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Peer Review File

XFEL Crystallography Reveals Catalytic Cycle Dynamics during Non-Native Substrate Oxidation by Cytochrome P450BM3

Corresponding Author: Dr Minoru Kubo

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors report an X-ray free electron laser (XFEL) crystallographic study of cytochrome P450BM3, which catalyzes the monooxygenation of styrene and related substrates to produce a chiral epoxide product. The major finding of this work is that binding of molecular oxygen and substrate, as well as changes in Fe oxidation state, result in changes in active site conformational heterogeneity that facilitate adoption of catalytically productive binding poses. The use of serial femtosecond crystallography (SFX) is a major strength of the work, as it avoids X-ray photoreduction of the heme site and therefore allows the authors to compare authentic oxidized and reduced crystal structures. The combination of using different oxidation states, site-directed mutagenesis, and addition of substrate allowed the authors to obtain structures representing different states along the catalytic cycle. Therefore, although this is not a time-resolved SFX study, it does permit characterization of representative states in catalysis. Overall, the manuscript is well-written and illustrated. My suggestions for improvement are predominantly minor and are provided below.

Minor comments:
1. Turnover frequency (TOF) is a term mostly commonly used in inorganic catalysis, while turnover number is more common when referring to enzymes. The authors should consider changing TOF to turnover number given the system and audience.
2. “obstructed by numerous technically challenges” should be “technical… ”
3. A comment that borders on major is that it is unclear what “decoy molecules” means in the manuscript. The Introduction mentions C7ProPhe but this is not shown in any figure or further explained. More should be provided for the reader about this molecule and its role in the study.
4. “ununiform” is not a word. Please use either “non-uniform” or, better, “conformationally heterogeneous”.
5. “which may have been acquired through molecular evolution.” There is no other mechanism by which a functionally important trait would be acquired by a protein, so this should be a stronger statement.
6. This sentence from the Discussion summarizes a key aspect of the study: “We emphasise that our data demonstrates how structural dynamics that occur during the catalytic cycle are important for determining enzymatic activity and enantioselectivity, and that structures of the oxidised state do not represent an accurate depiction of the catalytically active state of the enzyme, particularly for the positioning of non-native substrate in the active site.” However, there are at least two other studies using XFEL crystallography in unrelated systems that have shown that functionally important, catalysis-activated motions occur in enzymes. The authors should consider citing those preceding studies.
7. The XFEL crystallographic portion of the Methods states that data were collected at 100K. As XFELs allow for damage-free room temperature data collection, why was cryocrystallography preferred here?

Reviewer #2

(Remarks to the Author)
Cytochrome P450 monooxygenases are an important family of heme enzymes which can form highly oxidising heme intermediates capable of activating inert C-H bonds of a substrate molecule in a regio- and stereo-selective manner. The value of such biotransformations cannot be understated, both in terms of drug metabolism pathways in humans and in the biocatalysis of high value chemicals.

The catalytic cycle of P450s as presented in scheme 1of the submission has been the focus of intensive research, with much of the mechanistic information of the various catalytic states in the cycle being derived from spectroscopic information. Capturing intermediate catalytic states has proven challenging from a structural perspective owing to their transient nature

and the recognition that conventional cryo-rotational crystallography induces photoreduction of the heme, that can result in changes to the structural architecture within the active site. The advent of X-ray free electron lasers (XFEL) somewhat mitigates against the problems of photoreduction, as the femtosecond X-ray pulse allows diffraction to be collected before the effects of photoreduction can manifest. Thus, if catalytic intermediates can be generated and stabilised, then by using an XFEL source coupled with spectroscopic validation of the heme redox state, structure determination of true catalytic states within the cycle can be accomplished.

The manuscript by Nagao et al. reports the careful preparation and cryo-trapping of three catalytic states in microcrystals of a P450 monooxygenase. Each state generated contained a bound decoy molecule (its use explained elsewhere in the literature) and a non-native substrate, styrene. The ferric, ferrous (reduced) and oxygen bound ferrous/ferric states, are the first three states in the catalytic cycle, and were cryo-trapped. These were subjected to measurement in a microspectrophotometer, to firstly validate that the state trapped was as expected, followed by collection of XFEL data and structure determination. From these data the authors present a structure guided mechanism that explains the enzymatic and stereoselectivity data they present in Figure 1.

Overall, the work provides a significant advance in obtaining structure guided mechanism of P450s members. It will be interesting to see in the future if the authors tailor these approaches to capture the more challenging ferryl heme intermediate states of the P450 cycle.

