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Redesign of a mononuclear zinc metalloenzyme for organophosphate hydrolysis

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Competing financial interests

The authors declare no competing financial interests.

Author Contributions

SDK developed the computational method for active site redesign, performed computational design and kinetic characterization of PT1-PT12, analyzed the data and wrote the paper. YK designed and performed the directed evolution and library screening, wild type activity measurement, substrate selectivity and inhibition experiments, analyzed the data and wrote the paper. PJG implemented the computational redesign method, performed the computational design of PT1-PT12 and analyzed the data. RT determined the crystal structure of PT3.1. JLG expressed and purified the designed proteins PT1-PT12. YS performed pKa calculations. YA, MG, IS, HL and JLS synthesized nerve agents and nerve agent analogues, screened these with PT3, and determined its stereoselectivity. BLS performed structural analysis and wrote the paper. DST designed the experiments, and analyzed the data. DB designed the computational method and the experiments, analyzed the data, and wrote the paper.

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Abstract

The ability to redesign enzymes to catalyze non-cognate chemical transformations would have wide-ranging applications. We developed a computational method for repurposing the reactivity of active site functional groups of metalloenzymes to catalyze new reactions. Using this method, we engineered a zinc-containing murine adenosine deaminase to catalyze the hydrolysis of a model organophosphate with a catalytic efficiency $k_{\text{cat}}/K_m \sim 10^4 \text{ M}^{-1}\text{s}^{-1}$ after directed evolution. In the high-resolution crystal structure of the enzyme, all but one of the designed residues adopt the designed conformation. The designed enzyme efficiently catalyzed the hydrolysis of the *R*-isomer of a coumarinyl analog of the nerve agent cyclosarin, and showed striking substrate selectivity for coumarinyl leaving-groups. Computational redesign of native enzyme active sites complements directed evolution methods and offers a general approach for exploring their untapped catalytic potential for new reactivities.

Introduction

The redeployment of catalytic machinery in naturally occurring enzyme active sites for non-cognate reactions holds considerable promise for obtaining novel biocatalysts^{1,2}. Alterations of substrate- and stereo-specificity, and the enhancement of pre-existing promiscuous activities of enzymes have been accomplished by library-based directed evolution approaches in several instances³. However, introducing completely new catalytic activities by exploiting native functional groups remains a challenge with these approaches because a starting point with some activity is typically required for directed evolution. Mechanistically related activities have been introduced into enzymes but these approaches require homologs with the desired activity to borrow sequence or structural features from^{4–12}.

Organophosphate (OP) pesticides and nerve agents, introduced in the environment in the last century, are highly toxic threat agents and there is considerable interest in treating and mitigating their effects. Enzymes that rapidly hydrolyze these compounds with high proficiency when reacting with non-chiral OP pesticides, and with strong stereo-preference when reacting with the more toxic isomers of chiral nerve agents would be valuable as prophylactics and post-exposure treatments¹³. These enzymes may also serve as useful reagents for the bioremediation of contaminated sites. Naturally occurring enzymes with cognate or promiscuous OP hydrolysis activity have been evolved to target the toxic isomers of some OP compounds^{14,15}.

Computational enzyme design methods have been used to alter enzyme substrate specificity¹⁶ and for designing catalysts for reactions that are not known to be catalyzed by natural enzymes^{17–19}. In *de novo* computational enzyme design, constellations of backbones that can support idealized active sites for the target reaction are identified in a set of protein scaffolds, and the sequence of the rest of the binding pocket is optimized for transition state affinity²⁰. Because these efforts have been aimed at building active sites *de novo*, the reactivity of wild type functional groups already present in the scaffold proteins is not utilized in design.

Metal ions in enzyme active sites are highly reactive, and metal catalysis plays a key role in both enzymatic and abiological reactions. In particular, zinc ions serve as powerful catalysts

in several hydrolase enzymes by (a) providing an activated water molecule for nucleophilic attack, (b) polarizing the scissile bond, (c) stabilizing the developing negative charge on the transition state, or a combination of the above²¹.

