nature portfolio

Peer Review File

Decoding the Substrate Specificity Landscape of a Promiscuous Enzyme through Multi-Substrate Mutational Scanning

Corresponding Author: Professor Michael Nash

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)
This study used the enzyme-proximity sequencing method (EP-Seq) developed by the authors to comprehensively map, at single-mutation resolution, the substrate specificity of Rhodotorula gracilis D-amino acid oxidase (RgDAOx), and to identify mutations that confer preferences for particular D-amino acid substrates together with their structural implications. The results indicate that, at least in this enzyme system, EP-Seq can serve as a powerful platform for high-throughput enzyme design.
The strengths of the study are threefold: (i) a comprehensive, large-scale screening dataset is carefully supported by rigorous statistical processing and assessments of reproducibility; (ii) the D-factor obtained from single-clone kinetic measurements agrees well with the Sp-score derived from deep mutational scanning (DMS), thereby reinforcing the validity of the measurement approach; and (iii) the work illuminates not only hotspots near the active site but also distal mutations, demonstrating that substrate specificity can be governed through diverse proximal and distal routes.
On the other hand, for some of the key mutations that drive substrate preferences, the structural interpretation remains ambiguous or is not fully presented. In representative cases, further concretizing the mechanistic hypotheses, based on three-dimensional models, interaction networks, and conformational changes, and, where appropriate, supplementing them with in silico modeling or validation using substrate analogs would deepen readers' understanding.
Overall, the manuscript offers strong methodological contributions and high-quality data, providing important advances for the practical utility of EP-Seq and for understanding the evolution of substrate specificity. A deeper examination of the structural mechanisms underlying the major mutations would further strengthen the paper.

Comments:
1. The authors used five types of D-amino acids as substrates. Three of these are hydrophobic (D-Ala, D-Met, D-Phe), and two are polar/hydrophilic (D-Asn, D-Gln). Supplementary Material S2 outlines the rationale for these choices, but can the authors explain why the substrates were limited to five types and why other valid candidates (e.g., moderately sized hydrophobic substrates like D-Val or D-Ile, polar substrates like D-Thr, or acidic D-amino acids) were not included? Additionally, why was the contribution of salt bridge effects to substrate specificity not investigated using an acidic D-amino acids?

2. It appears that coenzyme FAD was not added to the enzyme assay system. Could the effect of the amino acid mutation on FAD binding (i.e., the difference in catalytic activity) be influencing the results?

3. Regarding the discussion of the structural mechanisms by which amino acid mutations affect substrate specificity, several points remain ambiguous. For example, concerning S341I, I believe a detailed explanation should be added, such as: "S341 forms a hydrogen bond with the main-chain carbonyl group of Y338, which is adjacent to Q339 forming the substrate-binding site; therefore, it may influence the orientation of Q339." Is it possible to provide a more detailed analysis of the structural mechanism by which the S341I mutation confers absolute activity toward D-Gln, or the structural mechanism by which the T93M mutation enhances binding affinity for D-Met? Readers are likely to seek an understanding of the structural mechanisms by which amino acid mutations located away from the active site can influence substrate specificity.

4．Fig. 7. Why weren't combinations of amino acid mutations at positions distant from the active site verified?

Minor comments:
1. As shown below, “Fig. 2C” should be added to the following sentence.
Nonsense mutations had strongly negative effect on activity (average score -0.58 ± 0.05; n = 316 in all five datasets), indicating that loss of the C-terminal region beginning anywhere from position 1 to position 354 had a deleterious effect on the catalytic activity of the enzyme, regardless of the substrate used (Table S3, Fig. 2C)

2. The data for M213D do not appear to be present in Fig. 5 iv and Table S5.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The authors present compelling work exploring enzyme substrate specificity in the model oxidoreductase D-amino acid oxidase (DAOx). They apply their previously developed enzyme proximity sequencing (EP-Seq) method to perform deep mutational scanning of DAOx across five diverse D-amino acid substrates. The high-throughput fluorescence-based measurements are highly reproducible and show excellent agreement with clonal measurements of activity and specificity. The resulting data provide a detailed, quantitative map of activity and specificity across the DAOx sequence, revealing a complex substrate-specificity landscape. The primary contribution of this work is the rigorous and insightful analysis of this large dataset. The authors identify hundreds of mutations throughout the enzyme that significantly impact substrate preference. They find that active-site mutations can cause large specificity shifts but often reduce catalytic activity, whereas distal mutations exert subtler effects that fine-tune specificity. They introduce a quantitative substrate-specificity score (Sp-score) that correlates strongly with experimentally determined discrimination factors, enabling large-scale analysis of specificity directly from high-throughput data. They identify structural factors driving specificity and a key mutational hotspot along helix 10 that modulates substrate preference via long-range interactions. Additionally, the authors show individual mutations can be stacked into multi-mutants, leading to highly specific enzymes. Overall, this study provides a comprehensive single-mutation resolution map of enzyme specificity and demonstrates the power of EP-Seq for decoding and engineering enzyme function.