Minor changes:

A brief comment on why the F393H variant affects the redox potential – (Ref14) is warranted and would be useful to general readers. Also, please mention where in the structure His393 is located, and in the supporting information make clear its location in the structures that are presented.

In all Figure legends of XFEL structures include the resolution.

The authors state the chain B monomer in the asymmetric unit was used for structural analysis as the electron density was clearer than for monomer A; what were the differences?

Add a coordinate error to distances in Table S1 for Fe-O separations e.g. consider using the diffraction precision index (DPI server).

Reviewer #3

(Remarks to the Author)
This manuscript describes freeze trap XFEL serial femtosecond crystallography studies from the reaction cycle of cytochrome P-450 BM3. Short lived intermediates or those very sensitive to oxygen or radiation damage are trapped by cryo-cooling and validated using spectroscopic means before the structures are determined by SFX to avoid radiation damage. Work is very well conducted and described and is suitable for publication in this journal. An advance is the production of structures the identity of which may be confidently assigned and whether radiation damage is not an issue.

A number of minor corrections are required prior to publication:

Page 4: it is described that radiation damage can lead to abberant coordination geometries. This is of course correct but it should be explicitly noted that the major phenomenon of concern for heme proteins is reduction caused by x-ray generated electrons. I wonder if dose dependence of reduction by X-rays has been investigated in this study or previous work?

The authors state correctly that radiation damage effects in electron density maps can be avoided by using SFX. It would be interesting to discuss in the manuscript how much radiation damage would be expected to occur is very low dose serial synchrotron crystallography was used, often this is associated with doses below 25-50 kGy where only very minimal electron density changes might be expected.
Line 144: ununiform is quite an obscure phrasing, please replace with non-uniform
Page seven: ligands have been modelled with round number fractional occupancies e.g. 0.3-0.6. How were these occupancies determined? Was occupancy refinement undertaken or have they been set based on B factors?
are the fractional occupancy ligands associated with partial occupancy different confirmations of the protein structure? This should be discussed and if present partial occupancies of the protein should be shown and described in the paper.

Page nine: where previous structures of P-450 enzymes are discussed, in the context of the present work the authors should comment on whether they suspect these previous structures to have electronic states altered by the effects of the X-ray beam
Page 12 line 376 - please correct the typo in the spelling of microspectrophotometer
Page 13: please comment on the number of mesh crystal mounts that were required to collect the full datasets described and how much time this needed

Scheme one: there appears to be a formatting error in the word monooxygenases
figures 2,3 and associated text: the position of residue 393 should be included in these figures because they are showing mutations of this residue. For the modelling of the ligand's the text should discuss whether different binding moments and orientations were tried and what validation was undertaken that the model orientation is correct. For example in figure 3 the

fit of the electron density in the centre and right panels appears significantly better than that in the left-hand panel. Can a suitable metric be included for the correspondence of electron density and model, for example the real space correlation coefficients or LLDF?

Supplementary data table S1: it is not reasonable to state angles to 1 decimal place as this is beyond the accuracy of structures. Please give these as whole numbers only

In figure S1: it is commendable that spectra from microcrystals are shown books for comparative purposes please either show spectra from solution data for comparative purposes or state the absorbance maxima in a table.

Figure S2: please comment on the apparent poor density coverage for the styrene molecule, and how this relates to occupancy

Figure S4: it may be helpful to show a zoomed in panel showing the solving channel directly in the vicinity of the heme pocket for the right-hand figure
Figure S8: please add labels for amino acid residues

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my concerns.

Reviewer #2

(Remarks to the Author)
The authors have addressed all my original comments, and have made clear in the revised version of the manuscript where the requested changes have been made. I am now satisfied that this manuscript is suitable for publication

Reviewer #3

(Remarks to the Author)
The revised version of the manuscript addresses all the points made in my original review and I now recommend acceptance without further revision

[Reviewer #1]

The authors report an X-ray free electron laser (XFEL) crystallographic study of cytochrome P450BM3, which catalyzes the monooxygenation of styrene and related substrates to produce a chiral epoxide product. The major finding of this work is that binding of molecular oxygen and substrate, as well as changes in Fe oxidation state, result in changes in active site conformational heterogeneity that facilitate adoption of catalytically productive binding poses. The use of serial femtosecond crystallography (SFX) is a major strength of the work, as it avoids X-ray photoreduction of the heme site and therefore allows the authors to compare authentic oxidized and reduced crystal structures. The combination of using different oxidation states, site-directed mutagenesis, and addition of substrate allowed the authors to obtain structures representing different states along the catalytic cycle. Therefore, although this is not a time-resolved SFX study, it does permit characterization of representative states in catalysis. Overall, the manuscript is well-written and illustrated. My suggestions for improvement are predominantly minor and are provided below.

