

De Novo Design of Miniature and Efficient Metallo-Ketoreductases

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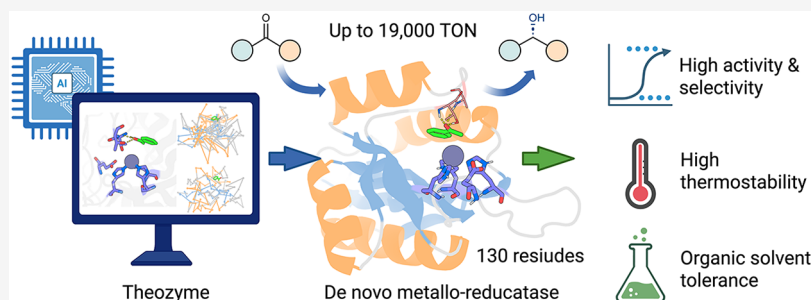
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ABSTRACT: The rational design of enzymes capable of catalyzing abiotic transformations represents a frontier in synthetic biocatalysis. While computational strategies have successfully produced enzymes for acid-base chemistry, examples of de novo designed oxidoreductases are still rare. Here, we report a deep learning-guided workflow for designing metallo-ketoreductases from theoretical active sites, enabling asymmetric reduction of ketones via an abiotic hydride-transfer mechanism. The resulting miniature enzyme contains only 130 residues, while exhibiting high catalytic performance under whole-cell conditions, achieving $k_{\text{cat}}/k_{\text{uncat}}$ up to 1.4×10^6 , turnover numbers (TON) up to 19,000, enantiomeric excess (e.e.) values of up to 98%, broad substrate scope, and regioselective reduction of diketones. Notably, the designed scaffold shows exceptional thermal stability toward 90 °C treatment, outperforming natural promiscuous reductases, and exhibits tolerance to various organic solvents. This work demonstrates the power of de novo enzyme design to access non-natural catalytic functions, offering a scalable and sustainable route to engineer tailor-made biocatalysts for asymmetric synthesis.

INTRODUCTION

Oxidoreductases are of particular importance in the synthetic chemical industry, especially for the production of chiral intermediates of pharmaceutical and fine chemicals,¹ where the enantiomeric purity critically determines bioactivity. Reductases are especially valuable for the asymmetric reduction of alkenes,^{2,3} ketones, and imines,^{4–6} offering an attractive alternative to traditional chemical hydrogenation methods,⁷ which often rely on expensive and nonrenewable noble metals (e.g., Ru, Pd, Rh) and complex chiral ligands.⁸ Biocatalytic reductive processes are more efficient and more sustainable, operating under mild reaction conditions with aqueous media. Most natural reductases are limited to flavin-dependent mechanisms that require reduced nicotinamide adenine dinucleotide (NADPH) as a hydride donor.^{3,9,10} Recent studies have shown that abiotic hydride donors, such as silanes, could be adopted by enzymatic systems to efficiently catalyze the asymmetric reduction of ketones, alkenes, and amines via zinc-hydride or iron hydride intermediates,^{11–16} providing a complementing route for building robust and cofactor-independent reductases. Natural scaffolds that enable this abiotic chemistry remain limited and require extensive directed evolution.

Computational enzyme design offers the potential to generate diverse protein scaffolds to enable this abiotic metal

hydride-mediated reduction chemistry.^{17–19} By constructing theoretical active sites, it becomes possible to create novel enzymes from scratch for enabling entirely new catalytic functions, with successful examples including retro-aldolases,^{20,21} Kemp eliminases,^{22,23} Diels–Alderase,^{24,25} hydrolases,^{26–28} and luciferases.²⁹ Moreover, the de novo design of metal-containing proteins and enzymes has been demonstrated in recent work.^{27,30–32} Recent breakthroughs in diffusion models (e.g., RFDiffusion³³) and deep learning-based structure prediction (e.g., AlphaFold^{34,35}) have greatly improved the accuracy of de novo protein design, opening new opportunities for expanding biocatalytic functions through de novo design.^{28,32,36}

In this study, we report the computational design of metallo-ketoreductases by constructing a theoretical active-site model to guide hydride-transfer catalysis. The designed miniature enzyme only contains 130 residues (13.8 kDa), while

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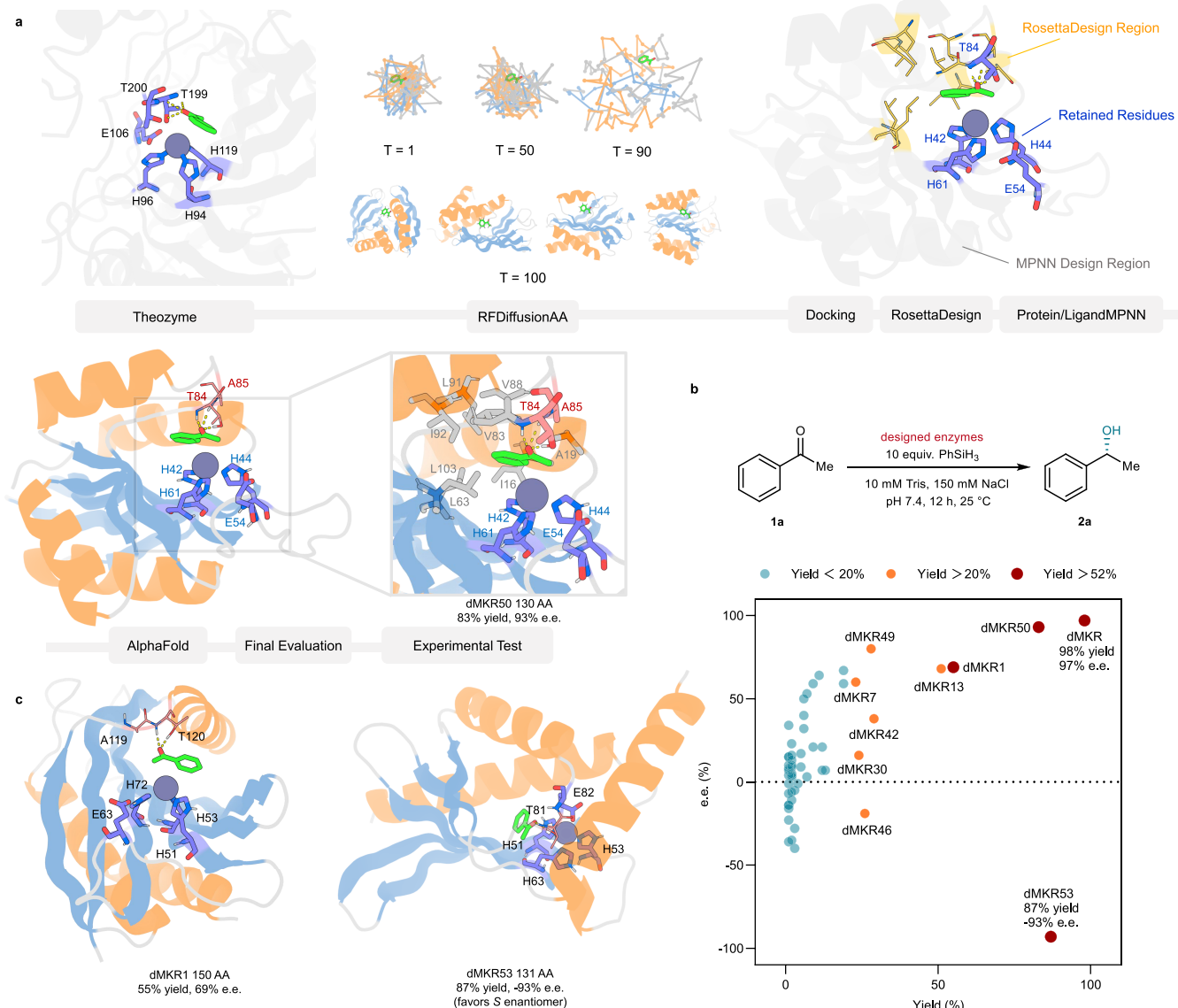


