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Manipulating Conformational Dynamics To Repurpose Ancient Proteins for Modern Catalytic Functions

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Cite This: *ACS Catal.* 2020, 10, 4863−4870

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INTRODUCTION

Conformational flexibility plays a critical role in enzyme function and regulation. Conformational changes, which can occur on multiple length and time scales, facilitate, for example, the binding of multiple substrates to the same enzyme, or allow for allosteric regulation or product release.[1] Such conformational dynamics can range from fluctuations of side chains and local conformational changes such as loop motion, through to secondary structure exchanges and protein unfolding/refolding.[2] Such dynamics plays an important role in allowing for enzyme promiscuity, (i.e., the ability of an enzyme to catalyze multiple, chemically distinct reactions, through either substrate, condition or catalytic promiscuity) and protein moonlighting (i.e., the ability of a protein to perform multiple chemically distinct functions).[2−5] This, in turn, facilitates enzyme evolvability (the ability of enzymes to acquire new functions), because the introduction of mutations along an evolutionary trajectory can shift the ensemble of conformational states available to an enzyme, allowing it to bind new substrates and facilitate new chemistry.[6−8] This is significant also in artificial enzyme evolution, since, frequently, directed evolution studies identify residues far from the active site that have significant impact on activity and function,[9−15] likely by changing the conformational ensemble of the enzyme.[2,3] In addition, many enzyme scaffolds (in particular, in the case of TIM-barrel fold proteins[16,17]) possess decorating loops that cover the active site, and there is increasing awareness of the role modulating the dynamics of these loops plays in facilitating enzyme evolvability and the emergence of new functions.[3,18−20] However, there is a caveat to this: a highly "floppy" enzyme can, on the one hand, sample multiple conformational states, allowing for new chemistry to evolve.[2,3] On the other hand, if there is too much "floppiness" in the system, it becomes very hard to achieve specificity in transition-state binding. Therefore, optimizing conformational dynamics during evolution requires both allowing the system to have enough flexibility to allow for new chemistry, while simultaneously dampening nonproductive dynamics that can impair the catalytic activity of the enzyme.[21,22]

In parallel to improved understanding of the role of conformational dynamics in enzyme evolvability, there has been significant research effort into understanding the physiochemical properties of ancestral enzymes, identified through ancestral sequence reconstruction and resurrected in the laboratory, before being subjected to extensive biochemical and biophysical characterization. Curiously, such enzymes have a tendency to have greater thermostability,[23−25] which likely leads to higher evolvability than their modern counterparts (since higher stability inherently would be expected to lead to higher evolvability[26]). As a result of this, we[27] and other researchers[28,29] have suggested that ancestral scaffolds provide powerful starting points for the evolution of new enzyme activities, going back to Jensen's original model for enzyme promiscuity and evolution.[6]

The focus of our research activities is to bring together these two features for enzyme engineering, taking advantage of the desirable physiochemical properties of ancestral protein scaffolds, and using conformational dynamics as a feature that can be manipulated for the engineering of new enzyme activities. This Viewpoint article will provide a brief overview of the current state-of-the-art in the field, as well as potential future directions. However, we note that, despite its tremendous promise, engineering protein dynamics,[21,30] whether on an ancestral or modern scaffold, remains a significant challenge to both theory and experiment, not least due to the multiple ways in which functionally relevant conformational dynamics can manifest itself in different systems, and the general complexity of the problem.

CONFORMATIONAL DYNAMICS AND ENZYME EVOLVABILITY

Our interest in understanding the role of conformational dynamics in evolution stemmed from a broader interest in understanding catalytic promiscuity, with a particular focus on enzymes that hydrolyze phosphoryl transfer reactions, which, in turn, has a tendency to be highly catalytically promiscuous.[31] For example, our initial work on understanding the selectivity of alkaline phosphatases suggested that the promiscuity (and, thus, evolvability) of these enzymes is driven by the high plasticity (ability to be easily molded) of their active sites, which allows the enzyme to adapt the electrostatic environment of its active site to accommodate multiple substrates with different requirements for efficient catalysis.[32] This led to