We are grateful for your careful reading and valuable suggestions, which have helped improve and strengthen our manuscript. Our responses to all your comments are provided below.

Minor comments:

Comment 1. *Turnover frequency (TOF) is a term mostly commonly used in inorganic catalysis, while turnover number is more common when referring to enzymes. The authors should consider changing TOF to turnover number given the system and audience.*

Response 1. We have considered the potential use of "turnover number" but have opted for "turnover frequency (TOF)" in our manuscript, as "turnover number" can have two distinct meanings. While "turnover number" is indeed often used synonymously with TOF in enzymology, it also has a separate definition related to the lifetime (robustness) of a catalyst. This alternative definition is also widely recognised and could cause confusion for potential readers in the fields of inorganic and bioinorganic chemistry. Furthermore, TOF has been primarily used in our previous studies of P450BM3, and its use in this context enables a direct comparison of the reaction efficiency of the current P450BM3 system with that of previously developed P450BM3 systems, which involved various combinations of decoys and non-native substrates. For these reasons, we would like to use TOF in our manuscript.

Comment 2. *"obstructed by numerous technically challenges" should be "technical… "*

Response 2. Thank you for the correction. We have corrected the grammatical error, changing "technically challenges" to "technical challenges." (page 4)

Comment 3. *A comment that borders on major is that it is unclear what "decoy molecules" means in the manuscript. The Introduction mentions C7ProPhe but this is not shown in any figure or further explained. More should be provided for the reader about this molecule and its role in the study.*

Response 3. This is a critical point for our manuscript. The function of "decoy molecules" (slightly smaller pseudo-substrates) is to create a small reaction space for non-native substrates within the active site. "C7ProPhe" is an effective decoy that has been shown to facilitate enantioselective styrene oxidation. We have added this explanation to the Introduction as follows. Additionally, we have included the structural formula of "C7ProPhe" in **Fig. 1**.

(Page 3)
"As an alternative, a chemical approach utilising pseudo substrates (decoy molecules), which are substrate mimics that are slightly smaller than the natural fatty acid substrates, has been developed for manipulating P450BM3 reactivity. When P450BM3 misrecognises and binds a decoy molecule, a cavity is left within the active site, which serves as a reaction space for non-native substrates. So far, a variety of decoy molecules have been developed[8], and this approach has proven effective for changing P450BM3 into a versatile biocatalyst for the oxidation of challenging non-native substrates, for example the gaseous alkane methane, which possesses a high energetic barrier for oxidation.[8-10] A combination of mutagenesis with decoy molecules, such as *N*-enanthyl-L-prolyl-L-phenylalanine (C7ProPhe), has enabled the epoxidation of styrene with high enantioselectivity.[11,12]"

Comment 4. *"ununiform" is not a word. Please use either "non-uniform" or, better, "conformationally heterogeneous".*

Response 4. Thank you for pointing that out. We have replaced "ununiform" with "conformationally heterogeneous" in the revised manuscript. (Page 6)

Comment 5. *"which may have been acquired through molecular evolution." There is no other mechanism by which a functionally important trait would be acquired by a protein, so this should be a stronger statement.*

Response 5. I agree with your point, and we have deleted "may" to make the statement stronger. (Page 10)

Comment 6. *This sentence from the Discussion summarizes a key aspect of the study: "We*

emphasise that our data demonstrates how structural dynamics that occur during the catalytic cycle are important for determining enzymatic activity and enantioselectivity, and that structures of the oxidised state do not represent an accurate depiction of the catalytically active state of the enzyme, particularly for the positioning of non-native substrate in the active site." However, there are at least two other studies using XFEL crystallography in unrelated systems that have shown that functionally important, catalysis-activated motions occur in enzymes. The authors should consider citing those preceding studies.

