

Computational design of metalloproteases

Corresponding Author: Dr David Baker

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

This study focuses on the de novo design of zinc metalloproteases. The authors employ the deep learning-based RFD2-MI diffusion framework to generate both the peptidase and its target peptide substrate. Initial screening was conducted in a so-called “cis” configuration, where the peptide substrate was covalently linked to the protease, followed by trans assays using soluble substrates. Among 135 designed variants, 14 displayed catalytic activity and originated from five distinct structural scaffolds. The most efficient enzyme exhibited a k_{cat}/K_M of up to $900 \text{ M}^{-1} \text{ s}^{-1}$ and a k_{cat} of 0.025 s^{-1} . The two-sided protease–substrate design conferred a measurable degree of substrate specificity, and one scaffold was further optimized to cleave the human protein TDP-43, though no kinetic parameters or specificity measures were provided for it. The authors justifiably argue that the design of novel peptidases is much more challenging than previous applications due to the high stability of the peptide bond. There are, however, concerns regarding the low catalytic rates, the lack of structural validation of the designs, and the limited kinetic data on the designs. Furthermore, the presentation makes claims about the potential uses of the methodology that are not supported by the low reported levels of activity.

Major comments

The authors claim (lines 239-240) that the best-performing design achieves kinetic parameters comparable to some natural metalloproteases. However, they cite reactions on synthetic peptide substrates, not natural protein substrates. Native metalloproteinases, such as MMP2, exhibit much higher catalytic efficiencies ($k_{cat} \approx 10 \text{ s}^{-1}$, $k_{cat}/K_M \approx 24,000 \text{ M}^{-1} \text{ s}^{-1}$) than measured in this study. The subsequent comparison to TEV protease is not necessarily relevant, as TEV lacks a metal cofactor and works through a different mechanism. A more appropriate benchmark would be aminopeptidase N, which is the enzyme from which the active-site geometry was derived in the submitted study, and this enzyme has reported values of $k_{cat} \approx 300 \text{ s}^{-1}$ and $k_{cat}/K_M \approx 3,500 \text{ M}^{-1} \text{ s}^{-1}$, dramatically higher than the values of the designs. The catalytic rates and efficiencies achieved by the designed enzymes are instead more comparable, though orders of magnitude below, those of proteolytic antibodies ($k_{cat} 0.13 \text{ s}^{-1}$ and $k_{cat}/K_M 55,000 \text{ M}^{-1} \text{ s}^{-1}$ (Urusov AE et al. *Molecules*. 2023; 28(3):1330)). Overall, the observed kinetic performance of the designed enzymes appears largely driven by a favorable K_M toward their specifically designed substrates. Although the Discussion makes the point about the significant gap with respect to natural peptidases, the main text compares to enzymes of a different class that are not directly comparable to the designed ones.

Given the problems observed in the past in computational enzyme design where crystal structures did not align with the designed models, the manuscript would benefit substantially from structural validation. Experimental determination of the protease structure, demonstrating proper zinc coordination and active-site geometry, would provide critical support for the claimed design accuracy. Reliance solely on AF3-predicted models is insufficient for validating such mechanistic details, especially when it was used as a filter for design selection.

The recent papers on serine hydrolases, metallohydrolases and the companion on cysteine proteases are conceptually similar. The main distinction in this submission is the two-sided generative design of enzyme and substrate, and the focus on the energetically more challenging protease reaction (though the latter point is shared with the cysteine protease companion). By approaching the problem as a two-sided design challenge, the authors make the problem easier than a realistic scenario where one is targeting a natural protein. Despite this, the authors argue that this study establishes the generality of de novo peptidase designs, suggesting a path towards therapeutic use. Given the artificial nature of two-sided design, the very low catalytic rates and the lack of demonstrations on selectivity with respect to the human proteome, such

statements are premature.

Furthermore, the current submission and the recent serine hydrolase paper raise a similar set of concerns: in both, while K_M values are favorable, the turnover numbers are very low by comparison to natural enzymes that carry out similar reactions. Furthermore, experimental structures are either lacking (in the current submission) or do not provide clear-cut validation of design accuracy within the active site. These problems are reminiscent of those seen with catalytic antibodies, where efficiencies were driven largely by binding rather than by turnover numbers. One of the conclusions stemming from the catalytic antibody studies was that the active sites were not precisely organized for catalysis. Given that the reactivities seen in the current papers are, in fact, below those of comparable catalytic antibodies, a detailed structural and mechanistic verification of design accuracy is crucial to support the claims of the paper on the efficacy of the design strategy. Needless to say, catalytic antibodies have largely been abandoned as a path for developing efficient biotechnological or medical tools due to their poor performance.

Minor comments

Line 60: esters are also resonance-stabilized.

Line 60: The statement “Diffusive denoising methods such as RoseTTAFold Diffusion (RFDiffusion) have proven powerful in generating a wide range of new functional proteins” should be referenced.

Line 95: The authors state “Starting from a choice of the length of the designed enzyme and the substrate...” — please indicate the range of lengths tested for both enzyme and substrate.

Line 98: The sentence “Many of the RFD2-MI trajectories produced scaffolds with all the desired structural features” is vague. Please clarify what specific structural features were considered “desired” and describe what occurred in the unsuccessful trajectories.

Lines 104–106: The description – “Designs that AF3 predicted with high confidence... were fed back to RFD2-MI for two more rounds of finer sampling...” — is ambiguous. From the manuscript, it appears that this stage involves sequence design only rather than backbone redesign. Please clarify which parameters are being refined and whether any backbone changes are introduced during these additional iterations.

Lines 146–147: The reported overall success rate of 36.3% reflects only the cis-cleavage mode, in which the substrate is covalently tethered to the protease. As this setup does not rigorously test substrate recognition, the actual catalytic success that should be reported in the abstract section is the one under trans conditions.

Line 207: Targeting arbitrary peptides wasn’t shown. The peptides were designed.

Section “Reprogramming to cleave human TDP-43”: It would be helpful to explain the rationale for reusing an existing scaffold (ZnO36) rather than designing a new protease de novo for this target. Also, please provide kinetic parameters of the best designs from this group.

Please describe the differences in terms of the structure and number of mutations among the designs.

For all figures: please indicate clearly that the structures shown are models.

Referee #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #3

(Remarks to the Author)

This manuscript describes de novo designed metalloproteases: metalloenzymes containing a zinc(II) active site, capable of hydrolyzing peptide bonds. The metalloproteases, as well as their peptide substrates, were computationally designed in a two-sided process and a selection of the designs was experimentally validated. The predicted structures of top designs proved to be accurate and had unprecedented peptide folds. Moreover, they showed impressive activity, both in cis and in trans reactions, of up to 10^8 fold rate acceleration compared to the uncatalyzed reaction and the k_{cat} approaches that of some Zn-dependent proteases. It was shown that in all cases indeed the peptide was cleaved at the designed site. As a consequence of the two-sided design process, most enzymes showed limited cross-reactivity, i.e. they were selective for their designed substrate peptide sequence, albeit with notable exceptions of some very substrate promiscuous designs. Finally, one of the successful designs, ZnO36, was redesigned to target a biologically relevant substrate: the TAR DNA-binding protein 43 (TDP-43), protein which aggregation and fibrillization are linked to neurodegenerative processes. Encouraging activity was found that raises the prospect of biological applications, albeit that for this purpose the substrate specificity needs to be further improved.

This work builds on two prior works by the same group(s). Reference 2 (listed as preprint, but published in the meantime: Nature (2025). <https://doi.org/10.1038/s41586-025-09746-w>) in the manuscript describes the de novo design of a zinc esterase: a similar type of metalloenzyme that hydrolyzed activated esters. A similar type of reaction, but on much more

reactive and, hence, easier substrates. These zinc esterases were designed using RFDiffusion2 (RFD2). The other preprint describes an adaptation of RF Diffusion 2, called RFDiffusion2 Molecular Interfaces (RFD2 MI), which was used in the current work to design the Zn protease.

The key innovations of this work, compared to these reported works are:

- This work achieves the successful design of active proteases. Peptide bonds are notoriously unreactive and hence present a much bigger challenge for enzyme design than, for example, esters (reported before). Moreover, designer proteases could have important applications, for example in biomedicine and bioremediation. This is suggested by the application to the hydrolysis of RDP-43. Therefore, this work represents a milestone in enzyme design.

- A water molecule, which is needed as the second substrate for the proteolysis, was included in the design. This significantly increased the frequency of successful, i.e. active, designs. The success rate of ~87% active designs, compared to ~15% without the water included, is impressive. It also shows, as the authors note in the manuscript, that inclusion of key molecules for the reaction in the design process can greatly increase design success.

- A two-sided design strategy was used to design both the metalloprotease and its peptide substrate. This means that the substrate sequence was unique for each design. A result of this was that many (most) designs displayed a significant substrate selectivity (peptide sequence).

The work presented is of high quality. Data is generally presented well, with replicates and error margins (one exception, see below). The conclusions are supported by the data. The manuscript is well-written: it was a pleasure to read.

General comments:

1. The computational design techniques used, RFD2-MI, as well as the design of a zinc hydrolase were established by the same group in recent work. So, the main novelty here is the application towards the creation of metalloproteases and achieving peptide bond hydrolysis.

2. This is maybe considered semantics, but I am not sure if the claim of “de novo design”, i.e. design from first principles, of metalloproteases is warranted here. The active site structure, i.e. the ligation of the catalytic metal, as well as the positioning of the oxy anion stabilizing residues was not designed, but taken from an existing metalloproteases, aminopeptidase N and astacin, and precisely reproduced in the designed proteins. So, one could argue that while the protein environment was designed de novo, the part of the structure that makes it an enzyme, the catalytic site, was not and, thus, that this work represents a (very impressive) redesign of aminopeptidase N and/or astacin. I think calling it a “designer enzyme” or “designer metalloprotease” is probably better justified than calling it a “de novo designed enzyme/metalloprotease”.

3. The design was modeled in three states: apo, substrate bound and transition state. This kind of multistate design is clearly important to achieve proficient enzymes. However, only one transition state (TS) was modeled, whereas there are more along the reaction coordinate. The Baker lab has shown in recent work (Science 2025, <https://doi.org/10.1126/science.adu2454>) that including all TS's along the reaction coordinate in the design improves the quality of the outcome. The authors should comment on why this approach was not used here; it might have delivered enzymes that are even closer to the catalytic activity observed for natural metalloproteases.

Other comments,

4. While the manuscript in general is very well-written and clear, the section explaining the design choices could be improved a bit. A bit more explanation on why choosing the Zn site from one enzyme (aminopeptidase N) and the substrate binding site of another (astacin) would help the reader understand the thought process better.

5. To probe the necessity and contribution of the active site residues for both activity and zinc binding, they were sequentially mutated. In almost all cases this resulted in a significant drop of activity, thus confirming the essential role of this particular residue (Fig 3.). An exception is the R79A mutation in ZnO36: this seems to give rise to a significantly increased activity (see bar graph in 3rd row of Fig 3). This surprising result is completely ignored in the manuscript. Here clearly the design was not optimal, which should be commented on.

6. While it is nice to see there is limited cross-reactivity of the enzymes towards the peptides for the other selected design, I am hesitant to call this “considerable” specificity. The design sequences tested are very dissimilar. See below for the sequences, which I had to retrieve from the SI; it would have been better to have these available in the manuscript. To assess specificity, seeing how selective the designed protease is when mutating single amino acids in the substrate peptide

Sequences substrates:

SZn5: VIRLRMR

SZn07: ALTLTFRLR

SZn025: SLTLTFRLR

SZn036: ARILRVNFKI

SZn039: SRVIEVKYLK

SZn45: VLEFTGRR

7. Kinetics: a comparison of the kinetics is made to matrix metalloproteinases and TEV protease. The latter is a cysteine

protease, not a Zn protease. How do the kinetics compare to the Zn proteases that the design was based on, i.e. aminopeptidase A and astacin? I feel that this comparison would be much more valuable.

The highest reported rate acceleration was achieved for an enzyme (Zn45) on a substrate for which it was not designed (SZnO36). What are the kinetic parameters for its designed substrate (SZn45)? Figure 4a suggests it is significantly less active on its designed substrate: what does this mean for the design process?

8. In the discussion section, the authors state: "there is still room for improvement—turnover straight out of the computer is still $\sim 10^3$ – 10^4 -fold below those of the most efficient natural proteases under similar conditions". While the rate enhancements achieved straight out of the computer are absolutely impressive, it is indeed still a significant gap. Where does the current approach still fall short? Can the authors speculate on this?

9. Figure S26: information of number of replicates and error margins for TDP cutting experiments is missing.

Minor comments:

Typo (first paragraph of computational design section): "The fourth ligand of Zn(II), a water molecule, is activated by a glutamate (E1)" - should be "E2".

Figure 4a: in the matrix are listed the relative reactivities of the enzymes for the various substrates. From the caption it is unclear what is compared here: catalytic efficiencies? k_{cat} ? Yields?

Referee #4

(Remarks to the Author)

In this study, Chen and coworkers report the computational design of zinc-dependent proteases capable of breaking the amide bonds of some designed peptide sequences. The authors used an enhanced version of RoseTTAFold Diffusion 2 for Molecular Interfaces (RFD2-MI) tailored for enzyme and protein–protein interface design to generate multiple protease–peptide pairs. The best design accelerated peptide bond hydrolysis by more than 10^8 -fold compared to the uncatalyzed reaction, however, it should be highlighted that the kinetic characterization showed a poor k_{cat} of ca. 0.0025 s^{-1} , and a good K_M of ca. $26\text{ }\mu\text{M}$. These values clearly indicate that there is much room for improvement, as the turnover is very low if compared with those of natural proteases. While we agree with the authors that this study shows that de novo enzyme design can now tackle more difficult, chemically demanding reactions (as shown also in their previous work on serine hydrolases, DOI:10.1126/science.adu2454), the catalytic activities of the obtained designs are still far below those of natural enzymes. The authors also used some of the designed enzymes as template scaffold for optimizing it for cleaving a therapeutically relevant human peptide, but again the activities obtained need further improvement and refinement. Considering that the method applied in the study has already been developed in another work, and also the level of catalytic improvements achieved, we do not believe the results obtained in this study have the novelty and impact required for its publication in Nature journal.

We have the following comments and concerns about the work:

1. The authors state that: "We modeled each resulting design in three states: 1) the apo protease state, to confirm correct folding; 2) the ES complex state, to assess substrate binding and positioning of the target amide bond; and 3) the transition state to evaluate stabilization." However, a few sentences later they mention that: "For modeling the transition state, we used a phosphoramidate TSA; many crystal structures have been solved for similar TSA complexes, so they are better modeled by ML software trained on the PDB than the actual transition state". We agree with the authors that the ML software trained on X-ray structures will provide better results with TSA than the actual TS, however, we disagree with the authors when they state that the TS is used to evaluate stabilization. Although the charge distribution of TSA might resemble that of TS, TSA is not the TS, so it should not be stated that the TS is used in the modeling to evaluate stabilization. This is not accurate and should be rephrased.

2. The authors used two different strategies for modeling the zinc and coordination sphere, which by the way, it is directly taken from a natural protease scaffold and not generated de novo via theozyme calculations. In one of the approaches a water molecule is added close to the zinc metal ion. As this is constrained during the design process, this does not guarantee that the water will be in reality there and neither that the pocket environment would favor the proper positioning of the water molecule.

3. The authors used two different strategies for screening: the cis screen in which the peptide and protein are fused together, and the trans screen in which a (GS)₁₅ linker is added to the N-terminus and an mScarlet tag at the C-terminus of the substrate peptide. In both cases, additional protein-protein interactions imposed by either the fusion or especially the addition of a rather big protein at the terminal regions of the peptide might substantially affect the binding affinity of the peptide, and thus dramatically affect the obtained results.

4. In their assessment of active and inactive designs (shown in Figure 2d, 2e, 3a, S10 and S16), there is a lack of geometrical features, which makes the quantification of the preorganization of the binding pose and so the evaluation of the catalytic potential of the designed variant hard (we would say impossible) to assess. In Figure S10 only the distance between R79 and carbonyl group at the ES and TSA complex is shown. This is again completely insufficient.

5. In Figure S11, the tyrosine hydrogen bond angles found in X-ray structures are shown. The hydrogen bond distances should also be included and compared.

6. It is also mentioned: "In the prediction of Zn45 with the Zn45 substrate, the angle between the attacking water oxygen, the scissile carbonyl carbon, and the carbonyl oxygen is 51.8°, smaller than the ideal 90° geometry, potentially interfering with the nucleophilic attack." While the angle is important for a catalytically competent pose, the distance is essential even if the perfect angle is achieved, the distance has to be short enough for allowing catalysis.
7. "Using a previously published side-chain pocket scoring metric to assess compatibility between substrate windows and designed active sites by logos, we identified ZnO36 with a targeting window spanning residues 329–340 as a suitable starting scaffold." More details should be given on how this selection was made. It is also not clear for us why the authors used as starting scaffold one of the previous designs and not a new one based on only optimizing the protease sequence.
8. The correlation between experimentally measured normalized digested fraction and computational metrics shown in Figure S18 is extremely poor.
9. The novelty of the generated folds with respect to natural scaffolds (including PDB or AF databases) is not presented neither investigated.
10. "While these results are encouraging for programming proteases against therapeutic targets, there is still considerable room for improvement as the designs retained activity on the original substrate (Fig. S24)." We agree that the results are promising, but they should be substantially improved for their publication in Nature.

The references are appropriate and the whole manuscript is well-written and easy to read.

Referee #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #6

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

Given the similarities between the papers, below we assess both together.

The authors have considerably revised the manuscripts, adding data on structures that exhibit high accuracy, mutations that improve catalytic rates, and molecular dynamics simulations that help explain the partial improvement in catalytic rates in mutants based on active-site preorganization. These are important additions.