Received: February 11, 2020
Published: March 31, 2020

https://dx.doi.org/10.1021/acscatal.0c00722
ACS Catal. 2020, 10, 4863−4870

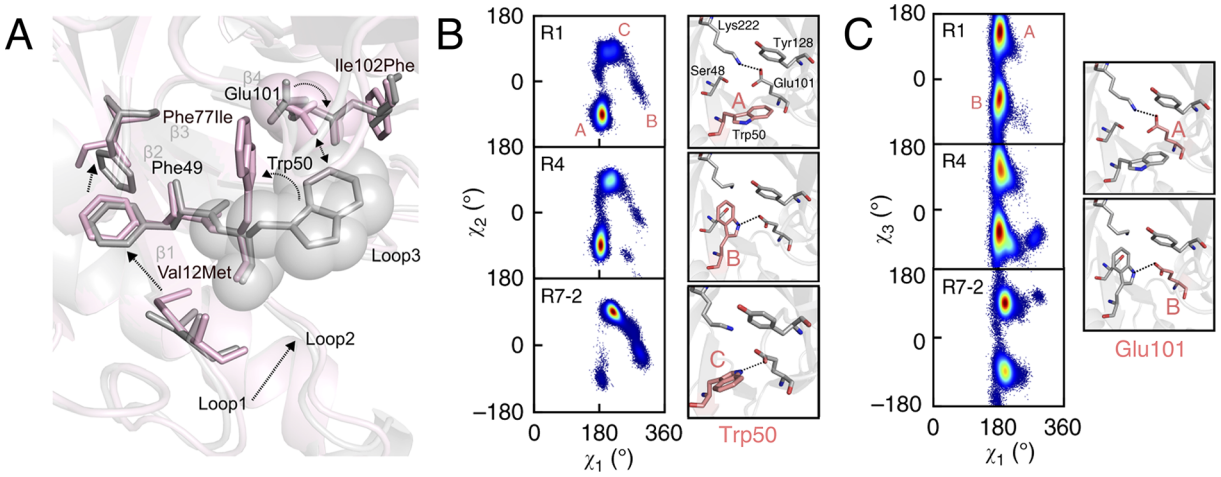

Figure 1. Remote mutations introduce new catalytic configurations to a designed Kemp eliminase (KE07) that has been optimized by directed evolution.[40] (A) Remote mutations of second-shell residues cause active site rearrangement, including rotation in catalytic residue Glu101 and stabilizing residue Trp50 (for a list of mutations, see Supplementary Table 2 in ref 41. In the originating orientation (R1), Trp50 stabilizes Glu101 through hydrogen bonding, maintaining the catalytic orientation. After four rounds of evolution involving primarily active site mutations (R4), electrostatics of the binding pocket are enhanced by removing Glu101-Trp50 hydrogen bonding. R5 and R6 (where conformational mixing first occurs) include remote mutations of residues I7, V12, K146, G202, and N224 (not shown in this figure, for the sake of clarity), After three additional rounds of directed evolution involving mutations Val12Met, Phe77Ile, and Ile102Phe (R7−2), Trp50 rotates into a secondary position, which orients the substrate for optimal catalytic activity. (B) Conformational sampling of Trp50 in Hamiltonian replica exchange molecular dynamics (HREX-MD) simulations[43] for the R1, R4, and R7-2 variants. Dihedral angle distributions are plotted. Three independent configurations of Trp50 are shown. (C) Conformational sampling of Glu101 in Hamiltonian replica exchange molecular dynamics (HREX-MD) simulations for the R1, R4, and R7-2 variants. Dihedral angle distributions are plotted. Two independent configurations of Glu101 are shown. [Adapted with permission from ref 41. Copyright 2018, Springer Nature. Published under a CC-BY license (http: //creativecommons.org/licenses/by/4.0/).]

similar conclusions as that from experimental work on an organophosphate hydrolase, serum paraoxonase 1 (PON1), where it was suggested that catalytic backups in the active site lead to the promiscuity of this enzyme.[33] Subsequently, we performed detailed combined experimental and computational studies on both PON1[34−36] and computational work on a related organophosphate hydrolase, diisopropyl fluorophosphatase (DFPase),[37] where we showed multiple ways in which conformational dynamics affects the selectivity and evolvability of these enzymes, from dampening of nonproductive conformations for catalysis through membrane association,[34] to regulation of active-site loop dynamics and flexibility,[35,37] through to an epistatic ratchet, created by structural remolding of the active site, that blocks reversibility in a laboratory evolution trajectory of PON1.[36]