Figure 1. Computational design workflow of zinc-based metallo-ketoreductases and initial screening of catalytic reductions. (a) Computational design pipeline of dMKR, including identification of critical catalytic residues, de novo diffusion protein scaffold generation using RFDiffusionAA, docking of ligand and metal, sequence design with Rosetta and MPNN-based method, structure prediction using Colab version of AlphaFold2, final evaluation with FastRelax, and experimental test. (b) Evaluation of the reduction activity of dMKR designs from different diffusion families for acetophenone. Reactions were performed using 4 mL of whole-cell culture, except for dMKR53 (1 mol % loading of purified enzyme). Whole cells (4 mL) were centrifuged, resuspended in 0.4 mL of 10 mM Tris and 150 mM NaCl buffer (pH 7.4), and reacted with 8.6 μ mol of acetophenone (**1a**) and 86 μ mol of PhSiH₃ (10 equiv relative to **1a**) at 25 °C for 12 h. Yields and e.e. values were determined by GC. (c) Designs from various diffusion families other than dMKR50 that achieved >50% yield and e.e. are displayed. Zinc-coordinating residues are highlighted in blue, and residues forming hydrogen bonds with the substrate are displayed in pink.

exhibiting excellent catalytic properties, including high turnover numbers (TON, up to 19,000), $k_{\text{cat}}/k_{\text{uncat}}$ up to 1.4×10^6 , outstanding enantioselectivity (up to 98% enantiomeric excess (e.e.) value), broad substrate scope under whole-cell conditions, robust tolerance to diverse organic solvents (DMSO, 1,4-dioxane, etc.), and superior thermal stability (melting temperature, T_m of 93.8 °C). These attributes make the designed enzymes highly attractive for synthetic applications, particularly in asymmetric synthesis. By successfully integrating abiotic mechanisms into de novo protein scaffolds, this work highlights the power of de novo enzyme design to develop next-generation biocatalysts.

RESULTS AND DISCUSSION

Computational Design of Zinc-Based Metallo-Ketoreductases

Native human carbonic anhydrase II (hCAII) catalyzes zinc-hydride-mediated ketoreduction with high activity¹¹ but contains structural features that are not ideal, including long loops, a trefoil knot, and a large overall molecular weight of 29 kDa. We employed RFDiffusion to generate small and robust protein backbones, starting simply from predefined catalytic residues.³³ Acetophenone was chosen as the model substrate due to the high synthetic value of the resulting chiral alcohol. Based on a reported quantum mechanics/molecular mechanics