Here, we extend our *de novo* enzyme design methodology to exploit the reactivity of metal ions in existing enzyme active sites, and use this strategy to design an OP hydrolase starting from a functionally diverse set of mononuclear zinc-containing metalloenzyme scaffolds. Starting from a native adenosine deaminase with catalytic efficiency $k_{\text{cat}}/K_{\text{m}} < 10^{-3} \text{ M}^{-1}\text{s}^{-1}$ for the hydrolysis of an OP substrate, we computationally engineered an OP hydrolase with catalytic efficiency $k_{\text{cat}}/K_{\text{m}} \sim 10^4 \text{ M}^{-1}\text{s}^{-1}$ after activity maturation using directed evolution, a net $>10^7$ -fold increase in activity.

Results

Computational design of OP hydrolysis activity

We extracted a set of mononuclear zinc enzyme scaffolds, with diverse wild type functions and at least one open coordination site on the zinc atom from the Protein Databank (PDB). The requirement of at least one open zinc coordination site ensured that structural zinc sites were excluded from the scaffold set. The zinc coordination geometry in the scaffold set was either tetrahedral or trigonal bipyramidal.

The OP hydrolysis reaction proceeds by a $\text{S}_{\text{N}}2$ mechanism such that the TS geometry is trigonal bipyramidal around the phosphorous center²². We obtained the bond length and bond angles around the phosphorous reaction center in the TS from previously reported gas-phase quantum mechanical calculations²³ of the hydrolysis of paraoxon with the modification that the lengths of the bonds being formed and broken around the phosphorous center were set to 1.6Å and 2.0Å, respectively. We created a TS ensemble by sampling the torsional degrees of freedom in the R1, R2 and R3 substituents (Fig. 1) of the TS model while avoiding internal steric clashes. We superimposed models of the TS corresponding to two substrates, methyl paraoxon and DECP respectively, into the active site of each member of the scaffold set. In hydrolytic metallo-enzymes, metal ions act as activating agents for a hydroxyl ion nucleophile, or as Lewis acids for stabilizing the charge on the transition state, or both. Therefore, we generated three corresponding alignments (Fig 1a–c) of the TS model with the wild type metal center. In each case, we used RosettaMatch²⁰ to search for additional hydrogen bonding interactions to the phosphoryl oxygen, the nucleophilic hydroxyl moiety, and the leaving group oxygen. In cases where at least two of these three hydrogen bonding interactions were found, we introduced shape complementary interactions to the TS using RosettaDesign²⁴ (Fig. 1d). We maintained the coordination geometry around the metal ion during the design procedure by enforcing pseudo-covalent restraints between the metal ion and the protein sidechains coordinating the metal. We selected a set of twelve designed proteins (Table I) for experimental characterization based on the number of hydrogen bond interactions to the TS (more than 2), TS shape complementarity²⁵ ($S_{\text{c}} > 0.6$), and the presence of a docking funnel²⁶ ensuring that alternative orientations of the TS were higher in energy compared to the designed pose. We found that one of these twelve proteins, a redesigned adenosine deaminase (scaffold PDB accession code 1A4L), which we called PT3, hydrolyzed the substrate diethyl 7-hydroxycoumarinyl phosphate (DECP). While OP hydrolysis and adenosine deamination are both hydrolytic reactions (Fig 1e), the transition state geometry, leaving group character and the inherent reactivity of the substrate electrophilic center are quite distinct.