Yet, even after optimization by systematic mutagenesis, the designed enzymes remain 2-3 orders of magnitude less active than the natural counterpart from which the active site was derived. This is the most relevant comparison, particularly because several recent studies have already demonstrated that RFDiffusion can produce de novo enzymes with low kcat. We agree that the catalytic parameters of natural enzymes vary, but it is important to remember that much of this variation is due to trade-offs: TEV trades off rate for very high specificity, and the specificity tests carried out by the authors are far from sufficient to suggest they have achieved similar levels. We do not doubt that hydrolysing amides presents a greater challenge in terms of activation barriers than previous studies on hydrolases. But the methods are very similar to those of the previous studies and exactly the same in the two companion papers, limiting novelty and questioning why the authors did not integrate them into one paper.

Furthermore, the gap from the natural enzymes from which the active site was derived limits confidence in the authors' conclusions that low rates in the final designs are due to low preorganization. Given that all recent de novo hydrolases have shown low rates, there may be a ceiling to the rates that are feasible using the current generation of de novo fold design methods. Identifying such missing factors may explain what makes natural enzymes so proficient, beyond the accuracy of the active site, and suggest ways to break the rate ceiling.

Regarding generality, except for one designed enzyme, all were generated to target custom-designed substrates (two-sided designs), and the design process relied on the simplifying assumption that the substrate associates with the enzyme through β -sheet interactions. It is not clear from the writing whether the targeted human protein (one-sided design) was chosen because of similarity to some of the previously designed substrates, adding another limitation to generality.

Given the concerns on rates and generality, the authors' assertions that the work opens the way to therapeutic and industrial applications are not tenable.

Referee #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #3

(Remarks to the Author)

The authors have made substantive changes to the manuscript. Especially the discussion about the discrepancy between designed and native metalloproteins is a valuable addition that really adds something to the manuscript (including a further improved variant of the metalloprotease).

I am not fully convinced by the response on whether this can be called "de novo enzymes", but I think we can "agree to disagree" here; it is not a major issue.

Overall I am fully satisfied with the response from the authors and I congratulate them on this beautiful work.

Referee #4

(Remarks to the Author)

In this revised version of the manuscript, the authors have added structural X-ray data, performed MLFF-based calculations and MD simulations to rationalize the observed differences in activity, designed proteases capable of cleaving human TDP-43, and further enhanced the catalytic activity of their designs through targeted experimental mutagenesis around the active site. Although the reported 30-fold improvement in activity is substantial, the fact that this gain was achieved through experimental saturation mutagenesis focused on the active site somewhat reduces its impact and novelty. Nevertheless, the revised manuscript is considerably stronger than the original submission. Based on the new data presented, we have the following comments:

1. In the X-ray data, why does Q32 adopt a different side-chain rotamer only in ZnO7? In the Supplemental Information, the authors state: "Despite the potential crystallography artifact, this same Q-to-E mutation in TDP-43, and in a few other crystal structures we obtained (Extended Data Fig. 5), did not result in complete flipping of the rotamer, potentially due to better stabilization in the catalytically competent conformation." However, this explanation remains unclear. Fig. S20 shows the new hydrogen-bonding interactions adopted by Q32, but it is not evident why the same side-chain conformation is not observed in the other variants. Can the hydrogen-bonding interactions shown in Fig. S20 not also be maintained in those cases?
2. "To compare entire reaction pathways at the active sites of the designs, we used machine learning force fields⁴⁶ (MLFF) to compute the activation barriers for the *likely rate-limiting* proton abstraction and nucleophilic attack steps⁴⁷ for Zn45, ZnO7, and native astacin (Fig. 1b, Supplementary Discussion 4). All catalytic residues, the substrate and all residues within 5 Å of the substrate were included in the simulation." As the authors themselves note in Supplementary Discussion Note 4, even metalloproteases with very similar active-site catalytic machinery can display markedly different reaction profiles and rate-limiting steps. Therefore, the authors' decision to focus solely on this particular step, using astacin as a reference, is not sufficiently justified. In addition, the apparent correlation with the experimental trends based on only three data points (variants) is not especially compelling. At a minimum, the analysis should be extended to include at least one additional variant, such as the improved Zn45_v2, which exhibits a 30-fold increase in activity.

Referee #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #6

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Version 3:

Reviewer comments:

Referee #1

(Remarks to the Author)

We emphasize again that we recognize that amide-bond hydrolysis is more challenging than the reactions achieved to date using de novo design methodology. But the revision does not contain new data to counter the main criticisms of our previous reviews: namely, that the *k*_{cats} (even after experimental optimization) are significantly lower than those of the enzymes based on which the active sites were built, and that the approach is very similar to previous work on hydrolases.

The authors' response to the reviewers compares the performance of the best two-sided designs to some therapeutically relevant enzymes, but when applying the design methods to plausible targets (one-sided design), the performance of the designs fell drastically short of the values the authors cite for therapeutically relevant enzymes. Reiterating points from the previous review rounds, two-sided enzyme design is highly artificial (probably irrelevant beyond proof of concept) and

dramatically improves the chances of success. With respect to industrial applications, we agree that some manufacturing properties of de novo designs are excellent. But we are unaware of a study that systematically tested the performance of de novo enzymes in such contexts. Regarding methodological novelty, in the response letter, the authors merely contrasted with past failures in designing metalloproteases. Again, we do not doubt that this submission represents progress, but we see no conceptual breakthrough in the described methods relative to previous studies.

In conclusion, we agree with the authors that the work extends the scope of de novo design to more challenging reactions, but we do not find the methodological novelty or the experimental results to merit publication in Nature.

Referee #3

(Remarks to the Author)

I was already satisfied with the previous version. The rebuttal by the authors to the other reviewer's comments is convincing and the additions to the manuscript as well.

Referee #7

(Remarks to the Author)

- HC12 - Overall the details are right and well explained in the manuscript. A minor detail: Gly91 adjacent to Cys94 (catalytic residue) has an unmodeled peptide flip; this may be related to the small difference density accumulating on Cys94, which may be indicative of the start of radiation damage. The related structure Kal C103A complex_D_1000301017_model-annotate_P1 was supplied but not mentioned in main text the manuscript. It is the same complex with the Cys mutated and indeed has the peptide flip as the main/only conformation.

- Gawi_09 - Overall I agree with the interpretation. Only one chain has alt conf for catalytic Cys, justified by slightly more pronounced density than the other chain. Overall the active site density features are very similar between the two chains, further supporting the interpretation and the Zn displacement hypothesis.

- HC34/Dokki WT apo_D_1000301020_model-annotate_P1 looks more like Dokki_15 C100A complex_D_1000300811_model-annotate_P1. This despite the cysteine sulfonic acid on HC34. This suggests that the larger His shift of the Dokki_15_WT structure may be due to the absence of substrate, not just the damaged Cys. Furthermore, the absence of the substrate and the Cys sulfonic are accompanied by not just a side chain shift. Rather, the whole beta hairpin His45 sits on shifts. None of this materially changes any conclusions of the paper or even this section; I would just would reword as I think it is a bit more complicated than what they are explaining here.

- For the Cys sulfonic, the authors may want to speculate whether this is caused by radiation damage or accumulates during the purification/crystallization. There may be ways to salvage the dataset and resolve this if it is radiation damage, which can address some of the issues they are seeing. For example, reprocessing the first half of the data, etc.

James Fraser (UCSF)

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We thank the reviewers for their constructive feedback and helpful comments on our manuscript. Below, we address these comments point-by-point. Our responses are provided in blue regular font and are interspersed with the reviewers' comments, which are shown in black, italic and underlined text.

Reviewer 1 and 2:

The authors claim (lines 239-240) that the best-performing design achieves kinetic parameters comparable to some natural metalloproteases. However, they cite reactions on synthetic peptide substrates, not natural protein substrates. Native metalloproteinases, such as MMP2, exhibit much higher catalytic efficiencies ($k_{cat} \approx 10 \text{ s}^{-1}$, $k_{cat}/K_M \approx 24,000 \text{ M}^{-1} \text{ s}^{-1}$) than measured in this study.

We thank the reviewer for pointing out that the MMP2 reference cited in our original submission only reported reactions on synthetic peptides. We have replaced that reference with another on MMP1, which lists kinetic parameters for both short peptides and native collagens, as a better comparison with a biologically relevant natural metalloprotease. More generally, native metalloproteinases exhibit a wide range of k_{cat} values, many on the order of $0.01\text{--}100 \text{ s}^{-1}$ (Pahwa 2019, Brownell 1994, Wang 1999, Lauer-Fields 2007, Kunugi 1982, Henkel 2009, Tokioka-Terao 1984), depending strongly on substrate sequence, assay pH, and construct (catalytic domain vs full-length). For these same substrates, k_{cat}/K_M values are commonly in the $10^3\text{--}10^5 \text{ M}^{-1} \text{ s}^{-1}$ range. In the revised manuscript, we have additionally provided Extended Data Table 3 with representative examples.

Although the designed proteases are still orders of magnitude slower than the best native proteases, we don't claim to achieve such activities in the manuscript. However, we think it is notable that our designs are quite close to the activities of natural enzymes on the lower end of the scale. Moreover, as described in the revised text, our best variant could be improved 30-fold through targeted mutagenesis of residues surrounding the active site, yielding an improved catalyst with steady-state parameters ($k_{cat} = 0.75 \text{ s}^{-1}$ and $k_{cat}/K_M = 31,000 \text{ M}^{-1} \text{ s}^{-1}$) approaching or exceeding those cited for MMP2. This variant accelerates the rate of amide bond hydrolysis by an extraordinary ten orders of magnitude over background.

The subsequent comparison to TEV protease is not necessarily relevant, as TEV lacks a metal cofactor and works through a different mechanism. A more appropriate benchmark would be aminopeptidase N, which is the enzyme from which the active-site geometry was derived in the submitted study, and this enzyme has reported values of $k_{cat} \approx 300 \text{ s}^{-1}$ and $k_{cat}/K_M \approx 3,500 \text{ M}^{-1} \text{ s}^{-1}$, dramatically higher than the values of the designs.

The comparison to TEV, a broadly used tool protease, was intended to show the potential utility of our designs for practical applications rather than suggest that they approach the activity of the best mechanistically related native metalloproteases. However, we appreciate the reviewers' point and have clarified this comparison in the main text. We have also added a new section explicitly discussing differences between our designs and native metalloprotease from which their theozyme was derived, along with additional experiments investigating potential origins of the observed activity gaps.

The catalytic rates and efficiencies achieved by the designed enzymes are instead more comparable, though orders of magnitude below, those of proteolytic antibodies (k_{cat} 0.13 s⁻¹ and k_{cat}/K_M 55,000 M⁻¹ s⁻¹ (Urusov AE et al. *Molecules*, 2023; 28(3):1330)). Overall, the observed kinetic performance of the designed enzymes appears largely driven by a favorable K_M toward their specifically designed substrates. Although the Discussion makes the point about the significant gap with respect to natural peptidases, the main text compares to enzymes of a different class that are not directly comparable to the designed ones.

Although our designs are less active than the best native metalloproteases, we don't believe they are directly comparable to catalytic antibodies with putative protease activity. The large majority of these antibodies were not designed. Of the two that were, one catalyzed cleavage of an activated nitroanilide amide (Janda et al. *Science* 1988), and the other required a triethylenetetramine-metal ion complex as an external cofactor (Iverson & Lerner, *Science* 1989). Both are (much) less active than the present computational designs. In the cofactor-dependent case, biochemical characterization was minimal, and neither sequence nor structural information is available, precluding verification of the claims.

Numerous "natural" antibodies have been described as proteolytic, spanning a wide range of apparent k_{cat} values (a non-exhaustive list is provided in Table R1). However, these studies generally lack detailed biochemical characterization, convincing structural data, or even sequence determination. For the few that have been studied in greater detail, significant questions remain. For example, in the crystal structure of one putative proteolytic antibody (Ramsland 2006, Figure R1b), an arginine is proposed to replace a histidine in a classic Ser-His-Asp triad (Figure R1a). Given its pKa of ~13, an arginine is almost always protonated under physiological condition and thus unlikely to abstract a proton from a serine. In the other reported structure (Uda 2025, Figure R1c), a classic Ser-His-Asp triad is proposed. However, the distance between the His NE2 atom and the Ser OG atom is 12.66 Å, much too long for productive hydrogen bonding much less proton abstraction. These data are therefore not sufficient to exclude the possibility that the

observed activity originates from a contaminating native protease in the preparations.

Hydrolysis of an unactivated amide bond is the most energetically demanding reaction catalyzed by a computationally designed enzyme to date. For example, this reaction is five orders of magnitude more difficult than the recently published cleavage of activated coumarin esters by computationally designed esterases (Kim 2025), yet the rate accelerations achieved by our metalloproteases, produced directly by design, are several orders of magnitude higher. Moreover, in contrast to the proteolytic antibodies, the detailed mechanistic analyses presented here firmly link the observed catalytic activities to the designed proteins themselves.

[Note: the reviewers reference kinetic parameters from Urosov 2023; however, the cited work concerns catalytic antibodies with peroxidase, not protease, activity—an entirely different reaction type. Was a different reference intended?]

Given the problems observed in the past in computational enzyme design where crystal structures did not align with the designed models, the manuscript would benefit substantially from structural validation. Experimental determination of the protease structure, demonstrating proper zinc coordination and active-site geometry, would provide critical support for the claimed design accuracy. Reliance solely on AF3-predicted models is insufficient for validating such mechanistic details, especially when it was used as a filter for design selection.

We agree with the reviewers on the importance of structural validation of the computational designs. Since the original submission, we obtained seven crystal structures of the designed zinc proteases, and have added the structural data to the manuscript. These include three structures presented in main figures (Zn5-Zn(II), ZnO7 E32Q-Zn(II) in complex with substrate, and apo TDP3) and four structures in extended figures (apo Zn5, Zn5 E75Q-Zn(II), TDP3 E45Q in complex with substrate, and Zn48 E75Q-Zn(II) in complex with substrate). Six of the seven structures show an all-residue C α RMSD of < 1 Å relative to their design models, underscoring the accuracy of the design and, in combination with the catalytic-residue knockout experiments, validation of the proposed mechanism. In the structures of the Zn5 and ZnO7 complexes, the zinc cofactor is coordinated exactly as designed, in agreement with the zinc binding affinity experiments.

The recent papers on serine hydrolases, metallohydrolases and the companion on cysteine proteases are conceptually similar. The main distinction in this submission is the two-sided generative design of enzyme and substrate, and the focus on the energetically more challenging protease reaction (though the latter point is shared with the cysteine protease companion). By approaching the problem as a two-sided

design challenge, the authors make the problem easier than a realistic scenario where one is targeting a natural protein. Despite this, the authors argue that this study establishes the generality of de novo peptidase designs, suggesting a path towards therapeutic use. Given the artificial nature of two-sided design, the very low catalytic rates and the lack of demonstrations on selectivity with respect to the human proteome, such statements are premature.

As the reviewers note, simultaneously designing the sequences of the substrates and the proteases simplifies the problem, as more sampling freedom in sequence space can potentially enable more accurate positioning of the substrate with respect to the active site. In the original manuscript, activity towards a specific peptide sequence in human TDP-43 was achieved by redesigning an enzyme that had been obtained through two-sided design. To address the reviewers' concern, we have added new experiments in which we designed a new, even more efficient de novo metalloprotease from scratch that selectively cleaves the same peptide sequence in TDP-43. We also provide demonstrations of substrate selectivity, and show cleavage of the full-length TDP-43 protein in vitro.

Furthermore, the current submission and the recent serine hydrolase paper raise a similar set of concerns: in both, while K_M values are favorable, the turnover numbers are very low by comparison to natural enzymes that carry out similar reactions.

While the turnover numbers are low, amide bond hydrolysis is a very challenging reaction. Moreover, as noted above, the steady-state parameters of some natural metalloproteases fall within a similar range as our best catalysts.

Furthermore, experimental structures are either lacking (in the current submission) or do not provide clear-cut validation of design accuracy within the active site. These problems are reminiscent of those seen with catalytic antibodies, where efficiencies were driven largely by binding rather than by turnover numbers. One of the conclusions stemming from the catalytic antibody studies was that the active sites were not precisely organized for catalysis. Given that the reactivities seen in the current papers are, in fact, below those of comparable catalytic antibodies, a detailed structural and mechanistic verification of design accuracy is crucial to support the claims of the paper on the efficacy of the design strategy.

The major problem with catalytic antibodies elicited to transition state analogs was the relative simplicity of their active sites. The most active catalysts possessed at most one or two functional groups in their binding pocket, in contrast to natural enzymes like metalloproteases and cysteine proteases, which have complex arrays of functional groups, so it is unsurprising that their activities lagged far behind those

of their natural counterparts. As shown in this and the companion paper on cysteine proteases, deep learning now allows us to recapitulate these complex active geometries, successfully placing four or five residues in catalytically productive poses around a substrate. As a consequence, the one-shot catalytic efficiencies that we achieve (10^7 - 10^8 -fold over background) are orders of magnitude higher than those of typical catalytic antibodies. In addition, as described in the revision, we have succeeded in improving our best variant by an additional 30-fold through a round of medium-throughput mutagenesis of residues within 4 Å of the catalytic site, achieving a 10^{10} -fold rate enhancement over background. These results demonstrate the potential for optimization of these designed proteases, which despite much effort, proved extremely challenging for catalytic antibodies due to production challenges and intolerance to mutation. Together with the additional structural and mechanistic studies we have provided, we believe that the revised manuscript provides strong support for the efficacy of our design strategy.

Needless to say, catalytic antibodies have largely been abandoned as a path for developing efficient biotechnological or medical tools due to their poor performance.

