nature portfolio

Peer Review File

Coordinated conformational changes in P450 decarboxylases enable hydrocarbons production from renewable feedstocks

Corresponding Author: Dr Leticia Zanphorlin

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)
Opinion: Accept with major revisions

Summary: This original research article presents the mechanism of fatty acid peroxygenases (CYP152 decarboxylase) based on a combination of crystallographic structure elucidation of oleic-acid-bound new enzymes (OleTCL, OleTKM), their structure-guided protein engineering, molecular dynamics simulations, and unsupervised machine learning. Specifically, the authors reveal that OleTCL has unique features compared to other fatty acid decarboxylases (OleTKM and OleTPRN), including the absence of the aromatic residue from the Phe-His-Arg triad at the heme proximal site, and concerted structural alterations in the hydrophobic cradle to orient the substrate Cβ atom towards the catalytic heme-iron. These two unique features provide a distinct perspective compared to previous reports regarding the necessity of the triad and a rigid hydrophobic cradle. The results are impressive and provide unparalleled mechanistic insights into unsaturated fatty acid decarboxylation by P450 peroxygenases that may foster further development of sustainable and biobased routes for the synthesis of olefins. However, some data need to be described more clearly, and the discussions should be improved to show the broader impact of this study.

Major revisions
1) OleTCL has high specificity for olefin decarboxylation compared to other fatty acid decarboxylases (OleTKM and OleTPRN), which may be related to unique concerted structural alterations in the hydrophobic cradle (rotating F19 and L26 rather than F). Please clarify whether this property is unique to only the OleTCL enzyme, or if it is conserved in other CYP152 decarboxylases possessing a similar hydrophobic cradle environment. It may be important to provide the universality or specificity of each CYP152 decarboxylase mechanism (OleTCL, OleTPRN, and OleTKM) for the impact of this study.
2) Please provide a graphic summary of the proposed fatty acid decarboxylase mechanism including OleTPRN and OleCL. This would make it easier to understand for a broader audience.
3) Throughout the manuscript, the discussion about bifurcated chemoselectivity towards decarboxylation rather than α or β hydroxylation is relatively scarce compared to unsaturated substrate selectivity.
4) Please emphasize the impact of oleic acid decarboxylation rather than saturated acid decarboxylation in terms of industrial application. If important, the decarboxylation activity of other unsaturated fatty acids should also be tested.
5) Figure 5A and Figure S16, the substrate of OleTPRN structure seems to be palmitic acid rather than oleic acid. Oleic acid bound OleTPRN structure may be required to describe the results.

Minor revisions
1) Line 85, please correspond “unsupervised machine learning approaches” to the main result data, because it is unclear which data is indicated.
2) Figure 1A, what is the main sequence difference among different clusters? It would be helpful to show this as an additional figure.
3) Line 109~112, please provide table or figure (same as above) for the sentence, “members from cluster I were classified as acting mainly as hydroxylases” and “Cluster II and cluster III members act mainly as fatty acid decarboxylases”.
4) Figure 1B and Line 124-126, it is unclear what the author wants to be emphasized. It may be better to move to

supplementary figure.
5) Line 135-138, please discuss about the possible explanation about the absence of inhibition by oleic acid in OleTCL.
6) Line 177, Fig 3G should be Fig 2G.
7) Line 193-195, please discuss about author's opinion about further aspects.
8) Line 301-303 and Figure 5B, it would be helpful to indicate how each mutation affects to cradle distance and hydrophobicity.
9) Line 387, OleT"KM" should be subscript.
10) Table S1, table legend seems to be changed (Corynebacterium e Korcuria?)

Reviewer #2

(Remarks to the Author)
In the present manuscript, Generoso et al performed a mechanistic study based on the structures of CYP450 peroxygenase, particularly based on the structure of OleTCL and OleTKM. The authors used crystal structure, MD simulations, and supervised machine learning to support their study. This work is very specialized, and it represents one more work in the list of several alkene-producing Cytochrome 450 works. I don't see its scope for the general audience of Nature Communication. Indeed, its scope is also limited to readers of general chemistry as the importance of the hydrophobic cradle is already published in the PNAS, 2023 by the same group. Therefore it is just an extension.
Apart from the above opinion about the scope of the work in Nat Comm, I have several other concerns that don't allow me to recommend this work for publication.

Scholarly presentations: The work presentation is poor. There are several confusing statements and incorrect words (see details below). Several typos and incorrect citations of the figure in the text, e.g., line 114 'genera' ; line 188 citation of Fig 3G, which should be either 2G or 3C.

Technical issue: 1) The MD simulations of 100ns in just one replica are not recommended on any platform. This becomes of high importance when your entire story is structure-dependent. You are emphasizing conformational changes (e.g., rotation of Phe etc) and 100 ns is too short to validate the claim.
2) Parameterisation: You are using the resting state of the heme-porphyrin, but the reactive state is Cpd I. There is no MD simulation in this state. During the catalytic cycle, many conformation changes may happen (see Science, 2000 by Schlitching 2000).
3) Supervised machine learning for data generated by 100 ns is misleading and is of no sense.

Detailed report:
1. The use of proximal and distal terms is confusing in the entire manuscript. Usually, the Thio-ligated side is termed as proximal, and the substrate side is termed as distal. However, somewhere in the manuscript, Phenylalanine is mentioned as distal (Page 2, Introduction line 83), and somewhere, it is proximal (abstract, line 43).
2. In most places, authors discuss the role of Phe, Val, etc, without mentioning the residue ID, which shows poor scholarly presentations. For example: in line 119 and there are several more.
3. Line 129-130, the product percentage or ratio (hydroxylation or decarboxylation) is needed. The use of 'major product' is ambiguous.
4. In Lines 137-141, the Authors say- OleTCL metabolized both substrates, but OleTKM was inhibited. Here, readers will assume that OleTKM does not show activity. However, in the proceeding sentence, the authors say that the three enzymes, including OleTKM, have broader substrate specificities. This is confusing.
5. Line 163, "OleTCL functions as monomer most likely due to…." This again confusing. The substitution of K299, Q404, E407 may be structurally important but not necessarily functionally important. When we use the function, we automatically think of the catalytic function, but here, I assume the authors want to talk about dimerization and monomer, which are structural properties not anyhow related to catalytic function.
6. Line 179 to 181, Histidine 85 in OleTJE is on the distal side close to the substrate side. Therefore, the use of 'proximal' is incorrect here.
7. Line 188, Figure 3G, doesn't say anything about the reactivity of the H92Q mutant. I guess the authors want to cite Figure 2G.
8. The flexibility of F19 has been exaggerated in the manuscript. I can see the rotation of F19 only in Figure 4B and Figure 4C. Therefore, I don't think it is an intrinsic property, as claimed by the authors in line 225. In fact, the rotation of the Phe19, as mentioned by the authors, was also not found in the crystal structure (line 228). Rotation, seen in the simulation, might be an artifact of the simulation.
9. MD simulation of 100 ns and only in one replica is unacceptable at any scale.
10. Authors mentioned that they co-crystalized the enzyme complex, but to my surprise, the Phe19 is nowhere in any crystal structure. It will be intriguing to compare the simulated structure and the crystal structure.
10. Supervised machine learning with 100ns of simulation and in one replica is recommended. The simulated data is too preliminary to be used for Machine learning, and the results are of no sense.