Characterization and directed evolution of PT3

PT3 has 8 mutations compared to the parent adenosine deaminase – the substitutions F65W, A183I, F61T, D19S, L62I, I299V and D296A provide shape complementarity, and L58Q introduces a hydrogen bond to the nucleophile in the computational model (Fig. 1d). The zinc coordinating residues H15, H17, H214 and D295, and the catalytic residues E217 and H238 were retained from the wild type adenosine deaminase. Kinetic measurements showed that the activity of PT3 was ~7-fold higher than the buffer background with protein and substrate concentrations of 2.5 and 50 μM , respectively. The products of the reaction were identical to those obtained with the wild type bacterial phosphotriesterase enzyme (Fig. S1, Supplementary Information), but the catalytic efficiency of PT3, measured by Michaelis-Menten analysis, was modest, $k_{\text{cat}}/K_{\text{m}} = 4 \text{ M}^{-1}\text{s}^{-1}$ (Fig. 2a). Wild type adenosine deaminase exhibited no acceleration of OP hydrolysis at $<20 \mu\text{M}$ enzyme concentration, suggesting a $k_{\text{cat}}/K_{\text{m}}$ below $10^{-3} \text{ M}^{-1} \text{ s}^{-1}$ (Fig. S2, Supplementary Information). The E217Q variant of an activity-matured variant of PT3 (PT3.1, see below) showed an OP hydrolysis activity with DECP identical to the wild type deaminase (Fig. 2b), indicating that E217 – a residue that is involved in proton shuttling in the deamination reaction²⁷ – is also crucial for catalysis of the newly introduced OP hydrolysis reaction. The wild type murine adenosine deaminase enzyme belongs to the amidohydrolase superfamily²⁸, and while some member enzymes of this superfamily catalyze OP hydrolysis, they use dinuclear rather than mononuclear metal sites²⁹, and do not contain the substitutions introduced to obtain PT3.

To gain insight into the features missing from the computational design protocol, we performed directed evolution experiments aimed at enhancing OP hydrolysis activity. We chose twelve positions – including five computationally designed positions, but excluding the putative catalytic residues – surrounding the active site for saturation mutagenesis, and screened variants at each position separately (see Methods). We found activity-enhancing mutations at three of the twelve positions. By recombining these substitutions, we obtained the variant PT3.1 (PT3 I62L, V218F, V299E) with a ~40-fold higher catalytic efficiency (Table II). The I62L substitution reverted a computationally designed residue to its wild type identity, V299E changed the identity of a computationally designed residue, and the largest single contributor to the enhancement, the V218F substitution (~20-fold increase), was at a position that was not considered during the computational design process.

We subsequently introduced random mutations in PT3.1 using error-prone PCR, and found increased activity upon substitutions at three positions (S57, P59 and E186). Saturation mutagenesis at these positions led to the identification of the variant PT3.2 (PT3.1 S57D, P59K, E186D) with a ~10-fold higher catalytic efficiency compared to PT3.1 (Table II). Finally, modeling the interaction of the TS with the crystal structure of apo-PT3.1 (see below) suggested that Q58 was suboptimal for activity. We performed saturation mutagenesis at this position and identified the variant PT3.3 (PT3.2 Q58V) with a ~10-fold higher catalytic efficiency compared to PT3.2. The variant PT3.3 had a catalytic efficiency ($k_{\text{cat}}/K_{\text{m}} = 9750 \text{ M}^{-1}\text{s}^{-1}$) approximately 2500-fold higher than the initial PT3 variant (Table II). Product inhibition leading to low total turnover numbers can be an issue for metallohydrolases, but PT3 proceeded through multiple turnovers – at an enzyme concentration of 350 nM, PT3.3 completely hydrolyzed 50 μM DECP indicating >140 catalytic turnovers per enzyme molecule (Fig. 2c).

As the designed OP hydrolysis activity increased during directed evolution, there was a concomitant decrease in the wild type deamination activity. The variant PT3 had an adenosine deamination activity that was ~50,000-fold less than the wild type deaminase whereas in the evolved variant PT3.1 deamination activity was undetectable (Fig. S3, Supplementary information). To uncover the minimal set of mutations required to endow the deaminase with the designed OP hydrolysis activity, we reverted each position in PT3.3 to

its wild type identity, one at a time (PT3toWT set). In parallel, starting from the wild type adenosine deaminase we generated a library of variants (WTtoPT3 set) in which the substitutions corresponding to PT3 were present in random combinations. We screened these libraries as cell lysates for OP hydrolysis activity with DECP, and sequenced select variants. In both cases, we found that four substitutions in the wild type deaminase (D19S, F61T, A183I, and D296A) were required to be simultaneously present for the emergence of OP hydrolysis activity. All variants with detectable DECP hydrolysis activity from the WTtoPT3 set (Table S2, Supplementary Information) had these four substitutions simultaneously.