Both we and others have also examined the role of conformational dynamics in the laboratory evolution of designed enzymes, such as designer retroaldolases and Kemp eliminases. For example, Osuna and co-workers[38] have demonstrated that both distal and active site mutations modify the conformational landscape of designed retroaldolases, causing population shifts to catalytically more favorable conformations. Mutations that cause such shifts were demonstrated by the authors to be computationally predictable.[4] Similar conclusions were drawn through Markov state modeling of a monoamine oxidase from *Aspergillus niger*, which identified hidden conformations, stabilized by mutations, that are critical for activity. In the case of Kemp elimination, it had been argued that precision in the placement of key active site residues is critical for enhancing the activity of a designed Kemp eliminase.[39] We demonstrated that, in the case of the Kemp eliminase KE07,[40] in fact, directed evolution led to the emergence of multiple new active site configurations (Figure 1), with greater catalytic efficiency than the initial design, and which were gradually stabilized by evolutionary conformational selection across the directed evolution trajectory trajectory.[41] Similar evolutionary conformational selection has also been experimentally observed during the evolution of a phosphotriesterase to an arylesterase.[42]

Following from this, there has been increasing interest in probing the emergence of new enzymes, completely *de novo*, on scaffolds that were previously noncatalytic. There are an increasing number of studies that demonstrate that this occurs frequently on scaffolds that were formerly merely solute binding proteins (e.g., refs 42 and 44, among others). One example of such enzymes are chalcone isomerases (CHI), which play an important role in plant flavonoid biosynthesis and have likely evolved from a nonenzymatic ancestor related both to a nonenzymatic chalcone-isomerase-like plant protein family (CHILs), both of which are, in turn, related to fatty-acid binding proteins, via a common noncatalytic ancestor.[42] We have demonstrated, through a combination of structural, biochemical, spectroscopic, and computational characterizations, that the emergence of CHI activity on this scaffold occurred through a combination of reshaping of the active site to allow a productive substrate binding conformation, as well as changes in the conformational flexibility of a key catalytic residue in the active site.[42] It is critical to bear in mind that *all necessary catalytic residues* were already present in the noncatalytic ancestor, and fine-tuning of the dynamical properties of the protein was critical for imparting enzymatic activity to the scaffold.

Finally, several experimental studies have increasingly demonstrated that it is possible to perform laboratory evolution that targets enzyme conformational dynamics, opening up great potential in this area (for a detailed review, see, e.g., ref 21; for more general reviews on the role of