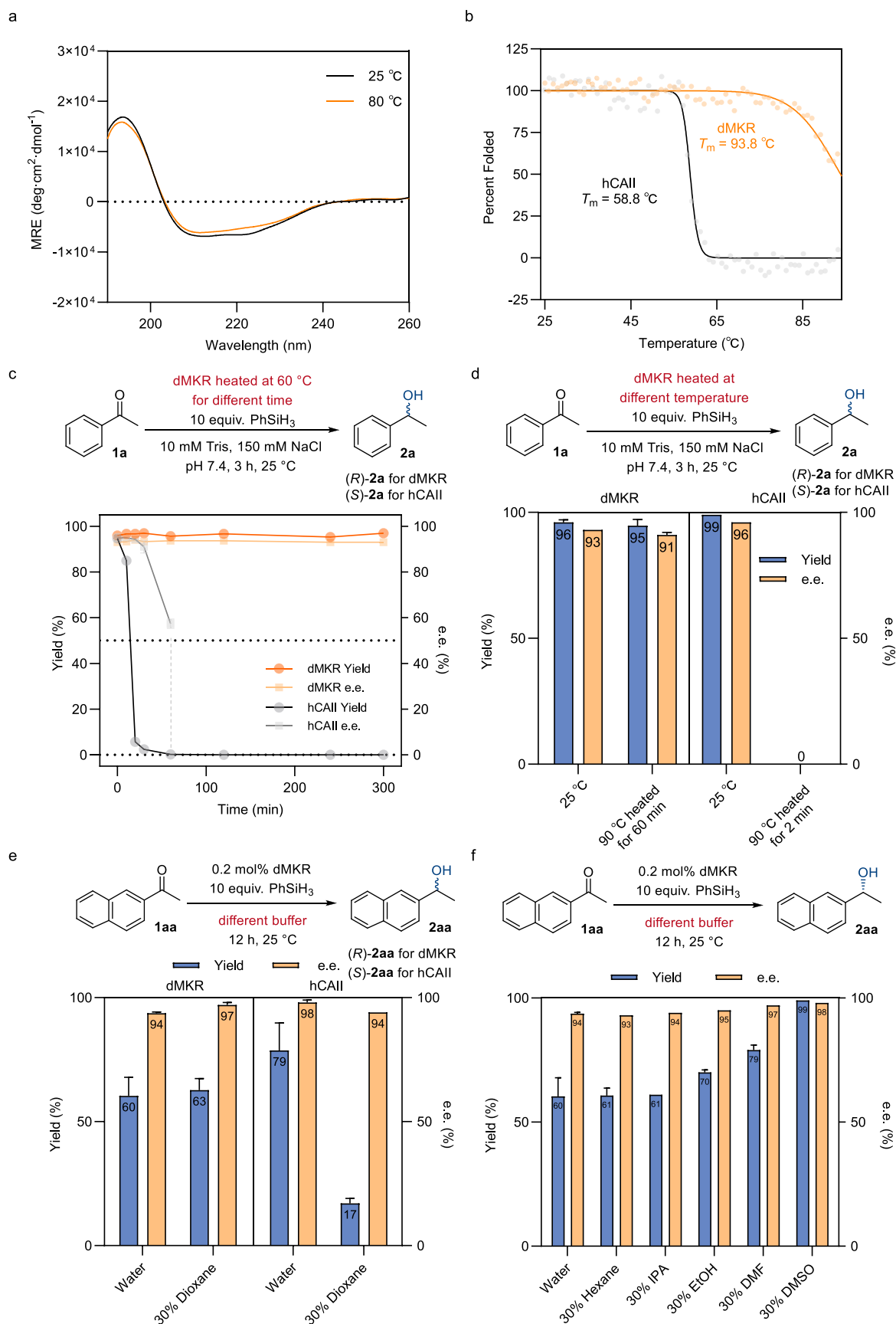


Figure 2. Thermal stability and organic solvent tolerance of dMKR. (a) Far-ultraviolet Circular dichroism spectra for dMKR at 25 and 80 °C. MRE, molar residue ellipticity. (b) Measurement of melting temperature by the change in signal at 193 nm for dMKR (212 nm for hCAII) over a range of temperatures; All constructs show a folded baseline followed by a sigmoidal two-state transition to the unfolded state. At lower temperatures, the baseline plateau corresponded to an assignment of 100% folded protein. (c) Residual activity for **1a** of the dMKR and hCAII after incubation at 60

Figure 2. continued

°C for different times. (d) Residual activity for **1a** of the dMKR and hCAII after incubation at 90 °C for different times. (e) Reduction of **1aa** catalyzed by dMKR in 10 mM Tris and 150 mM NaCl buffer (pH 7.4) and hCAII in 50 mM Tris buffer (pH 8.0), with or without 30% (v/v) 1,4-dioxane. (f) Reduction of **1aa** catalyzed by dMKR in 10 mM Tris and 150 mM NaCl buffer (pH 7.4) containing 30% (v/v) different organic solvents.

(QM/MM) optimized model of the zinc-hydride hCAII-acetophenone complex,¹¹ we fixed the positions of the critical residues and the substrate. Three sets of catalytic residues from hCAII were selected, including His94, His96, and His119 for Zn²⁺ coordination; Glu106 for electrostatic stabilization and assisting deprotonation of the Zn-bound water to generate the nucleophilic hydroxide species; and Thr199 and Thr200 for the oxyanion hole to stabilize the alkoxide intermediate (Figure 1a). Using RFDiffusion All-Atom (RFDiffusionAA),³⁷ 8000 de novo protein backbones with diverse mixed α/β secondary structures, each with fewer than 155 residues, were generated (Figure 1a). In the first sequence design step, 1833 backbone models with superior scaffold quality metrics were selected (Figure S2, Supporting Data 1) to generate a total of 54,990 sequences via ProteinMPNN, which were folded with AlphaFold2 and filtered based on predicted local distance difference test (pLDDT) scores,^{34,38} C α root-mean-square deviation (RMSD), and retention of the critical Glu side chain.

To refine the catalytic active site of the designed structures, we docked acetophenone using RIFDock,³⁹ and evaluated hydrogen bonding interactions involving Thr199 and neighboring backbone donors, as well as overall binding strength and shape complementarity to the substrate. Docking complexes favoring both the *S*- and *R*-stereochemistry were generated at this step. We intentionally selected examples favoring each stereochemistry for design. This resulted in 777 selected complexes, which were further filtered for ideal substrate burial (relative SASA between 0.05 and 0.20) to give 148 designs. After introducing a zinc ion by geometry constraints to the three His residues with Metal3D,⁴⁰ active-site residues that are within 5.5 Å of the ligand were designed using the FastDesign module in RosettaScripts to produce a total of 7350 designs,^{41–43} which were further filtered for hydrogen bonding to the substrate carbonyl, the placement of Glu residue, and optimal ligand orientation toward hydride insertion (Figures 1a and S4, Supporting Data 2).

The second sequence design step was focused on peripheral regions beyond 4 Å from the ligand by using ProteinMPNN and LigandMPNN in parallel to optimize the protein fold.⁴⁴ After sequence optimization, the designs were filtered on the basis of structural confidence, proper Glu placement, and ligand binding using the same methods as in the first iteration of design, and 91 designs were selected. These designs clustered into seven families, all featuring α -helix and β -sheet hybrids. At least one candidate was selected from each family, resulting in 24 metallo-ketoreductases (denoted as dMKR1–24) for experimental testing (Figure S6).

Experimental Evaluation of Designed Ketoreductases

To identify active ketoreductases, genes encoding 24 de novo designed dMKRs were cloned into pET29a and expressed in *Escherichia coli* BL21(DE3) with zinc sulfate supplementation. SDS-PAGE analysis of cell lysate confirmed high levels of soluble protein expression for all the 24 designs (Figure S8). Whole-cell catalysis was employed to test asymmetric reduction of acetophenone (**1a**), using phenylsilane

(PhSiH₃) as the terminal reductant (Figure 1b). Three designs, including dMKR1, dMKR7, and dMKR13, showed notable activity, each yielding >20% product yields, with e.e. values >50% (Figure 1b; data listed in Supporting Information, Table S1). Notably, dMKR7 and dMKR13 share a common scaffold from diffusion Family VII, featuring a Rossmann-like fold with six β -sheets flanked by two α -helices on each side (Figure S6). In contrast, the dMKR1 scaffold was derived from the diffusion family I, consisting of six β -sheets and three α -helices (Figure 1c).

On the basis of these findings, an additional 28 sequences (dMKR25–dMKR52) from family VII were expressed and tested for catalytic activity in whole cells. Most of the 28 designs exhibited high soluble expressions as confirmed by SDS-PAGE (Figure S8). Two new highly active designs, dMKR49 and dMKR50, were identified. dMKR50 displayed the best performance, achieving an 83% yield and 93% e.e. in the asymmetric reduction of acetophenone. A series of control experiments showed that the catalytic activity originated from the designed protein, rather than from the cellular background or nonbound zinc species (Figure S7). Replacing His-tag to Strep-tag further improved catalysis to 98% yield and 97% e.e. in whole-cell catalysis (Figure 1b), likely due to avoiding undesired zinc coordination to the His-tag. Strep-tagged dMKR50 was purified with Streptactin Beads at an impressive yield of 175 mg per liter of cell culture. Mass spectrometry confirmed the molecular weight of dMKR to be 15,165.83 Da (Figure S18), consistent with the designed sequence (15,166.82 Da, without the N-terminal methionine). The protein sequence obtained from the tandem mass spectrometry analysis was also completely consistent with the designed sequence (Figure S19). The purified protein showed 96% yield and 94% e.e. for the reduction at 0.5 mol % enzyme loading. The product, 1-phenylethanol (**2a**), was confirmed to be the *R*-enantiomer, complementing the *S*-enantiomer produced by the promiscuous catalytic reactivity of hCAII. Additionally, another designed enzyme dMKR53 (Figure 1c) catalyzed the formation of the *S*-enantiomer of **2a** with 87% yield and 93% e.e. at 1 mol % loading (Figure 1b; design method in Supporting Information, Section 1.6). Notably, the stereochemical outcome of the designed enzyme matched well with the design model. Among all 12 designs that have yielded greater than 5% and absolute e.e. values greater than 50%, 11 of them exhibited stereochemistry consistent with the designed configuration, which corresponds to a success rate of approximately 91.7%.

