Comparison of designed and randomly generated catalysts for simple chemical reactions

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Abstract: There has been recent success in designing enzymes for simple chemical reactions using a two-step protocol. In the first step, a geometric matching algorithm is used to identify naturally occurring protein scaffolds at which predefined idealized active sites can be realized. In the second step, the residues surrounding the transition state model are optimized to increase transition state binding affinity and to bolster the primary catalytic side chains. To improve the design methodology, we investigated how the set of solutions identified by the design calculations relate to the overall set of solutions for two different chemical reactions. Using a TIM barrel scaffold in which catalytically active Kemp eliminase and retroaldolase designs were obtained previously, we carried out activity screens of random libraries made to be compositionally similar to active designs. A small number of active catalysts were found in screens of 10^3 variants for each of the two reactions, which differ from the computational designs in that they reuse charged residues already present in the native scaffold. The results suggest that computational design considerably increases the frequency of catalyst generation for active sites involving newly introduced catalytic residues, highlighting the importance of interaction cooperativity in enzyme active sites.

Keywords: computational protein design; computational enzyme design; enzyme engineering; directed evolution; enzyme; aldolase; Kemp eliminase; rational design

Introduction

Computationally designed protein catalysts for several model chemical reactions have been described recently.[1–3] The two reactions for which the most catalysts have been designed are the Kemp elimination (KE)[4,5] and a (retro)aldol condensation (RA).[6] Both sets of catalysts were designed using a two-step protocol in which locations in protein scaffolds at which ideal active sites (theozymes[7]) could be realized are first identified using RosettaMatch,[8] and the resultant sites are then further optimized for transition state binding affinity. While the two reactions are very different, the designed sites are similar in that a charged residue (lysine for RA, aspartate/glutamate for the KE) is responsible for much of the chemistry and the overall binding pocket is quite hydrophobic.

To identify the strengths and weaknesses in the design methodology and possible avenues for improvement, it would be desirable to compare the space of catalytically active solutions identified by the design methodology to the space of catalytically active solutions overall. The latter is of course unknown, but we can use random selection experiments to identify a subset of catalytically active solutions and compare their properties to the designs. We decided to

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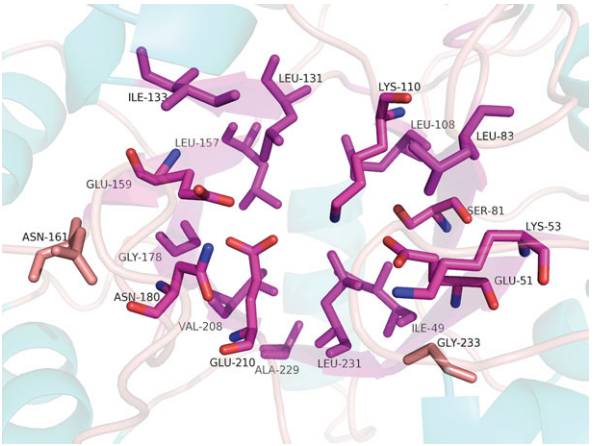

Figure 1. Positions of the 1A53 scaffold diversified in the combinatorial libraries. The native residue identities are shown.

focus our investigations on a region identified as having appropriate geometry for active site construction using RosettaMatch since it is not feasible to produce comprehensive combinatorial libraries in large numbers of locations in large numbers of scaffolds (we did not test completely random sequence libraries as the likelihood of folding alone is extremely small, and did not survey random sites in randomly selected scaffolds as the likelihood of obtaining enzyme activity also seemed likely to be very small).

We took advantage of the fact that RosettaMatch had identified active site placements for both the Kemp eliminase reaction and the retroaldol reaction in the TIM barrel scaffold 1A53, and the subsequent design had yielded active enzymes in both cases. Rather than fixing the positions of the catalytic residues, we designed randomized libraries to recapitulate the overall catalytic residue distributions and amino acid composition in the computational designs. Our previous studies had identified active catalysts with catalytic residues (Lys for the retroaldolase and Asp/Glu for Kemp eliminase) at many positions along the rim formed by the C-termini of the 8 β-strands (Fig. 1). To maximize the chance of observing Kemp or retroaldolase enzyme activity and enable the most direct comparison with the designed catalysts, we designed combinatorial libraries with these potentially catalytic amino acids along the rim, and with an overall amino acid distribution similar to that of the active computational designs. Here we describe the comparison of the properties of catalytically active variants obtained from screening the libraries with those of the computational designs.