Reviewer #3

(Remarks to the Author)

This manuscript describes new crystal structures of P450 peroxygenases in complex with (un)saturated fatty acids, aiming to provide further insights into this biotechnologically interesting family of enzymes that have considerable scope for olefin production. Of particular interest is understanding both substrate and product specificity in this enzyme family, with the need for biocatalysts that can convert readily available fatty acids (which includes unsaturated FA) to corresponding olfin in absence of hydroxylation reactions.

The manuscript would significantly benefit from editing to improve English language/clarity. From the main title, abstract to the main text, language used is not concise enough and often resorts to cumbersome/convoluted sentences/vocabulary.

As a brief example see beginning of abstract:

"Fatty acid peroxygenases have been underscored in hydrocarbon biosynthesis due to their capacity to perform C-C scission en route, producing olefins, a central building block for the production of sustainable plastics, polymers, and fuels. These biocatalysts possess non-canonical and complex mechanisms, which involve redox partners, co-factors, hydrogen abstraction, controlled electrons and protons delivery that culminate in bifurcated chemoselectivity into hydroxylation or decarboxylation."

Which i suggest could be:

P450 fatty acid peroxygenases are able to produce olfins, a central building block for production of sustainable polymers and fuels, through oxidative decarboxylation. In addition, they also catalyse the more canonical fatty acid hydroxylation, with product specificity dependent on identity of both substrate and peroxygenase used.

The abstract finishes with:

"Taken together, these findings untangle key molecular factors governing the tunable biocatalytic selectivity of P450 peroxygenases that are central for the sustainable production of olefins from oleic acid, the most abundant and relevant renewable fatty acids in nature."

While i agree the authors provide compelling data for how oleic acid is accepted by OleTcl through structural studies combined with simulation, various mutagenic studies provide little in way of clear effect on product specificity (see for examples Figs 2,3,5 where only minor changes are observed for various mutants made). Therefore, it is not clear how key these molecular factors actually are, nor whether these could be use to predictively "tune" P450 peroxygenases.

A few other suggestions:

A schematic figure detailed the chemistry catalysed by P450s peroxygenases would assist the non-expert reader, while it would also help to clearly clarify these are peroxide dependent (ie not your classical redox partner one electron at a time P450s).

Fig 2 could perhaps be more clear if it included the active site solvent accessible surface to provide context to the fatty acid conformation. Please clarify for this fig and other containing product analysis how many replicates and whether these were technical replicates or not.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Opinion: Accept with minor revisions

Summary: The authors have addressed most of the questions raised by the reviewers, and the revised version of the manuscript appears to have a greater impact, particularly by presenting olefin production data from renewable feedstocks using OleTPCL. Additionally, the authors provide high oleic acid conversion data for OleTPCD and OleTPMA, which may also possess a flexible phenylalanine residue in the hydrophobic cradle. This suggests the potential to discover more diverse P450 decarboxylases similar to OleTPCL in the future. Therefore, the manuscript is recommended for acceptance

with a few additional minor revisions.

Minor revisions
1) The authors' opinion is understood to be that this work focuses on decarboxylation and does not extensively discuss the α or β regioselectivity of the studied enzymes. Nevertheless, it may be beneficial to briefly discuss the differences in hydroxylated product yield between samples in some graphs, such as the increased βOH of the C12:0 product in the L26F mutant shown in Figure 3c.
2) Additionally, there are significant differences in hydroxylated product yield among different substrates in Figures 1b and 1c. For example, C10:0 does not yield any hydroxylated products, compared to the OleTPRN data from a previous PNAS paper. It would enhance the quality of the paper if the authors suggested or discussed the reasons for the small proportion of hydroxylated products and possible engineering approaches to achieve higher selectivity for decarboxylation.
3) The authors' response regarding the difficulty of obtaining substrate/product crystal complexes with OleTP enzymes is noted. Are there any specific or important methods to successfully obtain the structures of the enzymes discussed in this paper? If so, it would be helpful for further structural studies. Additionally, it would be beneficial to discuss this limitation in the discussion section.
4) In line 171, OleTPCL is found to be a monomer. Does this property make any difference in substrate specificity and decarboxylation activity compared to dimeric OleTPs? Please discuss.
5) In lines 274-276, please indicate the amino acids of OleTPCD and OleTPMA that correspond to L26 of OleTPCL for better readability.
6) OleTCL and OleTPCL are both used in the manuscript, which is confusing. Please unify them into one term.
7) In Figure 4b, what is the significance of the two different dihedral angle distributions with similar frequencies?

Reviewer #2

(Remarks to the Author)
I appreciate the careful and comprehensive response to my previous comments. The authors have extended the simulations and revised the previous ambiguity. The manuscript is fairly clear relative to the original version. I believe the revised version can be accepted for publication.

Reviewer #3

(Remarks to the Author)
This manuscript is now much improved with constructive response to the various review raised. The additional data with respect to DCO oil conversion provides a useful new perspective on the potential for future application.

I have a few minor points:

1) the language throughout will need careful editing, while improved at places, it remains cumbersome and at points not quite grammatically or factually correct. As a minimum, the abstract should be entirely rewritten with that in mind.

2) please provide R/Rfree values for outer resolution shell.