The reviewers are correct that catalytic antibodies have been largely abandoned as a path for developing biotechnological or medical tools. This can be ascribed to three major problems not shared by the designed proteases presented in our manuscript: 1) Transition state analogs are imperfect mimics of true transition states; 2) Generating antibodies containing sophisticated arrays of functional groups is extremely unlikely by immunization; and 3) Optimization of the catalytic performance first generation catalytic antibodies by rational design or directed evolution proved extremely difficult. Moreover, as mentioned above, for most of the autoantibodies with putative proteolytic activities, it's difficult to rule out the presence of contaminating natural proteases.

Our results show that machine learning provides a powerful alternative to the immune system for generating tailor-made catalysts with complex active sites embedded in robust, optimizable scaffolds, offering a more reliable route for developing efficient biotechnological and medical tools.

Minor comments

Line 60: esters are also resonance-stabilized.

Esters are indeed resonance-stabilized, but to a lesser extent than amides. Because nitrogen is less electronegative than oxygen, the nitrogen lone pair in amides delocalizes more effectively into the carbonyl, giving the C-N bond partial double-bond character and resulting in greater resonance stabilization. We have modified the original sentence to clarify this.

Line 60: The statement “Diffusive denoising methods such as RoseTTAFold Diffusion (RFdiffusion) have proven powerful in generating a wide range of new functional proteins” should be referenced.

We added the references per the reviewers’ request.

Line 95: The authors state “Starting from a choice of the length of the designed enzyme and the substrate...” — please indicate the range of lengths tested for both enzyme and substrate.

We added the range of lengths tested for both enzyme and substrate as requested.

Line 98: The sentence “Many of the RFD2-MI trajectories produced scaffolds with all the desired structural features” is vague. Please clarify what specific structural features were considered “desired” and describe what occurred in the unsuccessful trajectories.

The features considered “desired” in the backbones are described in the Supplementary Methods section 1.2 section. We have added a reference to this section in the main text.

Lines 104–106: The description – “Designs that AF3 predicted with high confidence... were fed back to RFD2-MI for two more rounds of finer sampling...” — is ambiguous. From the manuscript, it appears that this stage involves sequence design only rather than backbone redesign. Please clarify which parameters are being refined and whether any backbone changes are introduced during these additional iterations.

We apologize for the ambiguity and have clarified backbone-level versus sequence-level refinement in the main text. The overall resampling workflow is shown in Extended Data Figure 1, and the detailed methods for backbone-level diversification and refinement are described in Supplementary Methods section 1.5. Briefly, we used Partial Diffusion, a method to partially noise and de-noise a structure, diversifying the original design around a general fold (Vazquez Torres, 2024). The difference between the Partial Diffusion output and the input backbone can be controlled by an input parameter called Partial Diffusion temperature, where higher temperatures result in more dramatic differentiation from the original backbone. The exact parameters used and the filtering thresholds are described in

Supplementary Methods 1.4, 1.5 and 1.8, and summarized at the high level in Extended Data Figure 1.

Lines 146–147: The reported overall success rate of 36.3% reflects only the cis-cleavage mode, in which the substrate is covalently tethered to the protease. As this setup does not rigorously test substrate recognition, the actual catalytic success that should be reported in the abstract section is the one under trans conditions.

The reviewer is correct that the success rate refers to the cis-cleavage assay. Since rescreening each design in the trans mode would have required the re-synthesis of all DNA sequences, entailing substantial additional costs, we only screened 22 hits from the cis assay, spanning a range of cleavage levels, in the trans assay (see the section entitled “Experimental validation of two-sided designed proteases”). The selected designs and their performance in both the cis and trans assays are shown in Supplementary Table 2. The cis and trans cleavage fractions at the same time point (1 h at 37 C) are well-correlated, as shown in Fig. 2h, indicating that substrate recognition is not a key challenge for our approach. Therefore, we believe reporting the overall success rate in the cis-screen is appropriate.

Line 207: Targeting arbitrary peptides wasn’t shown. The peptides were designed.

We have added a new design, TDPn3, which was generated from scratch to target the same TDP-43 sequence(ALQSSWGMMG) that was targeted in the redesign of ZnO36. The results have been incorporated in the “Design of proteases cleaving human TDP-43 ” section of the main text, and the data are shown in Figure 4.

Section “Reprogramming to cleave human TDP-43”: It would be helpful to explain the rationale for reusing an existing scaffold (ZnO36) rather than designing a new protease de novo for this target. Also, please provide kinetic parameters of the best designs from this group.

We initially chose to reuse an existing scaffold to assess whether its substrate specificity could be reprogrammed to recognize an arbitrary peptide sequence in a disease relevant protein. Prompted by the reviewers’ query, we also explored the alternative strategy of designing a new protease de novo for the same target. The design and kinetic characterization of TDP-n3, the most effective TDP-43 protease, are now described in the main text and in Figure 4e.

Please describe the differences in terms of the structure and number of mutations among the designs.

The TM-score between any pair of active designs shown in Fig.3 is less than 0.23 (calculated using <https://aideepmed.com/TM-score/>, where a TM-score > 0.5 suggests fold-level similarity). We have clarified this in the figure caption. We have also added the number of mutations in the designs sharing the same backbone. For example, sequences of ZnO7 and ZnO25 (Fig.3d) which originate from the same backbone, differ by 39/170 amino acids.

For all figures: please indicate clearly that the structures shown are models.

We have indicated clearly in figures or figure captions where the structures shown are models.

We clarify, again, that we do not argue that the authors should present a practically useful enzyme, but they should not trivialize the gaps towards such enzymes. Instead, they should clarify that the gaps are significant, characterize the underlying causes, and perhaps improve the performance of the designs.

Given the above, the main scientific question that arises is why activity levels are so low despite structural accuracy (in some cases)? What is missing? This question is neither posed nor addressed in the papers, though the points we and other reviewers raised make it extremely pertinent.

We thank Reviewers 1 and 2 for clarifying their major concerns. In response, we have added a new section in the main text detailing how we addressed the observed activity differences between our designs and the best natural metalloproteases. Our analysis indicates that despite overall structural accuracy, suboptimal preorganization of the catalytic residues largely limits catalytic efficiency. We further show that combining four individually beneficial mutations partially alleviated these limitations, resulting in a substantial improvement in the activity of our best design. The reviewers' questions have greatly strengthened the clarity and impact of the revised manuscript.

Referee #3 (Remarks to the Author)

This manuscript describes de novo designed metalloproteases: metalloenzymes containing a zinc(II) active site, capable of hydrolyzing peptide bonds. The metalloproteases, as well as their peptide substrates, were computationally

designed in a two-sided process and a selection of the designs was experimentally validated. The predicted structures of top designs proved to be accurate and had unprecedented peptide folds. Moreover, they showed impressive activity, both in cis and in trans reactions, of up to 10^8 fold rate acceleration compared to the uncatalyzed reaction and the k_{cat} approaches that of some Zn-dependent proteases. It was shown that in all cases indeed the peptide was cleaved at the designed site. As a consequence of the two-sided design process, most enzymes showed limited cross-reactivity, i.e. they were selective for their designed substrate peptide sequence, albeit with notable exceptions of some very substrate promiscuous designs. Finally, one of the successful designs, ZnO36, was redesigned to target a biologically relevant substrate: the TAR DNA-binding protein 43 (TDP-43), protein which aggregation and fibrillization are linked to neurodegenerative processes. Encouraging activity was found that raises the prospect of biological applications, albeit that for this purpose the substrate specificity needs to be further improved.

We appreciate the reviewer's support. Regarding the final point, we have included in the revised manuscript a newly generated TDP-43 protease, designed from scratch, which shows enhanced activity and substrate specificity toward the target TDP-43 peptide compared to the redesigned ZnO36 protease.

This work builds on two prior works by the same group(s). Reference 2 (listed as preprint, but published in the meantime: Nature (2025). <https://doi.org/10.1038/s41586-025-09746-w>) in the manuscript describes the de novo design of a zinc esterase: a similar type of metalloenzyme that hydrolyzed activated esters. A similar type of reaction, but on much more reactive and, hence, easier substrates. These zinc esterases were designed using RFdiffusion2 (RFD2). The other preprint describes an adaptation of RF Diffusion 2, called RFdiffusion2 Molecular Interfaces (RFD2 MI), which was used in the current work to design the Zn protease.

The key innovations of this work, compared to these reported works are:
- This work achieves the successful design of active proteases. Peptide bonds are notoriously unreactive and hence present a much bigger challenge for enzyme design than, for example, esters (reported before). Moreover, designer proteases could have important applications, for example in biomedicine and bioremediation. This is suggested by the application to the hydrolysis of RDP-43. Therefore, this work represents a milestone in enzyme design.

- A water molecule, which is needed as the second substrate for the proteolysis, was included in the design. This significantly increased the frequency of successful, i.e. active, designs. The success rate of ~87% active designs, compared to ~15% without the water included, is impressive. It also shows, as the authors note in the

manuscript, that inclusion of key molecules for the reaction in the design process can greatly increase design success.

- A two-sided design strategy was used to design both the metalloprotease and its peptide substrate. This means that the substrate sequence was unique for each design. A result of this was that many (most) designs displayed a significant substrate selectivity (peptide sequence).

The work presented is of high quality. Data is generally presented well, with replicates and error margins (one exception, see below). The conclusions are supported by the data. The manuscript is well-written: it was a pleasure to read.

We thank the reviewer for the positive comments.

General comments:

1. The computational design techniques used, RFD2-MI, as well as the design of a zinc hydrolase were established by the same group in recent work. So, the main novelty here is the application towards the creation of metalloproteases and achieving peptide bond hydrolysis.

We agree with the reviewer on this comment.

2. This is maybe considered semantics, but I am not sure if the claim of “de novo design”, i.e. design from first principles, of metalloproteases is warranted here. The active site structure, i.e. the ligation of the catalytic metal, as well as the positioning of the oxy anion stabilizing residues was not designed, but taken from an existing metalloproteases, aminopeptidase N and astacin, and precisely reproduced in the designed proteins. So, one could argue that while the protein environment was designed de novo, the part of the structure that makes it an enzyme, the catalytic site, was not and, thus, that this work represents a (very impressive) redesign of aminopeptidase N and/or astacin. I think calling it a “designer enzyme” or “designer metalloprotease” is probably better justified than calling it a “de novo designed enzyme/metalloprotease”.

The definition of *de novo* protein design can vary across publications and contexts (Huang 2016, Korendovych 2020, Kortemme 2024). However, a consistent message across these definitions is that *de novo* designed proteins have amino acid sequences that are not derived from or homologous to naturally occurring proteins, distinguishing them from traditional protein engineering approaches such as directed evolution or rational redesign of existing scaffolds.

In our work, while the catalytic motif geometry was informed by known metalloprotease active sites, the overall protein sequence and structural scaffold were designed independently and do not share sequence homology with natural metalloproteases (Extended Data Table 2). The catalytic residues were incorporated into a newly designed structural context rather than by modifying an existing natural protein scaffold (Extended Data Fig. 6). This approach is consistent with widely accepted definitions of *de novo* protein design, which emphasize the creation of novel protein sequences and folds rather than strict adherence to “first-principles” design of every atomic feature.

Furthermore, although earlier definitions of *de novo* design (Huang 2016) sometimes include “physical principles”, mainly because alternative methods were lacking, recent advances in computational protein design—including deep learning–based structure prediction and generative methods—have broadened the operational meaning of this term. For example, *de novo* binders can be readily generated from deep learning based pipelines without the input of physical principles.

For these reasons, we believe that the term “*de novo* designed metalloprotease” accurately reflects the nature of this work and is consistent with current conventions in the field. While terms such as “designer enzyme” are also valid descriptively, they do not provide greater clarity or align more closely with established terminology. Therefore, we have retained the original wording in the manuscript.

3. The design was modeled in three states: apo, substrate bound and transition state. This kind of multistate design is clearly important to achieve proficient enzymes. However, only one transition state (TS) was modeled, whereas there are more along the reaction coordinate. The Baker lab has shown in recent work (Science 2025, <https://doi.org/10.1126/science.adu2454>) that including all TS's along the reaction coordinate in the design improves the quality of the outcome. The authors should comment on why this approach was not used here; it might have delivered enzymes that are even closer to the catalytic activity observed for natural metalloproteases.

The mechanism of metalloprotease hydrolysis is different from that of serine or cysteine hydrolases, which involves acylation and de-acylation steps because the nucleophile is an amino acid in the enzyme and needs to be regenerated after acylation. For metallohydrolases, where a water molecule directly performs the nucleophilic attack, the tetrahedral intermediate formed after the nucleophilic attack is the only intermediate in the current-established mechanism that involves bond formation or breaking between heavy atoms (Fig. 1b). Subsequent intermediate steps proposed in the literature, including hydrogen bond rearrangement and proton shuttling (Pelmenschikov 2002, Vasilevskaya 2015, Varghese 2023, Chen 2021)

don't involve bond formation or breaking between heavy atoms and are therefore beyond the capability of AF3. PLACER, which was used in Lauko et al, Science, 2025 as the primary tool to assess rotamer ensemble geometries across different states in catalytic side chains, was not trained on ion coordination, and was therefore not suitable for the design of metalloproteases.

Other comments

4. While the manuscript in general is very well-written and clear, the section explaining the design choices could be improved a bit. A bit more explanation on why choosing the Zn site from one enzyme (aminopeptidase N) and the substrate binding site of another (astacin) would help the reader understand the thought process better.

In response to the reviewer's request, we have added a section "Choice of the theozyme" in Supplementary Discussions to explain our rationale for theozyme generation.

5. To probe the necessity and contribution of the active site residues for both activity and zinc binding, they were sequentially mutated. In almost all cases this resulted in a significant drop of activity, thus confirming the essential role of this particular residue (Fig 3.). An exception is the R79A mutation in ZnO36: this seems to give rise to a significantly increased activity (see bar graph in 3rd row of Fig 3). This surprising result is completely ignored in the manuscript. Here clearly the design was not optimal, which should be commented on.

As now explained in the Fig. 3 caption, we speculated that R79, which was introduced by ligandMPNN during sequence optimization, might provide beneficial interactions with the oxyanionic intermediate and flanking transition states. However, the increase in activity observed when this residue was mutated to alanine suggests that such an interaction may instead disrupt productive oxyanion stabilization by the catalytic tyrosine. We observed a similar result in our mutagenesis experiments on Zn45, where activity increased when Y152 was mutated to isoleucine. Both of these results as well as our interpretation are described in the main text and in the Supplementary Discussion 5 of the revised manuscript.

6. While it is nice to see there is limited cross-reactivity of the enzymes towards the peptides for the other selected design, I am hesitant to call this "considerable" specificity. The design sequences tested are very dissimilar. See below for the sequences, which I had to retrieve from the SI; it would have been better to have

these available in the manuscript. To assess specificity, seeing how selective the designed protease is when mutating single amino acids in the substrate peptide

Sequences substrates:

SZn5: VIRLRMRR

SZnO7: ALTLTFRLLR

SZnO25: SLTLTFRLLR

SZnO36: ARILRVNFKI

SZnO39: SRVIEVKYLK

SZn45: VLEFTGRR

We agree and have removed the adjective “considerable” from the discussion of specificity. As requested, we have also added the substrate sequences to Fig. 3d in the main text.

7. Kinetics: a comparison of the kinetics is made to matrix metalloproteinases and TEV protease. The latter is a cysteine protease, not a Zn protease. How do the kinetics compare to the Zn proteases that the design was based on, i.e. aminopeptidase A and astacin? I feel that this comparison would be much more valuable.

The comparison with TEV was intended to show that the designed zinc protease approaches the kinetics requirements necessary for practical applications. We now explicitly note in the main text that TEV operates via a different catalytic mechanism. Moreover, we have added Extended Data Table 3 summarizing the kinetic parameters of representative metalloproteases, included a new section in the main text analyzing the activity gap between our designs and the best natural metalloproteases, and discussed possible avenues for future improvement.

The highest reported rate acceleration was achieved for an enzyme (Zn45) on a substrate for which it was not designed (SZnO36). What are the kinetic parameters for its designed substrate (SZn45)? Figure 4a suggests it is significantly less active on its designed substrate: what does this mean for the design process?

The reviewer is correct that Zn45 is significantly less active on its designed substrate. In fact, the kinetics parameters of Zn45 for its designed substrate were too low to measure accurately. Unfortunately, the corresponding sfGFP-mScarlet construct gives a FRET signal with a signal-to-noise ratio less than two upon complete cleavage and exhibits a strong inner filter effect (Supplementary Method 3.9), further reducing the signal. As a consequence, we were unable to determine accurate kinetics. Interestingly, though, the lower activity of Zn45 on its cognate substrate compared to S_ZnO36 is reflected in the AF3 metrics. We have added a

sentence in the main manuscript discussing this point and clarified that implementing negative selection (filtering against off-targets) could further enhance specificity.

8. In the discussion section, the authors state: “there is still room for improvement—turnover straight out of the computer is still $\sim 10^3$ – 10^4 -fold below those of the most efficient natural proteases under similar conditions”. While the rate enhancements achieved straight of the computer are absolutely impressive, it is indeed still a significant gap. Where does the current approach still fall short? Can the authors speculate on this?

We have added a substantial section in the main text describing the experiments performed to address this issue and outlining potential directions for further improvement. We are grateful to all reviewers for raising this point, as addressing it led to the identification of an even more active Zn45 variant and has significantly strengthened the study.

9. Figure S26: information of number of replicates and error margins for TDP cutting experiments is missing.

Thank you for catching this. We have now repeated this experiment using a FRET-based assay (instead of the original gel assay) to enable a more direct comparison with the newly generated de novo TDP-43 protease. The updated data are presented in Fig. 4, and the number of replicates and associated error margins are reported in the figure caption.