Results

Library design

As described in the introduction, we designed combinatorial libraries biased to be enriched in KE and retroaldolase activities by favoring Asp/Glu or Lys residues at the top of the 1A53 TIM barrel where computational design efforts had previously succeeded in generating active catalysts for both reactions. In the design of the combinatorial libraries, we aimed at variants with one "catalytic" charged residue (Lys for RA, Asp, or Glu for Kemp) in an otherwise largely hydrophobic pocket. We chose the number and locations of residues to mutate to mimic typical computationally designed enzymes which differ from the starting scaffolds by 10–13 mutations distributed primarily within 12 Å of the designed active site. To maximize the recovery of folded proteins, we kept the distribution of residue types similar as much as possible to that in the original scaffold, without specifying the exact amino acid identity anywhere in the inward facing positions in the top two rungs of the barrel. Table I summarizes positions diversified in constructed libraries. At sites in the lower part of the barrel the degenerate codon NTT (which encodes large hydrophobic amino acids I, L, F, V) was used to prevent formation of openings at the bottom of the potential active site, while at sites in the middle and upper part of the barrel the NTT and GBT codons (which encodes smaller hydrophobic amino acids V, A, G) were both allowed. At positions in the upper part of the barrel, the GBT codon was used to free room for the substrate by favoring smaller hydrophobic residues upon mutation. Each position in the upper two rungs of the barrel was also spiked with a lower concentration of oligos encoding the relevant charged catalytic residues.

Library mutation rate and solubility of the proteins

Combinatorial libraries with different level of mutagenesis were constructed according to the guidelines described above using the procedure described in Material and Methods section. The more heavily mutagenized first set of libraries (libKE1 and libRA1, made with oligo ratios 1:1:1:0.2) had on average 13–17 mutations per gene. For this and the libraries described below, we sequenced ~ 10 clones to evaluate the extent of mutagenesis. The number of sequenced clones having 0, 1, 2 Asp/Glu in the KE library was 1, 8, and 1; and the number of clones carrying 0, 1, 2, 3 catalytic Lys for the RA library was 3, 2, 2, and 3, respectively. Three hundred eighty-four individual clones from the KE and 384 clones from the RA libraries were screened for corresponding enzymatic activities in crude lysate. No variants with activity higher than the background were identified for KE or for RA. Six randomly picked clones were grown on a larger scale and the His-tagged 1A53 variants were purified by His-tag affinity chromatography. The expression level of all variants was very low and none were detected by electrophoresis on denaturing acrylamide gel (SDS-PAGE) after purification. As this level of mutagenesis results in very few expressible and soluble protein variants, we decided to focus on libraries with a lower mutation level.