Point-by-point response to the reviewers' comments

Reviewer Comments:

Reviewer #1:

Opinion: Accept with major revisions

Summary: This original research article presents the mechanism of fatty acid peroxygenases (CYP152 decarboxylase) based on a combination of crystallographic structure elucidation of oleic-acid-bound new enzymes (OleTCL, OleTKM), their structure-guided protein engineering, molecular dynamics simulations, and unsupervised machine learning. Specifically, the authors reveal that OleTCL has unique features compared to other fatty acid decarboxylases (OleTKM and OleTPRN), including the absence of the aromatic residue from the Phe-His-Arg triad at the heme proximal site, and concerted structural alterations in the hydrophobic cradle to orient the substrate Cβ atom towards the catalytic heme-iron. These two unique features provide a distinct perspective compared to previous reports regarding the necessity of the triad and a rigid hydrophobic cradle. The results are impressive and provide unparalleled mechanistic insights into unsaturated fatty acid decarboxylation by P450 peroxygenases that may foster further development of sustainable and biobased routes for the synthesis of olefins. However, some data need to be described more clearly, and the discussions should be improved to show the broader impact of this study.

R: We appreciate the constructive comments from Reviewer #1 supporting our work. They will certainly increase the impact of our research and related studies on P450 peroxygenases.

Major revisions

1) OleTCL has high specificity for olefin decarboxylation compared to other fatty acid decarboxylases (OleTKM and OleTPRN), which may be related to unique concerted structural alterations in the hydrophobic cradle (rotating F19 and L26 rather than F). Please clarify whether this property is unique to only the OleTCL enzyme, or if it is conserved in other CYP152 decarboxylases possessing a similar hydrophobic cradle environment. It may be important to provide the universality or specificity of each CYP152 decarboxylase mechanism (OleTCL, OleTPRN, and OleTKM) for the impact of this study.

R: Reviewer #1 raised an interesting point, and following their recommendation, we performed oleic acid turnover assays with two additional $OleTP_{CL}$ orthologs that possess putative flexible phenylalanine residues. These enzymes had their DNA sequences synthesized, were then expressed in *E. coli*, purified, and finally assayed for activity

against oleic acid. The results showed that these orthologs also exhibited high oleic acid conversion, pointing that the ability to decarboxylate oleate is likely associated with the increased flexibility of phenylalanine, rather than being an exclusive feature of $OleTP_{CL}$. These findings are presented in Supplementary Fig. 21 and discussed in the main text (lines 270-280).

2) Please provide a graphic summary of the proposed fatty acid decarboxylase mechanism including OleTPRN and OleCL. This would make it easier to understand for a broader audience.

R: In response to Reviewer #1's recommendation, a graphic summary (Supplementary Fig. 28) was created to compare the proposed oleic acid activity mechanism of $OleTP_{RN}$ and $OleT_{KM}$ (with fixed phenylalanine) versus $OleTP_{CL}$ (with flexible phenylalanine) within this specific CYP152 cluster. In this work, the flexible phenylalanine (F19) in $OleTP_{CL}$ was shown to be important for pushing the oleic acid Cβ towards the heme group, positioning it productively to promote alkene production. Additionally, a hydrophobic cradle has been shown to play a significant role in pulling the product away from the enzyme's active site in $OleTP_{RN}$ [1]. Thus, both mechanisms suggest that substrate binding in a productive position — specifically, with Cβ closer to the heme group — is essential for catalysis, followed by a backward pull of the alkene to promote product release. In contrast, due to $OleT_{KM}$ having a constrained binding pocket with low hydrophobicity and lacking the flexible phenylalanine present in $OleTP_{CL}$, oleic acid cannot achieve productive positioning or proper product release, leading to an obstructed binding pocket that results in substrate inhibition.

3) Throughout the manuscript, the discussion about bifurcated chemoselectivity towards decarboxylation rather than α or β hydroxylation is relatively scarce compared to unsaturated substrate selectivity.

R: We acknowledge that this work does not extensively discuss the α or β regioselectivity of the studied enzymes. We agree that this is a relevant and still open question, although it has been deeply investigated in several studies [2–7]. In contrast, the conversion of unsaturated substrates has been overlooked for fatty acid decarboxylases until it was addressed by Rade et al. (2023) [1], principally by the fact the P450 decarboxylases are inhibited by this fatty acid. Therefore, in this manuscript, we focused on the elucidation of the basis of oleic acid conversion, which could have straightforward implications in the context of the sustainable production of biohydrocarbons and consequently to circular bioeconomy.

4) Please emphasize the impact of oleic acid decarboxylation rather than saturated acid decarboxylation in terms of industrial application. If important, the decarboxylation activity of other unsaturated fatty acids should also be tested.

R: We thank Reviewer #1 for pointing out this relevant aspect. As suggested, we evaluated $OleTP_{CL}$ performance against different mixtures of unsaturated substrates, including oleic, linoleic and linolenic acids, and results were included in Supplementary Fig. 11 and lines (134-143).

Given the promising role of enzymatic decarboxylation in olefin production, principally with the discovery of P450 decarboxylases active on unsaturated fatty acids (Rade et al. (2023) [1] and this work), we investigated the use of the enzyme $OleTP_{CL}$ to convert distillers corn oil (DCO) into alkenes. DCO is a lipid-rich byproduct produced in corn ethanol biorefineries (Supplementary Fig. 26), consisting mostly of unsaturated fatty acids (>80%).

Using a two-pot reaction, which included a previous enzymatic hydrolysis to maximize the content of FFAs for conversion into hydrocarbons by $OleTP_{CL}$, we achieved an olefin titer of 1.7 g/L from DCO. This was made possible by the robustness of $OleTP_{CL}$, which performed effectively under industrially relevant conditions, demonstrating a high total turnover number (TTN) of 1470 in a 50-mL reaction system. To date, the highest olefin production reported in the literature via enzymatic decarboxylation using P450 enzymes has achieved 0.49 g/L from coconut oil (in a 10-mL reaction), a more expensive oleaginous feedstock rich in saturated fatty acids, in contrast to DCO.

Therefore, our findings create new potentials for exploration of fatty acids decarboxylases, not only from a scientific perspective but also for biotechnological applications aimed at synthesizing green products from industrial byproducts.

5) Figure 5A and Figure S16, the substrate of OleTPRN structure seems to be palmitic acid rather than oleic acid. Oleic acid bound OleTPRN structure may be required to describe the results.