https://dx.doi.org/10.1021/acscatal.0c00722
ACS Catal. 2020, 10, 4863−4870

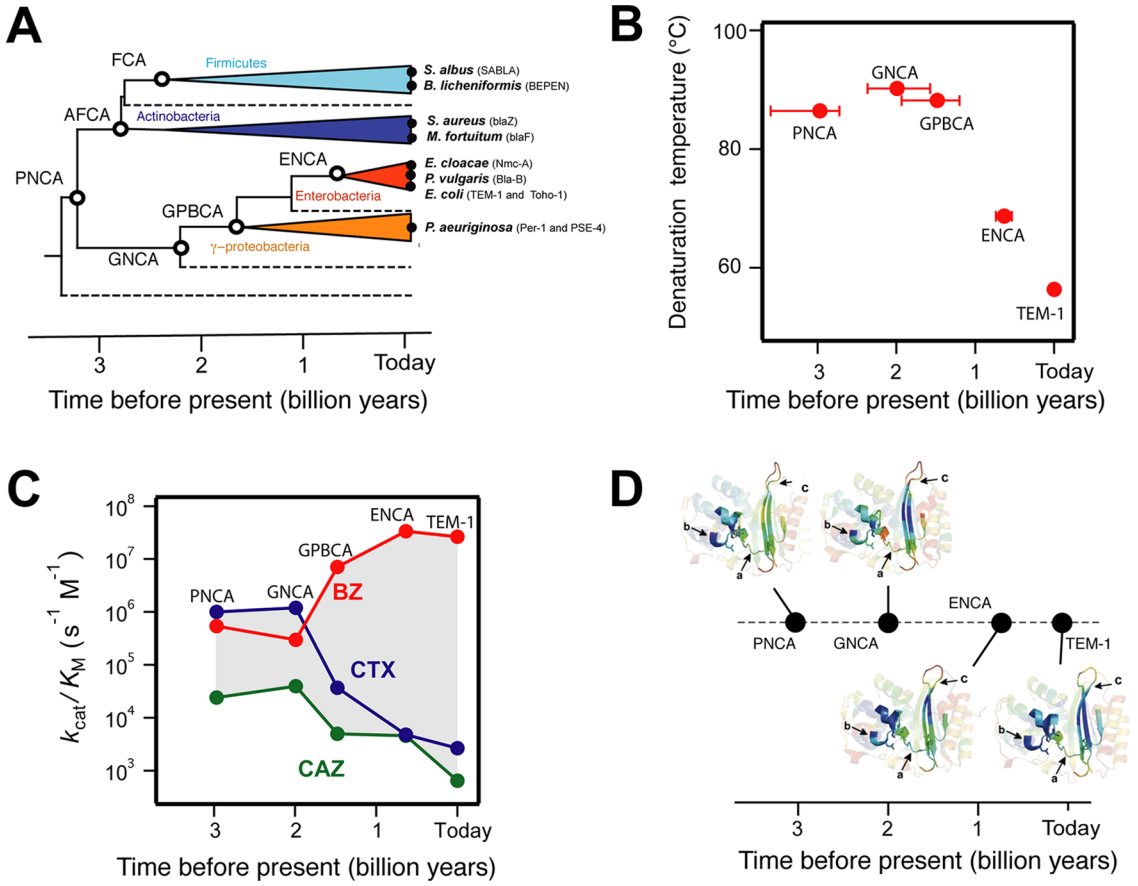

Figure 2. Simultaneous enhancement of stability, substrate promiscuity, and conformational flexibility in β-lactamases upon ancestral sequence reconstruction. (A) Schematic representation of the phylogenetic tree used for ancestral reconstruction of class A β-lactamases.[64] Ancestral nodes and modern proteins used as scaffolds for the de novo generation of a Kemp eliminase activity[27] are labeled. The ancestral nodes represent the common ancestors (CA) of Enterobacteria (ENCA), Gammaproteobacteria (GPBCA), various Gram-negative bacteria (GNCA), various Gram-positive and Gram-negative bacteria (PNCA), Firmicutes (FCA), and Actinobacteria and Firmicutes (AFCA). (B) Plot of denaturation temperature versus estimated age for the modern TEM-1 β-lactamase (at today) and the proteins at the ENCA, GPBCA, GNCA, and PNCA nodes.[64] A very large enhancement in denaturation temperature (more than 30°) is achieved upon ancestral sequence reconstruction. (C) Plot of catalytic efficiency versus estimated age for the degradation of several β-lactam antibiotics by the modern TEM-1 β-lactamase (at today) and the proteins at the ENCA, GPBCA, GNCA, and PNCA nodes.[64] Data for benzylpenicillin (BZ), and the third-generation antibiotics cefotaxime (CTX) and ceftazidime (CAZ). The modern enzyme is a penicillin specialist, while the resurrected Precambrian lactamases are moderately efficient promiscuous enzymes. (D) Evolution of conformational flexibility in β-lactamases. Regions with differences in differences in dynamic flexibility index[66] are highlighted and color coded (blue denotes rigid, red/orange denotes flexible). Critical residues at the antibiotic degradation active-site are shown with sticks. Panel (A) was adapted with permission from ref 27. Copyright 2017, Springer Nature. [Published under a CC-BY license (http://creativecommons.org/licenses/by/4.0/).] Panels (B) and (C) were adapted with permission from ref 64. Copyright 2013, American Chemical Society, Washington, DC. Panel (D) was adapted with permission from ref 66. Copyright 2014, Oxford University Press.

conformational dynamics in enzyme evolution, see e.g., refs 2−5 and 45−47.