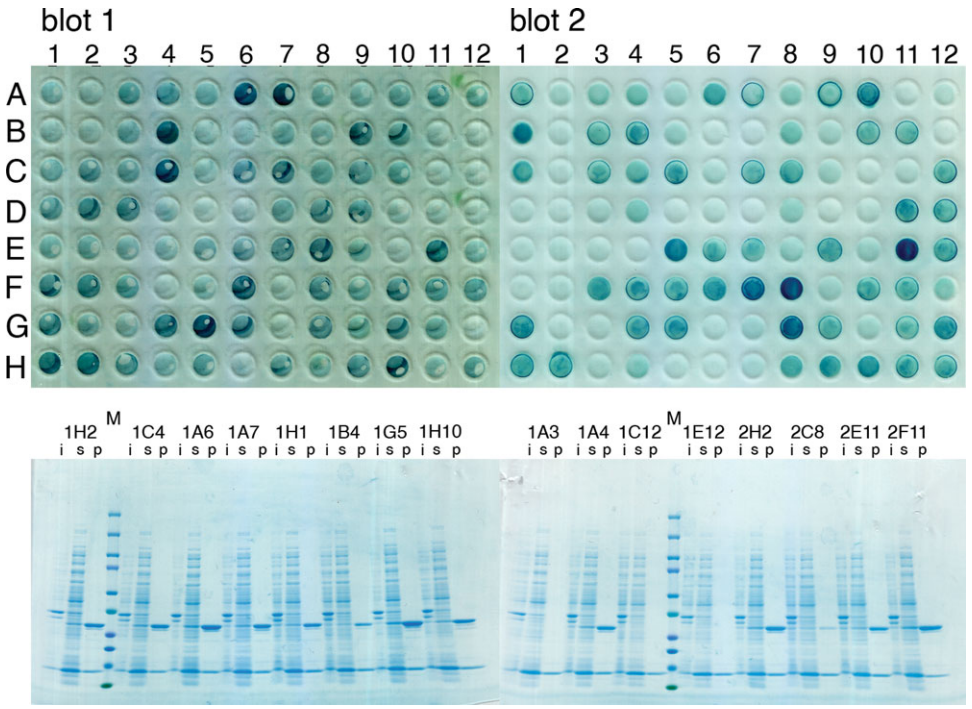

Figure 2. Assessment of protein solubility in random libraries. Top, Dot-blot; bottom SDS-PAGE. Left, low mutagenesis libraries libRA02; right, libKE02. No FLAG control is in 1B5, 1C5, 2B2, and 2C2; FLAG-1A53 is in 1F8, 1G8, 2F11, and 2G11. For each variant selected for SDS-PAGE analysis insoluble (i), soluble (s), and purified protein (p) fractions were loaded on the gel. "M" indicates position of a marker on the gels; the position of the 1A53 scaffold is indicated by the arrow.

In the second, less extensively mutagenized, set of libraries (libKE2 and libRA2, made with oligo ratios 1:1:1:2) the average mutation rate was 4.2 ± 3.4 mutation/gene for libKE2 and 4.0 ± 3.3 mutation/gene for libRA2. The number of clones having 0, 1, 2, or 3 catalytic D or E residues for the Kemp library was 4, 5, 0, and 1, and the number of clones having 0, 1, 2, and 3 catalytic lysine residues for the RA library was 5, 2, 0, and 1. The fraction of clones expressing soluble variants of the protein was investigated by measuring the amount of FLAG-tagged protein in 92 variants from each library using dot-blot analysis. As shown in Figure 2, more than 50% of variants appear to be at least partially soluble using the dot-blot assay. Analysis of selected purified variants showed a good correlation between the intensity of the spot on the dot-blot and the solubility of the variant as assayed by SDS-PAGE.

Additional libraries libRA3 (oligo ratios 1:1:1:1) and libRA4 (oligo ratios 3:0.5:0.5:1) were made to test variants with a higher frequency of catalytic Lys mutation. The average mutation rates were 7.6 ± 4.1 for libRA3 and 9.4 ± 4.1 mutation/gene for libRA4. The number of clones having 0, 1, 2, 3, 4 catalytic Lys was 3, 2, 2, 3, 1 in the libRA3; and for libRA4 the number of clones having 0, 1, 2, 3, 4, 5, 6 catalytic Lys was 2, 0, 1, 2, 3, 3, 1. The number of soluble variants in these libraries estimated by dot-blot analysis decreases significantly, going below 25%.

Kemp eliminase and retroaldolase activity measurement

Seven hundred forty-eight clones from the Kemp library libKE2 were screened for Kemp eliminase activity using a plate assay, and a number of variants with KE activity as much as 20-fold higher than the background were identified (initial computationally designed Kemp eliminases were 2- to 3-fold more active than the background under similar assay conditions). Sequencing data and corresponding activities for the library-identified variants are summarized in Table II. It is immediately evident from these data that none of the active variants contains the newly incorporated polar residues. The sequences fall in two classes. The first, exemplified by 1B6, 5C10, 3H8, 1E11, 2E3, and 5H6, involves large hydrophobic residues at positions 178 and 180 and is relatively abundant—there are evidently many combinations, which have activity. The second, exemplified by 1B8 and 5F5, involves substitutions at 49–53 and 208–210 in addition to 178–180.