Specific comments:

- The results are interesting and make sense. It would be helpful if the authors more explicitly discussed their findings in the context of related DMS studies from Mikkelsen, Whitehead, Tokuriki, and others. Are there general principles about enzyme specificity that emerge across these systems?

- The authors should also discuss the potential limitations of their approach. For example, in yeast surface display, it is common to find enzyme variants that perform well on the yeast surface but lose activity in soluble form. Could this influence the observed activity or specificity trends? Are there other technical or biological limitations that should be acknowledged?

- Figure S6 is one of the most informative figures in the paper and essential for understanding the definition and interpretation of the Sp-score. It would strengthen the manuscript to move all or at least part of this figure into the main text (perhaps alongside Fig. 3).

- Several figures could also benefit from clearer explanation. For instance, in Fig. 3, are the plotted Sp-scores for all 10 substrate pairs combined and summed? This should be explained in the caption. In Fig. 3C, is it meaningful to sum specificity scores across substrates given that each substrate has unique mutational preferences? In Fig. 5B, the substrate discrimination plots were not clearly explained--this information was only briefly mentioned in the SI and should be included directly in the legend. the authors should check to make sure all panels of all figures are clearly explained in the text or legends.

- A question on terminology because this is not my field. I normally think of allostery referring to a ligand or effector molecule binding at a site distinct from the active site and altering enzyme activity. This seems a little different than the allostery referred to in Fig 6 where mutations far from the active site in helix 10 alter specificity. Are these related? The former is an evolved conformational mechanism linking distant sites, wheras the later is simply structurally coupled regions of the enzyme.

- A question on terminology: I typically think of allostery as referring to a ligand or effector binding at a site distinct from the active site and altering enzyme activity. In Fig. 6, however, “allosteric hotspots” refer to mutations in helix 10 that influence specificity through structural coupling rather than ligand binding. Are these two uses of the term related? The classical definition implies an evolved conformational mechanism linking distant sites, whereas the paper’s use of the term seems to reflect structurally coupled regions of the enzyme.
The experimental reproducibility metrics are impressive. For Fig. 2A, the scatter plots would be more informative if they showed point density, either using transparent markers or density-based coloring (as commonly done in FACS plots).

- The authors also stated “We attribute the high level of agreement to the inherently promiscuous and generalist nature of R.

gracilis DAOx, a trait that is conserved in more evolutionarily recent homologs such as human DAOx." Is the human enzyme more recent than R. gracilis?

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors previously developed a yeast display assay to decouple enzymatic activity measurement from covariant factors (expression and folding stability) on a single pair of enzyme-substrate interaction. Here, they extended their work to the interaction between D-amino acid oxidase (DAOx) and five different substrates. Through a series of comparative analysis, they showed that while substrate-specific mutations can be distal from the active site, those within have stronger impacts at the cost of reduced activity.

Overall, the technical analysis of this submission is solid and innovative, but its experimental technique and findings are not novel. On yeast display assay, Amorosi et al. have already applied a similar technique to measure mutational impact on P450 activity [1]. On direct kinetic measurements, an excellent article from Markin et al. showcased a microfluidic platform to measure Michaelis-Menten constants for phosphatase [2]. These highly-relevant studies restrict the novelty of this submission to substrate specificity. Despite the thorough analysis presented, the finding is either already established in other DMS studies (on allosteric modulation) or too restrictive (structural insights on specific substrate-sidechain interactions). If possible, establishing key identifier(s) for allosteric modulation would be highly impactful in the field.

In conclusion, this submission is technically well-established, and I recommend referring it to other journals with stronger focus on technically sound research such as Scientific Reports.

References
1. Amorosi, C. J. et al. Massively parallel characterization of CYP2C9 variant enzyme activity and abundance. Am. J. Hum. Genet. 108, 1735–1751 (2021). doi:10.1016/j.ajhg.2021.07.001.
2. Markin, C.J. et al. Revealing enzyme functional architecture via high-throughput microfluidic enzyme kinetics. Science 373, eabf8761 (2021). doi:10.1126/science.abf8761.