■ ANCESTRAL PROTEINS AS SCAFFOLDS FOR PROTEIN ENGINEERING

Strictly speaking, ancestral proteins are proteins from extinct organisms. Obviously, neither extinct organisms nor their proteins exist anymore. However, while de-extinction of ancient organisms (such as the woolly mammoth) is being discussed mostly as a theoretical possibility,[48] de-extinction of ancestral proteins is, in some sense, actually possible, and, indeed, ancestral protein "resurrection" has been an active field of research for more than 20 years. The origin of the field can be traced back to the proposal by Linus Pauling and Emile Zuckerkandl,[49] that a set of sequences for several modern protein homologues can be processed to yield a reasonable approach to the sequence of their common protein ancestor. At the time of the Pauling−Zuckerkandl proposal (1963),[49] very few modern sequences were known and "ancestral sequence reconstruction" (ASR) was not a practical possibility. However, the exponential increase of the sequence databases in the genomic and post-genomic eras, together with advances in phylogenetics and bioinformatics, has transformed ASR into an almost-routine computational procedure.[50] Furthermore, standard molecular biology methodologies allow the preparation, in the laboratory, of proteins encoded by the reconstructed sequences. Of course, there is no way we can be absolutely certain that such "resurrected" ancestral proteins match the proteins that actually existed millions or billions of years ago. In addition, there is inherent uncertainty in the prediction,[24,51−53] in particular, in the case of residues with intermediate-to-high divergence, such as, for example, surface residues that are not involved in function.[24] Still, validation to some extent is often achieved at the phenotypic level,[54] since,

https://dx.doi.org/10.1021/acscatal.0c00722
ACS Catal. 2020, 10, 4863−4870

in many cases, the biophysical/biochemical properties of the resurrected proteins lead to convincing evolutionary narratives and rationalize evolutionary information from a diversity of sources. Resurrected ancestral proteins have thus been extensively used in the last 25 years or so as tools to address important problems in evolution.[52,55]

Moreover, recent work has highlighted their potential in biotechnological and protein-engineering applications. This potential arises because of the often unusual or extreme ancestral properties, which could reflect early stages in protein evolution and/or adaptation to ancestral intracellular and extracellular environments that differed from the environments hosting modern proteins. Properties that were required to guarantee functionality only under ancestral conditions may no longer be observed in the modern proteins, in keeping with the general evolutionary principle that features that become superfluous undergo evolutionary degradation.[56] Hyperstability is, for instance, a common result of ancestral protein resurrection that plausibly reflects the thermophilic nature of ancient life (see discussion in ref 23 and references cited therein), with enhancements in denaturation temperature of many degrees, with respect to modern mesophilic enzymes being often observed (see discussion in ref 57 and references cited therein). However, it has also been proposed that hyperstability is being predicted by so-called "consensus effects": that is, if, at a given position, a certain amino acid is highly conserved in the majority of consensus sequences, ASR will also assign this position to the ancestral state.[24] The extent of this bias is dependent, to some degree, on the ASR approach used: for example, maximum likelihood has been suggested to be more prone to consensus effects than Bayesian methods.[24,58] However, in the case of relatively conserved sites, the consensus amino acid is inevitably the most likely ancestral state. In addition, consensus effects are likely to be less pronounced in highly conserved functional sites, in very slowly evolving core residues, and in positions where exchanges are correlated with other positions.[24,59−62] Since replacing a rare amino at a given site with the consensus amino acid often increases the kinetic and/or thermodynamic stability of the proteins,[63] a bias toward the consensus in the inferred ancestor may also lead to a bias toward increased thermostability. An example of this is seen in the case of inferred ancestors of modern serum paraoxonases (including inferred mammalian ancestor), which display drastically increased thermostability, compared to their extant human counterpart (melting point (T_m) values up to 30 °C higher than those of the extant counterpart),[24] despite the fact that the environmental temperature was the same at the time of mammalian emergence as in the present day. This increased thermostability was argued to be due to two potential factors: a potential consensus effect that biased some (but not all) reconstructions, and other environmental factors, such as oxidative stress, high radiation levels, or intrinsic factors such as high rates of genetic mutations and less-efficient protein quality control systems.[24]

Following from this, promiscuity, that is, the capability to perform a diversity of more- or less-related molecular tasks, is also a common result of ancestral resurrection,[53,57] reflecting that the most ancient proteins were possibly generalists, capable of catalyzing the turnover of multiple substrates, as suggested many years ago by Jensen,[6] or simply that ancestral reconstruction often targets preduplication nodes in the evolution of new functions.[53] Both high stability and functional promiscuity are known contributors to evolvability, i.e., the capability to evolve new functionalities. High stability thus allows destabilizing but functionally useful mutations to be accepted,[26] while promiscuity is commonly linked to conformational flexibility,[3,45] which facilitates the emergence of new functions through the probing of a diversity of conformations, with minor conformations responsible for alternative functions being enriched by subsequent evolutionary process.