There was striking spatial clustering (Fig. 3) of the mutations that enhanced PT3 activity – primarily by increasing k_{cat} (Table II) – suggesting that these residues fine-tuned the alignment of the substrate in the active site and/or increased the pKa of E217 by increasing hydrophobicity (e.g. substitutions V218F and Q58V) around this putative catalytic base (Fig. 1d). This residue has an elevated pKa in the wild type deaminase²⁷, and the calculated pKa of E217 in the crystal structure of the evolved variant PT3.1 (see below) was higher by 1.6 ΔpH units than that in the design model (Table S3, Supplementary Information). These results suggest that the increase in activity observed during the course of directed evolution was related to an increase in the basicity of E217.

Crystal structure of apo-PT3.1

We determined the crystal structure of the variant PT3.1 (PT3 I62L, V218F, V299E) to 2.35 Å resolution. In the crystal structure, the TIM-barrel fold of the deaminase was maintained, and the overall backbone conformation was quite similar (backbone RMSD 0.65 Å) to the PT3 design model (Fig. 4a). The zinc-binding site and other elements of the wild type catalytic machinery retained in the designed protein (e.g. residues H238, E217, D295) maintained their wild type conformations. We observed clear density for a zinc-bound water molecule and found that the trigonal bipyramidal zinc coordination geometry from the wild type was maintained in PT3.1. All computationally designed residues of PT3.1 except Q58 adopted rotamers predicted by the computational model (Figs. 4b and 4c).

Two loops that border the active site and that connect sequential β–α elements within the core TIM barrel fold (β4–α4 and β5–α5, respectively) each displayed backbone conformational differences compared to the wild-type deaminase structure and to their predicted conformation in the original PT3 computational design model (Fig. 4a). Loop I, composed of residues 183–194, was displaced by approximately 2 Å away from the mouth of the β-barrel. This conformational difference involved only small alterations of backbone dihedral angles, such that the loop was displaced with little overall change in its peptide conformation, and likely resulted from electrostatic repulsion between the D185 and Q58 sidechains.

Loop II, composed of residues 217 to 221, contains the V218F mutation that provided a significant contribution (20-fold) to rate enhancement for the PT3.1 construct. The side chain for F218 was well ordered and was in close proximity to side chains of E217 and Q58. In contrast, the surrounding residues of loop II appeared to exhibit dynamic flexibility resulting in density consistent with two distinct conformations (one of which resembled the structure of the original wild-type deaminase). The side chain of Q58 was found in an alternate rotameric conformation relative to the original computational design, suggesting that the addition of V218F during directed evolution led to the inversion of the Q58 rotamer (Fig. 4d).

Substrate- and stereo-selectivity of PT3

Both the computational design and directed evolution of PT3 were performed with the non-chiral substrate DECP. To investigate the substrate specificity of the designed OP hydrolysis activity, we used several substrates, each with different substituents on the phosphate moiety (see Methods). Surprisingly, we found that PT3 efficiently hydrolyzed substrates with a coumarinyl leaving-group (DECP, DEPCyc and CMP-coumarin, Fig. S4 and Fig. 5) but did not hydrolyze substrates that have substituted phenyl-ring leaving-groups (e.g. paraoxon, 4-nitrophenyl diethyl phosphate), despite their similar intrinsic reactivity. We found that both paraoxon and 4-nitrophenol inhibited the reaction of PT3 with DECP ($K_i \sim 250 \mu\text{M}$ and $\sim 150 \mu\text{M}$, respectively; Fig. S5, Supplementary information), but there was no evidence of product inhibition with DECP (Fig. 2c). Docking calculations indicated that the smaller size of the nitrophenyl group allowed a greater variety of alternative binding modes for paraoxon in the active site pocket, greatly reducing the fraction of bound configurations productively aligned with the catalytic machinery (Fig. S6, Supplementary information). These alternative binding modes, presumably driven by the binding of the phenyl ring, also sterically precluded the simultaneous binding of DECP and may thereby inhibit the reaction of PT3 with DECP.

