sentences on Page 7-8 lines187-192.

Response to Reviewers References

1 Porta, J., Vahedi-Faridi, A. & Borgstahl, G. E. O. Structural analysis of peroxide-soaked MnSOD crystals reveals side-on binding of peroxide to active-site manganese. *J. Mol. Biol.* **399**, 377-384, (2010).

2 Azadmanesh, J., Trickel, S. R. & Borgstahl, G. E. O. Substrate-analog binding and electrostatic surfaces of human manganese superoxide dismutase. *J. Struct. Biol.* **199**, 68-75, (2017).

3 Azadmanesh, J. *et al.* Revealing the atomic and electronic mechanism of human manganese superoxide dismutase product inhibition. *Nat Commun* **15**, 5973, (2024).

4 Azadmanesh, J., Lutz, W. E., Coates, L., Weiss, K. L. & Borgstahl, G. E. O. Direct detection of coupled proton and electron transfers in human manganese superoxide dismutase. *Nat Commun* **12**, 2079, (2021).

We thank the reviewers for helpful feedback to further strengthen the manuscript! Below is our point-by-point response/corrections to the remaining reviewer comments. We have highlighted the places modified in yellow in the marked up copy we uploaded.

Reviewer #1 (Remarks to the Author):

This is the revised version of a study presenting neutron crystal structures, EXAFS and XANES measurements, as well as DFT calculations of the Tyr34Phe mutant of Mn SOD in the reduced, oxidized and H2O2-inhibited states. The article has been improved in many aspects following the suggestions by the reviewers and it can be published as it is now.

Thank you

Reviewer #2&#3 (Remarks to the Author):

Reviewers 2 and 3 have expressed their concern in the confidential comments to the editor that the debate about a 5-6-5 coordination change during the mechanism has not been properly discussed. Please amend this.

In the introduction we added discussion of the 5-6-5 coordination in the paragraph that discusses what is known or hypothesized about superoxide binding, lines 78-80 and added a citation for the study by Lah et al in 1995.

A few minor comments/corrections:
PCET is misspelled a couple times as CPET (example: lines 113, 169, 174)
Thank you for catching this. Fixed

Figure 1: mentions that in order for solvent and substrate to enter the active site they must pass through gateway residues H30 and Y34. Proceed to compare the distances of nearby residues and water when Mn is oxidized and reduced. Does not mention or show the distances between H30 and Y34 under those two conditions. This is important as they are the gateway residues and seems to be a controlling factor of when things can come in or leave the active site. (closed or opened).

The distance between the gateway residues is 5.4 Å and this was added in the text line 55-56 and the Fig. 1 figure legend lines 155-156.

In figure 1d oxidized, is that water WAT2? Based on 1a it seems that it is, but a label there would be nice. Also, where does the water go in the 1d reduced?

We labeled it WAT2 as this water is positioned between His30 and Tyr34. For the reduced structure, Y34 is protonated and there is not a water within H bond distance. We added this to Fig. 1 legend lines 163-164.

Discussion of protonation state of Tyr34 in lines 70 and 71 need to be referenced to Fig. 1d
Done

Line 114: Tyr34 protonates the dioxygen species twice to produce H2O2?
Thank you for catching this, it is thought to provide one of the protons. We rewrote that sentence on what is now line 116 to avoid this kind of confusion!

Line 122 is the first mention of Trp161 having any catalytic importance. Line 56 states Trp161 stabilizes the nearby hydrogen bonds. Comparing Trp161Phe to Tyr34Phe feels like apple to

oranges. Also, what was the “similar kinetics” of the two.
Our 2024 paper studied product inhibition using the Trp161Phe variant and this sentence was to convey the rationale, differences and similarities of the work. We expanded the discussion on lines 124-126.

Figure 2: overlay the two XANES regions of the superoxide and peroxide soaked since you are directly comparing them. (lines 206-207)
Done. Figure 2 is now a four-part figure with the addition of the overlay.

Line 286, 304, 309, 448, 453: use of the word “ablated” is not right in this context. Abrogated? Diminished?
Agreed, we replaced “ablated” with “greatly diminished”, “dramatically reduced”, “greatly diminished”, “very low”, “diminished”, and “nearly extinguished” in these places.

Lines 293-294. Sentence about the anionic deprotonated Tyr34 is missing is not needed when talking about Tyr34Phe. (its mutated, we know it’s not there)
OK, We deleted this sentence and rewrote the next sentence (lines 298-299).

Lines 291-298: The structure was identical to wildtype but are still trying to use it in their favor by bringing in kinetics data
I think what the reviewer is trying to say is that if the active site wasn’t nearly identical with wildtype then taking advantage of the mutant kinetics data to capture the product inhibited state would be invalid and interpreting the product inhibited structure of the mutant would not apply to the wildtype mechanism. So, We added the follow to the end of this paragraph on lines 302-304.

“Because the active sites are practically identical the use of Tyr34Phe MnSOD and its handy kinetic data to study the product inhibited state is valid and can help interpret the wildtype mechanism.”

Line 299 states “The neutron structures of reduced and oxidized Tyr34Phe Mn2+SOD…. ” but the information is only from reduced compared to WT (not oxidized mutant).
Not true, we analyzed both the reduced and oxidized neutron structures in the section.

Lines 331-361: Data from XANES in the HERFD mode should be presented here and not in the supplementary data. Since it is an improvement in energy resolution and sensitivity and presents the same results as the XANES data did earlier in the paper. Combine the two sections and reference then XANES data in the supplementary.
Sorry but there is a small mistake in the figure legend for Figure 2. These are also HERFD data!
Sorry for the confusion. We have edited the figure legend title to accurately reflect this high resolution methods. The HERFD-XANES data in the supplement were just measured on a different day.

Lines 363-377: A casual reader is going to have a very hard time following and understanding this paragraph. Feels like a large step up from previous writing with limited explanation of the new complex topics.
We performed a very detailed analysis of the electronic configuration of the Mn ion. That is why the bulk of the analysis was moved to the supplement for those interested to retrieve and a brief summary of the main results was left in this section. To clarify this, we added the following to lines 370-371.

“A detailed analysis of the electronic configuration of the Mn ion is presented in the Supplementary information and is summarized here. ”

Put bond distances on Fig 5

This figure is very busy so we did not put in bond distances. Instead, we used different line types to indicate the length of the bond. The meaning of the line types including distances for dashed, wide-dashed and round-dotted lines are described in the figure legend.

“Dashed lines represent normal hydrogen bonds (> 1.8 Å), wide-dashed represent SSHBs (hydrogen bonds <1.8 Å), and round-dotted lines represent a shared proton.”

Also on page 18, line 434 these hydrogen bond lengths are also given in the discussion.