Major edits on possible ways to introduce novelty
1. identify quantitative and statistically significant structural/biophysical features that
1.1 segregate impactful allosteric mutations from neglectible ones, or
1.2 segregate allosteric mutations by substrate specificity
2. establish generalizability of insights found in this work
2.1 engineer and test new beneficial/substrate-specific mutations, and
2.2 compare the success rate with other baselines

Minor edits
1. Figure 3A: highlight first-shell positions
2. Figure 3B: label the distribution explicitly as CDF/PDF
3. Heatmap S4: unify color scale and normalize to 1.0
4. Inconsistent wordings
4.1 “labeling” and “labelling”
4.2 “Sp-scores” and “SP-scores”
4.3 “gray” and “grey”

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have carefully addressed all of my previous comments, and the revised manuscript has been improved

accordingly.
The responses are appropriate, and the manuscript is now clear and scientifically sound.
I recommend acceptance of the manuscript without further revision.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The authors have satisfactorily addressed all my comments.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The primary concern on the novelty of this submission stems from the fact that the authors have previously published in the same journal on the same enzyme (DAOx) and one of the five substrates reported here (D-alanine) utilizing the same EP-Seq assay they developed [1]. Therefore, the scientific merit of this work should be based on the contrastive and comparative insights gained from these five substrates collectively. While the data collection is technically well-established, as noted in the previous reviewer comment, the current analysis relies heavily on qualitative case studies rather than establishing general principle(s) of enzyme specificity through quantifiable metrics. This needs not to be addressed through additional experiments.

While the authors highlighted notable mutations like those on α-helix 10, observations are generally descriptive and lack statistical analysis on quantifiable biophysical metrics. In fact, precedent quantitative analysis has been done in their previous publication [1]. Figure 3C of their prior work identified biophysical properties that concurrently or differentially impacted enzyme activity and expression. Similarly, Krustal-Wallis test can be performed here to identify outstanding structural features that explain the differential response amongst the substrates with statistical confidence. For instance, features can be changes in interactive energy with the substrates, conservation score, or distance from the active site. The community would benefit tremendously from either positive or negative results consolidated in a quantifiable manner.

References
Vanella, R., Küng, C., Schoepfer, A. A. et al. Understanding activity-stability tradeoffs in biocatalysts by enzyme proximity sequencing. Nat. Commun. 15, 1807 (2024). doi:10.1038/s41467-024-45630-3.

(Remarks on code availability)

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General comments for all the reviewers:

We thank the reviewers for their valuable comments and insight. Below we address each comment point-by-point. The reviewer's queries are written in black text, with our corresponding responses written in blue.

Reviewer #1:

This study used the enzyme-proximity sequencing method (EP-Seq) developed by the authors to comprehensively map, at single-mutation resolution, the substrate specificity of Rhodotorula gracilis D-amino acid oxidase (RgDAOx), and to identify mutations that confer preferences for particular D-amino acid substrates together with their structural implications. The results indicate that, at least in this enzyme system, EP-Seq can serve as a powerful platform for high-throughput enzyme design.

The strengths of the study are threefold: (i) a comprehensive, large-scale screening dataset is carefully supported by rigorous statistical processing and assessments of reproducibility; (ii) the D-factor obtained from single-clone kinetic measurements agrees well with the Sp-score derived from deep mutational scanning (DMS), thereby reinforcing the validity of the measurement approach; and (iii) the work illuminates not only hotspots near the active site but also distal mutations, demonstrating that substrate specificity can be governed through diverse proximal and distal routes.

On the other hand, for some of the key mutations that drive substrate preferences, the structural interpretation remains ambiguous or is not fully presented. In representative cases, further concretizing the mechanistic hypotheses, based on three-dimensional models, interaction networks, and conformational changes, and, where appropriate, supplementing them with in silico modeling or validation using substrate analogs would deepen readers' understanding.

Overall, the manuscript offers strong methodological contributions and high-quality data, providing important advances for the practical utility of EP-Seq and for understanding the evolution of substrate specificity. A deeper examination of the structural mechanisms underlying the major mutations would further strengthen the paper.

We thank the reviewer for the overall positive evaluation of the work.

Comments:

1. The authors used five types of D-amino acids as substrates. Three of these are hydrophobic (D-Ala, D-Met, D-Phe), and two are polar/hydrophilic (D-Asn, D-Gln). Supplementary Material S2 outlines the rationale for these choices, but can the authors explain why the substrates were limited to five types and why other valid candidates (e.g., moderately sized hydrophobic substrates like D-Val or D-Ile, polar substrates like D-Thr, or acidic D-amino acids) were not included? Additionally, why was the contribution of salt bridge effects to substrate specificity not investigated using an acidic D-amino acids?

Acidic D-amino acids are poorly processed by *Rg*DAOx, and other substrates, such as positively charged D-amino acids, are turned over at activity levels that fall below the detection limit of our tyramide-based high-throughput assay (https://doi.org/10.1021/acsbiomaterials.4c00895). Our

choice of these particular five substrates represents a practical compromise, where we examined small, medium, and large hydrophobic side chains (D-Ala, D-Met, D-Phe) and two polar substrates of different sizes (D-Asn, D-Gln) for which activity could be reliably measured by EP-Seq. This substrate set allowed us to cover a range of chemical properties and steric sizes while ensuring a good dynamic range of measurable activity for each substrate, which in turn makes the comparisons across the various screening feasible.