Response 6. Thank you for this important suggestion. We have cited previous studies using XFEL crystallography for the intermediate analysis of haem enzymes as follows:

(Page 10-11)
"We emphasise that our data demonstrates how structural dynamics that occur during the catalytic cycle are important for determining enzymatic activity and enantioselectivity, and that structures of the oxidised state do not represent an accurate depiction of the catalytically active state of the enzyme, particularly for the positioning of substrate in the active site, as demonstrated for other haem enzymes.[30,33-36]"

30. Nguyen RC, et al. In situ structural observation of a substrate- and peroxide-bound high-spin ferric-hydroperoxo intermediate in the P450 enzyme CYP121. *J. Am. Chem. Soc.* **145**, 25120-25133 (2023).
33. Tosha T, et al. Capturing an initial intermediate during the P450nor enzymatic reaction using time-resolved XFEL crystallography and caged-substrate. *Nat. Commun.* **8**, 1585 (2017).
34. Ishigami I, et al. Snapshot of an oxygen intermediate in the catalytic reaction of cytochrome *c* oxidase. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 3572-3577 (2019).
35. Lučić M. et al. Serial femtosecond zero dose crystallography captures a water-free distal heme site in a dye-decolorising peroxidase to reveal a catalytic role for an arginine in Fe^{IV} =O formation. *Angew. Chem. Int. Ed.* **59**, 21656-21662 (2020).
36. Kwon H, et al. XFEL crystal structures of peroxidase compound II. *Angew. Chem. Int. Ed.* **60**, 14578-14585 (2021).

*Refs. 34 and 35 have been newly cited in the revised manuscript.

Comment 7. *The XFEL crystallographic portion of the Methods states that data were collected at 100K. As XFELs allow for damage-free room temperature data collection, why was cryocrystallography preferred here?*

Response 7. XFEL indeed enables damage-free analysis at room temperature, as suggested by

the reviewer. However, in this study, the cryogenic conditions were necessary to prevent auto-oxidation of the oxygen-bound form. The preparation of the oxygen-bound form required the removal of dithionite (reductant), as detailed in the Results and Methods sections. In addition to the low redox potential of haem, this preparation condition further made it difficult to prevent auto-oxidation at room temperature. In the revised manuscript, we have included the following statement in the Methods section:

(Page 13)

"Notably, maintaining a low temperature below −12 °C was crucial to prevent auto-oxidation of the oxygen-bound form. Consequently, the fixed-target X-ray data collection method at 100 K was chosen over the room-temperature data collection method using an injector device with liquid or viscous media."

[Reviewer #2]

Cytochrome P450 monooxygenases are an important family of heme enzymes which can form highly oxidising heme intermediates capable of activating inert C-H bonds of a substrate molecule in a regio- and stereo-selective manner. The value of such biotransformations cannot be understated, both in terms of drug metabolism pathways in humans and in the biocatalysis of high value chemicals.

The catalytic cycle of P450s as presented in scheme 1of the submission has been the focus of intensive research, with much of the mechanistic information of the various catalytic states in the cycle being derived from spectroscopic information. Capturing intermediate catalytic states has proven challenging from a structural perspective owing to their transient nature and the recognition that conventional cryo-rotational crystallography induces photoreduction of the heme, that can result in changes to the structural architecture within the active site. The advent of X-ray free electron lasers (XFEL) somewhat mitigates against the problems of photoreduction, as the femtosecond X-ray pulse allows diffraction to be collected before the effects of photoreduction can manifest. Thus, if catalytic intermediates can be generated and stabilised, then by using an XFEL source coupled with spectroscopic validation of the heme redox state, structure determination of true catalytic states within the cycle can be accomplished.

The manuscript by Nagao et al. reports the careful preparation and cryo-trapping of three catalytic states in microcrystals of a P450 monooxygenase. Each state generated contained a bound decoy molecule (its use explained elsewhere in the literature) and a non-native substrate, styrene. The ferric, ferrous (reduced) and oxygen bound ferrous/ferric states, are the first three states in the catalytic cycle, and were cryo-trapped. These were subjected to measurement in a microspectrophotometer, to firstly validate that the state trapped was as expected, followed by collection of XFEL data and structure determination. From these data the authors present a structure guided mechanism that explains the enzymatic and stereoselectivity data they present in Figure 1.

Overall, the work provides a significant advance in obtaining structure guided mechanism of P450s members. It will be interesting to see in the future if the authors tailor these approaches to capture the more challenging ferryl heme intermediate states of the P450 cycle.

We are very pleased to hear that you recognise the importance of our work and its significant contribution to the research area of P450s, as well as your encouragement for further investigation of the subsequent reaction steps. Our responses to all your comments are below.