Thermal Stability and Organic Solvent Tolerance of dMKR

dMKR50Strep (abbreviated as dMKR) was selected as the final candidate of designed metallo-ketoreductase and evaluated for its thermostability. Circular dichroism (CD) spectroscopy demonstrated that the protein maintains a well-folded α/β structure, as evidenced by characteristic signals in the 190–260 nm range, remaining stable between 25 and 80 °C (Figures 2a and S20). Thermal unfolding analysis characterized by CD signal at 193 nm gave a melting

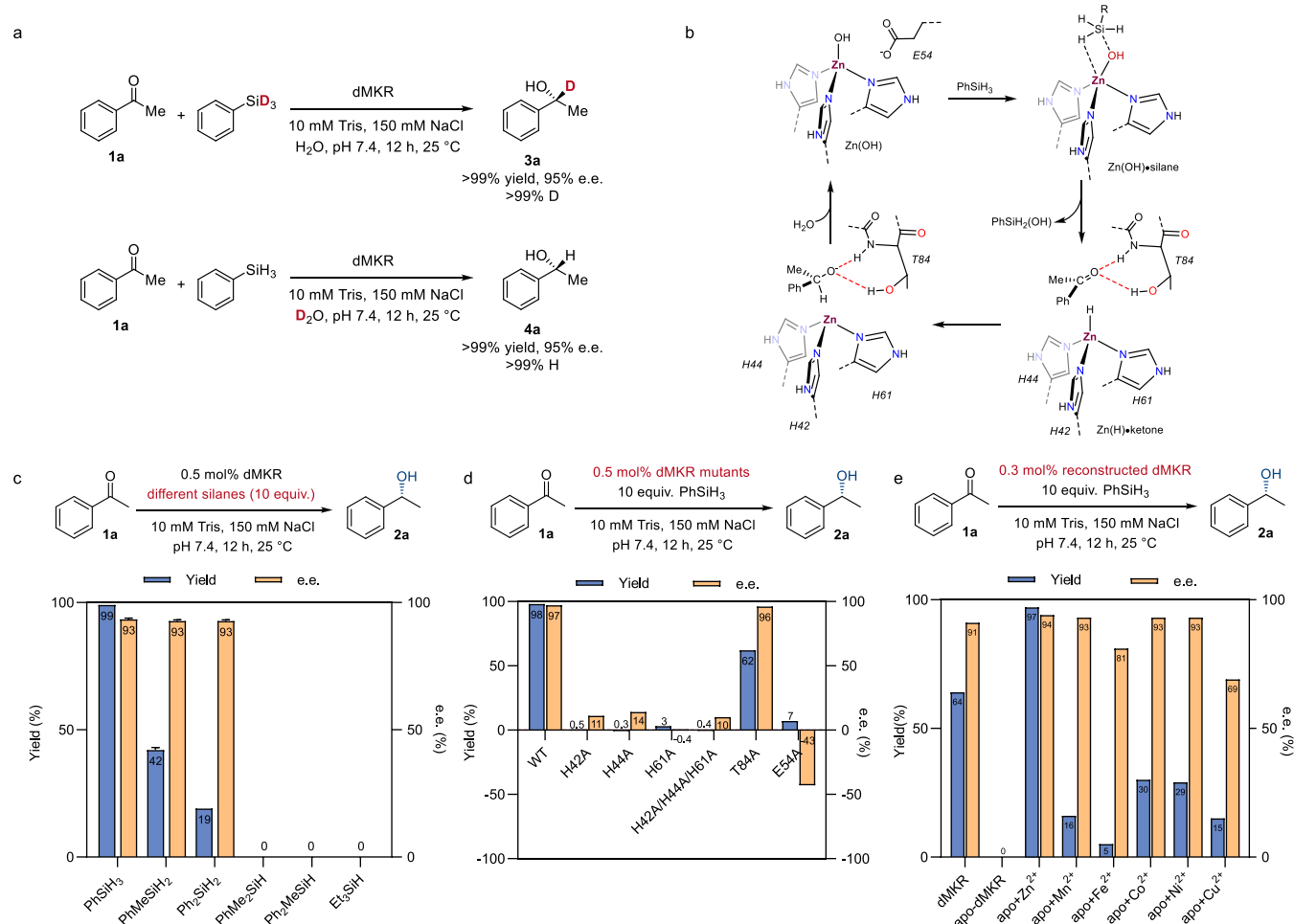


Figure 3. Mechanistic study. (a) Deuterium isotope-labeling experiment for tracing the source of hydride. (b) Proposed overall reaction mechanism for the dMKR-catalyzed reduction of acetophenone. (c) Influence of the types of silanes on the e.e. values and yields of 2a. (d) dMKR with key catalytic residues mutated into alanine catalyzed the reduction of 1a in the presence of PhSiH3. Reactions were performed under whole-cell conditions. (e) dMKR with Zn²⁺ substituted by other metal ions catalyzed the reduction of 1a in the presence of PhSiH3. Dialyze with dipicolinic acid to remove Zn²⁺, and add other metal sulfate solutions for substitution. All the yields and e.e. values were determined by GC.

temperature (T_m) of 93.8 °C (Figure 2b; method in Supporting Information, Section 2.14), which is significantly higher than the T_m of native hCAII (58.8 °C; Figure 2b). The structure of dMKR began to gradually unfold above 80 °C, and still retained about 30% of its structure at 100 °C, while hCAII was completely unfolded at 65 °C.

Moreover, the enzyme was heated at 60 °C for a varied amount of time and then tested for catalyzing the reduction of 1a (Figure 2c). After preheating at 60 °C, hCAII rapidly lost catalytic activity, with yields decreasing from 95% to only 6% after 20 min, and almost no product was detected following 30 min of heating. In stark contrast, dMKR displayed exceptional thermal resilience, maintaining almost 96% yield and 94% e.e. even after 300 min preincubation at 60 °C. This divergence became even more pronounced at 90 °C. hCAII completely lost catalytic activity after only 2 min of incubation, whereas dMKR maintained full catalytic competence even after 60 min at 90 °C, still delivering 95% yield and 91% e.e. (Figure 2d). Benefiting from the excellent thermal stability, dMKR could be easily purified from *E. coli* by heating the bacterial lysate at 80 °C for 10 min, followed by removing the sediment by centrifugation (Figure S21). These results highlight the thermal robustness of enzymes achieved through computa-

tional design, providing the potential to extend this enzymatic system to large-scale industrial applications.