R: We recognize that a crystallographic $OleTP_{RN}$ complex with oleic acid would be more beneficial for binding pocket comparisons, which our group has extensively attempted to obtain. However, it is notorious the sheer complexity and challenging nature of obtaining substrate/product crystal complexes with these enzymes principally due to the high hydrophobicity of their substrates. Supporting this, apart from the structures deposited by our group ($OleTP_{RN}$, $OleT_{KM}$, and $OleTP_{CL}$), only one additional structure of the $OleT_{JE}$ have been reported in complex with eicosanoic acid (C20), a rare substrate in nature. Therefore, our result represents the first crystallographic structure complexed with oleic acid, an economically relevant substrate, which was only achieved with $OleTP_{CL}$, despite of exhaustive attempts with all other P450 decarboxylases. Remarkably, the newly obtained oleic acid complexed structure allowed us to infer new roles for the binding pocket and particularly conformational changes in driving catalysis selectivity. This

outcome opens up new possibilities for future investigations with P450 peroxygenases, such as selecting CYP152 members with activity against unsaturated substrates for further bioprocess studies.

Minor revisions

1) Line 85, please correspond “unsupervised machine learning approaches” to the main result data, because it is unclear which data is indicated.

R: Sentence was rewritten to better convey our results and hypotheses to the reader in a more direct speech (lines 80-86).

2) Figure 1A, what is the main sequence difference among different clusters? It would be helpful to show this as an additional figure.

R: We thank the reviewer for pointing this out. It is worth noting that the SSN was constructed to emphasize isofunctional clusters, ensuring that sequences from different clusters share no more than 50% of identity. To further highlight the main sequence differences among the clusters, we selected representatives’ sequences from each cluster, including reference sequences with demonstrated activity, to perform a phylogenetic analysis (Supplementary Fig. 2) and a multiple sequence alignment (MSA) of those sequences (Supplementary Fig. 3). As can be seen from those analyses, intra-cluster sequences share high residue conservation, however, this cannot be seen inter-cluster due to sequence distances. To further highlight inter-cluster low conservation, a sequence logo of the representative sequences is here provided to highlight the differences in the hydrophobic cradle, dimeric interface and catalytic residues among the different clusters (Fig. R1).

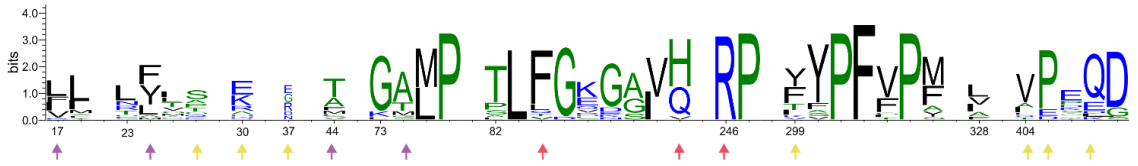

Fig. R1. Sequence logo generated using WebLogo 3.7.12 of the representatives’ sequences highlighting the catalytic site forming amino acids (red arrows), the hydrophobic cradle (pink), and the dimeric interface (yellow).

3) Line 109~112, please provide table or figure (same as above) for the sentence, “members from cluster I were classified as acting mainly as hydroxylases” and “Cluster II and cluster III members act mainly as fatty acid decarboxylases”.

R: The members of cluster I were classified as acting mainly as hydroxylases since they are closely related to *Bacillus subtilis* (G8IRJ8) and *Sphingomonas paucimobilis* (A0A0C9N8Y6), enzymes previously characterized as mainly hydroxylases. Similarly, members from clusters II and III were classified as acting mainly as fatty acid

decarboxylases, sharing sequence similarity with the peroxygenases $OleTP_{RN}$ from *Rothia nasimurum* and $OleT_{JE}$ *Jeotgalicoccus* sp. ATCC 8456, respectively. These references sequences were included in the phylogenetic tree and MSA shown in the Supplementary Figs. 2-3.

4) Figure 1B and Line 124-126, it is unclear what the author wants to be emphasized. It may be better to move to supplementary figure.
R: Fig. 1B was moved to SM as suggested (Supplementary Fig. 6). The intent of the paragraph was to emphasize the challenges associated with predicting CYP152 activity. Even when considering genomic context, members of this family deviate from the patterns observed in other CYP152 decarboxylases, making predictions difficult. The sentence has been modified to better articulate this perspective (lines 122-125).

5) Line 135-138, please discuss about the possible explanation about the absence of inhibition by oleic acid in OleTCL.
R: Oleic acid, being a longer substrate with unsaturation, requires a specific binding for the productive catalysis within the P450 catalytic pocket. The conformational change induced by F19 in $OleTP_{CL}$ enables this productive binding, allowing catalysis to proceed and the product to be released. Once the product is released, the pocket is free to bind other substrates. In contrast, other P450 enzymes, such as $OleT_{JE}$ and $OleT_{KM}$, lack a mechanism that facilitates oleic acid catalysis, either impaired productive binding or product release. As a result, oleic acid and/or alkene may occupy the pocket without undergoing complete catalysis, thereby preventing other substrates from accessing the pocket and leading to competitive inhibition. This characteristic was also included in the mechanism scheme represented in Supplementary Fig. 28.

6) Line 177, Fig 3G should be Fig 2G.
R: Typo was accordingly corrected.

7) Line 193-195, please discuss about author's opinion about further aspects.
R: As requested, we have included further discussion on possible aspects that preserve oleic acid decarboxylation capacity of the H92Q mutant (lines 197-210).

8) Line 301-303 and Figure 5B, it would be helpful to indicate how each mutation affects to cradle distance and hydrophobicity.
R: As suggested, a table was included in Fig. 5b, highlighting how mutations increase or decrease binding pocket length and hydrophobic cradle hydrophobicity.

9) Line 387, OleT”KM” should be subscript.

R: Typo was accordingly corrected.

10) Table S1, table legend seems to be changed (Corynebacterium e Korcuria?)

R: Supplementary Table 1 legend was changed to clarify the content.

Reviewer #2:

In the present manuscript, Generoso et al performed a mechanistic study based on the structures of CYP450 peroxygenase, particularly based on the structure of OleTCL and OleTKM. The authors used crystal structure, MD simulations, and supervised machine learning to support their study. This work is very specialized, and it represents one more work in the list of several alkene-producing Cytochrome 450 works. I don't see its scope for the general audience of Nature Communication. Indeed, its scope is also limited to readers of general chemistry as the importance of the hydrophobic cradle is already published in the PNAS, 2023 by the same group. Therefore it is just an extension.