Importantly, high stability and functional promiscuity (with the concomitant conformational flexibility) are not necessarily incompatible features, as our ancestral resurrection studies on the antibiotic-resistant enzymes β-lactamases show.[64] The modern TEM-1 β-lactamase is moderately stable (denaturation temperature of ∼55 °C) and is a catalytic specialist (i.e., an enzyme with catalytic activity focused toward a single substrate or reaction), being able to degrade penicillin at near the diffusion-limit rate, but showing very low levels of catalysis for the degradation or other β-lactam antibiotics. In contrast, resurrected ancestral enzymes corresponding to Precambrian nodes in the evolution of β-lactamases have denaturation temperatures of ∼90 °C, while displaying substantial levels of catalysis levels with the diversity of β-lactam antibiotics (Figure 2). Such ancestral substrate promiscuity is linked to enhanced conformational flexibility,[27] as shown by molecular dynamics simulations and by experimental nuclear magnetic resonance (NMR) relaxation studies (Figure 2). As another example of simultaneous enhancement of stability and promiscuity, Kazlauskas and co-workers[65] found that a catalytically promiscuous ancestor of esterases and hydroxynitrile lyases displayed a denaturation temperature of 80 °C, which is substantially higher than that of their modern descendant (54−70 °C). Overall, the biophysical properties of high stability and conformational flexibility often displayed by resurrected ancestral proteins should make them excellent scaffolds for engineering efforts such as the design or laboratory evolution of new enzymes.

USING CONFORMATIONAL DYNAMICS TO ENGINEER KEMP ELIMINASE ACTIVITY IN A DE NOVO ACTIVE SITE ON A PRECAMBRIAN SCAFFOLD

Having discussed both the role of conformational dynamics in enzyme evolvability and the potential of ancestral proteins as starting scaffolds for protein engineering, we would like to highlight here a recent success story from our laboratories, focusing on engineering and optimizing a de novo active site, capable of highly proficient Kemp eliminase activity, onto a Precambrian lactamase scaffold resurrected through ancestral sequence reconstruction.[27] Specifically, a computational comparison of Precambrian β-lactamases corresponding to 2- to 3-Gy-old Precambrian nodes in the evolution of Class A β-lactamases indicated that, despite minimal structural differences between the ancestral and modern enzymes, the ancestral enzymes showed significantly greater flexibility and deformability (ability to change shape from the native structure), particularly in the active site region.[66] This, in turn, likely accounts for the greater promiscuity of the ancestral proteins toward a range of antibiotics, compared to their modern counterparts.

However, enhanced flexibility in the ancestral proteins was not restricted to the antibiotic-degradation active site. Indeed,