We conducted 300 ns molecular dynamics (MD) simulations at 90 and 150 °C with 3 replicates to computationally probe the conformational dynamics of dMKR at high temperature. At 90 °C, the protein backbone equilibrated at an RMSD of around 2.0 Å and remained stable throughout the simulation (black line, Figure S26), indicating limited backbone fluctuations under these conditions. The α -helix (residues 9–18) and β -sheet (residues 24–31) structure were determined to unfold at 150 °C (orange line, Figures S26 and S28), but maintained their secondary structure integrity for the entire 300 ns at 90 °C (Figure S27). This conformational robustness is consistent with experimental CD spectra, which show well-preserved secondary structure features under high temperature.

Many industrial-scale bioengineering production processes were carried out in the presence of organic cosolvents to improve the solubility of hydrophobic substrates and products, which presented a significant challenge for enzyme stability, as many enzymes denatured and lost activity when the solvent proportion exceeded 10%.⁴⁵ We tested the activity of dMKR toward challenging substrate 1a (low solubility in water) in

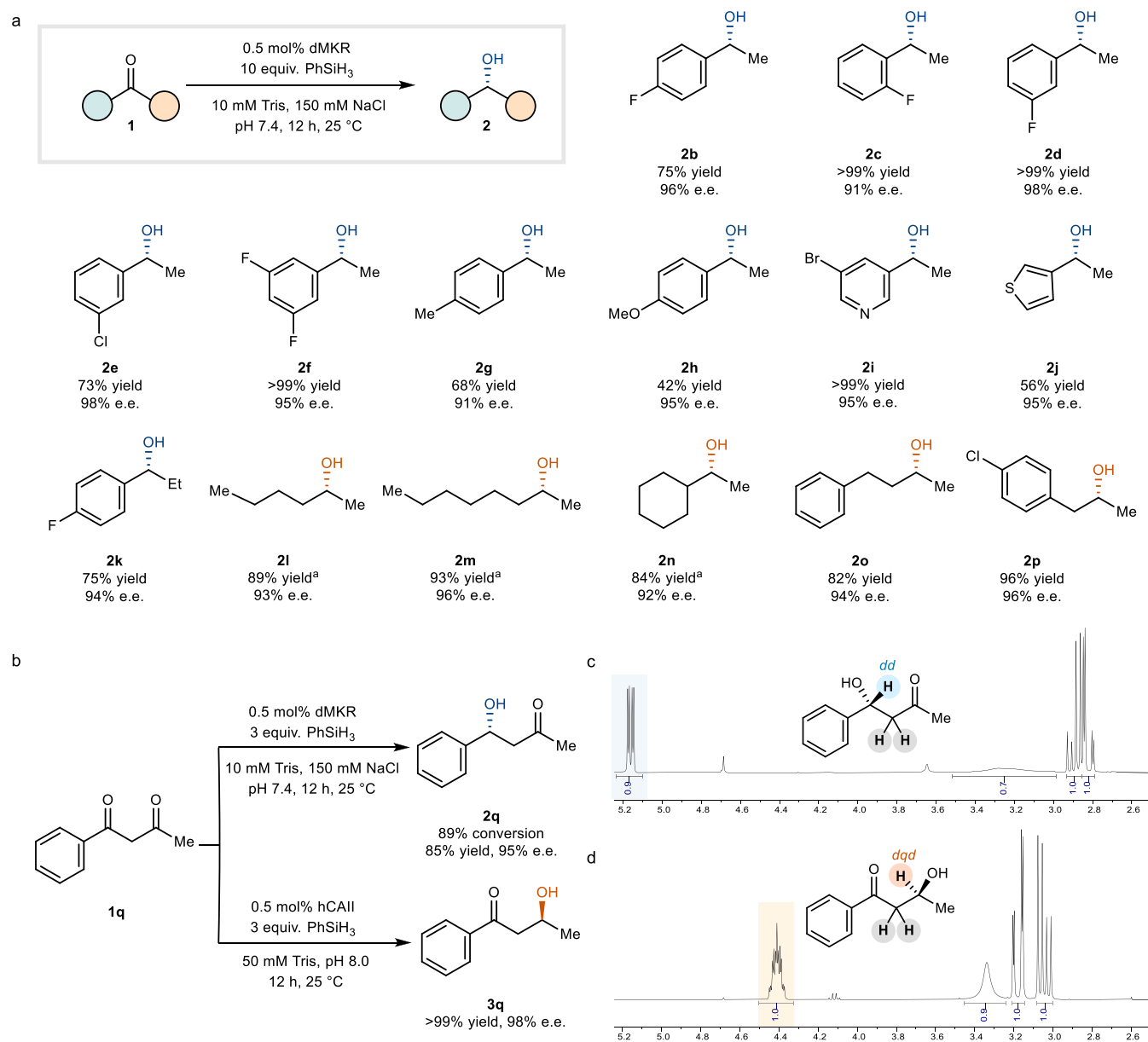


Figure 4. Substrate scope studies. (a) General substrate scope for the reduction of ketones by a designed metallo-ketoreductase. Standard reaction conditions: 0.046 μmol of dMKR in whole cells, 0.4 mL of 10 mM Tris and 150 mM NaCl buffer (pH 7.4), 8.6 μmol of ketone, 86 μmol of PhSiH₃ (10 equiv relative to ketone), reacted at 25 °C for 12 h. ^aThe e.e. values of these alcohol products were difficult to determine by GC, and were thus determined with their corresponding acetylated product. (b) Regioselective reduction of a diketone substrate. Reaction conditions: 0.046 μmol of dMKR in whole cells, 0.4 mL of 10 mM Tris and 150 mM NaCl buffer (pH 7.4), or 0.046 μmol purified hCAII in 50 mM Tris buffer (pH 8.0), 8.6 μmol of ketone, 25.8 μmol of PhSiH₃ (3 equiv relative to ketone), reacted at 25 °C for 12 h. Yield and e.e. values were determined by GC or HPLC. (c, d) ¹H NMR spectrum of **2q** and **3q** at 5.2–2.6 ppm.