2. It appears that coenzyme FAD was not added to the enzyme assay system. Could the effect of the amino acid mutation on FAD binding (i.e., the difference in catalytic activity) be influencing the results?

The wild-type enzyme binds FAD tightly (Kd ≈ 20 nM) and contemporaneously folds upon FAD binding. In our experience, we have never observed any difference in activity with or without added FAD. It is true that overall catalytic activity can depend on FAD binding, and in our previous publication we showed that mutations that interfere with FAD binding led to a dramatic loss of activity (https://doi.org/10.1038/s41467-024-45630-3). In the context of this work, however, we do not expect global FAD affinity to influence changes in substrate preference. Our measurements are made for the same variant library on all 5 substrates, therefore the differences in substrate specificity that we observe are unlikely to arise from weakened FAD binding. It is possible that variants exhibiting unchanged substrate preferences but altered (overall lower) catalytic activity could be affected by differences in FAD binding affinity, although this was not examined in detail here.

3. Regarding the discussion of the structural mechanisms by which amino acid mutations affect substrate specificity, several points remain ambiguous. For example, concerning S341I, I believe a detailed explanation should be added, such as: “S341 forms a hydrogen bond with the main-chain carbonyl group of Y338, which is adjacent to Q339 forming the substrate-binding site; therefore, it may influence the orientation of Q339.” Is it possible to provide a more detailed analysis of the structural mechanism by which the S341I mutation confers absolute activity toward D-Gln, or the structural mechanism by which the T93M mutation enhances binding affinity for D-Met? Readers are likely to seek an understanding of the structural mechanisms by which amino acid mutations located away from the active site can influence substrate specificity.

We thank the reviewer for this thoughtful suggestion. We agree that a deeper understanding of the structural mechanisms underlying the specificity changes observed for variants such as S341I and T93M would be valuable. However, providing such detailed mechanistic insight would require extensive molecular dynamics simulations together with high-resolution structural data (e.g., from crystallography), which are beyond the scope of the present study. Where the available structural information allowed a clear interpretation, we have provided it in the manuscript (p. 13-14). For S341I, we appreciate the reviewer’s suggestion and are happy to include the proposed explanation regarding its possible effect on the orientation of Q339. We have included the following in the main text:

“The mutation at position S341 can alter the orientation of nearby residues Y338 and Q339, which are directly involved in substrate coordination”

4. Fig. 7. Why weren't combinations of amino acid mutations at positions distant from the active site verified?

The relative impact of distal mutations on substrate specificity is generally lower than that of mutations in the active site first shell (Fig. 3C, bottom). Our goal in combining single mutations was to test for additive effects on specificity, and we focused on mutations that individually caused strong changes. This approach allowed us to maximize the chance of observing meaningful combinatorial effects.

1. As shown below, “Fig. 2C” should be added to the following sentence.

Nonsense mutations had strongly negative effect on activity (average score -0.58 ± 0.05; n = 316 in all five datasets), indicating that loss of the C-terminal region beginning anywhere from position 1 to position 354 had a deleterious effect on the catalytic activity of the enzyme, regardless of the substrate used (Table S3, Fig. 2C)

We thank the reviewer for this suggestion. We have added the reference to Fig. 2C in the sentence as recommended.

2. The data for M213D do not appear to be present in Fig. 5 iv and Table S5.

Thank you for pointing this out. The data for M213D are presented only in Table S5 (raw 10). The reference to Fig. 5 iv in the main text was intended to compare with the M213E variant. We have removed the reference to Fig. 5 iv in this context to avoid any confusion.

Reviewer #2:

The authors present compelling work exploring enzyme substrate specificity in the model oxidoreductase D-amino acid oxidase (DAOx). They apply their previously developed enzyme proximity sequencing (EP-Seq) method to perform deep mutational scanning of DAOx across five diverse D-amino acid substrates. The high-throughput fluorescence-based measurements are highly reproducible and show excellent agreement with clonal measurements of activity and specificity. The resulting data provide a detailed, quantitative map of activity and specificity across the DAOx sequence, revealing a complex substrate-specificity landscape. The primary contribution of this work is the rigorous and insightful analysis of this large dataset. The authors identify hundreds of mutations throughout the enzyme that significantly impact substrate preference. They find that active-site mutations can cause large specificity shifts but often reduce catalytic activity, whereas distal mutations exert subtler effects that fine-tune specificity. They introduce a quantitative substrate-specificity score (Sp-score) that correlates strongly with experimentally determined discrimination factors, enabling large-scale analysis of specificity directly from high-throughput data. They identify structural factors driving specificity and a key mutational hotspot along helix 10 that modulates substrate preference via long-range interactions. Additionally, the authors show individual mutations can be stacked into multi-mutants, leading to highly specific enzymes. Overall, this study provides a comprehensive single-mutation resolution map of enzyme specificity and demonstrates the power of EP-Seq for decoding and engineering enzyme function.