The variants PT3.1-PT3.3 efficiently hydrolyzed the coumarinyl analog of the racemic nerve agent surrogate of cyclosarin, CMP-coumarin. PT3.3 had a marked stereo-preference for the R_P isomer of CMP-coumarin, whereas the wild type deaminase showed no detectable activity with the either isomer of CMP-coumarin at the same concentration (Fig. 5a). Despite the pronounced stereo-preference of PT3.3 for the R_P isomer, we also observed slow hydrolysis of the S_P isomer (a R_P/S_P kinetic ratio >150). These results are consistent with the modeled alignment of CMP-coumarin in the PT3.3 active site (Fig. 5b). For the actual nerve agent cyclosarin, the more toxic S_P -isomer was not hydrolyzed by any PT3 variant (data not shown). However, since the design model is consistent with the observed stereo-preference, and in view of the slow but detectable hydrolysis of the S_P -isomer of the cyclosarin surrogate, it should be possible to use the same approach to explicitly design catalysts to hydrolyze the toxic S_P -isomer of nerve agents.

Discussion

The high reactivity and large diversity of reactions carried out by metalloenzymes makes them attractive starting points for the introduction of new activities. We chose zinc-containing enzymes as templates for computational design of OP hydrolysis activity because zinc performs diverse mechanistic roles in enzymes, is redox-stable and is used as a Lewis acid-type catalyst in many natural hydrolases²¹ including the dinuclear bacterial OP hydrolases. Computational design of mononuclear zinc-containing active sites successfully identified a set of mutations in an adenosine deaminase that endowed it with the target OP hydrolysis activity. We considered only the geometric compatibility of the TS with the active site structure during design, and did not use *a priori* knowledge about the wild type activity in scaffold identification. Because we found that four simultaneous mutations were required for the emergence of OP hydrolysis activity in the deaminase (Fig. 3), identification of the computationally designed variant by library screening would likely require generating and screening a library of an exceedingly large size. Having obtained the initial low-activity lead from computational design, activity levels were enhanced by approximately 2500-fold using directed evolution, demonstrating the power of computational design followed by directed evolution for enzyme redesign.

In contrast with the dinuclear zinc-containing bacterial phosphotriesterases, PT3 is a mononuclear zinc enzyme. In the dinuclear phosphotriesterases, one zinc ion polarizes the scissile P-O bond and the μ -hydroxyl moiety bridging the zinc ions acts as the

nucleophile²². Because we observed only one free zinc coordination site in the crystal structure of apo-PT3, the zinc ion could act either to polarize the P-O bond (Fig. 1b) or to activate the nucleophile (Fig. 1a). In the wild type adenosine deaminase, the zinc ion activates the hydroxyl nucleophile²⁷ whereas in the design model it polarizes the P-O bond along with the sidechain of H238, and E217 activates the nucleophile by functioning as a general base. The increased hydrophobicity around the E217 sidechain during directed evolution, the stereo-preference for the *R*_p-isomer of CMP-coumarin (Fig. 5) and the lack of activity of the E217Q variant (Fig. 2b) of PT3 are consistent with the placement of the TS in the design model (Fig. 1b) and the role of E217 as a general base. The residue E217 shuttles protons in the wild type deaminase²⁷ and metal ion polarization of the P-O bond has been proposed for the OP hydrolase diisopropyl fluorophosphatase which has a single calcium ion in the active site³⁰. However, in the absence of a substrate- or TS-analogue bound structure of PT3, the possibility that the zinc ion and the residues H238 and D295 activate the nucleophile while the protonated form of E217 helps polarize the P-O bond (Fig. 1a) cannot be definitively ruled out.

Shortcomings of the computational enzyme design protocol³¹, and avenues for further improvement were highlighted by the sequence changes accrued during directed evolution and by comparing the crystal structure of the evolved variant PT3.1 to the design model. Most improvements in catalytic efficiency during directed evolution were increases in k_{cat} , and the largest single increase arose from the substitution V218F (variant PT3.1). The sidechain at position 218 is not expected to directly contact the TS, but V218F increases the hydrophobic bulk around the catalytically critical E217 sidechain, which likely modulates its pK_a and reactivity. Furthermore, the crystal structure of PT3.1 shows that the backbone structure of the loop composed of residues 217–221 needs to change conformation to accommodate the bulkier phenylalanine sidechain introduced by the V218F mutation. Since the design procedure is carried out on a fixed (wild type) backbone, the V218F substitution would cause steric clashes in the design model. Allowing backbone flexibility in the design procedure, and incorporating pK_a effects using a more accurate electrostatic interaction model are avenues for improving computational enzyme design.