R: We thank Reviewer #2 for the comments regarding our work. Fatty acid decarboxylation by P450 members is still a nascent field, started with the discovery of the first P450 enzyme decarboxylating saturated fatty acids [8], which is inhibited by unsaturated fatty acids. Later on, our group [1] discovered the first P450 member ($OleTP_{RN}$) able to efficiently decarboxylate both saturated and unsaturated fatty acids. Remembering that unsaturated fatty acids are abundant and economically relevant for biotechnological applications such as in biofuels production.

This present work brings findings about P450 decarboxylases that we respectfully disagree that is a merely extension of our previous publications including the elucidation of the first crystal structure of a complex between a P450 decarboxylase and an unsaturated fatty acid and the uncovering of a novel mode of action among P450 decarboxylases that involves intricated and concerted conformational changes associated with the productive biocatalytic reaction with unsaturated fatty acids leading to alkene production. Particularly, the novel mode of action described in the present work does not follow the same hydrophobic cradle-guided catalysis observed in $OleTP_{RN}$. Instead, it encompasses a compelling binding pocket rearrangement in $OleT_{CL}$, discovered by the visualization of a phenylalanine residue (F19) adopting two distinct conformations in crystal structures complexed with two different substrates (palmitic and oleic acids).

This manuscript, therefore, introduces a new layer of complexity to the understanding of fatty acid decarboxylation, particularly for oleic acid, grounded in extensive and interdisciplinary experimentation encompassing biochemical methods, analytical chemistry, chemical biophysics, structural and computational biology, and structure-guided mutagenesis. Our work challenges the assumption of mechanistic homogeneity within P450 decarboxylases, highlighting the critical need for continued research to unravel the factors governing specificity and catalytic mechanisms. A deep mechanistic understanding of P450 decarboxylases is instrumental to enabling their rational design and to advancing their industrial applications.

Besides, given the promising role of enzymatic decarboxylation in olefin production principally with the discovery of P450 decarboxylases active on unsaturated

fatty acids (Rade et al. (2023) [1] and this work), we investigated the use of $OleTP_{CL}$ to convert distillers corn oil (DCO) into alkenes. DCO is a lipid-rich byproduct produced in corn ethanol biorefineries (Supplementary Fig. 26), consisting mostly of unsaturated fatty acids (>80%). Using a two-pot reaction, which included a previous enzymatic hydrolysis to maximize FFA content for conversion into hydrocarbons by $OleTP_{CL}$, we achieved an olefin titer of 1.7 g/L from DCO. This was made possible by the robustness of $OleTP_{CL}$, which performed effectively under industrially relevant conditions, demonstrating a high total turnover number (TTN) of 1470 in a 50-mL reaction system. To date, the highest olefin production reported in the literature via enzymatic decarboxylation using P450 enzymes has achieved 0.49 g/L from coconut oil (in a 10-mL reaction; Supplementary Table 8), a more expensive oleaginous rich in saturated fatty acids, in contrast to DCO.

Therefore, our findings create new potentials for exploration of fatty acids decarboxylases, not only from a scientific perspective but also for biotechnological applications aimed at synthesizing green products from industrial byproducts.

In conclusion, we believe our work offers valuable insights into the mechanistic diversity of P450 decarboxylases and their potential for sustainable bio-olefin production. We hope this revised manuscript, with its expanded scope and additional data, addresses the reviewer's concerns and highlights the broader significance of our research.

Apart from the above opinion about the scope of the work in Nat Comm, I have several other concerns that don't allow me to recommend this work for publication.

R: We believe the extensive changes we have made, including the incorporation of longer MD simulations and assays on industrially relevant materials, substantially strengthen the work and address the reviewer's initial reservations. We are confident that these improvements make a compelling case for the publication of our work, and we look forward to the reviewer's reevaluation of our manuscript and are hopeful that these substantial revisions will lead to a positive recommendation.

The work presentation is poor. There are several confusing statements and incorrect words (see details below). Several typos and incorrect citations of the figure in the text, e.g., line 114 'genera' ; line 188 citation of Fig 3G, which should be either 2G or 3C.

R: We thank the reviewer for pointing that out. Typos were accordingly corrected.

Technical issue: 1) The MD simulations of 100ns in just one replica are not recommended on any platform. This becomes of high importance when your entire story is structure-dependent. You are emphasizing conformational changes (e.g., rotation of Phe etc) and 100 ns is too short to validate the claim.

R: As had already been discriminated in Fig. 4 in the original submitted manuscript, we performed four independent simulations of 100 ns (considering only the production stage) for each system. Each independent simulation started from independents initial heating

stages (in which initial velocities were assigned randomly according to a Maxwell-Boltzmann distribution at the temperature). Even though, to further address this point, we have now extended our simulations to 250 ns considering the production stage per independent simulation (n = 4), totalizing 1 microsecond of simulation for each system. It is crucial to emphasize that the conformational change in the phenylalanine residue was primarily observed and established based on the crystal structures of the complex, not solely on the MD simulations. The simulations added insights into the dynamics and potential mechanisms underlying this conformational change. In the revised manuscript, we explicitly state the number of independent simulations performed (n=4) and the extended duration of 250 ns for each system. We also reiterate that the primary evidence for the phenylalanine conformational change stems from crystallographic data, with the MD simulations providing complementary dynamic information.

2) Parameterisation: You are using the resting state of the heme-porphyrin, but the reactive state is Cpd I. There is no MD simulation in this state. During the catalytic cycle, many conformation changes may happen (see Science, 2000 by Schlitching 2000).
R: Simulations were performed considering the heme-porphirin resting state, as contained in Charmm36 force-field. Since a ns–µs scale simulations were necessary to sample the dynamical events associated to F19 mobility, we adopted the classical MD approach, therefore, not considering electronic rearrangements. However, considering Reviewer #2 comment, for this new version, we performed additional simulations using the Cpd I, as a control, for the system $OleTP_{CL}$ with oleic acid. As expected, the same F19 flexibility was observed (Supplementary Fig. 30), corroborating previous results.