We thank the reviewer for the overall positive evaluation of the work.

Specific comments:

- The results are interesting and make sense. It would be helpful if the authors more explicitly discussed their findings in the context of related DMS studies from Mikkelsen, Whitehead, Tokuriki, and others. Are there general principles about enzyme specificity that emerge across these systems?

We thank the reviewer for this comment. We have touched on this point on page 16 of main text (first and second paragraphs), where we compare our findings with previous DMS studies from Mikkelsen, Whitehead, Tokuriki, and others. As reported there:

"*Previous deep mutational scanning studies have reported contrasting patterns. In some cases, specificity-determining mutations are mainly distal (>9 Å from the catalytic site)[16], while in others, they cluster within the first-shell residues around the active site[13,15,44]. In DAOx, we found that the strongest specificity-shifting mutations were predominantly located within 8 Å of the catalytic site (Fig. 3C), yet these accounted for only 30% (n = 101) of all such mutations. The remaining 70% (n = 231) were scattered across more distal regions of the enzyme.*"

This comparison highlights that the classical view, where first-shell residues drive the largest changes in substrate specificity, is consistent with our findings and with those of Mikkelsen and Tokuriki. At the same time, our data also show that distal mutations can modulate specificity in a more gradual manner and generally with a lower impact on overall enzyme fitness. This is in agreement with observations from Whitehead and colleagues. In our view, the evidence of this dual behaviour is one of the most interesting findings of the study, as it suggests that multiple evolutionary trajectories are available to achieve substrate specificity, depending on the selective pressure acting on substrate preference and on the fitness costs that the enzyme can tolerate.

- The authors should also discuss the potential limitations of their approach. For example, in yeast surface display, it is common to find enzyme variants that perform well on the yeast surface but lose activity in soluble form. Could this influence the observed activity or specificity trends? Are there other technical or biological limitations that should be acknowledged?

We thank the reviewer for this comment. Our approach is compatible only with enzymes that can be functionally expressed on the yeast surface, and it is essential to verify that surface-displayed activity reflects the behavior of the soluble enzyme. In the present work, we have confirmed this for *Rg*DAOx: the catalytic K_M values on several substrates agree closely between the soluble and yeast-displayed forms (Supplementary Fig. S1). Furthermore, for the five representative single mutants with altered specificity, we compared activity on the yeast surface with that of the corresponding purified soluble proteins and observed full agreement (Supplementary Fig. S9). We nevertheless acknowledge that this correspondence is not guaranteed and must be evaluated case by case for other enzymes.

A detailed discussion of the EP-Seq method and its technical limitations was provided in our previous publication (https://doi.org/10.1038/s41467-024-45630-3), where we stated: "*Our workflow is compatible with enzymes that can be functionally displayed on the cell surface and whose activity can be directly or indirectly (via enzymatic cascade) linked to the production of peroxide. This includes enzymes with immediate therapeutic relevance such as Arginase and Asparaginase, as well as biocatalysts with diagnostic or industrial applications, like Glucose Oxidase.*"

Following the reviewer's suggestion, we have added the following sentence also to the conclusion paragraph of the current manuscript:

"Beyond these evolutionary insights, our work establishes EP-Seq as a robust platform for high-throughput engineering of enzymes that can be functionally displayed on the cell surface and whose activity can be coupled, directly or via a cascade, to peroxide production."

- Figure S6 is one of the most informative figures in the paper and essential for understanding the definition and interpretation of the Sp-score. It would strengthen the manuscript to move all or at least part of this figure into the main text (perhaps alongside Fig. 3).

We thank the reviewer for this suggestion. We did consider putting Figure S6 in the main text, but in our view, it would be somewhat repetitive since it shows all 10 possible combinations of substrates and cannot be partially shown without losing important information. Panel A of Figure 3 combines information on the regions that give specificity across all pairs, providing an overview of the main points. Keeping Figure S6 in the Supplementary Information allows readers to examine all pairwise comparisons of the substrate screening outcomes in detail, but we feel it is too much information for the main text.

- Several figures could also benefit from clearer explanation. For instance, in Fig. 3, are the plotted Sp-scores for all 10 substrate pairs combined and summed? This should be explained in the caption.

In Fig. 3A, the plot provides an overview of all individual z-scores for missense and synonymous variants across the library for each of the 10 substrate pairs. The plot shows how the z-score varies along the enzyme sequence and highlights that the z-scores of synonymous variants remain consistently below 3, which was the threshold we used to define mutations that cause a change in substrate specificity. To improve the clarity of the caption we have modified to the following:

"Scatter plot of absolute specificity scores across DAOx positions for all missense and synonymous variants and for each of the 10 substrate pairs. Missense variants with $|Sp\text{-score}| \geq 3$ are shown as yellow dots."