Minor comments

Typo (first paragraph of computational design section): “The fourth ligand of Zn(II), a water molecule, is activated by a glutamate (E1)” - should be “E2”.

We have removed the subscripted labels used to define the catalytic residues throughout the manuscript, as we believe they do not aid clarity. Instead, we now refer to these residues by their catalytic roles, such as “the general base glutamate” where appropriate.

Figure 4a: in the matrix are listed the relative reactivities of the enzymes for the various substrates. From the caption it is unclear what is compared here: catalytic efficiencies? K_{cat} ? Yields?

The fractions of the substrates digested, normalized to each enzyme's cognate substrate, are compared in the matrix. A sentence has been added to the figure caption to clarify the measurements.

Reviewer 4:

In this study, Chen and coworkers report the computational design of zinc-dependent proteases capable of breaking the amide bonds of some designed peptide sequences. The authors used an enhanced version of RoseTTAFold Diffusion 2 for Molecular Interfaces (RFD2-MI) tailored for enzyme and protein–protein interface design to generate multiple protease-peptide pairs. The best design accelerated peptide bond hydrolysis by more than 10^8 -fold compared to the uncatalyzed reaction, however, it should be highlighted that the kinetic characterization showed a poor k_{cat} of ca. 0.0025 s^{-1} , and a good K_M of ca. $26\text{ }\mu\text{M}$. These values clearly indicate that there is much room for improvement, as the turnover is very low if compared with those of natural proteases. While we agree with the authors that this study shows that de novo enzyme design can now tackle more difficult, chemically demanding reactions (as shown also in their previous work on serine hydrolases, DOI:10.1126/science.adu2454), the catalytic activities of the obtained designs are still far below those of natural enzymes.

Although the turnover numbers achieved by the current designs are below those of the best native proteases acting on their most preferred substrate, the activity of native proteases spans a wide range, exhibiting k_{cat} values on the order of $0.01\text{--}100\text{ s}^{-1}$ (Pahwa 2019, Brownell 1994, Wang 1999, Lauer-Fields 2007, Kunugi 1982, Henkel 2009, Tokioka-Terao 1984). For comparison, our best catalyst straight-out-of-computer has a k_{cat} of 0.025 s^{-1} (not 0.0025 s^{-1} as stated by the reviewer), comparable to the k_{cat} values reported for native proteases at the lower end of this scale, such as MT1 MMP (Pahwa 2019), MT2 MMP (Pahwa 2019), MT4-MMPCD (Wang 1999), ADAMTS-4 (Lauer-Fields 2007) when evaluated on substrates reported in the corresponding reference. Moreover, we have shown in the revised manuscript that we could improve the k_{cat} value of Zn45 to $0.75 \pm 0.04\text{ s}^{-1}$ by introducing four point mutations.

The authors also used some of the designed enzymes as template scaffold for optimizing it for cleaving a therapeutically relevant human peptide, but again the activities obtained need further improvement and refinement.

As noted in our responses to the other reviewers, we have included a newly designed de novo protease targeting the same TDP-43 peptide epitope as the previously redesigned protease. This new enzyme exhibits enhanced activity and

improved specificity against all other synthetic substrates tested in the manuscript (new Fig. 4).

Considering that the method applied in the study has already been developed in another work, and also the level of catalytic improvements achieved, we do not believe the results obtained in this study have the novelty and impact required for its publication in Nature journal.

We respectfully disagree with the reviewer's assessment. Prior to our work on metalloproteases and cysteine proteases, the RFD2- MI framework had *never* been applied to enzyme design. Our study therefore demonstrates, for the first time, that this method can generate active and evolvable proteases with measurable catalytic improvement, validated through medium- throughput experimental characterization. Importantly, the scientific contribution of this work extends beyond the computational framework itself: it provides a new, generalizable toolbox of designed proteases that can be readily repurposed for diverse targets. Thus, the novelty of our study lies not only in the methodological extension of RFD2- MI to enzyme catalysis but also in the resulting catalytic scaffolds, which have broad practical and conceptual impact for the enzyme design community.

1. The authors state that: "We modeled each resulting design in three states: 1) the apo protease state, to confirm correct folding; 2) the ES complex state, to assess substrate binding and positioning of the target amide bond; and 3) the transition state to evaluate stabilization." However, a few sentence later they mention that: "For modeling the transition state, we used a phosphoramidate TSA; many crystal structures have been solved for similar TSA complexes, so they are better modeled by ML software trained on the PDB than the actual transition state". We agree with the authors that the ML software trained on X-ray structures will provide better results with TSA than the actual TS, however, we disagree with the authors when they state that the TS is used to evaluate stabilization. Although the charge distribution of TSA might resemble that of TS, TSA is not the TS, so it should not be stated that the TS is used in the modeling to evaluate stabilization. This is not accurate and should be rephrased.

The reviewer is correct; our statement that the TS, rather than a TSA, was used to evaluate transition state stabilization was an oversight on our part. We have corrected and updated the main text accordingly.

2. The authors used two different strategies for modeling the zinc and coordination sphere, which by the way, it is directly taken from a natural protease scaffold and not generated de novo via theozyme calculations. In one of the approaches a water

molecule is added close to the zinc metal ion. As this is constrained during the design process, this does not guarantee that the water will be in reality there and neither that the pocket environment would favor the proper positioning of the water molecule.

We agree with the reviewer that computation modeling of a coordinated water molecule may not fully capture the properties of the pocket environment and does not guarantee accurate positioning of a water as a Zn(II) ligand. Nonetheless, we hypothesized that adding water in the complex prediction would better reproduce the catalytic site geometry during catalysis, given water's established mechanistic role. To evaluate this assumption, we conducted design campaigns using both the "zinc-only" and "zinc-water" strategies in parallel. The experimental hit rates were 14.7% for the zinc-only strategy and 87.5% for the zinc-water approach, consistent with the proposed benefit of expliciting modeling water.

To address the reviewer's concern that the water molecule was "constrained" during design, we performed AF3 predictions on a set of designs where the Zn(II) and water were modeled as separate entities, effectively unconstraining the water position. Although we initially avoided this setup due to concerns that additional ligands might reduce AF3's accuracy, the predictions obtained with constrained and unconstrained water were remarkably similar across both active and inactive designs (Supplementary Table 5). Because only ordered designs were carried forward, our dataset does not include poorly scoring variants that might demonstrate failure cases. Nevertheless, our results suggest that the unconstrained approach could better exploit AF3's ability to model Zn(II)-water coordination and may represent a useful refinement for future design efforts.

We have added a Supplementary Discussion section elaborating on this point and thank the reviewer identifying this potential direction for improvement.

3. The authors used two different strategies for screening: the cis screen in which the peptide and protein are fused together, and the trans screen in which a (GS)15 linker is added to the N-terminus and an mScarlet tag at the C-terminus of the substrate peptide. In both cases, additional protein-protein interactions imposed by either the fusion or especially the addition of a rather big protein at the terminal regions of the peptide might substantially affect the binding affinity of the peptide, and thus dramatically affect the obtained results.

We agree with the reviewer on the potential effects of fusing the protein to a short peptide substrate. However, as our end goal for this technology is to develop designer proteases to cleave human proteins of interest, it is important to validate our designs as "proteases" instead of "peptidases" for feasibility. We only designed the interactions between the proteases and the peptide substrate (i.e. the linker was not considered during design). Therefore, the fact that the change in constructs did

not qualitatively alter cleavage suggests that the protein-protein interaction is dominated by the designed interface. This is further supported by the specificity matrix in Fig. 4a where cleavage preference is entirely grouped by the short substrate preferences.

4. In their assessment of active and inactive designs (shown in Figure 2d, 2e, 3a, S10 and S16), there is a lack of geometrical features, which makes the quantification of the preorganization of the binding pose and so the evaluation of the catalytic potential of the designed variant hard (we would say impossible) to assess. In Figure S10 only the distance between R79 and carbonyl group at the ES and TSA complex is shown. This is again completely insufficient.

The active site geometry of the designs were quantified and filtered using catalytic residue RMSD and other geometric constraints, as discussed in Supplementary Method section 1.8 and in the new section “Origin of activity discrepancies between the designs and native metalloproteases” in the main text.

The R79 residue is not part of the original catalytic motif, but was instead introduced by MPNN during sequence design; therefore the purpose of Figure S10 was to establish the motivation for analyzing the R79A single amino acid substitution, rather than to assess activity. This was included in the Methods section but left out of the main text and the figure caption. We have now clarified this in the figure caption and discussed this mutation in the section “Origin of activity discrepancies between the designs and native metalloproteases” in the main text as well as Supplementary Discussion.

5. In Figure S11, the tyrosine hydrogen bond angles found in X-ray structures are shown. The hydrogen bond distances should also be included and compared.

We agree that the hydrogen bond length is a critical metric to assess this interaction and have added these distances to the Figure (current Fig.S9)

6. It is also mentioned: “In the prediction of Zn45 with the Zn45 substrate, the angle between the attacking water oxygen, the scissile carbonyl carbon, and the carbonyl oxygen is 51.8°, smaller than the ideal 90° geometry, potentially interfering with the nucleophilic attack.” While the angle is important for a catalytically competent pose, the distance is essential even if the perfect angle is achieved, the distance has to be short enough for allowing catalysis.

In all the AF3 predictions, the distances between the modeled water molecules and the substrate carbonyl carbon atoms were consistently < 3.5 Å in all our AF3

complex predictions, irrespective of the angle. Specifically, the predicted distance was 2.5 Å for Zn45 with its cognate substrate and 2.4 Å with ZnO36 substrate. Because these distances were similarly short, we did not emphasize this point in the original discussion. Upon further examination, however, we found that in the Zn45-ZnO36 substrate complex, the positioning of the substrate appears suboptimal, an observation independently supported by molecular dynamics simulations and mutagenesis analysis of the Y152 position (revised main text section “Origin of activity discrepancies between the designs and native metalloproteases”). Accordingly, we have removed this premature interpretation from the main text and the corresponding Supplementary Figure.

7. “Using a previously published side-chain pocket scoring metric to assess compatibility between substrate windows and designed active sites by logos, we identified ZnO36 with a targeting window spanning residues 329–340 as a suitable starting scaffold.” More details should be given on how this selection was made. It is also not clear for us why the authors used as starting scaffold one of the previous designs and not a new one based on only optimizing the protease sequence.

The window selection criteria used in our study are essentially identical to the IDR binding window selection criteria described in the cited paper; we have added a brief summary of this in the main text. Our primary objective was to demonstrate that de novo proteases can be structurally robust and designable, thereby establishing them as potential future therapeutic agents. A detailed exploration of designing clinically viable therapeutic proteases, however, lies beyond the scope of the current work. Additionally, the redesigns presented here were generated through partial diffusion rather than sequence optimization alone.

Inspired by the reviewer’s suggestion, we also conducted a de novo design campaign targeting the same TDP-43 sequence. This effort yielded a more specific protease, for which we report kinetic parameters and confirm full-length TDP-43 cleavage. The corresponding data are included in the new Fig.4, where the de novo and redesign approaches are directly compared. We are grateful to the reviewers for this suggestion, which has significantly strengthened the manuscript.

8. The correlation between experimentally measured normalized digested fraction and computational metrics shown in Figure S18 is extremely poor.

The Pearson correlation coefficient quantifies the strength and direction of a linear relationship. However, the purpose of this figure is to demonstrate the ability of each metric to distinguish between active and inactive designs, rather than to evaluate linear correlation. Thus, the use of the Pearson correlation coefficient is

not appropriate in this context. We thank the reviewer for highlighting this point. Despite this issue, several computational metrics—such as complex pLDDT, ipae_min, ipTM, and pae_interface—still show clear predictive power for substrate cross-reactivity, as reflected in the data distributions. To avoid ambiguity, we have removed the Pearson correlation values from the figure.

9. The novelty of the generated folds with respect to natural scaffolds (including PDB or AF databases) is not presented neither investigated.

Structural diversity among enzyme scaffolds is particularly critical in protease design, as the ultimate goal is to accommodate specific target epitopes within appropriately shaped and chemically complementary active site clefts. Accordingly, we have analyzed the structural novelty of our designs relative to natural protease scaffolds and now include a summary at the end of the “Structural validation” section.

10. “While these results are encouraging for programming proteases against therapeutic targets, there is still considerable room for improvement as the designs retained activity on the original substrate (Fig. S24).” We agree that the results are promising, but they should be substantially improved for their publication in Nature.

As described above, we reprogrammed one of the variants for TDP-43 cleavage to show the potential of our design approach. In the revised manuscript, we now include a newly generated de novo TDP-43 protease, created from scratch, compared to the redesign strategy and showed markedly improved and specificity compared to the previous redesign strategy. While further optimization may be required, for example through directed evolution, these results provide a strong proof of principle.

Table R1. Reported proteolytic catalytic antibodies. Catalytic antibodies with reported crystal structures are shaded in blue. In both reported structures, catalytically-competent active sites are lacking.

Important First and Last Authors	Year	k_{cat}	Title	Sequence published?
Iverson, Lerner	1989	$6 \times 10^{-4} \text{ s}^{-1}$	Sequence-Specific Peptide Cleavage Catalyzed by an Antibody	No
Paul	1989	0.26 s^{-1}	Catalytic hydrolysis of vasoactive intestinal peptide by human autoantibody	
Nevinsky	2005	$1.0 \times 10^{-2} \text{ s}^{-1}$	Casein-hydrolyzing activity of sIgA antibodies from human milk	
Gabibov	2006	$6 \times 10^{-3} \text{ s}^{-1}$	Autoantibodies to myelin basic protein catalyze site-specific degradation of their antigen	
Nevinsky	2010	up to 2.6 s^{-1}	Affinity and catalytic heterogeneity of polyclonal myelin basic protein-hydrolyzing IgGs from sera of patients with multiple sclerosis	
Paul	1994	$1.8 \times 10^{-4} \text{ s}^{-1}$	Molecular cloning of a proteolytic antibody light chain	Yes
Hifumi, Uda	2003	$1.0 \times 10^{-2} \text{ s}^{-1}$	Catalytic features of monoclonal antibody i41SL1-2 subunits	
Paul	2008	$2.9 \times 10^{-4} \text{ s}^{-1}$	Autoantibody-catalyzed hydrolysis of Amyloid β peptide	
Gabibov	2008	$2.6 \times 10^{-5} \text{ s}^{-1}$	Strategies for induction of catalytic antibodies toward HIV-1 glycoprotein gp120 in autoimmune prone mice	
Hifumi, Uda	2018	$3 \times 10^{-4} \text{ s}^{-1}$	New technologies to introduce a catalytic function into antibodies: a unique human catalytic antibody light chain showing degradation of β -amyloid molecule along with peptidase activity.	
Hifumi, Uda	2020	$6 \times 10^{-5} \text{ s}^{-1}$	A new algorithm to convert a normal antibody into the corresponding catalytic antibody	
Hifumi, Uda	2022	$4.5 \times 10^{-5} \text{ s}^{-1}$	A new catalytic site functioning in antigen cleavage by H34 catalytic antibody light chain	

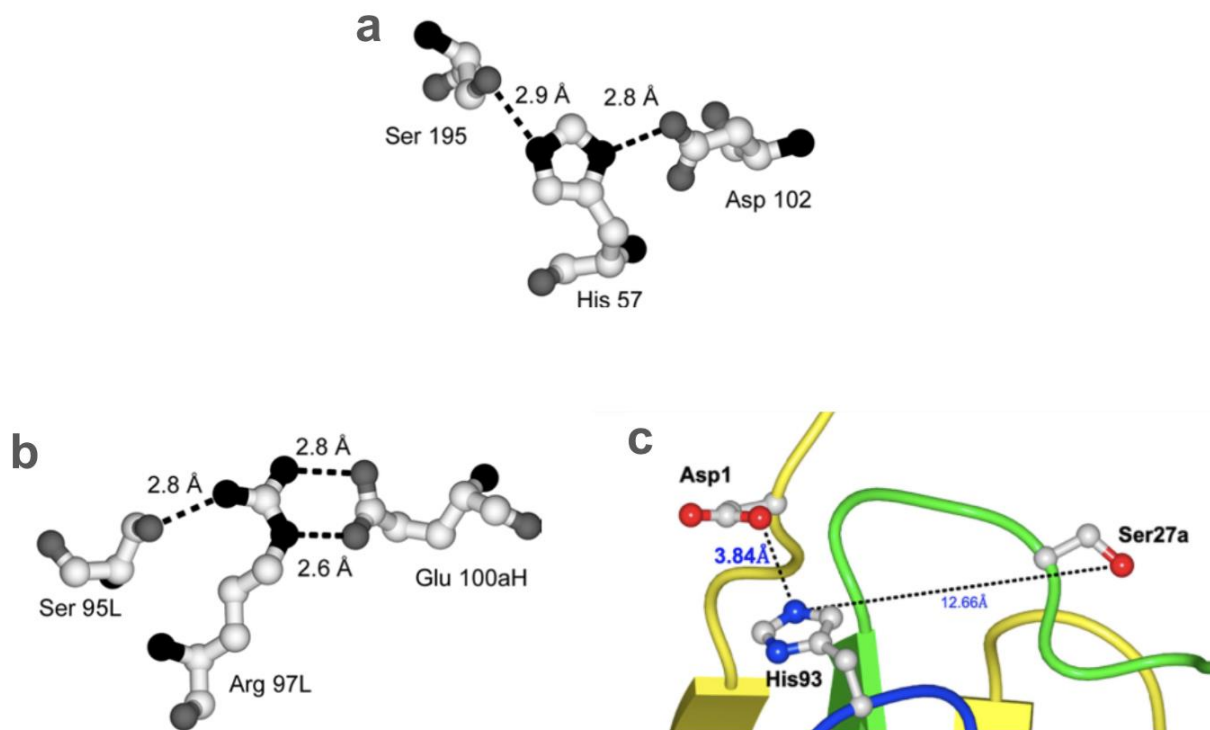


Figure R1. Crystal structures of previously reported proteolytic catalytic antibodies. a, the classical Ser-His-Asp triad in bovine trypsin (PDB 1BJU). b, the proposed putative active site of the catalytic antibody reported in Ramsland 2006. c, the proposed putative active site of the catalytic antibody reported in Uda 2025. Panels a, b adapted from Ramsland 2006; panel c adapted from Uda 2025

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We thank the reviewers for their constructive feedback and helpful comments on our manuscript. Below, we address these comments point-by-point. Our responses are provided in blue regular font and are interspersed with the reviewers' comments, which are shown in black, italic and underlined text.