Minor changes:

Comment 1. *A brief comment on why the F393H variant affects the redox potential – (Ref14) is warranted and would be useful to general readers. Also, please mention where in the structure His393 is located, and in the supporting information make clear its location in the structures that are presented.*

Response 1. Phe393 is one of the highly conserved residues throughout the P450 superfamily and is located near the proximal ligand (Cys400) of the haem iron. The interaction between Phe393 and the haem-Cys400 moiety is considered essential for controlling the reactivity of haem. However, the mechanism by which the F393H mutation elevates the redox potential of haem remains unclear. While ref. 14 found the change in redox potential caused by this mutation, the underlying mechanism was not elucidated. Very recently, it has been proposed that His393 in the F393H mutant might form a hydrogen bond with Cys400, potentially increasing the haem redox potential (*J. Inorg. Biochem.* **259**, 112660 (2024)). However, no such hydrogen bond was observed in our current crystal structures. Further studies, such as a QM/MM analysis using our F393H structures, are needed to understand the effect of the F393H mutation on the electronic structure of haem.

Nevertheless, we should at least mention the location of Phe393 (His393) in the structure as the reviewer pointed out, and comment on the effect of this residue on the haem redox potential. In the revised manuscript, we have added the following sentences to the Introduction, and the location of His393 is shown in **Figs. 2** and **4**. In the oxygen-bonded form of F87A/F393H, His393 exhibits double conformations, but as this has no functional implications, it is only shown in the figures and not discussed in the manuscript.

(One page 4)
"Phe393 is located near the proximal ligand Cys400, and substitution of this residue with His elevates the redox potential of haem, though the underlying mechanism remains unclear.[14,20]"
*Refs. 20 (*J. Inorg. Biochem.* **259**, 112660 (2024)) was newly cited in the revised manuscript.

Comment 2. *In all Figure legends of XFEL structures include the resolution.*

Response 2. We agree that resolution is important when viewing the structures. We have included the resolution in the legends of **Figs. 2**, **3**, **S2**, **S6**, **S8**, and **S9**.

Comment 3. *The authors state the chain B monomer in the asymmetric unit was used for structural analysis as the electron density was clearer than for monomer A; what were the differences?*

Response 3. The overall structure of chains A and B was very similar, with RMSD values below 0.5 Å for all six structures (F393H and F87A/F393H in the oxidised, reduced, and

oxygen-bound forms). In the oxygen-bound form (both F393H and F87A/F393H), the haem-O_2 geometry was comparable between chains A and B. A difference was observed in the electron density of styrene in the oxidised form of F393H, where the density was poorer in chain A than in chain B. As a result, the second orientation of styrene could not be modelled for chain A.

In the revised manuscript, we have addressed this difference in the electron density of styrene between chains A and B by adding a comment to the Methods section. Additionally, we have included a new figure (**Fig. S13**) showing the superposition of the overall structure of chains A and B for all six structures with the RMSD values, and the haem-O_2 geometry in chain A is provided in **Table S1** to illustrate the similarity between chains A and B.

(Page 14)
"The asymmetric unit contained two monomers of P450BM3 (chains A and B). Although the structures of chains A and B were essentially the same (**Fig. S13**, **Table S1**), the electron density of styrene in the oxidised form of F393H was poorer in chain A than in chain B, which prevented the modelling of the second orientation of styrene for chain A. Thus, all figures and active site geometries were calculated from the atomic coordinates of chain B."

Comment 4. *Add a coordinate error to distances in Table S1 for Fe-O separations e.g. consider using the diffraction precision index (DPI server).*

Response 4. As the reviewer suggested, we have calculated the values of the diffraction precision index (DPI) using the online server,[50] and added them as estimates of coordinate error to Tables **S4** and **S5** in the revised manuscript (Tables S2 and S3 in the original manuscript).

Ref. 50: Kumar KS, et al. *Online_DPI*: a web server to calculate the diffraction precision index for a protein structure. *J. Appl. Cryst.* **48**, 939-942 (2015).

[Reviewer #3]

This manuscript describes freeze trap XFEL serial femtosecond crystallography studies from the reaction cycle of cytochrome P-450 BM3. Short lived intermediates or those very sensitive to oxygen or radiation damage are trapped by cryo-cooling and validated using spectroscopic means before the structures are determined by SFX to avoid radiation damage. Work is very well conducted and described and is suitable for publication in this journal. An advance is the production of structures the identity of which may be confidently assigned and whether radiation damage is not an issue.

A number of minor corrections are required prior to publication:

We are grateful for your positive evaluation of our experimental approach and for your constructive comments. In response, we have addressed all of them.