In Fig. 3C, is it meaningful to sum specificity scores across substrates given that each substrate has unique mutational preferences?

We agree that each substrate screen reveals its own mutational patterns, driven by the biochemical features of the substrate used. However, our goal in Figure 3C is to highlight broader trends in substrate specificity that depend on the position of the mutation rather than on the substrate itself. For this reason, we summed all specificity scores >3 at each DAOx position to estimate the overall contribution of individual positions to specificity. This analysis shows that positions directly interacting with the substrates (first-shell residues) contribute most strongly to specificity, independent of the substrate considered. To improve clarity, we have revised the figure caption accordingly and added the missing summation symbol (Σ) to the color bar next to the 3D structure.

" (Top) DAOx crystal structure (PDB ID: 1C0P) with substrate-specific positions highlighted as sticks. Color intensity (grey to yellow) represents the cumulative absolute Sp-score per position, considering

only Sp-scores >3. This reflects the contribution of each site to the global substrate-specificity landscape. The FAD cofactor and D-Ala substrate found in the catalytic site of the enzyme are shown as orange sticks. Bottom: bar plot showing the relationship between the distance of substrate-specific mutations from the catalytic site and their cumulative absolute specificity scores."

In Fig. 5B, the substrate discrimination plots were not clearly explained--this information was only briefly mentioned in the SI and should be included directly in the legend. the authors should check to make sure all panels of all figures are clearly explained in the text or legends.

A detailed explanation of how the discrimination score was calculated is provided on page 12 of the Supplementary Information ("Discrimination factor calculation"). To make the figure captions more self-explanatory, we have now added the following lines to the captions of Fig. 5B, Fig. 6C, and Supplementary Fig. S9, where the discrimination-factor heatmaps are shown.

"*Heatmaps of substrate discrimination factor (D) for all pairwise combinations of primary (y-axis) and secondary (x-axis) substrates, calculated as the catalytic efficiency (V_{max}/ K_M) for the primary substrate divided by that for the secondary substrate*."

We also note that we have checked all figure captions and modified them according to the reviewer's suggestions to improve readability and clarity. All changes are highlighted in the submitted manuscript with tracked changes.

- A question on terminology because this is not my field. I normally think of allostery referring to a ligand or effector molecule binding at a site distinct from the active site and altering enzyme activity. This seems a little different than the allostery referred to in Fig 6 where mutations far from the active site in helix 10 alter specificity. Are these related? The former is an evolved conformational mechanism linking distant sites, wheras the later is simply structurally coupled regions of the enzyme.

This point appears to be a duplicated comment from the reviewer, and we address it in detail in our response below.

- A question on terminology: I typically think of allostery as referring to a ligand or effector binding at a site distinct from the active site and altering enzyme activity. In Fig. 6, however, "allosteric hotspots" refer to mutations in helix 10 that influence specificity through structural coupling rather than ligand binding. Are these two uses of the term related? The classical definition implies an evolved conformational mechanism linking distant sites, whereas the paper's use of the term seems to reflect structurally coupled regions of the enzyme.

Classical allostery refers to dynamic, ligand-driven regulation, but in our work, "allosteric hotspots" describe regions where mutations can influence enzyme activity or specificity through long-range structural coupling, even without ligand binding. Mutations can cause allostery by changing a protein's structure and function at a site distant from the original mutation, a phenomenon known as allosteric mutation. In other words, these mutations act at a distance through similar structural contact networks that could enable classical allosteric regulation through ligand binding.

The experimental reproducibility metrics are impressive. For Fig. 2A, the scatter plots would be more informative if they showed point density, either using transparent markers or density-based coloring (as commonly done in FACS plots).

We thank the reviewer for this helpful suggestion. We have updated Fig. 2A to use transparent markers, which makes the point density easier to see, and believe that the plot is now more informative.

The authors also stated "We attribute the high level of agreement to the inherently promiscuous and generalist nature of R. gracilis DAOx, a trait that is conserved in more evolutionarily recent homologs such as human DAOx." Is the human enzyme more recent than R. gracilis?

We thank the reviewer for this comment, which helped us clarify the sentence. The human DAOx sequence represents a lineage that diverged more recently from the common ancestor shared with other eukaryotic DAOx, whereas *R. gracilis* branched off earlier in evolution. We have revised the sentence in the manuscript accordingly to make this point clear.

"We attribute the high level of agreement to the inherently promiscuous and generalist nature of R. gracilis DAOx, a trait that is also observed in more recently diverged eukaryotic homologs such as human DAOx."

Reviewer #3:

The authors previously developed a yeast display assay to decouple enzymatic activity measurement from covariant factors (expression and folding stability) on a single pair of enzyme-substrate interaction. Here, they extended their work to the interaction between D-amino acid oxidase (DAOx) and five different substrates. Through a series of comparative analysis, they showed that while substrate-specific mutations can be distal from the active site, those within have stronger impacts at the cost of reduced activity.