The computational active site redesign method described here is general and can be readily extended to obtaining biocatalytic leads for other reactions of interest. The method complements current library-based directed evolution approaches for enzyme engineering. Optimization of the computationally generated leads with directed evolution approaches has the potential to both significantly enhance the desired activities, and provide valuable feedback for incorporating key missing elements in the computational design methodology. The vast diversity of highly reactive catalytic microenvironments in structurally well-characterized natural enzymes represents a rich resource of reactivity for obtaining novel biocatalysts using computational enzyme redesign.

Methods

Protein expression, purification and kinetic measurements

Designed proteins were expressed in *Escherichia coli* BL21 (DE3) using the pET29b plasmid (Novagene), and purified over a Ni-NTA affinity column. Protein purity and integrity was verified by SDS-PAGE. For PT3 variants, protein concentration was estimated spectrophotometrically using a calculated molar extinction coefficient of 50,000 M⁻¹cm⁻¹. Reaction progress was monitored by following increase in fluorescence of 7-hydroxycoumarin product Excitation (365nm)/Emission(445nm) in 10 mM HEPES, 150 mM NaCl buffer (pH 7.5) at a protein concentration of 2.5 μM. Initial rates of DECP decomposition were determined at 10–500 μM of substrate and fit to the Michaelis-Menten

equation $v_0 = k_{\text{cat}}[E]_0[S]_0/(K_m + [S]_0)$ to obtain k_{cat} and K_m . Results of at least three independent measurements were used to calculate reported kinetic parameters and estimate errors.

Library construction and screening

Desired positions in the gene were changed or diversified using either the Kunkel method³² or QuickChange mutagenesis (Stratagene), using mutagenic oligonucleotides (IDT). Error prone PCR was performed using GeneMorphII kit (Stratagene) according to the manufacturer's instructions. Genes were cloned into the pET29b expression vector, propagated, and constructed libraries were transformed into the E. coli BL21 (DE3) strain. Depending on the estimated complexity of the library 100–800 variants were assayed in screening experiments. Single colonies were grown in 96-deep-well plates. After cell lysis, activity of the protein variants was assayed in clarified lysate with 50 μM DECP. Improved variants were sequenced and mutations responsible for improvements were recombined before proceeding to the next round.

Crystal structure determination

After PT3.1 expression, purification and 6xHis tag cleavage (see Supplementary Information), the purified protein was concentrated to 0.2 mM in 20 mM HEPES-NaOH, 100 mM sodium glutamate (pH 7.5), 0.1 mM zinc acetate, and mixed with a 2.5-fold excess of sodium orthovanadate and 7-hydroxycoumarin. The crystal was obtained in 0.1 M Tris-HCl (pH 7.0), 0.2 M calcium acetate, and 20 % PEG 3000, and frozen by looping and submersion into liquid nitrogen. The crystal diffracted up to approximately 2.35 Å resolution at the ALS beam line 5.0.1. The data set was processed using HKL 2000 package³³, and a murine adenosine deaminase (D295E; PDB accession code 1FKW) was used as a search model for molecular replacement. A single copy of the search model was found by PHASER³⁴, and refined using REFMAC5³⁵. The final model was deposited in RCSB Protein Data Bank with ID code 3T1G. Statistics for the crystallographic data are shown in Table S4 (Supplementary Information). The anomalous difference Fourier map was used to conclude that PT3.1 contained a heavy metal atom in its active site (Fig. S7, Supplementary Information).

Determination of substrate-specificity

Substrate specificity of the PT3 variants was tested using 2,4-dinitrophenyl diethyl phosphate, 4-diethyl phosphate benzaldehyde, 4-nitrophenyl diethyl phosphate (paraoxon), 4-nitrophenyl dimethyl phosphate (methylparaoxon), and 7-O-diethylphosphoryl-3-cyano-7-hydroxycoumarin (DEPCyC). 0.5–1 mM of the OP compound were incubated with 5–10 μM of protein for 30 min. Change in absorbance or fluorescence at appropriate wavelength was recorded. Inhibitory effect of 2,4-dinitrophenyl diethyl phosphate, paraoxon or 4-nitrophenol on decomposition of DECP was studied by recording rate of hydrolysis of 50 μM DECP in the presence of 0.025–1 mM inhibitor. IC_{50} was calculated by fitting into equation $V_i = V_0/(1 + I_i/\text{IC}_{50})$.