Comment 1. *Page 4: it is described that radiation damage can lead to abberant coordination geometries. This is of course correct but it should be explicitly noted that the major phenomenon of concern for heme proteins is reduction caused by x-ray generated electrons. I wonder if dose dependence of reduction by X-rays has been investigated in this study or previous work?*

Response 1. Although the dose-dependence of X-ray photoreduction has not been investigated, we determined a structure of the oxygen-bound form of F87A/F393H with an X-ray dose of 2 MGy using SPring-8, where we confirmed the effect of X-ray photoreduction (new **Fig. S9**). X-ray photoreduction clearly affects the Fe-O_2 geometry (new **Table S2**). In the revised manuscript, we explicitly address the issue of X-ray damage as "the reduction of haem iron by X-ray generated electrons" on page 4. Additionally, we have added the synchrotron data to supporting information (**Fig. S9** and **Table S2**), and included a comment on the effect of X-ray photoreduction on the heme-O_2 geometry as follows:

(Page 8)
"It is noteworthy that the haem-O_2 geometry determined using XFEL is damage-free. In fact, synchrotron crystallography with an X-ray dose of 2 MGy confirmed the X-ray photoreduction effect, resulting in a more bent Fe-O-O angle and an elongated Fe-O bond length for F87A/F393H (**Fig. S9**, **Table S2**)."

Comment 2. *The authors state correctly that radiation damage effects in electron density maps can be avoided by using SFX. It would be interesting to discuss in the manuscript how much radiation damage would be expected to occur is very low dose serial synchrotron crystallography*

was used, often this is associated with doses below 25-50 kGy where only very minimal electron density changes might be expected.

Response 2. While radiation damage in serial synchrotron crystallography (SSX) is not the main focus of this study, recent advances in SSX techniques would be of interest to readers of our manuscript. We have included the following discussion of SSX in the revised manuscript.

(Page 11)
"Although the work presented herein delivers a model for the structural dynamics of P450BM3, our experimental approach using XFEL for probing reduced and oxygenated intermediates is expected to work on a wide range of different P450s. It is worth noting that recent advances in serial synchrotron crystallography have made it possible to analyse haem pocket structures with a very low dose of around 100 kGy or less, making it a practical tool, provided that the coordination structure of haem is carefully considered.[37-39] The application of these latest techniques to intermediates of various types of P450s will provide a much needed understanding of enzyme and substrate dynamics at atomic resolutions, and…"

37. Ebrahim A. et al. Dose-resolved serial synchrotron and XFEL structures of radiation-sensitive metalloproteins. *IUCrJ* **6**, 543-551 (2019).
38. de la Mora E. et al. Radiation damage and dose limits in serial synchrotron crystallography at cryo- and room temperatures. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 4142-4151 (2020).
39. Mehrabi P. et al. Serial femtosecond and serial synchrotron crystallography can yield data of equivalent quality: A systematic comparison. *Sci. Adv.* **7**, eabf1380 (2021).

Comment 3. Line 144: ununiform is quite an obscure phrasing, please replace with non-uniform.

Response 3. We have replaced "ununiform" with "conformationally heterogeneous" in the revised manuscript. (Page 6)

Comment 4. Page seven: ligands have been modelled with round number fractional occupancies e.g. 0.3-0.6. How were these occupancies determined? Was occupancy refinement undertaken or have they been set based on B factors?

Response 4. A description of the method used to determine occupancy values should have been included in the Methods section. Initial occupancy values were obtained using the occupancy refinement function implemented in phenix.refine, and were then manually adjusted based on the $F_o - F_c$ map and *B*-factor analyses. Given the precision, the occupancy

values presented in the manuscript were rounded to one decimal place. In the revised manuscript, we have added the method for occupancy refinement to the Methods section as follows:

(Page 14)
"The partial occupancies of styrene, haem-bound O_2, water molecules, and Phe87 were determined through the following steps. Initial occupancy values for these molecules and the residue were obtained using the occupancy refinement function in phenix.refine. Subsequently, models with occupancy values varied in increments of 0.05 around the initial estimates were generated, and phenix.refine was run without occupancy refinement. The validity of the occupancy values was assessed based on the $F_o - F_c$ maps and *B*-factors, and final occupancies were determined where no residual density appeared in the $F_o - F_c$ map and the refined *B*-factors of the atoms in the target molecules and residue most closely matched those of the surrounding atoms. The final occupancy values were rounded to one decimal place."