3) Supervised machine learning for data generated by 100 ns is misleading and is of no sense.
R: We would like to clarify that the method employed was unsupervised learning, not supervised learning as mentioned in the reviewer's comment.

Regarding the concern about data sufficiency, we believe that the 100 ns simulations from a single replica generated an adequate number of frames (n = 5000) for PCA and clustering analysis. This allowed us to effectively sample the conformational space and identify relevant clusters. The fact that representative structures close to the cluster centers were found within the original trajectories further supports the adequacy of our sampling.

Even though, to strengthen this analysis, we are pleased to provide a new analysis considering the full 1 microsecond trajectory obtained by concatenating the four independent 250 ns trajectories of each system, which amounted to a total of 50000 frames to support our findings. The convergence test showed that the same result was

observed for the simulations of 250 ns compared to those of 100 ns, supporting that the results obtained in the original submission was statistically significant.

In the revised manuscript, we explicitly state that we used unsupervised machine learning techniques and provide further details about the specific methods and hyperparameters employed. We also emphasize the adequacy of our data sampling, and the validation provided by the presence of representative structures within the original trajectories.

We are confident that these clarifications will address the reviewer's concerns and demonstrate the validity of our unsupervised machine learning analysis in the context of our study.

Detailed report:

1. The use of proximal and distal terms is confusing in the entire manuscript. Usually, the Thio-ligated side is termed as proximal, and the substrate side is termed as distal. However, somewhere in the manuscript, Phenylalanine is mentioned as distal (Page 2, Introduction line 83), and somewhere, it is proximal (abstract, line 43).

R: Indeed, proximal and distal terms in P450 enzymes are unintuitive, not following a single, universal rule. Nonetheless, we were following previous publications of P450 decarboxylases that classify residues closer to heme group as proximal and residues at solvent-end extremity of the binding-pocket as distal. As an example, publication by Amaya et al. named as "A Distal Loop Controls Product Release and Chemo- and Regioselectivity in Cytochrome P450 Decarboxylases" [5], which describes the importance of F/G loop, located at the solvent-end extremity of OleTs binding-pocket, as an important player in activity of $OleT_{JE}$. Nevertheless, we omitted most "distal" and "proximal" residue indications to avoid miscomprehension.

2. In most places, authors discuss the role of Phe, Val, etc, without mentioning the residue ID, which shows poor scholarly presentations. For example: in line 119 and there are several more.

R: We acknowledge the importance of providing precise residue numbering. In the revised manuscript, we have carefully reviewed the text and added residue IDs wherever necessary to avoid any ambiguity or confusion. We believe this improvement will enhance the clarity and readability of our manuscript.

3. Line 129-130, the product percentage or ratio (hydroxylation or decarboxylation) is needed. The use of 'major product' is ambiguous.

R: The sentence was rewritten to avoid ambiguity and citation of Supplementary Table 3 was added, in which the percentage of decarboxylation is described.

4. In Lines 137-141, the Authors say- OleTCL metabolized both substrates, but OleTKM was inhibited. Here, readers will assume that OleTKM does not show activity. However, in the proceeding sentence, the authors say that the three enzymes, including OleTKM, have broader substrate specificities. This is confusing.

R: We acknowledge the potential for confusion in the original text. The statement that $OleT_{KM}$ was "inhibited" might lead readers to assume a complete lack of activity. We have revised the text to clarify that $OleT_{KM}$ is indeed active, but its activity is specific to saturated fatty acids. The previous statement referred specifically to its lack of activity towards oleic acid, an unsaturated fatty acid. The revised text now explicitly states this distinction, ensuring readers understand the nuances of $OleT_{KM}$'s substrate specificity. Furthermore, we included a schematic representation of oleic acid activity among investigated cluster-II CYP152 (Supplementary Fig. 28).

5. Line 163, "OleTCL functions as monomer most likely due to…." This again confusing. The substitution of K299, Q404, E407 may be structurally important but not necessarily functionally important. When we use the function, we automatically think of the catalytic function, but here, I assume the authors want to talk about dimerization and monomer, which are structural properties not anyhow related to catalytic function.

R: Sentence was modified to clarify our point of view (lines 171-173).

6. Line 179 to 181, Histidine 85 in OleTJE is on the distal side close to the substrate side. Therefore, the use of 'proximal' is incorrect here.

R: As stated previously, we were following previous publications of P450 decarboxylases that classify residues closer to heme group as proximal and residues at solvent-end extremity of the binding-pocket as distal. Nevertheless, the sentence was modified to avoid misunderstanding (line 181).

7. Line 188, Figure 3G, doesn't say anything about the reactivity of the H92Q mutant. I guess the authors want to cite Figure 2G.

R: This typo was accordingly corrected, thank you.

8. The flexibility of F19 has been exaggerated in the manuscript. I can see the rotation of F19 only in Figure 4B and Figure 4C. Therefore, I don't think it is an intrinsic property, as claimed by the authors in line 225. In fact, the rotation of the Phe19, as mentioned by the authors, was also not found in the crystal structure (line 228). Rotation, seen in the simulation, might be an artifact of the simulation.

R: We apologize if in any instance of the original text, it was not clear that the observation of F19 conformational change is primarily derived from crystallographic data of

complexes, and not from the MD simulations. The electron density map of this residue, clearly depicted in Fig. 3a and Supplementary Fig. 12, provides the experimental support for the existence of these conformations in the crystal structure itself. While the MD simulations offer additional insights into the dynamics of this flexibility, they are not the primary basis for our claims. Note in the following parts of the original submitted manuscript that refer to the crystallographic observations of this conformational change of F19.

"Such conformational change of F19 is confirmed by the clear electron density maps of the F19 side chain in both crystal structure complexes obtained with saturated or unsaturated substrates (Supplementary Fig. 12)."

9. MD simulation of 100 ns and only in one replica is unacceptable at any scale.
R: As aforementioned, MD simulations were carried out considering four independent replicas (considering only the production stage) for each system, each starting independently from the initial heating stage. For the revised version of the manuscript, we extended them to four independent simulations to 250 ns, yielding a total of 1 microsecond per system.

10. Authors mentioned that they co-crystalized the enzyme complex, but to my surprise, the Phe19 is nowhere in any crystal structure. It will be intriguing to compare the simulated structure and the crystal structure.
R: We appreciate the reviewer's careful attention to our manuscript. As mentioned earlier in our response, the crystal structure and corresponding electron density map showcasing the F19 residue were indeed included in the original submission as Supplementary Fig. 12. We have also revised the main text to ensure clearer referencing and highlighting of the crystallographic evidence supporting our observations regarding F19.