https://dx.doi.org/10.1021/acscatal.0c00722
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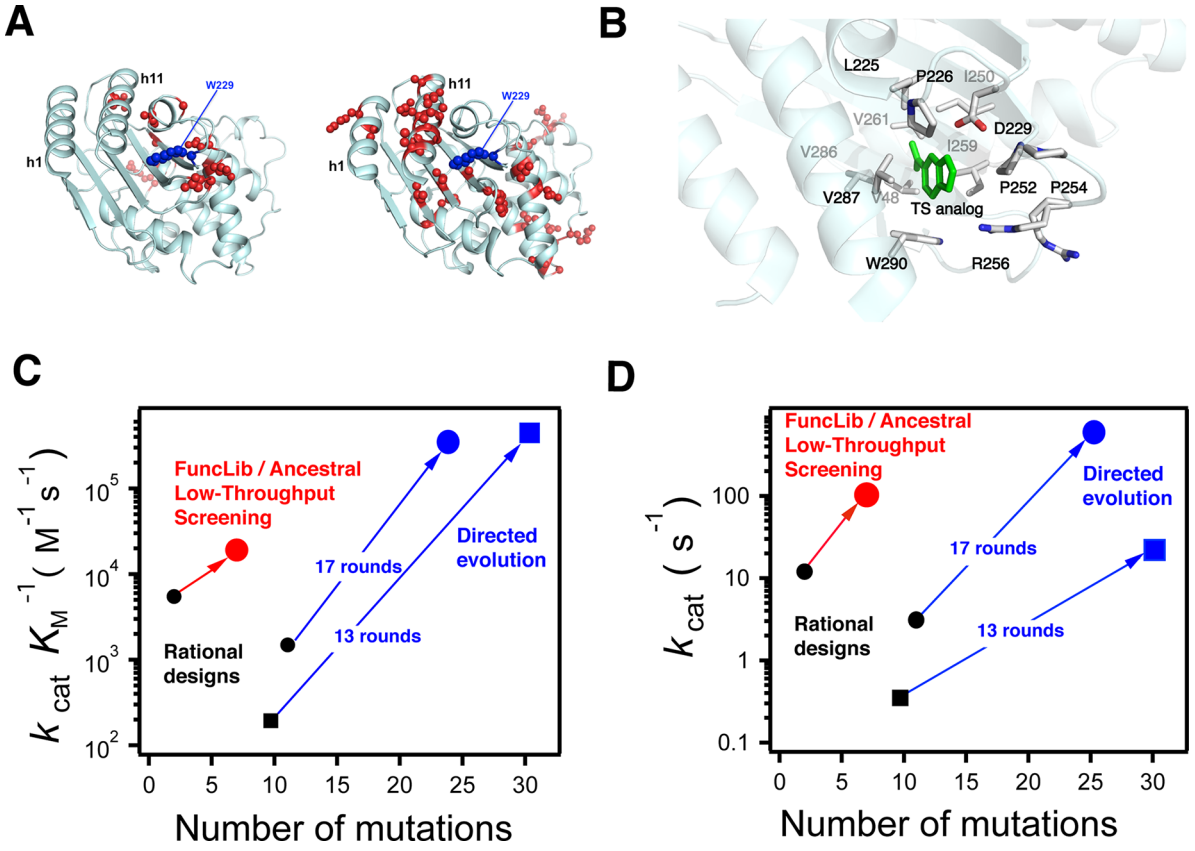

Figure 3. Using resurrected ancestral proteins as scaffolds for a de novo Kemp elimination activity. (A) Three-dimensional (3D) structures for the modern TEM1 β-lactamase (left) and an ancestral β-lactamase at the GNCA node (right; see Figure 2A). Residues for which NMR-relaxation rates cannot be explained without invoking a conformational exchange contribution are labeled in red.[27] The residue targeted for de novo active-site generation is labeled in blue. (B) The de novo active-site for Kemp elimination in an ancestral GNCA-lactamase generated by a single W229D mutation.[27] A bound transition-state analogue is shown in green. Panels (C) and (D) show the optimization of the Kemp elimination catalysis through computationally focused ultralow throughput screening. The results obtained for the catalytic parameters (red) are compared with the values for the best Kemp eliminases resulting from extensive directed evolution efforts (blue) taken from refs 39 and 68. Catalytic efficiencies (C) and turnover numbers (D) are plotted versus the number of mutational changes, with respect to the initial, noncatalytic scaffold used. Panel (A) was adapted with permission from ref 27. Copyright 2017, Springer Nature. [Published under a CC-BY license (http://creativecommons.org/licenses/by/4.0/).]