Reviewer 1 and 2:

The authors claim (lines 239-240) that the best-performing design achieves kinetic parameters comparable to some natural metalloproteases. However, they cite reactions on synthetic peptide substrates, not natural protein substrates. Native metalloproteinases, such as MMP2, exhibit much higher catalytic efficiencies ($k_{cat} \approx 10 \text{ s}^{-1}$, $k_{cat}/K_M \approx 24,000 \text{ M}^{-1} \text{ s}^{-1}$) than measured in this study.

We thank the reviewer for pointing out that the MMP2 reference cited in our original submission only reported reactions on synthetic peptides. We have replaced that reference with another on MMP1, which lists kinetic parameters for both short peptides and native collagens, as a better comparison with a biologically relevant natural metalloprotease. More generally, native metalloproteinases exhibit a wide range of k_{cat} values, many on the order of $0.01\text{--}100 \text{ s}^{-1}$ (Pahwa 2019, Brownell 1994, Wang 1999, Lauer-Fields 2007, Kunugi 1982, Henkel 2009, Tokioka-Terao 1984), depending strongly on substrate sequence, assay pH, and construct (catalytic domain vs full-length). For these same substrates, k_{cat}/K_M values are commonly in the $10^3\text{--}10^5 \text{ M}^{-1} \text{ s}^{-1}$ range. In the revised manuscript, we have additionally provided Extended Data Table 3 with representative examples.

Although the designed proteases are still orders of magnitude slower than the best native proteases, we don't claim to achieve such activities in the manuscript. However, we think it is notable that our designs are quite close to the activities of natural enzymes on the lower end of the scale. Moreover, as described in the revised text, our best variant could be improved 30-fold through targeted mutagenesis of residues surrounding the active site, yielding an improved catalyst with steady-state parameters ($k_{cat} = 0.75 \text{ s}^{-1}$ and $k_{cat}/K_M = 31,000 \text{ M}^{-1} \text{ s}^{-1}$) approaching or exceeding those cited for MMP2. This variant accelerates the rate of amide bond hydrolysis by an extraordinary ten orders of magnitude over background.

The subsequent comparison to TEV protease is not necessarily relevant, as TEV lacks a metal cofactor and works through a different mechanism. A more appropriate benchmark would be aminopeptidase N, which is the enzyme from which the active-site geometry was derived in the submitted study, and this enzyme has reported values of $k_{cat} \approx 300 \text{ s}^{-1}$ and $k_{cat}/K_M \approx 3,500 \text{ M}^{-1} \text{ s}^{-1}$, dramatically higher than the values of the designs.

The comparison to TEV, a broadly used tool protease, was intended to show the potential utility of our designs for practical applications rather than suggest that they approach the activity of the best mechanistically related native metalloproteases. However, we appreciate the reviewers' point and have clarified this comparison in the main text. We have also added a new section explicitly discussing differences between our designs and native metalloprotease from which their theozyme was derived, along with additional experiments investigating potential origins of the observed activity gaps.

The catalytic rates and efficiencies achieved by the designed enzymes are instead more comparable, though orders of magnitude below, those of proteolytic antibodies (k_{cat} 0.13 s⁻¹ and k_{cat}/K_M 55,000 M⁻¹ s⁻¹ (Urusov AE et al. *Molecules*, 2023; 28(3):1330)). Overall, the observed kinetic performance of the designed enzymes appears largely driven by a favorable K_M toward their specifically designed substrates. Although the Discussion makes the point about the significant gap with respect to natural peptidases, the main text compares to enzymes of a different class that are not directly comparable to the designed ones.

Although our designs are less active than the best native metalloproteases, we don't believe they are directly comparable to catalytic antibodies with putative protease activity. The large majority of these antibodies were not designed. Of the two that were, one catalyzed cleavage of an activated nitroanilide amide (Janda et al. *Science* 1988), and the other required a triethylenetetramine-metal ion complex as an external cofactor (Iverson & Lerner, *Science* 1989). Both are (much) less active than the present computational designs. In the cofactor-dependent case, biochemical characterization was minimal, and neither sequence nor structural information is available, precluding verification of the claims.

Numerous "natural" antibodies have been described as proteolytic, spanning a wide range of apparent k_{cat} values (a non-exhaustive list is provided in Table R1). However, these studies generally lack detailed biochemical characterization, convincing structural data, or even sequence determination. For the few that have been studied in greater detail, significant questions remain. For example, in the crystal structure of one putative proteolytic antibody (Ramsland 2006, Figure R1b), an arginine is proposed to replace a histidine in a classic Ser-His-Asp triad (Figure R1a). Given its pKa of ~13, an arginine is almost always protonated under physiological condition and thus unlikely to abstract a proton from a serine. In the other reported structure (Uda 2025, Figure R1c), a classic Ser-His-Asp triad is proposed. However, the distance between the His NE2 atom and the Ser OG atom is 12.66 Å, much too long for productive hydrogen bonding much less proton abstraction. These data are therefore not sufficient to exclude the possibility that the

observed activity originates from a contaminating native protease in the preparations.

Hydrolysis of an unactivated amide bond is the most energetically demanding reaction catalyzed by a computationally designed enzyme to date. For example, this reaction is five orders of magnitude more difficult than the recently published cleavage of activated coumarin esters by computationally designed esterases (Kim 2025), yet the rate accelerations achieved by our metalloproteases, produced directly by design, are several orders of magnitude higher. Moreover, in contrast to the proteolytic antibodies, the detailed mechanistic analyses presented here firmly link the observed catalytic activities to the designed proteins themselves.

[Note: the reviewers reference kinetic parameters from Urosov 2023; however, the cited work concerns catalytic antibodies with peroxidase, not protease, activity—an entirely different reaction type. Was a different reference intended?]

Given the problems observed in the past in computational enzyme design where crystal structures did not align with the designed models, the manuscript would benefit substantially from structural validation. Experimental determination of the protease structure, demonstrating proper zinc coordination and active-site geometry, would provide critical support for the claimed design accuracy. Reliance solely on AF3-predicted models is insufficient for validating such mechanistic details, especially when it was used as a filter for design selection.

We agree with the reviewers on the importance of structural validation of the computational designs. Since the original submission, we obtained seven crystal structures of the designed zinc proteases, and have added the structural data to the manuscript. These include three structures presented in main figures (Zn5-Zn(II), ZnO7 E32Q-Zn(II) in complex with substrate, and apo TDP3) and four structures in extended figures (apo Zn5, Zn5 E75Q-Zn(II), TDP3 E45Q in complex with substrate, and Zn48 E75Q-Zn(II) in complex with substrate). Six of the seven structures show an all-residue C α RMSD of < 1 Å relative to their design models, underscoring the accuracy of the design and, in combination with the catalytic-residue knockout experiments, validation of the proposed mechanism. In the structures of the Zn5 and ZnO7 complexes, the zinc cofactor is coordinated exactly as designed, in agreement with the zinc binding affinity experiments.

The recent papers on serine hydrolases, metallohydrolases and the companion on cysteine proteases are conceptually similar. The main distinction in this submission is the two-sided generative design of enzyme and substrate, and the focus on the energetically more challenging protease reaction (though the latter point is shared with the cysteine protease companion). By approaching the problem as a two-sided

design challenge, the authors make the problem easier than a realistic scenario where one is targeting a natural protein. Despite this, the authors argue that this study establishes the generality of de novo peptidase designs, suggesting a path towards therapeutic use. Given the artificial nature of two-sided design, the very low catalytic rates and the lack of demonstrations on selectivity with respect to the human proteome, such statements are premature.

As the reviewers note, simultaneously designing the sequences of the substrates and the proteases simplifies the problem, as more sampling freedom in sequence space can potentially enable more accurate positioning of the substrate with respect to the active site. In the original manuscript, activity towards a specific peptide sequence in human TDP-43 was achieved by redesigning an enzyme that had been obtained through two-sided design. To address the reviewers' concern, we have added new experiments in which we designed a new, even more efficient de novo metalloprotease from scratch that selectively cleaves the same peptide sequence in TDP-43. We also provide demonstrations of substrate selectivity, and show cleavage of the full-length TDP-43 protein in vitro.

Furthermore, the current submission and the recent serine hydrolase paper raise a similar set of concerns: in both, while K_M values are favorable, the turnover numbers are very low by comparison to natural enzymes that carry out similar reactions.

While the turnover numbers are low, amide bond hydrolysis is a very challenging reaction. Moreover, as noted above, the steady-state parameters of some natural metalloproteases fall within a similar range as our best catalysts.

Furthermore, experimental structures are either lacking (in the current submission) or do not provide clear-cut validation of design accuracy within the active site. These problems are reminiscent of those seen with catalytic antibodies, where efficiencies were driven largely by binding rather than by turnover numbers. One of the conclusions stemming from the catalytic antibody studies was that the active sites were not precisely organized for catalysis. Given that the reactivities seen in the current papers are, in fact, below those of comparable catalytic antibodies, a detailed structural and mechanistic verification of design accuracy is crucial to support the claims of the paper on the efficacy of the design strategy.

The major problem with catalytic antibodies elicited to transition state analogs was the relative simplicity of their active sites. The most active catalysts possessed at most one or two functional groups in their binding pocket, in contrast to natural enzymes like metalloproteases and cysteine proteases, which have complex arrays of functional groups, so it is unsurprising that their activities lagged far behind those

of their natural counterparts. As shown in this and the companion paper on cysteine proteases, deep learning now allows us to recapitulate these complex active geometries, successfully placing four or five residues in catalytically productive poses around a substrate. As a consequence, the one-shot catalytic efficiencies that we achieve (10^7 - 10^8 -fold over background) are orders of magnitude higher than those of typical catalytic antibodies. In addition, as described in the revision, we have succeeded in improving our best variant by an additional 30-fold through a round of medium-throughput mutagenesis of residues within 4 Å of the catalytic site, achieving a 10^{10} -fold rate enhancement over background. These results demonstrate the potential for optimization of these designed proteases, which despite much effort, proved extremely challenging for catalytic antibodies due to production challenges and intolerance to mutation. Together with the additional structural and mechanistic studies we have provided, we believe that the revised manuscript provides strong support for the efficacy of our design strategy.

Needless to say, catalytic antibodies have largely been abandoned as a path for developing efficient biotechnological or medical tools due to their poor performance.

The reviewers are correct that catalytic antibodies have been largely abandoned as a path for developing biotechnological or medical tools. This can be ascribed to three major problems not shared by the designed proteases presented in our manuscript: 1) Transition state analogs are imperfect mimics of true transition states; 2) Generating antibodies containing sophisticated arrays of functional groups is extremely unlikely by immunization; and 3) Optimization of the catalytic performance first generation catalytic antibodies by rational design or directed evolution proved extremely difficult. Moreover, as mentioned above, for most of the autoantibodies with putative proteolytic activities, it's difficult to rule out the presence of contaminating natural proteases.

Our results show that machine learning provides a powerful alternative to the immune system for generating tailor-made catalysts with complex active sites embedded in robust, optimizable scaffolds, offering a more reliable route for developing efficient biotechnological and medical tools.

Minor comments

Line 60: esters are also resonance-stabilized.

Esters are indeed resonance-stabilized, but to a lesser extent than amides. Because nitrogen is less electronegative than oxygen, the nitrogen lone pair in amides delocalizes more effectively into the carbonyl, giving the C-N bond partial double-bond character and resulting in greater resonance stabilization. We have modified the original sentence to clarify this.

Line 60: The statement “Diffusive denoising methods such as RoseTTAFold Diffusion (RFdiffusion) have proven powerful in generating a wide range of new functional proteins” should be referenced.

We added the references per the reviewers’ request.

Line 95: The authors state “Starting from a choice of the length of the designed enzyme and the substrate...” — please indicate the range of lengths tested for both enzyme and substrate.

We added the range of lengths tested for both enzyme and substrate as requested.

Line 98: The sentence “Many of the RFD2-MI trajectories produced scaffolds with all the desired structural features” is vague. Please clarify what specific structural features were considered “desired” and describe what occurred in the unsuccessful trajectories.

The features considered “desired” in the backbones are described in the Supplementary Methods section 1.2 section. We have added a reference to this section in the main text.

Lines 104–106: The description – “Designs that AF3 predicted with high confidence... were fed back to RFD2-MI for two more rounds of finer sampling...” — is ambiguous. From the manuscript, it appears that this stage involves sequence design only rather than backbone redesign. Please clarify which parameters are being refined and whether any backbone changes are introduced during these additional iterations.

We apologize for the ambiguity and have clarified backbone-level versus sequence-level refinement in the main text. The overall resampling workflow is shown in Extended Data Figure 1, and the detailed methods for backbone-level diversification and refinement are described in Supplementary Methods section 1.5. Briefly, we used Partial Diffusion, a method to partially noise and de-noise a structure, diversifying the original design around a general fold (Vazquez Torres, 2024). The difference between the Partial Diffusion output and the input backbone can be controlled by an input parameter called Partial Diffusion temperature, where higher temperatures result in more dramatic differentiation from the original backbone. The exact parameters used and the filtering thresholds are described in

Supplementary Methods 1.4, 1.5 and 1.8, and summarized at the high level in Extended Data Figure 1.

Lines 146–147: The reported overall success rate of 36.3% reflects only the cis-cleavage mode, in which the substrate is covalently tethered to the protease. As this setup does not rigorously test substrate recognition, the actual catalytic success that should be reported in the abstract section is the one under trans conditions.

The reviewer is correct that the success rate refers to the cis-cleavage assay. Since rescreening each design in the trans mode would have required the re-synthesis of all DNA sequences, entailing substantial additional costs, we only screened 22 hits from the cis assay, spanning a range of cleavage levels, in the trans assay (see the section entitled “Experimental validation of two-sided designed proteases”). The selected designs and their performance in both the cis and trans assays are shown in Supplementary Table 2. The cis and trans cleavage fractions at the same time point (1 h at 37 C) are well-correlated, as shown in Fig. 2h, indicating that substrate recognition is not a key challenge for our approach. Therefore, we believe reporting the overall success rate in the cis-screen is appropriate.

Line 207: Targeting arbitrary peptides wasn’t shown. The peptides were designed.

We have added a new design, TDPn3, which was generated from scratch to target the same TDP-43 sequence(ALQSSWGMMG) that was targeted in the redesign of ZnO36. The results have been incorporated in the “Design of proteases cleaving human TDP-43 ” section of the main text, and the data are shown in Figure 4.

Section “Reprogramming to cleave human TDP-43”: It would be helpful to explain the rationale for reusing an existing scaffold (ZnO36) rather than designing a new protease de novo for this target. Also, please provide kinetic parameters of the best designs from this group.

We initially chose to reuse an existing scaffold to assess whether its substrate specificity could be reprogrammed to recognize an arbitrary peptide sequence in a disease relevant protein. Prompted by the reviewers’ query, we also explored the alternative strategy of designing a new protease de novo for the same target. The design and kinetic characterization of TDP-n3, the most effective TDP-43 protease, are now described in the main text and in Figure 4e.

Please describe the differences in terms of the structure and number of mutations among the designs.

The TM-score between any pair of active designs shown in Fig.3 is less than 0.23 (calculated using <https://aideepmed.com/TM-score/>, where a TM-score > 0.5 suggests fold-level similarity). We have clarified this in the figure caption. We have also added the number of mutations in the designs sharing the same backbone. For example, sequences of ZnO7 and ZnO25 (Fig.3d) which originate from the same backbone, differ by 39/170 amino acids.

For all figures: please indicate clearly that the structures shown are models.

We have indicated clearly in figures or figure captions where the structures shown are models.

We clarify, again, that we do not argue that the authors should present a practically useful enzyme, but they should not trivialize the gaps towards such enzymes. Instead, they should clarify that the gaps are significant, characterize the underlying causes, and perhaps improve the performance of the designs.

Given the above, the main scientific question that arises is why activity levels are so low despite structural accuracy (in some cases)? What is missing? This question is neither posed nor addressed in the papers, though the points we and other reviewers raised make it extremely pertinent.

We thank Reviewers 1 and 2 for clarifying their major concerns. In response, we have added a new section in the main text detailing how we addressed the observed activity differences between our designs and the best natural metalloproteases. Our analysis indicates that despite overall structural accuracy, suboptimal preorganization of the catalytic residues largely limits catalytic efficiency. We further show that combining four individually beneficial mutations partially alleviated these limitations, resulting in a substantial improvement in the activity of our best design. The reviewers' questions have greatly strengthened the clarity and impact of the revised manuscript.