Comment 5. Are the fractional occupancy ligands associated with partial occupancy different confirmations of the protein structure? This should be discussed and if present partial occupancies of the protein should be shown and described in the paper.

Response 5. There is no partial occupancy observed for the protein structure, except for Phe87 in the oxidised form of F393H. Thus, the two orientations of styrene are attributed to the spacious substrate binding site, which can accommodate both orientations. Notably, the two orientations of styrene are not correlated with those of Phe87. In the reduced form of F393H, Phe87 adopts a single orientation, while styrene still exhibits two orientations. This discussion has been added to the Results section in the revised manuscript.

(Page 6)
"Comparison of the occupancies of Wat1 and Wat2 with those of the side chain of Phe87 revealed that the dual conformation of Phe87 may correlate with the partial expulsion of Wat1, although this does not appear to be associated with the two orientations of styrene (vide infra)."
"No multiple conformations were observed, except for Phe87 in the active site."
(Page 7)
"In F393H, Phe87 adopted a single conformation in the reduced form, while styrene retained its two orientations. This suggests that the dual conformations of Phe87 in the oxidised form were not correlated with the distinct binding modes of styrene."

Comment 6. *Page nine: where previous structures of P-450 enzymes are discussed, in the context of the present work the authors should comment on whether they suspect these previous structures*

to have electronic states altered by the effects of the X-ray beam.

Response 6. We discussed the proton shuttle pathway based on previous structural analyses of oxygen-bound P450cam, done by Ilme et al. and Nagano et al. Nagano et al. recognised the radiation damage issue and collected the diffraction data in-house, with a trade-off in resolution, which suppressed radiation damage to a minimum. Ilme et al. were also well aware of the radiation damage problem and employed a strategy to control it, by tuning the X-ray wavelength as well as observing the dose-dependent behaviour, where photo-reduction was actively used to drive the reaction. As a result, the structures and findings reported by both groups were consistent, ensuring the existence of the hydrogen bonding network in the proton shuttle pathway in P450cam. However, a recent SSX study (Ref. 37: *IUCrJ* **6**, 543-551 (2019)) indicated that the haem coordination structure can change even at a very low dose below 100 kGy. Thus, using XFEL is now a straightforward approach for damage-free determination of the coordination structure of haem. In the revised manuscript, we have included these comments in the Reference section (#26).

(Page 17)

26. The difference in the hydrogen bonding network between the reported and current structures is not due to the effect of radiation damage: The structural analysis in ref. 24 was based on the diffraction data collected in-house, where the radiation effect was suppressed to a minimum. In ref. 23, the radiation effect was carefully controlled by tuning the X-ray wavelength and observing dose-dependent structural alterations while using synchrotron radiation. Consequently, the structures and findings in refs. 23 and 24 were consistent, ensuring the existence of the hydrogen bonding network in P450cam, although the use of XFEL is now a straightforward approach for damage-free analysis, particularly for determining the coordination structure of haem.

Comment 7. *Page 12 line 376 - please correct the typo in the spelling of microspectrophotometer*

Response 7. The typo in the spelling of "microspectrophotometer" has been corrected in the revised manuscript. (page 13)

Comment 8. *Page 13: please comment on the number of mesh crystal mounts that were required to collect the full datasets described and how much time this needed.*

Response 8. This information would certainly be of benefit to readers. We have added the number of mesh loops used and the data collection time to **Tables S4** and **S5** in the revised

version (**Tables S2** and **S3** in the original version).

Comment 9. *Scheme one: there appears to be a formatting error in the word monooxygenases*

Response 9. The formatting error in the word "monooxygenases" has been corrected in the revised manuscript.

Comment 10. *figures 2,3 and associated text: the position of residue 393 should be included in these figures because they are showing mutations of this residue. For the modelling of the ligand's the text should discuss whether different binding moments and orientations were tried and what validation was undertaken that the model orientation is correct. For example in figure 3 the fit of the electron density in the centre and right panels appears significantly better than that in the left-hand panel. Can a suitable metric be included for the correspondence of electron density and model, for example the real space correlation coefficients or LLDF?*

Response 10. We have illustrated the location of His393 in **Figs. 2** and **4**, showing that this residue is located near the proximal ligand Cys400. His393 has not been included in **Fig. 3** (schematic of structural dynamics) as it is not located at the position directly involved in the reaction.