Determination of stereo-preference with CMP-coumarin

The stereo-preference of the PT3 variants when reacting with chiral OPs was determined with racemic CMP-coumarin as substrate using the previously reported stereo-preference of two engineered mammalian serum paraoxonase variants, rePON1s, 3B3 and 3D8^{14,36}. The exact concentration of the CMP-coumarin stock solution was chemically determined by use of NaF as described earlier³⁶. Approximately 10 μM of racemic CMP-coumarin were incubated with 2- to 100 nM PT3 variant in 50 mM Tris-100 mM NaCl, pH 8.0 at 25°C and the release of the leaving group 3-cyano-7-hydroxy-4-methylcoumarin was monitored by

measuring absorbance at 400 nm. To identify the configuration of the non-hydrolyzed isomer in solution after the reaction approached an end-point, the mixture was spiked first with 0.1 μM 3B3 and 1 mM CaCl_2 that induced the exclusive hydrolysis of residual R_p isomer. Additional spiking with 0.1 μM 3D8 revealed the presence and concentration of the intact S_p isomer. Detoxification of cyclosarin was carried out by monitoring the loss of anti-acetylcholine esterase potency as described previously¹⁴.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Computational active site redesign

Based on the mechanism of metal ion-catalyzed OP hydrolysis, we superimposed the model organophosphate TS²⁹ on a set of mononuclear zinc metalloenzyme active sites such that the zinc ion acts as (a) an activator of the nucleophilic hydroxyl moiety or (b) stabilizes the developing negative charge on the phosphate moiety, or (c) both. We generated similar alignments for tetrahedral zinc sites. (d) Additional hydrogen bonding interactions were found in the wild type (e.g. H238 in PT3) or introduced (e.g. Q58 in PT3) using the RosettaMatch algorithm. Shape complementary interactions to maximize TS affinity were introduced using RosettaDesign. Residue numbers shown correspond to the PT3 design (see text). (e) The wild type adenosine deamination and the designed OP hydrolysis reactions.

Figure 2. Kinetic characterization of PT3

(a) Michaelis-Menten analysis on the computationally designed PT3 variant and the wild type adenosine deaminase (1A4L) indicates a $k_{\text{cat}}/K_{\text{m}}=4 \text{ M}^{-1}\text{s}^{-1}$ for PT3. Measurements were made at an enzyme concentration of $2.5 \mu\text{M}$ for both the designed and wild type proteins. Measurements were made in triplicate, and a representative trace is shown (b) The E217Q mutation in the evolved variant PT3.1 shows 1A4L-level OP hydrolysis activity, pointing to the catalytic importance of E217. (c) Multiple turnovers (>140) are observed with PT3.3 with an enzyme concentration of 350 nM and DECP concentration of $50 \mu\text{M}$. For estimating the maximum fluorescence signal corresponding to complete hydrolysis of DECP, bacterial phosphotriesterase (PTE) at $10 \mu\text{M}$ was used to hydrolyze the substrate under identical conditions.

Figure 3. Spatial clustering of wild type and activity-enhancing residues

Residues where computationally designed simultaneous substitutions were essential for the emergence of OP hydrolysis activity are highlighted in blue. Wild type residues retained in the most active variant of PT3 (purple), and positions where activity-enhancing mutations occur during directed evolution (green) form two separate spatial clusters. Residue sidechain identities are from the wild type crystal structure (PDB accession code: 1A4L), and the TS model is shown in grey sticks.