30% (v/v) of 1,4-dioxane, for side-by-side comparison with hCAII (Figure 2e). The catalytic activity of hCAII significantly decreased from 79% yield to 17% yield, while dMKR maintained 63% yield at a higher 97% e.e., indicating the superior organic solvent tolerance of dMKR. We further evaluated the activity of dMKR toward **1aa** in five commonly used organic cosolvents (Figures 2f, S22, and S23), including hexane, isopropanol (IPA), ethanol (EtOH), dimethylformamide (DMF), and dimethyl sulfoxide (DMSO). Notably, dMKR maintained high catalytic efficiency in all tested organic cosolvents (30% v/v) with yields and e.e. values comparable to or even higher than those obtained in aqueous buffer alone. In hydrophobic solvents such as hexane and moderately polar

protic solvents such as IPA and EtOH, the activity of dMKR remained essentially unchanged (yield 61–70%, e.e. 93–95%), indicating excellent tolerance to both nonpolar and protic environments. More notably, its activity further increased in polar aprotic solvents, reaching 79% yield and 97% e.e. in DMF, and >99% yield and 98% e.e. in DMSO, demonstrating activity correlation with solvent polarity. We hypothesize that polar aprotic solvents (such as DMSO and DMF) are more favorable than protic solvents for improving substrate solubility without severely disrupting the hydrophobic core of the protein, thereby promoting a more favorable catalytic function. The resistance of dMKR toward protic solvents (such as IPA and EtOH), which are known to destabilize the native

structure of proteins through undesired hydrogen bonding interactions, indicates the exceptional solvent-resistance of this miniature enzyme.⁴⁶

Mechanistic Analysis of dMKR-Catalyzed Ketone Reduction

To identify the source of hydrogen in the catalytic reduction (Figure 3a), we used PhSiD₃ as the reductant. The dMKR converted **1a** to the corresponding deuterated product **3a** with >99% deuterium incorporation at the stereogenic carbon. In contrast, running the reaction with PhSiH₃ in a deuterated buffer resulted in no deuterium incorporation at this position. These deuterium labeling experiments confirm that the hydride is derived from the silane rather than from the solvent or any other source.

To distinguish between a zinc-hydride mechanism and a direct silane-hydride insertion (ternary mechanism), we tested the reduction of **1a** using various silanes as hydride donors (Figure 3c). If the silane directly inserts into the ketone through a ternary mechanism, the e.e. values of product **2a** would vary with silane structure. However, silanes including methylphenylsilane (PhMeSiH₂) and diphenylsilane (Ph₂SiH₂) gave identical e.e. values to PhSiH₃, suggesting that the silane does not participate in the enantio-determining hydride insertion step. This result supports a zinc-hydride mechanism where a silane donates a hydride to the zinc center before insertion into the ketone (Figure 3b). In addition, sterically hindered and less hydridic silanes such as dimethylphenylsilane (PhMe₂SiH), methylphenylsilane (PhMeSiH), and triethylsilane (Et₃SiH) produced no detectable alcohol products, suggesting that steric encumbrance and reduced hydride donor ability impair effective interaction with the zinc active site.¹¹

Alanine-scanning mutagenesis of active-site residues of dMKR, including His42, His44, His61, Glu54, and Thr84, was performed to assess their catalytic roles (Figure 3d). These residues correspond to His94, His96, His119, Glu106, and Thr199, respectively, in the hCAII-based theozyme. The mutation of any histidine led to a loss of catalytic activity. The coordination modes of the three histidines are different, as His42 and His44 coordinate Zn²⁺ through NE2, while His61 coordinates via ND1. His42 or His44 mutations abolished catalytic activity almost completely, while the His61 mutation reduced yield to 3% with undetectable e.e. These findings highlight stronger Zn²⁺ coordination of NE2-histidine and the cooperative nature of the histidine triad. Mutating Glu54 to Ala54 significantly reduced the catalytic activity and inverted the product configuration from *R* to *S*. This indicates that Glu is crucial for maintaining a stereoselective substrate-binding pocket. Mutating Thr84 to Ala84 slightly decreased the yield from 98 to 62%, without affecting e.e. This suggests that hydrogen bonding may be maintained via the backbone amide, as proposed for the role of Thr199 in hCAII for the binding of the CO₂ molecule.¹¹ Such backbone-amide hydrogen bonding interaction to the ligand was observed in the crystal structures of hCAII complexes (PDB 6WQ4, 1BN1) with different sulfonamide-like inhibitors.^{47,48}

We further tested the importance of Zn²⁺ for the catalytic function of dMKR (Figure 3e). Removal of Zn²⁺ by dipicolinic acid dialysis abolished dMKR activity. Supplementing Zn²⁺ to apo-dMKR restored full enzyme activity, giving the slightly higher yield and e.e. for the reduction of acetophenone. This suggests that dMKR may not have full zinc metalation, which was confirmed by native mass spectrometry analysis, showing

that approximately 70% of dMKR was zinc-loaded (Figure S17). By adding different equivalents of Zn²⁺ to dMKR, we observed slightly increased yield with increasing Zn²⁺ equivalence (Figure S16). The dissociation constant (*K_d*) for Zn²⁺ binding was measured to be 0.87 μM (Figure S25), which is higher than that of hCAII (0.5 nM),⁴⁹ but falls within the range for previously reported designed zinc enzymes,^{32,50,51} supporting the de novo designed protein has a moderate metal-binding affinity. Substitution with Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, or Cu²⁺ conferred partial catalytic activity for the reduction of acetophenone, with Zn²⁺-reconstituted enzyme being the most effective. These results confirm that dMKR is a zinc-dependent metalloenzyme and suggest potential for functional tuning via metal substitution.

Substrate Scope of Ketones

To evaluate the practical utility of dMKR, we examined the substrate scope using *E. coli* whole cells (Figure 4), because for large-scale biocatalytic processes, reactions are commonly carried out using whole cells or cell lysates to avoid the costly and lengthy steps of protein purification. Reactions were carried out by adding neat ketone (8.6 μmol) and PhSiH₃ (86 μmol) to 400 μL of whole-cell suspension in 10 mM Tris and 150 mM NaCl buffer (pH 7.4), followed by shaking at 25 °C for 12 h. dMKR efficiently reduced 16 types of ketones to the corresponding alcohols in yields up to 99%, with all examples showing >90% e.e. value (Figure 4a). A broad range of aryl ketones bearing electron-withdrawing or electron-donating groups at various positions (para-, meta-, or ortho-) were well tolerated, giving high yields and e.e. values of >90% (**2b–2h**). Electron-rich and electron-deficient heteroaryl ketones, including pyridines and thiophenes, were reduced in high enantioselectivity (**2i–2j**), with no sign of enzyme inhibition by the heterocycles. Notably, 1-(5-bromopyridin-3-yl)ethanol (**2i**), a precursor to aldosterone synthase inhibitors,⁵² was efficiently obtained in >95% yield and e.e. value. A substrate in which the methyl group was substituted with an ethyl (**2k**) group was also reduced with excellent enantioselectivity.