Referee #3 (Remarks to the Author)

This manuscript describes de novo designed metalloproteases: metalloenzymes containing a zinc(II) active site, capable of hydrolyzing peptide bonds. The metalloproteases, as well as their peptide substrates, were computationally

designed in a two-sided process and a selection of the designs was experimentally validated. The predicted structures of top designs proved to be accurate and had unprecedented peptide folds. Moreover, they showed impressive activity, both in cis and in trans reactions, of up to 10^8 fold rate acceleration compared to the uncatalyzed reaction and the k_{cat} approaches that of some Zn-dependent proteases. It was shown that in all cases indeed the peptide was cleaved at the designed site. As a consequence of the two-sided design process, most enzymes showed limited cross-reactivity, i.e. they were selective for their designed substrate peptide sequence, albeit with notable exceptions of some very substrate promiscuous designs. Finally, one of the successful designs, ZnO36, was redesigned to target a biologically relevant substrate: the TAR DNA-binding protein 43 (TDP-43), protein which aggregation and fibrillization are linked to neurodegenerative processes. Encouraging activity was found that raises the prospect of biological applications, albeit that for this purpose the substrate specificity needs to be further improved.

We appreciate the reviewer's support. Regarding the final point, we have included in the revised manuscript a newly generated TDP-43 protease, designed from scratch, which shows enhanced activity and substrate specificity toward the target TDP-43 peptide compared to the redesigned ZnO36 protease.

This work builds on two prior works by the same group(s). Reference 2 (listed as preprint, but published in the meantime: Nature (2025). <https://doi.org/10.1038/s41586-025-09746-w>) in the manuscript describes the de novo design of a zinc esterase: a similar type of metalloenzyme that hydrolyzed activated esters. A similar type of reaction, but on much more reactive and, hence, easier substrates. These zinc esterases were designed using RFdiffusion2 (RFD2). The other preprint describes an adaptation of RF Diffusion 2, called RFdiffusion2 Molecular Interfaces (RFD2 MI), which was used in the current work to design the Zn protease.

The key innovations of this work, compared to these reported works are:
- This work achieves the successful design of active proteases. Peptide bonds are notoriously unreactive and hence present a much bigger challenge for enzyme design than, for example, esters (reported before). Moreover, designer proteases could have important applications, for example in biomedicine and bioremediation. This is suggested by the application to the hydrolysis of RDP-43. Therefore, this work represents a milestone in enzyme design.

- A water molecule, which is needed as the second substrate for the proteolysis, was included in the design. This significantly increased the frequency of successful, i.e. active, designs. The success rate of ~87% active designs, compared to ~15% without the water included, is impressive. It also shows, as the authors note in the

manuscript, that inclusion of key molecules for the reaction in the design process can greatly increase design success.

- A two-sided design strategy was used to design both the metalloprotease and its peptide substrate. This means that the substrate sequence was unique for each design. A result of this was that many (most) designs displayed a significant substrate selectivity (peptide sequence).

The work presented is of high quality. Data is generally presented well, with replicates and error margins (one exception, see below). The conclusions are supported by the data. The manuscript is well-written: it was a pleasure to read.

We thank the reviewer for the positive comments.

General comments:

1. The computational design techniques used, RFD2-MI, as well as the design of a zinc hydrolase were established by the same group in recent work. So, the main novelty here is the application towards the creation of metalloproteases and achieving peptide bond hydrolysis.

We agree with the reviewer on this comment.

2. This is maybe considered semantics, but I am not sure if the claim of “de novo design”, i.e. design from first principles, of metalloproteases is warranted here. The active site structure, i.e. the ligation of the catalytic metal, as well as the positioning of the oxy anion stabilizing residues was not designed, but taken from an existing metalloproteases, aminopeptidase N and astacin, and precisely reproduced in the designed proteins. So, one could argue that while the protein environment was designed de novo, the part of the structure that makes it an enzyme, the catalytic site, was not and, thus, that this work represents a (very impressive) redesign of aminopeptidase N and/or astacin. I think calling it a “designer enzyme” or “designer metalloprotease” is probably better justified than calling it a “de novo designed enzyme/metalloprotease”.

The definition of *de novo* protein design can vary across publications and contexts (Huang 2016, Korendovych 2020, Kortemme 2024). However, a consistent message across these definitions is that *de novo* designed proteins have amino acid sequences that are not derived from or homologous to naturally occurring proteins, distinguishing them from traditional protein engineering approaches such as directed evolution or rational redesign of existing scaffolds.

In our work, while the catalytic motif geometry was informed by known metalloprotease active sites, the overall protein sequence and structural scaffold were designed independently and do not share sequence homology with natural metalloproteases (Extended Data Table 2). The catalytic residues were incorporated into a newly designed structural context rather than by modifying an existing natural protein scaffold (Extended Data Fig. 6). This approach is consistent with widely accepted definitions of *de novo* protein design, which emphasize the creation of novel protein sequences and folds rather than strict adherence to “first-principles” design of every atomic feature.

Furthermore, although earlier definitions of *de novo* design (Huang 2016) sometimes include “physical principles”, mainly because alternative methods were lacking, recent advances in computational protein design—including deep learning-based structure prediction and generative methods—have broadened the operational meaning of this term. For example, *de novo* binders can be readily generated from deep learning based pipelines without the input of physical principles.

For these reasons, we believe that the term “*de novo* designed metalloprotease” accurately reflects the nature of this work and is consistent with current conventions in the field. While terms such as “designer enzyme” are also valid descriptively, they do not provide greater clarity or align more closely with established terminology. Therefore, we have retained the original wording in the manuscript.

3. The design was modeled in three states: apo, substrate bound and transition state. This kind of multistate design is clearly important to achieve proficient enzymes. However, only one transition state (TS) was modeled, whereas there are more along the reaction coordinate. The Baker lab has shown in recent work (Science 2025, <https://doi.org/10.1126/science.adu2454>) that including all TS's along the reaction coordinate in the design improves the quality of the outcome. The authors should comment on why this approach was not used here; it might have delivered enzymes that are even closer to the catalytic activity observed for natural metalloproteases.

The mechanism of metalloprotease hydrolysis is different from that of serine or cysteine hydrolases, which involves acylation and de-acylation steps because the nucleophile is an amino acid in the enzyme and needs to be regenerated after acylation. For metallohydrolases, where a water molecule directly performs the nucleophilic attack, the tetrahedral intermediate formed after the nucleophilic attack is the only intermediate in the current-established mechanism that involves bond formation or breaking between heavy atoms (Fig. 1b). Subsequent intermediate steps proposed in the literature, including hydrogen bond rearrangement and proton shuttling (Pelmenschikov 2002, Vasilevskaya 2015, Varghese 2023, Chen 2021)

don't involve bond formation or breaking between heavy atoms and are therefore beyond the capability of AF3. PLACER, which was used in Lauko et al, Science, 2025 as the primary tool to assess rotamer ensemble geometries across different states in catalytic side chains, was not trained on ion coordination, and was therefore not suitable for the design of metalloproteases.

Other comments

4. While the manuscript in general is very well-written and clear, the section explaining the design choices could be improved a bit. A bit more explanation on why choosing the Zn site from one enzyme (aminopeptidase N) and the substrate binding site of another (astacin) would help the reader understand the thought process better.

In response to the reviewer's request, we have added a section "Choice of the theozyme" in Supplementary Discussions to explain our rationale for theozyme generation.

5. To probe the necessity and contribution of the active site residues for both activity and zinc binding, they were sequentially mutated. In almost all cases this resulted in a significant drop of activity, thus confirming the essential role of this particular residue (Fig 3.). An exception is the R79A mutation in ZnO36: this seems to give rise to a significantly increased activity (see bar graph in 3rd row of Fig 3). This surprising result is completely ignored in the manuscript. Here clearly the design was not optimal, which should be commented on.

As now explained in the Fig. 3 caption, we speculated that R79, which was introduced by ligandMPNN during sequence optimization, might provide beneficial interactions with the oxyanionic intermediate and flanking transition states. However, the increase in activity observed when this residue was mutated to alanine suggests that such an interaction may instead disrupt productive oxyanion stabilization by the catalytic tyrosine. We observed a similar result in our mutagenesis experiments on Zn45, where activity increased when Y152 was mutated to isoleucine. Both of these results as well as our interpretation are described in the main text and in the Supplementary Discussion 5 of the revised manuscript.

6. While it is nice to see there is limited cross-reactivity of the enzymes towards the peptides for the other selected design, I am hesitant to call this "considerable" specificity. The design sequences tested are very dissimilar. See below for the sequences, which I had to retrieve from the SI; it would have been better to have

these available in the manuscript. To assess specificity, seeing how selective the designed protease is when mutating single amino acids in the substrate peptide

Sequences substrates:

SZn5: VIRLRMRR

SZnO7: ALTLTFRLLR

SZnO25: SLTLTFRLLR

SZnO36: ARILRVNFKI

SZnO39: SRVIEVKYLK

SZn45: VLEFTGRR

We agree and have removed the adjective “considerable” from the discussion of specificity. As requested, we have also added the substrate sequences to Fig. 3d in the main text.

7. Kinetics: a comparison of the kinetics is made to matrix metalloproteinases and TEV protease. The latter is a cysteine protease, not a Zn protease. How do the kinetics compare to the Zn proteases that the design was based on, i.e. aminopeptidase A and astacin? I feel that this comparison would be much more valuable.

The comparison with TEV was intended to show that the designed zinc protease approaches the kinetics requirements necessary for practical applications. We now explicitly note in the main text that TEV operates via a different catalytic mechanism. Moreover, we have added Extended Data Table 3 summarizing the kinetic parameters of representative metalloproteases, included a new section in the main text analyzing the activity gap between our designs and the best natural metalloproteases, and discussed possible avenues for future improvement.

The highest reported rate acceleration was achieved for an enzyme (Zn45) on a substrate for which it was not designed (SZnO36). What are the kinetic parameters for its designed substrate (SZn45)? Figure 4a suggests it is significantly less active on its designed substrate: what does this mean for the design process?

The reviewer is correct that Zn45 is significantly less active on its designed substrate. In fact, the kinetics parameters of Zn45 for its designed substrate were too low to measure accurately. Unfortunately, the corresponding sfGFP-mScarlet construct gives a FRET signal with a signal-to-noise ratio less than two upon complete cleavage and exhibits a strong inner filter effect (Supplementary Method 3.9), further reducing the signal. As a consequence, we were unable to determine accurate kinetics. Interestingly, though, the lower activity of Zn45 on its cognate substrate compared to S_ZnO36 is reflected in the AF3 metrics. We have added a

sentence in the main manuscript discussing this point and clarified that implementing negative selection (filtering against off-targets) could further enhance specificity.

8. In the discussion section, the authors state: “there is still room for improvement—turnover straight out of the computer is still $\sim 10^3$ – 10^4 -fold below those of the most efficient natural proteases under similar conditions”. While the rate enhancements achieved straight of the computer are absolutely impressive, it is indeed still a significant gap. Where does the current approach still fall short? Can the authors speculate on this?

We have added a substantial section in the main text describing the experiments performed to address this issue and outlining potential directions for further improvement. We are grateful to all reviewers for raising this point, as addressing it led to the identification of an even more active Zn45 variant and has significantly strengthened the study.

9. Figure S26: information of number of replicates and error margins for TDP cutting experiments is missing.

Thank you for catching this. We have now repeated this experiment using a FRET-based assay (instead of the original gel assay) to enable a more direct comparison with the newly generated de novo TDP-43 protease. The updated data are presented in Fig. 4, and the number of replicates and associated error margins are reported in the figure caption.

Minor comments

Typo (first paragraph of computational design section): “The fourth ligand of Zn(II), a water molecule, is activated by a glutamate (E1)” - should be “E2”.

We have removed the subscripted labels used to define the catalytic residues throughout the manuscript, as we believe they do not aid clarity. Instead, we now refer to these residues by their catalytic roles, such as “the general base glutamate” where appropriate.

Figure 4a: in the matrix are listed the relative reactivities of the enzymes for the various substrates. From the caption it is unclear what is compared here: catalytic efficiencies? K_{cat} ? Yields?

The fractions of the substrates digested, normalized to each enzyme's cognate substrate, are compared in the matrix. A sentence has been added to the figure caption to clarify the measurements.

Reviewer 4:

In this study, Chen and coworkers report the computational design of zinc-dependent proteases capable of breaking the amide bonds of some designed peptide sequences. The authors used an enhanced version of RoseTTAFold Diffusion 2 for Molecular Interfaces (RFD2-MI) tailored for enzyme and protein–protein interface design to generate multiple protease-peptide pairs. The best design accelerated peptide bond hydrolysis by more than 10^8 -fold compared to the uncatalyzed reaction, however, it should be highlighted that the kinetic characterization showed a poor k_{cat} of ca. 0.0025 s^{-1} , and a good K_M of ca. $26\text{ }\mu\text{M}$. These values clearly indicate that there is much room for improvement, as the turnover is very low if compared with those of natural proteases. While we agree with the authors that this study shows that de novo enzyme design can now tackle more difficult, chemically demanding reactions (as shown also in their previous work on serine hydrolases, DOI:10.1126/science.adu2454), the catalytic activities of the obtained designs are still far below those of natural enzymes.

Although the turnover numbers achieved by the current designs are below those of the best native proteases acting on their most preferred substrate, the activity of native proteases spans a wide range, exhibiting k_{cat} values on the order of $0.01\text{--}100\text{ s}^{-1}$ (Pahwa 2019, Brownell 1994, Wang 1999, Lauer-Fields 2007, Kunugi 1982, Henkel 2009, Tokioka-Terao 1984). For comparison, our best catalyst straight-out-of-computer has a k_{cat} of 0.025 s^{-1} (not 0.0025 s^{-1} as stated by the reviewer), comparable to the k_{cat} values reported for native proteases at the lower end of this scale, such as MT1 MMP (Pahwa 2019), MT2 MMP (Pahwa 2019), MT4-MMPCD (Wang 1999), ADAMTS-4 (Lauer-Fields 2007) when evaluated on substrates reported in the corresponding reference. Moreover, we have shown in the revised manuscript that we could improve the k_{cat} value of Zn45 to $0.75 \pm 0.04\text{ s}^{-1}$ by introducing four point mutations.

The authors also used some of the designed enzymes as template scaffold for optimizing it for cleaving a therapeutically relevant human peptide, but again the activities obtained need further improvement and refinement.

As noted in our responses to the other reviewers, we have included a newly designed de novo protease targeting the same TDP-43 peptide epitope as the previously redesigned protease. This new enzyme exhibits enhanced activity and

improved specificity against all other synthetic substrates tested in the manuscript (new Fig. 4).

Considering that the method applied in the study has already been developed in another work, and also the level of catalytic improvements achieved, we do not believe the results obtained in this study have the novelty and impact required for its publication in Nature journal.

We respectfully disagree with the reviewer's assessment. Prior to our work on metalloproteases and cysteine proteases, the RFD2- MI framework had *never* been applied to enzyme design. Our study therefore demonstrates, for the first time, that this method can generate active and evolvable proteases with measurable catalytic improvement, validated through medium- throughput experimental characterization. Importantly, the scientific contribution of this work extends beyond the computational framework itself: it provides a new, generalizable toolbox of designed proteases that can be readily repurposed for diverse targets. Thus, the novelty of our study lies not only in the methodological extension of RFD2- MI to enzyme catalysis but also in the resulting catalytic scaffolds, which have broad practical and conceptual impact for the enzyme design community.

1. The authors state that: "We modeled each resulting design in three states: 1) the apo protease state, to confirm correct folding; 2) the ES complex state, to assess substrate binding and positioning of the target amide bond; and 3) the transition state to evaluate stabilization." However, a few sentence later they mention that: "For modeling the transition state, we used a phosphoramidate TSA; many crystal structures have been solved for similar TSA complexes, so they are better modeled by ML software trained on the PDB than the actual transition state". We agree with the authors that the ML software trained on X-ray structures will provide better results with TSA than the actual TS, however, we disagree with the authors when they state that the TS is used to evaluate stabilization. Although the charge distribution of TSA might resemble that of TS, TSA is not the TS, so it should not be stated that the TS is used in the modeling to evaluate stabilization. This is not accurate and should be rephrased.

The reviewer is correct; our statement that the TS, rather than a TSA, was used to evaluate transition state stabilization was an oversight on our part. We have corrected and updated the main text accordingly.

2. The authors used two different strategies for modeling the zinc and coordination sphere, which by the way, it is directly taken from a natural protease scaffold and not generated de novo via theozyme calculations. In one of the approaches a water

molecule is added close to the zinc metal ion. As this is constrained during the design process, this does not guarantee that the water will be in reality there and neither that the pocket environment would favor the proper positioning of the water molecule.

We agree with the reviewer that computation modeling of a coordinated water molecule may not fully capture the properties of the pocket environment and does not guarantee accurate positioning of a water as a Zn(II) ligand. Nonetheless, we hypothesized that adding water in the complex prediction would better reproduce the catalytic site geometry during catalysis, given water's established mechanistic role. To evaluate this assumption, we conducted design campaigns using both the "zinc-only" and "zinc-water" strategies in parallel. The experimental hit rates were 14.7% for the zinc-only strategy and 87.5% for the zinc-water approach, consistent with the proposed benefit of expliciting modeling water.

To address the reviewer's concern that the water molecule was "constrained" during design, we performed AF3 predictions on a set of designs where the Zn(II) and water were modeled as separate entities, effectively unconstraining the water position. Although we initially avoided this setup due to concerns that additional ligands might reduce AF3's accuracy, the predictions obtained with constrained and unconstrained water were remarkably similar across both active and inactive designs (Supplementary Table 5). Because only ordered designs were carried forward, our dataset does not include poorly scoring variants that might demonstrate failure cases. Nevertheless, our results suggest that the unconstrained approach could better exploit AF3's ability to model Zn(II)-water coordination and may represent a useful refinement for future design efforts.

We have added a Supplementary Discussion section elaborating on this point and thank the reviewer identifying this potential direction for improvement.

3. The authors used two different strategies for screening: the cis screen in which the peptide and protein are fused together, and the trans screen in which a (GS)15 linker is added to the N-terminus and an mScarlet tag at the C-terminus of the substrate peptide. In both cases, additional protein-protein interactions imposed by either the fusion or especially the addition of a rather big protein at the terminal regions of the peptide might substantially affect the binding affinity of the peptide, and thus dramatically affect the obtained results.