Figure 4. Design model and crystal structure of apo-PT3.1

(A) Superposition of the PT3.1 design model (gold) and the crystal structure (green) showing that while the overall backbone similarity is high (backbone RMSD=0.65 Å), two active site proximal loops show conformational differences. (B) The PT3 design model showing alignment of the TS in the redesigned enzyme. (C) Sidechains of designed residues S19, W65, I183 and A296 observed in the crystal structure (green) adopt rotamers predicted in the designed model (gold), whereas (D) the sidechain of residue Q58 adopts a different rotamer in the crystal structure (green) compared to the designed rotamer (gold) which corresponds to a change in its χ^2 dihedral angle.

Figure 5. PT3 variants stereo-selectively catalyze the hydrolysis of a cyclosarin analog

(a) Release of the coumarin leaving group from $\sim 10 \mu\text{M}$ racemic mixture of CMP-coumarin with the indicated enzyme in was monitored by measuring absorbance at 400nm (OD_{400}). After incubating $0.002 \mu\text{M}$ PT3 with CMP-coumarin so that OD_{400} reached a plateau, the variant 3B3 of the recombinant mammalian-like serum paraoxonase that selectively hydrolyzes the R_P form of CMP-coumarin, was added. When OD_{400} in presence of 3B3 reached the $\sim 0.2 \text{ OD}_{400}$ plateau, the reaction mixture was spiked with 3D8 variant of the paraoxonase that hydrolyzes both the R_P and S_P isomers^{14,36} to detect the presence of the intact S_P isomer. The resulting increase in OD_{400} to ~ 0.4 demonstrates the stereo-preference of PT3.3 for the R_P isomer. The observed burst phase (resulting in $\text{OD}_{400} \sim 0.2$) and biphasic behavior with a PT3 concentration of $0.1 \mu\text{M}$ suggests that the S_P isomer is hydrolyzed by PT3, albeit at a much slower rate compared to the R_P isomer. All data were collected in triplicate and a representative trace is shown. (b) Model of the CMP-coumarin TS docked into the PT3 crystal structure. The cyclohexyl moiety occupies the open pocket of PT3 consistent with the observed preference for the R_P -isomer.

List of designed proteins

Designs PT1-PT7 were made using methyl paraoxon, and PT8-PT12 using DECP as the model substrate, respectively. The expression of the designed proteins in *E. coli*, and their presence in the soluble fraction of the cell lysate were detected by SDS-PAGE analysis.

Table 1

Name (PDB ID)	Scaffold Function	Expressed	Soluble	Active	Number of mutations
PT1 (2PLM)	Deaminase	YES	NO	NO	10
PT2 (1N8K)	Alcohol dehydrogenase	YES	NO	NO	7
PT3 (1A4L)	Adenosine deaminase	YES	YES	YES	8
PT4 (1DQS)	Dehydroquinase synthase	YES	NO	NO	10
PT5 (2QW5)	Sugar phosphate isomerase	YES	YES	NO	11
PT6 (3H1W)	Mannose-6- phosphate isomerase	YES	NO	NO	15
PT7 (3COS)	Alcohol dehydrogenase	YES	NO	NO	5
PT8 (1H7N)	5-aminolaevulinic acid dehydratase	YES	YES	NO	8
PT9 (3HWP)	Diacetylphloroglucinol hydrolase	YES	NO	NO	9
PT10 (1KQ3)	Glycerol dehydrogenase	YES	YES	NO	11
PT11 (1ZSW)	Glyoxalase family protein	YES	YES	NO	11
PT12 (2HBV)	2-amino-3- carboxymuconate 6- semialdehyde decarboxylase	YES	YES	NO	7

Table II
**Kinetic parameters for the hydrolysis of DECP for the wild type deaminase (1A4L),
 designed variant PT3, and variants identified by activity maturation**

Standard deviations in k_{cat} and K_{m} from triplicate measurements were used to estimate errors.

Variant	k_{cat} ($\times 10^{-3} \text{ s}^{-1}$)	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1}\text{s}^{-1}$)
1A4L (wild type)	$5 \times 10^{-5} \pm 1 \times 10^{-5}$	>500	$<10^{-3}$
PT3	0.2 ± 0.1	43 ± 2	4 ± 2
PT3.1	4 ± 1	26 ± 1	154 ± 39
PT3.2	47 ± 5	49 ± 3	959 ± 118
PT3.3	351 ± 26	36 ± 5	9750 ± 1534