For the modelling of styrene, we explored various orientations for both STY_A and STY_B. To assess the validity of each orientation, we calculated polder omit maps, omitting each alternative orientation, and evaluated how well the models fit the maps. The real-space correlation coefficients (RSCCs) for styrene were also calculated using Phenix, yielding reasonable values, as presented in **Table S3** (a new table in the revised manuscript). In the revised manuscript, we have included the method for model validation in the **Methods** section, as shown below. Additionally, illustrations of the polder omit maps for each orientation of styrene have been provided in **Figs. S2** and **S6** to support the current styrene modelling.

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"The electron density for styrene exhibited a complex shape in the oxidised and reduced forms (**Figs. S2** and **S6**), indicating two distinct orientations of its structure. Thus, for the modelling of styrene, various orientations were explored. After structure refinement with the first orientation of styrene, with optimisation of its partial occupancy, the second orientation was built using the $F_o - F_c$ map and subjected to further refinement. The refined orientation, occupancy value, and *B*-factor of the alternative model were evaluated based on the fit to the polder omit map,[49] which was generated by omitting each of the two orientations. The real space correlation coefficients were computed from the final coordinates and the $2mF_o - DF_c$

maps using the validation tool (phenix.real_space_correlation) in PHENIX (**Table S3**).[47] The $2mF_o - DF_c$ maps were calculated without filling the missing reflections from the calculated structure factors."

Comment 11. *Supplementary data table S1: it is not reasonable to state angles to 1 decimal place as this is beyond the accuracy of structures. Please give these as whole numbers only*

Response 11. In the revised manuscript, the corresponding angles have been rounded to whole numbers.

Comment 12. *In figure S1: it is commendable that spectra from microcrystals are shown books for comparative purposes please either show spectra from solution data for comparative purposes or state the absorbance maxima in a table.*

Response 12. We have included the solution spectra of F393H and F87A/F393H in the oxidised, reduced, and oxygen-bound forms in **Fig. S1**. The peak positions are consistent under both solution and crystal conditions. In the oxidised form, the haem existed as a mixture of low-spin and high-spin states in microcrystals, whereas in solution, the low-spin haem was dominant due to the limited concentration of styrene. The addition of saturated styrene resulted in the solution becoming turbid. The methods of solution spectral measurements are provided in the Methods section.

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"The spectra were also measured under solution conditions using a quartz cuvette with a path length of 1 cm and a Nanophotometer-NP 80 (Implen) (**Fig. S1G–L**). Purified P450BM3 was dissolved in 125 mM Tris-HCl buffer (pH 8.3) containing 80 μM C7ProPhe and 200 μM styrene. The protein concentration was 10 μM for the oxidised and reduced forms and 5 μM for the oxygen-bound form. The reduced form was prepared by adding sodium dithionite (final concentration: 100 μM) to the oxidised protein. The oxygen-bound form was prepared by mixing the reduced protein with the oxygen-containing buffer at 4°C, and the measurement was performed immediately."

Comment 13. *Figure S2: please comment on the apparent poor density coverage for the styrene molecule, and how this relates to occupancy*

Response 13. As the reviewer pointed out, the weaker density of styrene in the oxidised form (**Fig. S2**) reflects its lower occupancies (total occupancy of 0.5 for F393H and 0.7 for F87A/F393H) compared to the other forms (0.8-0.9). To address the reviewer's concern regarding the poor

quality of the $2F_o - F_c$ map, we have added new panels showing the omit maps in **Fig. S2** (oxidised form) and **Fig. S6** (reduced form), to better illustrate the quality of model-to-map fitting, in the revised manuscript.

In this process, we updated the model of F393H by re-optimising the occupancies of the two orientations of styrene. Additionally, we revised the calculation method for the $2F_o - F_c$ map, because we noticed that the maps were not properly calculated. In the Fourier map calculation for previous figures, ~800 missing reflections in resolution lower than 10 Å were unintentionally filled from $\boldsymbol{F}_{calc}$ by Phenix, which resulted in very poor density for the substrate. As shown in the revised **Figs. S2** and **S6**, the quality of the recalculated $2F_o - F_c$ map, only using the observed reflections, improved significantly, particularly for the oxidised and reduced forms.

Comment 14. *Figure S4: it may be helpful to show a zoomed in panel showing the solving channel directly in the vicinity of the heme pocket for the right-hand figure*

Response 14. A magnified view of the channel end in F87A/F393H has been added to **Fig. S4**, showing the region in the vicinity of the haem pocket.

Comment 15. *Figure S8: please add labels for amino acid residues*

Response 15. We added the labels for amino acid residues in the revised version.