We agree with the reviewer on the potential effects of fusing the protein to a short peptide substrate. However, as our end goal for this technology is to develop designer proteases to cleave human proteins of interest, it is important to validate our designs as "proteases" instead of "peptidases" for feasibility. We only designed the interactions between the proteases and the peptide substrate (i.e. the linker was not considered during design). Therefore, the fact that the change in constructs did

not qualitatively alter cleavage suggests that the protein-protein interaction is dominated by the designed interface. This is further supported by the specificity matrix in Fig. 4a where cleavage preference is entirely grouped by the short substrate preferences.

4. In their assessment of active and inactive designs (shown in Figure 2d, 2e, 3a, S10 and S16), there is a lack of geometrical features, which makes the quantification of the preorganization of the binding pose and so the evaluation of the catalytic potential of the designed variant hard (we would say impossible) to assess. In Figure S10 only the distance between R79 and carbonyl group at the ES and TSA complex is shown. This is again completely insufficient.

The active site geometry of the designs were quantified and filtered using catalytic residue RMSD and other geometric constraints, as discussed in Supplementary Method section 1.8 and in the new section “Origin of activity discrepancies between the designs and native metalloproteases” in the main text.

The R79 residue is not part of the original catalytic motif, but was instead introduced by MPNN during sequence design; therefore the purpose of Figure S10 was to establish the motivation for analyzing the R79A single amino acid substitution, rather than to assess activity. This was included in the Methods section but left out of the main text and the figure caption. We have now clarified this in the figure caption and discussed this mutation in the section “Origin of activity discrepancies between the designs and native metalloproteases” in the main text as well as Supplementary Discussion.

5. In Figure S11, the tyrosine hydrogen bond angles found in X-ray structures are shown. The hydrogen bond distances should also be included and compared.

We agree that the hydrogen bond length is a critical metric to assess this interaction and have added these distances to the Figure (current Fig.S9)

6. It is also mentioned: “In the prediction of Zn45 with the Zn45 substrate, the angle between the attacking water oxygen, the scissile carbonyl carbon, and the carbonyl oxygen is 51.8°, smaller than the ideal 90° geometry, potentially interfering with the nucleophilic attack.” While the angle is important for a catalytically competent pose, the distance is essential even if the perfect angle is achieved, the distance has to be short enough for allowing catalysis.

In all the AF3 predictions, the distances between the modeled water molecules and the substrate carbonyl carbon atoms were consistently < 3.5 Å in all our AF3

complex predictions, irrespective of the angle. Specifically, the predicted distance was 2.5 Å for Zn45 with its cognate substrate and 2.4 Å with ZnO36 substrate. Because these distances were similarly short, we did not emphasize this point in the original discussion. Upon further examination, however, we found that in the Zn45-ZnO36 substrate complex, the positioning of the substrate appears suboptimal, an observation independently supported by molecular dynamics simulations and mutagenesis analysis of the Y152 position (revised main text section “Origin of activity discrepancies between the designs and native metalloproteases”). Accordingly, we have removed this premature interpretation from the main text and the corresponding Supplementary Figure.

7. “Using a previously published side-chain pocket scoring metric to assess compatibility between substrate windows and designed active sites by logos, we identified ZnO36 with a targeting window spanning residues 329–340 as a suitable starting scaffold.” More details should be given on how this selection was made. It is also not clear for us why the authors used as starting scaffold one of the previous designs and not a new one based on only optimizing the protease sequence.

The window selection criteria used in our study are essentially identical to the IDR binding window selection criteria described in the cited paper; we have added a brief summary of this in the main text. Our primary objective was to demonstrate that de novo proteases can be structurally robust and designable, thereby establishing them as potential future therapeutic agents. A detailed exploration of designing clinically viable therapeutic proteases, however, lies beyond the scope of the current work. Additionally, the redesigns presented here were generated through partial diffusion rather than sequence optimization alone.

Inspired by the reviewer’s suggestion, we also conducted a de novo design campaign targeting the same TDP-43 sequence. This effort yielded a more specific protease, for which we report kinetic parameters and confirm full-length TDP-43 cleavage. The corresponding data are included in the new Fig.4, where the de novo and redesign approaches are directly compared. We are grateful to the reviewers for this suggestion, which has significantly strengthened the manuscript.

8. The correlation between experimentally measured normalized digested fraction and computational metrics shown in Figure S18 is extremely poor.

The Pearson correlation coefficient quantifies the strength and direction of a linear relationship. However, the purpose of this figure is to demonstrate the ability of each metric to distinguish between active and inactive designs, rather than to evaluate linear correlation. Thus, the use of the Pearson correlation coefficient is

not appropriate in this context. We thank the reviewer for highlighting this point. Despite this issue, several computational metrics—such as complex pLDDT, ipae_min, ipTM, and pae_interface—still show clear predictive power for substrate cross-reactivity, as reflected in the data distributions. To avoid ambiguity, we have removed the Pearson correlation values from the figure.

9. The novelty of the generated folds with respect to natural scaffolds (including PDB or AF databases) is not presented neither investigated.

Structural diversity among enzyme scaffolds is particularly critical in protease design, as the ultimate goal is to accommodate specific target epitopes within appropriately shaped and chemically complementary active site clefts. Accordingly, we have analyzed the structural novelty of our designs relative to natural protease scaffolds and now include a summary at the end of the “Structural validation” section.

10. “While these results are encouraging for programming proteases against therapeutic targets, there is still considerable room for improvement as the designs retained activity on the original substrate (Fig. S24).” We agree that the results are promising, but they should be substantially improved for their publication in Nature.

As described above, we reprogrammed one of the variants for TDP-43 cleavage to show the potential of our design approach. In the revised manuscript, we now include a newly generated de novo TDP-43 protease, created from scratch, compared to the redesign strategy and showed markedly improved and specificity compared to the previous redesign strategy. While further optimization may be required, for example through directed evolution, these results provide a strong proof of principle.

Table R1. Reported proteolytic catalytic antibodies. Catalytic antibodies with reported crystal structures are shaded in blue. In both reported structures, catalytically-competent active sites are lacking.

Important First and Last Authors	Year	k_{cat}	Title	Sequence published?
Iverson, Lerner	1989	$6 \times 10^{-4} \text{ s}^{-1}$	Sequence-Specific Peptide Cleavage Catalyzed by an Antibody	No
Paul	1989	0.26 s^{-1}	Catalytic hydrolysis of vasoactive intestinal peptide by human autoantibody	
Nevinsky	2005	$1.0 \times 10^{-2} \text{ s}^{-1}$	Casein-hydrolyzing activity of sIgA antibodies from human milk	
Gabibov	2006	$6 \times 10^{-3} \text{ s}^{-1}$	Autoantibodies to myelin basic protein catalyze site-specific degradation of their antigen	
Nevinsky	2010	up to 2.6 s^{-1}	Affinity and catalytic heterogeneity of polyclonal myelin basic protein-hydrolyzing IgGs from sera of patients with multiple sclerosis	
Paul	1994	$1.8 \times 10^{-4} \text{ s}^{-1}$	Molecular cloning of a proteolytic antibody light chain	Yes
Hifumi, Uda	2003	$1.0 \times 10^{-2} \text{ s}^{-1}$	Catalytic features of monoclonal antibody i41SL1-2 subunits	
Paul	2008	$2.9 \times 10^{-4} \text{ s}^{-1}$	Autoantibody-catalyzed hydrolysis of Amyloid β peptide	
Gabibov	2008	$2.6 \times 10^{-5} \text{ s}^{-1}$	Strategies for induction of catalytic antibodies toward HIV-1 glycoprotein gp120 in autoimmune prone mice	
Hifumi, Uda	2018	$3 \times 10^{-4} \text{ s}^{-1}$	New technologies to introduce a catalytic function into antibodies: a unique human catalytic antibody light chain showing degradation of β -amyloid molecule along with peptidase activity.	
Hifumi, Uda	2020	$6 \times 10^{-5} \text{ s}^{-1}$	A new algorithm to convert a normal antibody into the corresponding catalytic antibody	
Hifumi, Uda	2022	$4.5 \times 10^{-5} \text{ s}^{-1}$	A new catalytic site functioning in antigen cleavage by H34 catalytic antibody light chain	

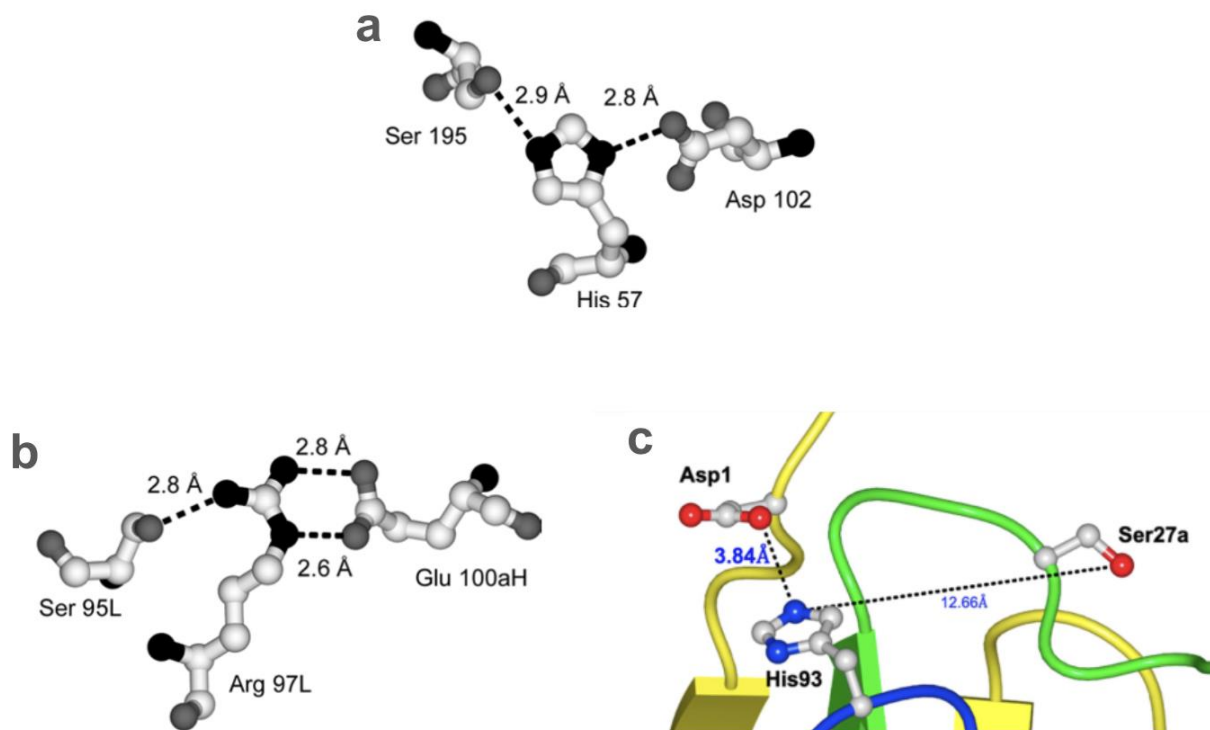


Figure R1. Crystal structures of previously reported proteolytic catalytic antibodies. a, the classical Ser-His-Asp triad in bovine trypsin (PDB 1BJU). b, the proposed putative active site of the catalytic antibody reported in Ramsland 2006. c, the proposed putative active site of the catalytic antibody reported in Uda 2025. Panels a, b adapted from Ramsland 2006; panel c adapted from Uda 2025

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Responses to Reviewers' Comments on the Metalloprotease Manuscript

Below we provide a point-by-point response to the reviewers. The reviewers' comments are shown in blue italics, and our responses are shown in regular black font.

Reviewer #1

Given the similarities between the papers, below we assess both together.

The authors have considerably revised the manuscripts, adding data on structures that exhibit high accuracy, mutations that improve catalytic rates, and molecular dynamics simulations that help explain the partial improvement in catalytic rates in mutants based on active-site preorganization. These are important additions.

We thank Reviewer #1 for suggesting many of these additions which have helped improve our manuscript significantly.

Yet, even after optimization by systematic mutagenesis, the designed enzymes remain 2-3 orders of magnitude less active than the natural counterpart from which the active site was derived. This is the most relevant comparison, particularly because several recent studies have already demonstrated that RFDiffusion can produce de novo enzymes with low k_{cat} .

We derived a minimal catalytic motif of five residues from a 967-amino acid protein and embedded it into entirely de novo scaffolds and sequences, without incorporating the broader, highly evolved active site environment. While similar k_{cat} values have been reported in previous enzyme design studies, the reaction we present here is orders of magnitude more challenging, as shown in Table R1.

For context, even native enzymes that share highly similar active site architectures with Aminopeptidase N exhibit considerable variation in catalytic rates. For example, angiotensin converting enzyme ($k_{cat} \sim 7 \text{ s}^{-1}$, Bünning 1986; active site RMSD = 0.95 Å, PDB 1O8A) and botulinum neurotoxin ($k_{cat} \sim 2.3 \text{ s}^{-1}$, Li 2000, active site RMSD = 1.53 Å, PDB 3BTA) both retain closely related active site geometries yet differ substantially in turnover from Aminopeptidase N.

Thus, preservation of catalytic residues alone does not ensure comparable k_{cat} values, even among native metalloproteases.

More broadly, even for native protease redesign problems, where both the scaffold and active site are held fixed and only sequence is varied, activity is often dramatically impaired upon sequence redesign. Maintaining activity can require greater than 70% sequence identity to the wild type. In addition, evolutionary information is needed to identify residues that must be preserved (Sumida, 2024). Against this backdrop, the activities achieved in our study do not indicate inherent limitations of de novo scaffolds.

We agree that the catalytic parameters of natural enzymes vary, but it is important to remember that much of this variation is due to trade-offs: TEV trades off rate for very high specificity, and the specificity tests carried out by the authors are far from sufficient to suggest they have achieved similar levels. We do not doubt that hydrolysing amides presents a greater challenge in terms of activation barriers than previous studies on hydrolases. But the methods are very similar to those of the previous studies and exactly the same in the two companion papers, limiting novelty, and questioning why the authors did not integrate them into one paper.

The computational methods used in this work differ substantially from those employed in previous enzyme design studies. For example, RFD1 does not support explicit ligand modeling, whereas RFD2 performs suboptimally for protein–protein interaction problems. In fact, our own attempts to design proteases using RFD1 and RFD2 were never successful. Although RFD2-MI is a general-purpose model trained on motif scaffolding as well as protein-protein interaction tasks, it had never been applied to enzyme design prior to this work. Its success in generating the first de novo proteases does not diminish the difficulty of the problem or the relevance of the approach. Rather, it highlights that protease design can be addressed through fundamental principles of protein design: namely, accurate positioning of catalytic motifs and the formation of high-quality binding interfaces.

An important advantage of using general-purpose models is that performance on specific tasks improves as the underlying models advance. In contrast, highly specialized tools often see more limited iterative development. Consistent with this, we showed in our previous revision that RFD3 generates proteases with higher activity and specificity than those generated by RFD2-MI. This progression highlights the rapid pace of computational protein design and its growing

capacity to tackle increasingly demanding and practically relevant challenges, such as the de novo cleavage of unactivated amide bonds. Therefore, we propose that success in addressing chemically challenging problems depends not only on a specific protein design method used, but also on decomposing the challenge into tractable design components and applying the most appropriate computational tools, as we described in the Introduction of the main text.

Although the scaffold generation model used in the manuscript had been reported previously, the computational design protocol and filtering strategies were established uniquely for the metalloprotease design problem, as described in the method section. For example, to promote efficient catalysis, we modeled three key states using AlphaFold3: the monomeric protease, the substrate-protease-Zn(II) complex with or without the Zn(II)-bound water, and the transition state analog complex. Incorporating these states contributed substantially to the high design success rate achieved in this study. Notably, prediction models with this level of accuracy and multimodal capability were also not available for earlier enzyme design studies.

Furthermore, the gap from the natural enzymes from which the active site was derived limits confidence in the authors' conclusions that low rates in the final designs are due to low preorganization.

Reviewer 1 previously noted that there is “a well-trodden path to improving the enzymes to reach close to practically useful levels of activity,” and further encouraged us to pursue this direction to better understand the limitations and improvement opportunities for AI-based design methods. In response, we extended our study in the revised manuscript and, through both experimental mutagenesis and computational simulation, achieved activities comparable to those of some native metalloproteases by improving active site and substrate preorganization. We emphasize that we are not proposing suboptimal active site and substrate preorganization as the sole determinant of the performance gap between the designed and the most efficient native proteases; electrostatic effects, conformational dynamics and other factors that are still poorly captured by the design tools presumably also play a role. The main text has been revised accordingly to more clearly reflect this point.

To gain further insight into factors potentially limiting the activities of the designed proteins, we have probed the local chemical environment of the glutamate-Zn(II)-water system by measuring

the pH-dependence of activity of the improved Zn45-v2 variant. The pH dependence of native metalloproteases often exhibits a bell-shaped curve, where the lower limb is often associated with the activation of the glutamate-Zn(II)-water system, with pKa1 values between 5-6 (Johnson 2000, Kunugi 1982). While the pH dependence of Zn45-v2 also exhibits a bell-like shape, the transition point of the lower limb is 6.7, one to two pH units above native metalloproteases, demonstrating that the local chemical environment of the active site may still be different from the native. We have modified the main text and Extended Data Fig.7 to include this information.