10. Supervised machine learning with 100ns of simulation and in one replica is recommended. The simulated data is too preliminary to be used for Machine learning, and the results are of no sense.
R: We would like to highlight that the method employed was unsupervised, not supervised as mentioned. Please see the response above regarding this point. Reinforcing that this analysis was carried out now with one microsecond of MD simulations.

Reviewer #3:

This manuscript describes new crystal structures of P450 peroxygenases in complex with (un)saturated fatty acids, aiming to provide further insights into this biotechnologically interesting family of enzymes that have considerable scope for olefin production. Of particular interest is understanding both substrate and product specificity in this enzyme family, with the need for biocatalysts that can convert readily available fatty acids (which includes unsaturated FA) to corresponding olfin in absence of hydroxylation reactions.
R: We appreciate Reviewer #3's constructive comments regarding our manuscript. Their insights on the importance of understanding substrate and product specificity in P450 peroxygenases are valuable and will undoubtedly enhance the impact of our research and contribute to the broader field.

The manuscript would significantly benefit from editing to improve English language/clarity. From the main title, abstract to the main text, language used is not concise enough and often resorts to cumbersome/convoluted sentences/vocabulary.

As a brief example see beginning of abstract:

"Fatty acid peroxygenases have been underscored in hydrocarbon biosynthesis due to their capacity to perform C-C scission en route, producing olefins, a central building block for the production of sustainable plastics, polymers, and fuels. These biocatalysts possess non-canonical and complex mechanisms, which involve redox partners, co-factors, hydrogen abstraction, controlled electrons and protons delivery that culminate in bifurcated chemoselectivity into hydroxylation or decarboxylation."

Which i suggest could be:

P450 fatty acid peroxygenases are able to produce olfins, a central building block for production of sustainable polymers and fuels, through oxidative decarboxylation. In addition, they also catalyse the more canonical fatty acid hydroxylation, with product specificity dependent on identity of both substrate and peroxygenase used.

The abstract finishes with:

"Taken together, these findings untangle key molecular factors governing the tunable biocatalytic selectivity of P450 peroxygenases that are central for the sustainable production of olefins from oleic acid, the most abundant and relevant renewable fatty acids in nature."

R: We appreciate the constructive remarks upon clarity of sentences, and therefore we altered these (and other) paragraphs aiming at a better understanding of the reader, employing less intricated language and more direct speeches.

While i agree the authors provide compelling data for how oleic acid is accepted by OleTcl through structural studies combined with simulation, various mutagenic studies provide little in way of clear effect on product specificity (see for examples Figs 2,3,5 where only minor changes are observed for various mutants made). Therefore, it is not clear how key these molecular factors actually are, nor whether these could be use to predictively "tune" P450 peroxygenases.

R: The discovery of $OleTP_{RN}$ in previous work highlighted that distinct molecular determinant can drive the unconventional decarboxylation reaction in P450 enzymes. Notably, it proposed this activity for unsaturated fatty acids, which have an inhibitory effect on $OleT_{JE}$, the founding member with decarboxylase activity. Now, we have introduced a new dimension to the understanding of this complex system — the flexibility of the phenylalanine residue (F19), which enabled oleate decarboxylation at the expense of reduced hydrophobicity of $OleTP_{CL}$ cradle. By integrating these molecular aspects into a broader context, predictability improves. Hence, we further validated this by examining two additional P450 orthologs with conserved microenvironment for the corresponding F19, confirming their ability to convert unsaturated substrates. These data were included in the revised manuscript in the Supplementary Fig. 21.

Besides, in the present work, we looked into the paradigm of the canonical Phe-His-Arg triad as a strictly necessary mark for alkene production. In the enzyme studied, $OleTP_{CL}$, the phenylalanine residue is naturally substituted by a valine (V86), and it still results in the productive decarboxylation of both saturated and unsaturated fatty acids, and besides, H92Q point mutation does not hinder oleic acid decarboxylation.

Moreover, as mentioned above in response to the other two reviewers, in the revised manuscript, we introduce the feasibility of converting distillers corn oil (DCO), a byproduct of corn ethanol biorefineries rich in unsaturated fatty acids, into olefins. By using an enzyme cascade in a cell-free system, we achieved an olefin titer of 1.7 g/L from DCO. This was made possible by the robustness of $OleTP_{CL}$, which performed effectively under industrially relevant conditions, demonstrating a high total turnover number (TTN) of 1470 in a 50-mL reaction system. To date, the highest olefin production reported in the literature via enzymatic decarboxylation using P450 enzymes has achieved 0.49 g/L from coconut oil (in a 10-mL reaction; Supplementary Table 8), a more expensive oleaginous rich in saturated fatty acids, in contrast to DCO.

Therefore, our findings open new avenues for exploration, not only from a scientific perspective but also for biotechnological applications aimed at synthesizing green products from industrial by-products.

A few other suggestions:

A schematic figure detailed the chemistry catalysed by P450s peroxygenases would assist the non-expert reader, while it would also help to clearly clarify these are peroxide dependent (ie not your classical redox partner one electron at a time P450s).
R: The classical P450 catalytic cycle has not been included in the previous manuscript, since no experimentation was performed to investigate heme electronic factors. However, following their suggestion, Supplementary Fig. 1 was included in the supplementary material schematizing direct peroxide shunt towards fatty acid decarboxylation.

Fig 2 could perhaps be more clear if it included the active site solvent accessible surface to provide context to the fatty acid conformation. Please clarify for this fig and other containing product analysis how many replicates and whether these were technical replicates or not.
R: Figure 2 was revised to include the binding pocket surface, as suggested. All these data consist of three independent experiments (n = 3), and this information has been added to each figure caption as recommended.

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Point-by-point response to the reviewers' comments

Reviewer Comments:

Reviewer #1:

Opinion: Accept with minor revisions

Summary: The authors have addressed most of the questions raised by the reviewers, and the revised version of the manuscript appears to have a greater impact, particularly by presenting olefin production data from renewable feedstocks using OleTPCL. Additionally, the authors provide high oleic acid conversion data for OleTPCD and OleTPMA, which may also possess a flexible phenylalanine residue in the hydrophobic cradle. This suggests the potential to discover more diverse P450 decarboxylases similar to OleTPCL in the future. Therefore, the manuscript is recommended for acceptance with a few additional minor revisions.