Overall, the technical analysis of this submission is solid and innovative, but its experimental technique and findings are not novel. On yeast display assay, Amorosi et al. have already applied a similar technique to measure mutational impact on P450 activity [1]. On direct kinetic measurements, an excellent article from Markin et al. showcased a microfluidic platform to measure Michaelis-Menten constants for phosphatase [2]. These highly-relevant studies restrict the novelty of this submission to substrate specificity. Despite the thorough analysis presented, the finding is either already established in other DMS studies (on allosteric modulation) or too restrictive (structural insights on specific substrate-sidechain interactions). If possible, establishing key identifier(s) for allosteric modulation would be highly impactful in the field.

In conclusion, this submission is technically well-established, and I recommend referring it to other journals with stronger focus on technically sound research such as Scientific Reports.

References

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2. Markin, C.J. et al. Revealing enzyme functional architecture via high-throughput microfluidic enzyme kinetics. Science 373, eabf8761 (2021). doi:10.1126/science.abf8761.

We thank the reviewer for the comment and assessment of related work. We have added the references to the main text of the manuscript (Ref. 10 and 11). Nevertheless, we want to specify that the two cited studies employ methodological approaches that are not really directly comparable to the platform used in our current study.

1. Regarding Amorosi et al.

This work does not use yeast display, but rather a fluorescence-based yeast assay employing CYP-specific activity probes that irreversibly stain enzymatically competent P450 variants. This platform cannot be used for steady-state kinetic measurements and operates inside cells under fixed reaction conditions. In contrast, our platform allows systematic control of key reaction parameters, including substrate concentration, buffer, temperature, and incubation time, which is essential for the quantitative kinetic characterization of substrate specificity. Although the overall workflows share conceptual elements, the experimental capabilities and aims of the two methods differ.

Regarding Markin et al.

This study was extensively acknowledged in our earlier Nature Communications publication (https://doi.org/10.1038/s41467-024-45630-3) describing the development of the EP-Seq method and its comparison to the microfluidic platform developed by Markin et al. Nevertheless, we can again acknowledge its relevance in the current work and have inserted the reference.

Major edits on possible ways to introduce novelty

1. identify quantitative and statistically significant structural/biophysical features that

1.1 segregate impactful allosteric mutations from neglectible ones, or

1.2 segregate allosteric mutations by substrate specificity

In the main text, we dedicate a full paragraph to the characterization of structural allosteric sites with an impact on substrate specificity. Specifically, α-helix 10 exhibits these features. We additionally tested the performance of six single-mutant variants (R254C, R254D, H258W, H258R, L262A, L262V) in this region toward each of the five substrates used in the study, demonstrating that each mutation results in a distinct specificity profile (Fig. 6C). Given this analysis and the existing experimental evidence, we believe the current data already address the reviewer's comment, and we respectfully note that further experimentation is not warranted within the scope of this work.

2. establish generalizability of insights found in this work

2.1 engineer and test new beneficial/substrate-specific mutations, and

2.2 compare the success rate with other baselines

Again, we feel what the reviewer is asking for was already done. In the current work, in addition to our high-throughput data, we characterized 36 single-mutant variants that were detected to have significantly altered substrate specificity. This included detailed measurements of Michaelis–Menten kinetic parameters on five substrates. For each variant, we report K_M and V_{max} values in supplementary Table S5. Moreover, the five most relevant substrate-specific mutants (F58W, N54L, T93M, M213E and S341I) were also expressed as soluble proteins, and their kinetic properties were re-evaluated, showing strong concordance with the yeast-display data (Table1, Supplementary Fig. S8, S9). Given the breadth and depth of these measurements, we believe this dataset sufficiently addresses the reviewer's request.

Minor edits

1. Figure 3A: highlight first-shell positions

We have added a heatmap along the x-axis of Figure 3A showing the distance of each residue from the catalytic site and have updated the figure caption accordingly.

Figure 3B: label the distribution explicitly as CDF/PDF

In Figure 3B, the y-axis represents the number of variants observed at each specificity score, i.e., a raw histogram, rather than a probability density or cumulative distribution.

3. Heatmap S4: unify color scale and normalize to 1.0

We thank the reviewer for this suggestion. In Figure S4, the color scale reflects the actual measured activity of each variant relative to the wild-type, with 1 corresponding to WT activity. The observed range of activities varies between different conditions and substrates, and normalizing each heatmap to 1.0 would obscure these biologically meaningful differences. Therefore, we have retained the current color scaling, which preserves the true variation and functional interpretation of the data.