Chemical catalysts often struggle to achieve high stereoselectivity in asymmetric reactions involving substrates with similar alkyl substituents.^{53,54} We evaluated the designed enzyme dMKR for the reduction of dialkyl ketones. dMKR catalyzed the asymmetric reduction of alkyl methyl ketones with excellent performance, affording the corresponding alcohols (**2l–2m**) in >80% yield and >90% e.e. Even substrates bearing bulky cycloalkyl groups, such as **2n**, were converted in 84% yield and 92% e.e. Furthermore, dMKR maintained high activity and selectivity for aryl-containing dialkyl ketones (**2o–2p**), showing tolerance toward bulky phenyl substituents.

Regioselective reduction of one targeted carbonyl group in diketones is particularly challenging for chemical catalysts due to their limited contact surface area to discriminate against similar functional groups. We hypothesized that the designed active-site cavity of dMKR could enable such regio- and enantioselectivity. Indeed, dMKR selectively reduced the internal carbonyl group of 1-phenylbutane-1,3-dione adjacent to the phenyl ring, producing **2q** in 85% yield and 95% e.e. value (Figure 4b). The regioselectivity of the reaction was confirmed by the ¹H NMR spectrum of the purified alcohol product (Figure 4c). This is because in the designed model for **1a** (Figure 1a), the phenyl group is buried in the enzyme cavity through hydrophobic interactions, the carbonyl group is hydrogen-bonded through Thr84, leaving the terminal carbon-

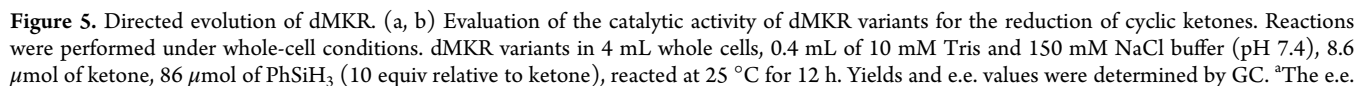


Figure 5. continued

values of these alcohol products were difficult to determine by GC, and were thus determined with their corresponding acetylated product. (c) Examples of chiral cyclic alcohol moieties in active intermediates and pharmaceutical drugs. (d) Reaction conditions: 0.019 μmol of dMKR_V88A or 0.032 μmol of dMKR_I92L in 4 mL whole cells, resuspended in 0.4 mL of 10 mM Tris and 150 mM NaCl buffer (pH 7.4), and reacted with 8.6 μmol of ketones and 86 μmol of PhSiH_3 (10 equiv relative to ketones) at 25 $^\circ\text{C}$ for 12 h. Yield and e.e. values were determined by GC or HPLC. (e) Reaction conditions: 0.032 μmol of dMKR_I92L in 4 mL whole cells, 2.0 mL of 10 mM Tris and 150 mM NaCl buffer (pH 7.4), 1 mmol of **1i**, 3 mmol of PhSiH_3 (3.0 equiv relative to ketone), reacted at 25 $^\circ\text{C}$ for 16 h. (f) Yield and enantioselectivity of reaction product from whole-cell reduction of **1i** on a gram scale. The picture shows the product **2i** that was obtained. (g) Kinetic parameters of dMKR_I92L or buffer as a catalyst when using **1i** as a substrate. Experiments were performed in three replicates independently.

yl group solvent-exposed and distal to the Zn center. This regioselectivity is in contrast to hCAII, which preferentially reduces the terminal carbonyl group of the same substrate, producing **3q** in >99% yield and 98% e.e. value (Figure 4b, 4d). These findings highlight the advantages of computational enzyme design in achieving regiospecific catalysis and its potential to access reaction spaces complementary to those of natural enzymes.

Directed Evolution of dMKR

To further improve the reactivity and enantioselectivity of dMKR toward a broader scope of substrates, we conducted directed evolution of dMKR by applying the focused rational iterative site-specific mutagenesis (FRISM) strategy.⁵⁵ Improvement in the substrate scope of dMKR is especially useful for enabling the synthesis of chiral cyclic alcohols, which are key components in or intermediate to pharmaceuticals and natural products, such as lovastatin and jardiance,⁵⁶ making their enantioselective synthesis highly valuable (Figure 5c).^{53,57}

We selected nine residues (I16, A19, L63, V83, A85, V88, L91, I92, and L103) in the active site that are within 4 Å to the ligand in the design model (Figure 5a) and mutated them to three residues with varied sizes, including Ala with little steric hindrance, Leu with moderate steric hindrance, and Phe with significant steric hindrance, respectively, thereby regulating the size of the active pocket and thus affecting substrate binding. We tested dMKR variants for the reduction of 2,2-dimethyloxan-4-one (**1r**) under whole-cell conditions. dMKR_V88A successfully produced the cyclic alcohol in 90% yield and 95% e.e. value, which was much higher than the 43% yield and 84% e.e. value of dMKR (Figure 5a). We also screened the mutant library for the reduction of acetophenone, among them, the dMKR_V88A and dMKR_I92L also showed improved catalytic activity and selectivity (Table S2). We extended the application of these variants to the reduction of three additional cyclic ketone substrates (**2s–2u**), where both variants continued to demonstrate excellent catalytic performance with high yields and e.e. values (Figure 5b). These results underscore the versatility and efficiency of the dMKR_V88A and dMKR_I92L variants in catalyzing the reduction of cyclic ketones, highlighting their broad applicability in this class of substrate.

Meanwhile, the catalytic activities of dMKR variants were evaluated toward several substrates that exhibited poor conversion by wild-type dMKR (Figure 5d), including a variety of aryls with electron-withdrawing or electron-donating groups (**2v–2aa**) and heteroaryl ketones (**2ab–2ac**). For these substrates, both yields and e.e. values increased significantly to mostly over 90% yield and over 90% e.e. when catalyzed by the V88A or I92L mutants. Notably, the sterically hindered substrate 2-acetonaphthone (**2aa**) showed a significantly enhanced yield of 95% and an e.e. value of 98%, which may be related to the reduction of steric hindrance at the binding

site for dMKR_V88A. Substrates with fused ring groups by replacing methyl with a phenyl-grafted alkyl group, such as tetralone (**2ad**), exhibited 98% e.e. and 55% yield. Notably, the experimentally observed stereochemistry of all the substrate scope is in the *R*-configuration, identical to the stereochemistry of acetophenone predicted from the designed active-site geometry.