NMR studies[27] revealed a large number of residues for which relaxation rates imply a conformational exchange contribution in the ancestral GNCA-lactamase, compared with a much smaller number of such residues in the modern TEM-1 lactamase (see Figure 3). Furthermore, in the ancestral protein, many residues with conformational exchange contribution clustered in a region including alpha helices h1 and h11, as well as loops 225−229 and 252−257. Therefore, we selected position 229 in that region as the target for the generation of a completely new active site, distinct from the antibiotic-degradation active site, through a minimalist design involving a single mutation (for a similar study, see ref 67). Thus, a simple hydrophobic-to-ionizable amino acid substitution (Trp → Asp) at position 229 in an ancestral lactamase scaffold generated a highly proficient Kemp eliminase activity (Figure 3), as well as promiscuous arylesterase activity in the de novo active site.[27] The highly simplistic design strategy used to generate a new function exploited both the fact that a Trp side chain is very similar to the substrate for Kemp elimination, allowing the substrate to bind in the de novo active site, as well as the fact that the new Asp side chain imparts the catalytic functionality necessary to perform the proton abstraction.

However, note that this design strategy is meant to probe the role of conformational flexibility in the emergence of new enzymes. That is, replacing the Trp residue does not immediately create a cavity with the shape of the substrate molecule, because the new Asp residue sits right in the middle of the cavity. Therefore, de novo catalysis relies on local flexibility that alters the cavity shape, so that substrate binding becomes possible. Indeed, the strategy only worked on the resurrected Precambrian lactamases and not on 10 different modern scaffolds (Figure 3), because of the differences in conformational flexibility between the two (the modern β-lactamase scaffolds were highly evolved and too rigid to bind the substrate), although low stability may have also played a role in the failure of the modern scaffolds tested to generate a new function, because the W229D mutation is highly disruptive. Overall, in our best Precambrian variant, GNCA4-W229D/F290W, the simple design strategy yielded Kemp eliminase activity with $k_{cat} \approx 10\ s^{-1}$ and $k_{cat}/K_M \approx 5 \times 10^3\ M^{-1}\ s^{-1}$,[27] which we have further enhanced to a $k_{cat} \approx 100\ s^{-1}$ and $k_{cat}/K_M \approx 2 \times 10^4\ M^{-1}\ s^{-1}$ using an ultralow-throughput screening strategy based on results from the FuncLib web server,[69] as described in ref 70. These values are well within the range of catalytic parameters spanned by modern natural

https://dx.doi.org/10.1021/acscatal.0c00722
ACS Catal. 2020, 10, 4863−4870

enzymes and approach the best Kemp eliminases to date, which were obtained through extensive library screening efforts and many rounds of directed evolution. Overall, our results suggest a simple evolutionary for the emergence of completely new enzymes and demonstrate the enhanced evolvability expected for resurrected ancestral protein scaffolds of high stability and conformational flexibility.

OVERVIEW AND OUTLOOK FOR THE FIELD

Recent years have witnessed an explosion of interest in understanding the physicochemical features that shape the evolution of new enzyme functions, with modulation of conformational ensembles by mutations becoming of increasing importance.[4,5,21] In parallel, ancestral sequence reconstruction has allowed us to resurrect ancestral proteins in the laboratory, and to explore their stabilities and activities, highlighting that these scaffolds have a tendency to both highly thermostable,[23,24] and also can be highly conformationally flexible[66] and highly evolvable.[71] In particular, it has been demonstrated that this conformational dynamics can be modulated to allow for the emergence of new activities and specificity patterns, both through natural evolution,[22,42,44,67,72] and through artificial design of de novo active sites.[20,27,73,74] This takes the field into two new directions: (1) incorporating conformational dynamics as an important component of the design process for protein engineering studies,[21,47] and (2) exploiting ancestral proteins as starting scaffolds for artificial enzyme design.[27,52,75,76] Combining these two aspects is likely to greatly expand our repertoire of enzymes with tailored physicochemical properties, including those capable of catalyzing reactions not otherwise observed in Nature.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Knut and Alice Wallenberg Foundation (Wallenberg Academy Fellowship to S.C.L.K., Grant No. 2018.0140), the Human Frontier Science Program (to J.M.S.-R. and S.C.L.K., Grant No. RGP0041/2017), FEDER-funds/Spanish Ministry of Economy and Competitiveness (to J.M.S.-R. Grant No. BIO2015-66426-R), FEDER Funds/Spanish Ministry of Science, Innovation and Universities (to J.M.S.-R., Grant No. RTI2018-097142-B-100), and FEDER Funds/Junta de Andalucia − Consejería de Economía, Conocimiento, Empresas y Universidad (to V.A.R. Grant No. E.FQM.113.UGR18).

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