4. Inconsistent wordings

4.1 "labeling" and "labelling"

4.2 "Sp-scores" and "SP-scores"

4.3 "gray" and "grey"

We have standardized spelling and terminology: "labeling," "Sp-scores," and "gray" are now used consistently. We have also standardized the method name to "EP-Seq" (instead of Ep-Seq) throughout the text and updated it in Figures 4 and S9.

General comments for all the reviewers:

We thank the reviewers for their comments. Below we address each comment point-by-point. The reviewer's queries are written in black text, with our corresponding responses written in blue.

Reviewer #1 (Remarks to the Author):

The authors have carefully addressed all of my previous comments, and the revised manuscript has been improved accordingly.
The responses are appropriate, and the manuscript is now clear and scientifically sound.
I recommend acceptance of the manuscript without further revision.

We thank reviewer for the positive evaluation of the work.

Reviewer #2 (Remarks to the Author):

The authors have satisfactorily addressed all my comments.

We thank reviewer for the positive evaluation of the work.

Reviewer #3 (Remarks to the Author):

The primary concern on the novelty of this submission stems from the fact that the authors have previously published in the same journal on the same enzyme (DAOx) and one of the five substrates reported here (D-alanine) utilizing the same EP-Seq assay they developed [1]. Therefore, the scientific merit of this work should be based on the contrastive and comparative insights gained from these five substrates collectively. comment, the current analysis relies heavily on qualitative case studies rather than establishing general principle(s) of enzyme specificity through quantifiable metrics. This needs not to be addressed through additional experiments. While the data collection is technically well-established, as noted in the previous reviewer

While the authors highlighted notable mutations like those on α-helix 10, observations are generally descriptive and lack statistical analysis on quantifiable biophysical metrics. In fact, precedent quantitative analysis has been done in their previous publication [1]. Figure 3C of their prior work identified biophysical properties that concurrently or differentially impacted enzyme activity and expression. Similarly, Krustal-Wallis test can be performed here to identify outstanding structural features that explain the differential response amongst the substrates with statistical confidence. For instance, features can be changes in interactive energy with the substrates, conservation score, or distance from the active site. The community would benefit tremendously from either positive or negative results consolidated in a quantifiable manner.

We thank the reviewer for raising this point regarding the identification of general principles of substrate specificity. While we agree that the scientific merit of this work lies in comparative insights across substrates, we respectfully disagree with the assessment that our study lacks quantitative metrics to address substrate specificity.

Our comparative analysis of high-throughput EP-Seq data was performed using stringent quantitative and statistical criteria. Internal reference populations (e.g., synonymous variants) controlled for experimental noise, excluding apparent substrate-specific effects arising from measurement variability (Fig. 3A, B). In addition, dozens of single-variant kinetic measurements were quantitatively characterized to validate the high-throughput data. The Sp-score metric demonstrates strong agreement between high-throughput measurements and single-clone enzymatic assays, providing a *robust quantitative basis* for assessing substrate specificity across all five substrates (Fig. 4A).

Building on this foundation, we established quantitative relationships between substrate specificity and multiple biophysical and functional features, including protein expression level, structural stability, mutation distance from the catalytic site, and catalytic performance. These analyses reveal a complex, interconnected landscape in which functional and structural parameters jointly influence specificity (Fig. 4E). Quantitative comparisons across substrates show that specificity effects depend strongly on substrate identity: smaller substrates exhibit the strongest and more straightforward effects, whereas larger substrates display complex specificity landscapes that likely arise from the coordinated action of multiple elements, such as steric factors and electrostatic interactions (Fig. 4B–C).

Regarding structural determinants, our data highlight the dominant role of catalytic-site mutations in driving specificity, often at the expense of overall activity, while distal mutations modulate specificity more subtly. This illustrates the primary importance of the catalytic site in determining enzyme specificity, as well as a secondary importance of distributed specificity-determining mutations across the protein structure (Fig. 3C). In line with this, we identified and quantitatively validated the contributions from α-helix 10, demonstrating how substitution in secondary structural elements outside the catalytic center can influence active-site geometry and substrate recognition (Fig. 6). While this feature is specific to DAOx, it exemplifies a broader principle of distributed structural influence on specificity.

As part of our original analysis, we also explored correlations between substrate-specific effects and individual structural and biophysical properties, including amino acid bulkiness, polarity, hydrophobicity, solvent exposure, and residue conservation. These analyses did not yield strong or consistent relationships for any of the features, and therefore we did not include them in the paper, as they offered no simple or intuitive trends for understanding specificity.

In conclusion, we believe that the inherent complexity of substrate recognition in enzyme catalysis highlights the high value of our approach. By combining high-throughput mutational mapping with cross-substrate comparative analysis, we provide a detailed, quantitative view of the DAOx specificity landscape and reveal evolutionary pathways toward highly specific yet functional enzyme variants, insights that are largely inaccessible through classical low-throughput methods.