The excellent catalytic efficiency of dMKR_I92L was demonstrated in the reduction of 3-acetyl-5-bromopyridine (**1i**), with the resulting product **2i** serving as a key precursor for synthesizing aldosterone-related drugs. Using only 0.0032 mol % of dMKR_I92L and a reduced amount of PhSiH_3 (3 equiv), **1i** was converted with a 61% yield and 94% e.e., corresponding to a calculated TON of 19,000 (Figure 5e). To further evaluate its potential for catalyzing large-scale reactions, we carried out a gram-scale asymmetric reduction of **1i** using *E. coli* whole cells. The reaction was performed in 10 mM Tris and 150 mM NaCl buffer (pH 7.4) at room temperature for 5 h, during which the starting ketone was fully converted to the corresponding chiral alcohol. The product was isolated by simple extraction and solvent evaporation, giving the alcohol in 96% yield (4.85 g) with 94% e.e. (Figure 5f). For this substrate, dMKR_I92L exhibited a K_M of $(2.1 \pm 0.2) \times 10^{-3}$ M, indicating higher substrate affinity than hCAII (K_M of $(1.1 \pm 0.2) \times 10^{-2}$ M, Figure S24) and a k_{cat} of $(3.4 \pm 0.1) \times 10^{-1} \text{ s}^{-1}$, giving a calculated k_{cat}/K_M of $160 \pm 20 \text{ M}^{-1} \text{ s}^{-1}$ (Figure 5g). When the reaction was performed in buffer without the enzyme, the k_{uncat} was only $(3.4 \pm 0.1) \times 10^{-7} \text{ s}^{-1}$. With a $k_{\text{cat}}/k_{\text{uncat}}$ ratio of 1.0×10^6 , the enzyme exhibits high rate enhancement, enabling efficient conversion of a substrate that would otherwise react extremely slowly without enzymatic assistance (Table S5), underscoring the high catalytic performance of our enzyme.

We further determined the kinetic parameters for acetophenone reduction catalyzed using the Michaelis–Menten kinetic model by dMKR, dMKR_V88A, and dMKR_I92L (Figure S24 and Table S4). Compared to dMKR, dMKR_V88A exhibited a 4.2-fold improvement in catalytic efficiency, as evaluated by k_{cat}/K_M ($88 \pm 8 \text{ M}^{-1} \text{ s}^{-1}$ for dMKR_V88A vs $21 \pm 3 \text{ M}^{-1} \text{ s}^{-1}$ for dMKR). For comparison, the k_{cat}/K_M of natural or evolved ketoreductases for acetophenone ranged from 10 to $600 \text{ M}^{-1} \text{ s}^{-1}$ (Table S5). The k_{cat}/K_M of dMKR_V88A was within the same order of magnitude compared with that of hCAII ($500 \pm 100 \text{ M}^{-1} \text{ s}^{-1}$). Moreover, the dMKR variants showed much better catalytic efficiency for substrate **1i** than the acetophenone substrate **1a**, with all dMKR and variants exhibiting k_{cat}/K_M over $60 \text{ M}^{-1} \text{ s}^{-1}$. The most active mutant, dMKR_V88A, gave an impressive k_{cat}/K_M of $250 \pm 30 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{uncat}}$ of 1.4×10^6 for **1i** (Tables S4 and S5). These findings demonstrate the potential of designed dMKR as a high-performance catalyst for asymmetric ketone reduction.

CONCLUSION

A high-efficiency miniature metallo-ketoreductase was designed from a theoretical catalytic active site. The deep learning-based methods expand beyond the restricted pool of native metallo-protein architectures and allow us to explore much broader sequence space. Among the screened 52 sequences, 36 of them showed activity with detectable enantioselectivity (e.e. value above 10%) for the asymmetric reduction of acetophenone using *E. coli* whole cells. The identified optimal dMKR showed up to 98% yield and e.e. of 97% toward the targeted substrate, and a very broad substrate scope toward various aryl-alkyl ketones, challenging dialkyl ketones, aliphatic cyclic ketones, and also diketones for regioselective reduction. The observed stereochemical outcomes were consistent with the designed model, highlighting the capability of this workflow to achieve predefined stereochemical preferences. This designed enzyme exhibits strong tolerance to various organic solvents and exceptional thermal stability toward 90 °C treatment. The variants dMKR_V88A and dMKR_I92L obtained by FRISM showed higher catalytic activity than dMKR and also had a wide range of substrate applicability. Mechanistic studies revealed that both Zn²⁺ and four introduced residues are indispensable for the catalytic activity. Overall, this study showcased the capability of computational rational design in building a metallo-reductase that functions through an abiotic metal hydride mechanism, offering fascinating perspectives toward the de novo design of oxidoreductases.

METHODS

Computational Design Workflow

Starting from the active site of a QM/MM-optimized hCAII-acetophenone model, RFDiffusionAA generated 8000 backbones filtered by structural criteria, yielding 1833 viable models (see Supporting Information Section 1.2 for details). ProteinMPNN designed sequences at three temperatures (0.1, 0.2, 0.3), producing 54,990 sequences. AlphaFold2 predictions were used to filter to 104 structures (pLDDT ≥ 88, RMSD ≤ 1.5 Å). Ligand docking with RIFDock and RosettaScripts refinement produced 777 docked models; filtering (CMS ≥ 120, ddG ≤ -10, dSASA 0.80–0.95) narrowed to 148. Metal3D predicted Zn²⁺ sites, integrated with constraints on His residues. RosettaScripts designed ligand interfaces with strict criteria (CMS ≥ 130, ddG ≤ -14, SC ≥ 0.75, see Supporting Information Section 1.4 for details), resulting in 193 designs after manual curation of 65. A second ProteinMPNN and LigandMPNN round generated 5850 sequences, folded by ColabFold;^{34,58} 767 passed filters (pLDDT ≥ 88, RMSD ≤ 1.2 Å). A final Rosetta FastRelax and manual selection yielded 91 structures,^{42,59} with 24 chosen for experimental testing.

Enzyme Expression and Standard Whole-Cell Catalysis Procedure

Proteins were overexpressed in chemically competent BL21(DE3) *E. coli* by transforming with plasmids, followed by incubation on ice for 30 min, heat shock at 42 °C for 90 s, and recovery in 1 mL 2YT medium. After 1 h at 37 °C, cultures were plated on kanamycin agar and incubated overnight. Single colonies were grown in 1 mL kanamycin/TB medium (50 mg/L) for 9 h at 37 °C, and 160 μL of the culture was used to inoculate 4 mL TB with kanamycin and shaken for 2 h at 37 °C. Expression was induced with 0.48 mg IPTG and 0.71 mg ZnSO₄·7H₂O at 20 °C for 24 h. Cells were harvested (3000 × g, 15 min), resuspended in 400 μL 10 mM Tris, 150 mM NaCl (pH 7.4), and reactions with ketones (8.6 μmol) and phenylsilane (86 μmol) were run at 25 °C, 600 rpm for 12 h. The reaction products were extracted with ethyl acetate, and the yield and

e.e. values of the resulting alcohols were analyzed by GC or high-performance liquid chromatography (HPLC).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.6c00732>.

Supporting Data 1 contains the filtered results from RFDiffusion; Supporting Data 2 presents the outcomes following RosettaDesign; Supporting Data 3 contains the design models for the experiment (<https://zenodo.org/records/15580524>) (PDF)

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[†]Y.X. and Y.L. are the first authors with equal contribution. All authors have reviewed and approved the manuscript.

Notes

The authors declare no competing financial interest.

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