R: We appreciate the constructive and positive comments from Reviewer #1 supporting our work. The subjects highlighted previously undoubtedly helped to increase the impact of our work.

Minor revisions

1) The authors' opinion is understood to be that this work focuses on decarboxylation and does not extensively discuss the α or β regioselectivity of the studied enzymes. Nevertheless, it may be beneficial to briefly discuss the differences in hydroxylated product yield between samples in some graphs, such as the increased βOH of the C12:0 product in the L26F mutant shown in Figure 3c.

R: Indeed, the bifurcation between hydroxylation and decarboxylation is one of the major open questions in this field since the discovery of the first predominantly decarboxylase. The set of techniques we have used here cannot fully explain the competition between oxygen rebound and decarboxylation in the catalytic cycle. Addressing this would require inherently complex and time-consuming approaches, for instance, QM/MM methods, and therefore could constitute itself an independent study. However, we agree that the increased β-hydroxylation of the C12:0 product by the L26F mutant should be briefly addressed in the text (lines 260–263).

2) Additionally, there are significant differences in hydroxylated product yield among different substrates in Figures 1b and 1c. For example, C10:0 does not yield any hydroxylated products, compared to the OleTPRN data from a previous PNAS paper. It would enhance the quality of the paper if the authors suggested or discussed the reasons for the small proportion of hydroxylated products and possible engineering approaches to achieve higher selectivity for decarboxylation.

R: Before the discovery of $OleTP_{RN}$, studies showed that for $OleT_{JE}$, a decrease in substrate chain length led to an increase in hydroxylation. However, as more decarboxylases have been characterized by our group, this trend no longer seems to be universal. Structural variations along with conformational changes in the active-site pocket of decarboxylases with differing specificities may impact substrate binding and accessibility to the heme iron center, thereby influencing the competition between decarboxylation and hydroxylation. Furthermore, variations in electron transfer pathways or the efficiency of electron delivery to the heme center could potentially influence the catalytic outcome. As several molecular and electronic factors can affect the regio and chemoselectivity of decarboxylases, a QM/MM study to investigate the catalytic itinerary would be instrumental to provide insight into structural determinants and therefore guide protein engineering to achieve higher selectivity for decarboxylation. It was included in the text (lines 415 – 420).

3) The authors' response regarding the difficulty of obtaining substrate/product crystal complexes with OleTP enzymes is noted. Are there any specific or important methods to successfully obtain the structures of the enzymes discussed in this paper? If so, it would be helpful for further structural studies. Additionally, it would be beneficial to discuss this limitation in the discussion section.

R: According to our experience acquired on the crystallization of P450 decarboxylases, we could never achieve any crystal of "free" enzymes (without any fatty acid), indicating that complexation with substrate is mandatory to achieve a stable and crystallizable state. Interestingly, some enzymes are more stable with specific substrates than others, which does not strictly correlate with substrate preference, and therefore, extensive experimentation is needed to achieve crystals of these enzymes. It is worth noting that these enzymes crystallized only at high concentrations (above 50 mg/mL), despite their activity on hydrophobic substrates. This information has been added to the experimental procedures section of the article.

4) In line 171, OleTPCL is found to be a monomer. Does this property make any difference in substrate specificity and decarboxylation activity compared to dimeric OleTPs? Please discuss.

R: Limited information is available about the oligomerization, substrate specificity and regioselectivity of CYPs overall, remembering that most enzymes belonging to the large

P450 family are predominantly monomeric. For $OleTP_{RN}$, we demonstrated that the dimer is related more to stabilizing the productive conformation of the enzyme instead of electronic aspects. Given this, we cannot discard a potential correlation between dimerization and substrate specificity and decarboxylase activity, which could only be clarified when additional structural data of both monomeric and dimeric decarboxylases become available.

5) In lines 274-276, please indicate the amino acids of OleTPCD and OleTPMA that correspond to L26 of OleTPCL for better readability.
R: Thank you for this suggestion. We have revised the manuscript to explicitly indicate the corresponding amino acids in $OleTP_{CD}$ and $OleTP_{MA}$ (lines 279-280).

6) OleTCL and OleTPCL are both used in the manuscript, which is confusing. Please unify them into one term.
R: Following our previous publication, we differentiated $OleTP_{RN}$ enzyme name by including a P (of polyunsaturated) due to its efficient conversion of unsaturated fatty acid. Therefore, following this rule, "$OleT_{CL}$" should be properly named as $OleTP_{CL}$ (same for $OleTP_{MA}$, $OleTP_{CD}$). The manuscript was revised to avoid typos in enzyme names.

7) In Figure 4b, what is the significance of the two different dihedral angle distributions with similar frequencies?
R: Data from Figure 4b correspond to the $OleTP_{CL}$ enzyme with palmitic acid as substrate. The bimodal histogram, with values centered around ~50° and ~160° and nearly equal frequencies (and therefore probabilities), indicates that both microstates — open and closed dihedral angles of the F19 residue — are equally accessible under these thermodynamic conditions, as observed across all independent replicas. This information has been added to the figure legend.

Reviewer #2:

I appreciate the careful and comprehensive response to my previous comments. The authors have extended the simulations and revised the previous ambiguity. The manuscript is fairly clear relative to the original version. I believe the revised version can be accepted for publication.

R: We appreciate the positive evaluation from Reviewer #2. The points raised previously undoubtedly helped to increase the impact of our work and aided a clearer comprehension of our achievements.

Reviewer #3:

This manuscript is now much improved with constructive response to the various review raised. The additional data with respect to DCO oil conversion provides a useful new perspective on the potential for future application.
R: We appreciate Reviewer #3's positive recommendation of our manuscript.

I have a few minor points:

1) the language throughout will need careful editing, while improved at places, it remains cumbersome and at points not quite grammatically or factually correct. As a minimum, the abstract should be entirely rewritten with that in mind.
R: We appreciate the constructive remarks upon clarity of sentences, and therefore we altered the abstract aiming at a better understanding of the reader.

2) please provide R/Rfree values for outer resolution shell.
R: The R/Rfree values for outer resolution shell were added in the Supplementary Table 4.