We also investigated the contributions of each catalytic residue in Zn45-v2 by introducing catalytic residue inactivating substitutions at all five positions. While knocking out the Zn(II)-chelating residues or the general base abolished detectable activity, mutating the oxyanion stabilizing tyrosine reduced activity to 9% of the wildtype level (revised Extended Data Fig. 7e). In native metalloproteases, mutating the oxyanion stabilizing residues typically results in a >50-fold decrease in k_{cat} (Yiallourous 2000, Beaumont 1995, Phillips 1990), suggesting that the positioning of this tyrosine in Zn45-v2 may be suboptimal. This inference is further supported by our active site mutagenesis studies: at residue 121, which is spatially proximal to the oxyanion-stabilizing tyrosine, a conservative change from valine to isoleucine doubled the activity (Extended Data Fig. 6).

Given that all recent de novo hydrolases have shown low rates, there may be a ceiling to the rates that are feasible using the current generation of de novo fold design methods. Identifying such missing factors may explain what makes natural enzymes so proficient, beyond the accuracy of the active site, and suggest ways to break the rate ceiling.

Understanding why natural enzymes are so highly effective has been a central challenge in enzymology for decades. A quantitative description of how mechanistic features translate into catalytic energetics remains difficult, largely due to the intrinsic complexity of enzymes and the non-additive nature of their energetic contributions (Herschlag 2014). Enzyme efficiency emerges from many individually modest effects that collectively produce large catalytic gains. These factors are strongly interdependent: structure, dynamics, electrostatics, solvation, and chemical reactivity are tightly coupled, so perturbations in one component often propagate through several others.

Although current computational methods cannot yet fully capture this level of coupled complexity, progress in extending enzyme design from energetically simple model reactions to more demanding transformations—such as amide bond cleavage—provides grounds for optimism that remaining gaps will be bridged. In the interim, integrating computational design with directed evolution offers a practical and powerful strategy for generating de novo enzymes with native-like catalytic activities.

In this context, a single round of mutagenesis and screening has already yielded a substantial boost in the catalytic efficiency of Zn45. As our exploration was limited to individual residues in the immediate vicinity of the active site, further gains are likely achievable through more extensive directed evolution. However, we consider this optimization beyond the scope of the present study, which focuses on the computational design of enzymes for an energetically demanding reaction. In the last revision, we presented an upper bound of the activity gap between metalloproteases with de novo folds and the best native metalloproteases from which the active site motifs were derived. This upper bound is approximately 2-3 orders of magnitude, out of the total 12-13 orders of magnitude rate acceleration attained by *the best* native metalloproteases over evolutionary timescales (Radzicka 1996, Beaumont 1995, Ryan 1993). Notably, the V121I mutation, which doubles the activity, as well as beneficial mutations in positions 73 and 75 were not included in the combinatorial library, underscoring that this upper bound likely underestimates the potential of the de novo scaffold.

Regarding generality, except for one designed enzyme, all were generated to target custom-designed substrates (two-sided designs), and the design process relied on the simplifying assumption that the substrate associates with the enzyme through β -sheet interactions.

It is universally observed that protease substrates adopt an extended beta strand conformation in all canonical protease classes (Tyndall 1995). In particular, many metalloproteases engage their substrates via the edge strand of a beta sheet, including astacin (PDB 1qji), matrix metalloproteases (MMPs; MMP1: PDB 3shi, MMP2: PDB 1qib, MMP3: PDB 2jt5, MMP9: PDB 6esm, MMP13: PDB 3tvc), and ADAM family members (ADAM10/17; ADAM10: PDB 6bdz, ADAM17: PDB 8snl). These enzymes fulfill central functional roles in diverse physiological processes in both prokaryotes and eukaryotes. Guided by this widely conserved recognition

mode, we generated proteases capable of cleaving multiple distinct substrate sequences, as reported in this study.

It is not clear from the writing whether the targeted human protein (one-sided design) was chosen because of similarity to some of the previously designed substrates, adding another limitation to generality.

In the original submission, we employed a redesign strategy to target predefined epitopes. Accordingly, the scaffold was selected based on its pocket geometry relative to the TDP-43 epitope. This selection criterion is explicitly stated in the second paragraph of the “Design of proteases cleaving human TDP-43” section. In the revised manuscript, we addressed Reviewer 4’s question (“why the authors used as starting scaffold one of the previous designs and not a new one based on only optimizing the protease sequence”), as well as Reviewer 1’s concern (“By approaching the problem as a two-sided design challenge, the authors make the problem easier than a realistic scenario where one is targeting a natural protein.”). Specifically, we implemented a one-sided design strategy, included a direct comparison of both approaches, and provided point-by-point responses in the rebuttal letter.

Designing proteases for prescribed sequences is a long-standing challenge. Historically, most approaches rely on re-engineering existing native proteases. However, such efforts are typically constrained to relatively minor sequence changes (~<10% of residues), limiting the extent to which substrate specificity can be altered (Gao 2026, Yi 2013), often resulting in reduced catalytic activity (Yi 2013, Hedstrom 2002, Blum 2021). In contrast, de novo protease design offers the opportunity to construct fully customized active site clefts tailored to target sequences, independent of native scaffolds. While we have not performed sufficiently broad screening to claim a generalizable method for designing de novo proteases against arbitrary peptide sequences, the pipeline reported in this manuscript has already enabled the generation of proteases targeting additional peptide epitopes from clinically relevant human proteins, including serum amyloid A, and the A β peptide, which we have included in Extended Data Fig.9

Given the concerns on rates and generality, the authors’ assertions that the work opens the way to therapeutic and industrial applications are not tenable.

For therapeutic applications, the relevant benchmark for catalytic rates is the timescale of physiological processes regulated by human proteases. Representative examples include ADAM10 ($k_{\text{cat}} \sim 0.04 \text{ s}^{-1}$, Moss 2016), MT1-MMP ($k_{\text{cat}} \sim 0.14 \text{ s}^{-1}$, Pahwa 2019), MMP1 ($k_{\text{cat}} \sim 0.47 \text{ s}^{-1}$, Fields 1991), MMP3 ($k_{\text{cat}} \sim 5.4 \text{ s}^{-1}$, Nagase 1994), and angiotensin-converting enzyme ($k_{\text{cat}} \sim 7 \text{ s}^{-1}$, Bünnig 1986). The activities of our designs fall within, and in some cases even exceed, the lower range of these naturally occurring metalloproteases. Given that such enzymes perform essential biological functions in humans, these comparisons suggest that our designed proteases operate on physiologically relevant timescales.

Notably, several approved or clinically used proteases exhibit similar turnover numbers, including tissue plasminogen activator ($k_{\text{cat}} \sim 0.28 \text{ s}^{-1}$, Zamarron 1984), used in acute ischemic stroke (Dewar 2019), and botulinum neurotoxin ($k_{\text{cat}} \sim 2.3 \text{ s}^{-1}$, Li 2000), used in movement disorders (Jabeen 2011). These values are comparable to those of our most active variant, further supporting the potential biological relevance of the observed cleavage rates.

We realize that practical therapeutic applications require properties beyond catalytic activity, and we do not claim that the current designs are directly translatable to clinical use. Nevertheless, we have included a demonstrative use case, conditional activation of masked therapeutics using the de novo protease, in Figure 6. For therapeutics with potential on-target off-tissue toxicity, the use of conditional protease-activated prodrugs may improve targeting specificity and reduce adverse effects (Sharma 2017).

For industrial applications, properties that determine manufacturability and process robustness, such as expression yield, solubility, and thermostability are often important considerations besides activity (Hilvert 2024). De novo scaffolds often excel in these crucial industry-relevant properties (Braun 2026, Juo 2026), supporting their potential for biotechnological applications.

Reviewer #3

The authors have made substantive changes to the manuscript. Especially the discussion about the discrepancy between designed and native metalloproteins is a valuable addition that really adds something to the manuscript (including a further improved variant of the metalloprotease).

I am not fully convinced by the response on whether this can be called "de novo enzymes", but I think we can "agree to disagree" here; it is not a major issue.

Overall I am fully satisfied with the response from the authors and I congratulate them on this beautiful work.

We thank Reviewer #3 for their positive and thoughtful comments. We greatly appreciate their careful review, which has significantly improved the quality of our study.

Reviewer #4

In this revised version of the manuscript, the authors have added structural X-ray data, performed MLFF-based calculations and MD simulations to rationalize the observed differences in activity, designed proteases capable of cleaving human TDP-43, and further enhanced the catalytic activity of their designs through targeted experimental mutagenesis around the active site. Although the reported 30-fold improvement in activity is substantial, the fact that this gain was achieved through experimental saturation mutagenesis focused on the active site somewhat reduces its impact and novelty.

We performed the site-saturation mutagenesis analysis around the active site mainly to investigate the origin of activity gap between the designs and the native metalloproteases, and to address Reviewer #1's concerns that enzymes with de novo scaffolds may have intrinsic limitations and be difficult to further improve. The original designs, including the one-shot design we added in the revised manuscript to target human TDP-43 were entirely generated by the computational design protocols we reported in this study.

Nevertheless, the revised manuscript is considerably stronger than the original submission.

We thank Reviewer #4 for this positive comment.

Based on the new data presented, we have the following comments:

1. In the X-ray data, why does Q32 adopt a different side-chain rotamer only in ZnO7? In the Supplemental Information, the authors state: “Despite the potential crystallography artifact, this same Q-to-E mutation in TDPPr3, and in a few other crystal structures we obtained (Extended Data Fig. 5), did not result in complete flipping of the rotamer, potentially due to better stabilization in the catalytically competent conformation.” However, this explanation remains unclear. Fig. S20 shows the new hydrogen-bonding interactions adopted by Q32, but it is not evident why the same side-chain conformation is not observed in the other variants. Can the hydrogen-bonding interactions shown in Fig. S20 not also be maintained in those cases?

The alternative rotamer state adopted by Q32 in the E32Q variant of ZnO7 is stabilized by three hydrogen bonds: one with the polar side chain of T68, one with the backbone carbonyl of D62 (an interaction not available to E32 in the WT protease), and one mediated by a solvent water molecule. Among all the de novo scaffolds described in this manuscript, ZnO7 is unique in that the catalytic base can access the protein surface upon adopting this flipped rotamer. MPNN-introduced polar residues in this region, including T68, both enhance solubility and provide favorable polar interactions with the flipped conformation of Q32. In addition, partial solvent exposure of residue 32 allows formation of the hydrogen bond with water, further stabilizing the alternative state. By contrast, in the other designs the catalytic base is more deeply buried within the hydrophobic core of the proteins, creating an environment that disfavors the catalytically unproductive rotamer due to the scarcity of nearby polar side chains and limited solvent access.

This observation suggests a potential design principle related to backbone geometry: the catalytic machinery should be sufficiently buried within the scaffold to shield it from competing polar interactions. A brief paragraph has been added to the Supplementary Discussion describing these observations and how they may inform the filtering of designs toward more active catalysts.

2. *“To compare entire reaction pathways at the active sites of the designs, we used machine learning force fields⁴⁶ (MLFF) to compute the activation barriers for the *likely rate-limiting* proton abstraction and nucleophilic attack steps⁴⁷ for Zn45, ZnO7, and native astacin (Fig. 1b, Supplementary Discussion 4). All catalytic residues, the substrate and all residues with at least one atom within 5 Å of the substrate were included in the simulation.” As the authors themselves note in Supplementary Discussion Note 4, even metalloproteases with very similar active-site catalytic machinery can display markedly different reaction profiles and rate-limiting steps. Therefore, the authors’ decision to focus solely on this particular step, using astacin as a reference, is not sufficiently justified. In addition, the apparent correlation with the experimental trends based on only three data points (variants) is not especially compelling. At a minimum, the analysis should be extended to include at least one additional variant, such as the improved Zn45_v2, which exhibits a 30-fold increase in activity.*

We have noted in the main text that the MLFF-based QM method we use is limited to a 5 Å truncated region within the substrate, and that only the enthalpic contribution of the free energy barrier is considered. Among the four residues where Zn45_v2 differs from Zn45, only one is within this truncated region. The MLFF-based NEB simulation we performed of Zn45_v2 yielded an activation energy barrier of 17.1 kcal/mol, very close to the value of Zn45 at 17.3 kcal/mol. Nevertheless, the molecular dynamics simulations nicely captured the effect of substrate and catalytic residue preorganization, which correspond to the factors missing from the NEB simulations. We added a paragraph in the Supplementary Discussion section to discuss this.

The first step of the reaction is bond-forming and the second step of the reaction is bond-breaking. Therefore, by estimating only the enthalpic contributions of the free energy barriers, we likely underestimate this barrier for the first step and overestimate this barrier for the second step. Thus, for the determination of the rate-limiting step of the reaction, the entropic effect cannot be ignored. A quantitative description of the free energy landscape along the full reaction pathway would require more sophisticated sampling methods that explicitly incorporate entropic contributions, which is beyond the scope of this study.

Although, as noted in both the main text and the Supplementary Discussion, these simulations have important limitations and are not suitable for quantitative prediction of activation free energy barriers, they are sufficient to provide a mechanistic explanation for the activity differences we experimentally observed despite the structural similarity of the active sites. This

question was raised by Reviewer 1 and, in our view, lies at the core of structure-based enzyme design, making it worthwhile to discuss. Therefore, we included these results in the main text. We have revised both the main text and the Supplementary Discussion to further clarify the purpose of this calculation and the limitations of this method.

Table R1. Rate accelerations of one-shot computational designs achieved in the current protease manuscripts in comparison to previously reported designed systems. The detailed calculations are shown in the Appendix.

Reaction name	Reference	Uncatalyzed half-life	$\log_{10}(k_{\text{cat}}/k_{\text{uncat}})$
<u>Activated ester hydrolysis</u>	Lauko et al. <i>Science</i> . 2025	14.8 hours	3.6
<u>Activated ester hydrolysis</u>	Kim et al. <i>Nature</i> . 2026	9.7 hours	4.9
<u>Kemp elimination</u>	Listov et al. <i>Nature</i> . 2025	6.7 days	6.4
<u>Retroaldol reaction</u>	Braun et al. <i>Nature</i> . 2026	3.4 years	6.7
<u>Unactivated amide hydrolysis</u>	Choi et al. companion cysteine protease manuscript	610 years	7.5
<u>Unactivated amide hydrolysis</u>	Chen et al. current metalloprotease manuscript	610 years	9.0

Appendix

Current manuscripts:

Chen et al. Metalloprotease (k_{uncat} = 3.6 x 10⁻¹¹ s⁻¹ at 25C; half-life = ~610 years)

Before optimization: k_{cat} = 0.033 s⁻¹, k_{cat}/K_m = 600 M⁻¹ s⁻¹

$$k_{cat}/k_{uncat} = 9.2 \times 10^8$$

After optimization: k_{cat} = 0.75 s⁻¹, k_{cat}/K_m = 3.1 x 10⁴ M⁻¹ s⁻¹

$$k_{cat}/k_{uncat} = 2.0 \times 10^{10}$$

Choi et al. Cysteine protease (k_{uncat} = 3.6 x 10⁻¹¹ s⁻¹ at 25C; half-life = ~610 years)

Before optimization: k_{cat} = 0.0011 s⁻¹, k_{cat}/K_m = 12 M⁻¹ s⁻¹

$$k_{cat}/k_{uncat} = 3.1 \times 10^7$$

After optimization: k_{cat} = 0.064 s⁻¹, k_{cat}/K_m = 413 M⁻¹ s⁻¹

$$k_{cat}/k_{uncat} = 1.8 \times 10^9$$

Best previous computational designs for substantially easier reactions

Kim et al. Computational design of metallohydrolases. Nature. 2026 Jan;649(8095):246-253. doi: 10.1038/s41586-025-09746-w.

Activated ester hydrolysis (k_{uncat} = 2.1 x 10⁻⁵ s⁻¹, half-life = 9.7 hours)

k_{cat} = 1.5 s⁻¹, k_{cat}/K_m = 53,000 M⁻¹ s⁻¹

$$k_{cat}/k_{uncat} = 7.1 \times 10^4$$

Lauko et al. Computational design of serine hydrolases. Science. 2025 Apr 18;388(6744):eadu2454. doi: 10.1126/science.adu2454.

Activated ester hydrolysis (k_{uncat} = 1.3 x 10⁻⁵ s⁻¹ at 30C; 14.8 h)

k_{cat} = 0.057 s⁻¹, k_{cat}/K_m = 2.2 x 10⁵ M⁻¹ s⁻¹

$$k_{cat}/k_{uncat} = 4.4 \times 10^3$$

Braun et al. Computational enzyme design by catalytic motif scaffolding. *Nature*. 2026 Jan;649(8095):237-245. doi: 10.1038/s41586-025-09747-9.

Retroaldolase ($k_{\text{uncat}} = 6.5 \times 10^{-9} \text{ s}^{-1}$; half-life = 3.4 years)

$k_{\text{cat}} = 0.036 \text{ s}^{-1}$, $k_{\text{cat}}/K_{\text{m}} = 12700 \text{ M}^{-1} \text{ s}^{-1}$

$k_{\text{cat}}/k_{\text{uncat}} = 5 \times 10^6$

(best evolved retroaldolase has $k_{\text{cat}} = 11 \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{uncat}} = 2 \times 10^9$)

Listov et al. Complete computational design of high-efficiency Kemp elimination enzymes. *Nature*. 2025 Jul;643(8074):1421-1427. doi: 10.1038/s41586-025-09136-2.

Kemp elimination ($k_{\text{uncat}} = 1.2 \times 10^{-6} \text{ s}^{-1}$; half-life = 6.7 days)

$k_{\text{cat}} = 3 \text{ s}^{-1}$, $k_{\text{cat}}/K_{\text{m}} = 12700 \text{ M}^{-1} \text{ s}^{-1}$

$k_{\text{cat}}/k_{\text{uncat}} = 2.5 \times 10^6$

(best evolved eliminase has $k_{\text{cat}} = 700 \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{uncat}} = 6 \times 10^8$)

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