None of the active variants acquired new polar residues to catalyze the eliminase reaction. As described earlier,[3] the 1A53 scaffold has a low level of Kemp eliminase activity, which can be detected using very high concentration of the purified protein (no retroaldolase activity is detected, however).[2] Since no new Asp/Glu residues were introduced, it is likely that the substitutions enhance the

Table I. *Library Design*

β-Strand	Position	Type 1	Type 2	Type 3	Type 4
1	I49	NTT	NTT	NTT	wt
	E51	NTT	GBT	GAN/AAA	wt
	K53	NTT	GBT	GBT	wt
2	S81	NTT	NTT	GAN/AAA	wt
	L83	NTT	GBT	GBT	wt
3	L108	NTT	NTT	GAN/AAA	wt
	K110	NTT	GBT	GBT	wt
4	L131	NTT	NTT	GAN/AAA	wt
	I133	NTT	GBT	GBT	wt
5	L157	NTT	NTT	NTT	wt
	E159	NTT	GBT	GAN/AAA	wt
	N161	NTT	GBT	GBT	wt
6	G178	NTT	NTT	GAN/AAA	wt
	N180	NTT	GBT	GBT	wt
7	V208	NTT	NTT	GAN/AAA	wt
	E210	NTT	GBT	GBT	wt
8	A229	NTT	NTT	NTT	wt
	L231	NTT	GBT	GAN/AAA	wt
	G233	NTT	GBT	GBT	wt

The NTT codon encodes I, L, V, or F; GBT encodes A, G or V; GAN encodes D or E; AAA encodes K; wt. residue remains unchanged. Residue numbering is that of the 1A53 scaffold.

promiscuous eliminase activity of the scaffold and that the catalytic base is one of several 1A53 scaffold Asp/Glu residues at the top of the barrel.

One thousand three hundred forty clones from retroaldolase libraries libRA2 (1116), libRA3 (180), and libRA4 (44) were screened for activity using a plate assay. Only two variants with RA activity higher than the background (~200 mAU/min, see Fig. 3) were found. Sequencing data and corresponding activities of these variants are summarized in Table III. The less active variant 6F5 (libRA3) has 12 substitutions; both native lysines were removed by K53A and K110V mutations, but the E51K substitution introduces a lysine in the immediate vicinity of the native K53. The more active 8D9 (libRA2) variant likely employs the native lysines of 1A53 scaffold to carry out the retroaldolase reaction; only four mutations are hydrophobic substitutions. In contrast, in the original enzyme design calculations, lysines were not introduced at one of the native polar residue positions in any of the top-scoring computational designs, which were chosen for experimental characterization. The 8D9 variant has level of activity comparable with previously published designs made on the same scaffold (k_{cat}/K_m(8D9)~0.7 M^{-1} s^{-1} and k_{cat}/K_m(RA34) = 0.11 ± 0.01 M^{-1} s^{-1} (b)[2]). To investigate the frequency of activities below the detection limit of the lysate assay, we tested the activity after purification of 10 randomly selected soluble variants identified by dot-blot analysis; none had activity higher than background.

Table II. *Comparison of Designed and Random Library Kemp Eliminases*

	1A53	KE59	1B6	1B8	5C10	3H8	5F5	1E11	2E3	5H6
KE, mOD/min	ND	10	68	60	28	27	17	16	13	11
49	I						L	F		
(51)	E	V	(*)	V	(*)	(*)	(*)	(*)	(*)	(*)
(53)	K			L			G			
81	S	V		A						
83	L			V						
108	L								A	
(110)	K	W							N	
131	L	S								
157	L	L								
(159)	E	V	(*)	(*)	(*)	(*)	(*)	(*)	(*)	(*)
178	G	I	I	A	F	L		F		I
180	N	S	I	G	L	L		F	V	F
208	V						A		A	I
(210)	E	A	(*)	F	(*)	(*)	V	(*)	V	V
231	L	**E**								

1A53 is the native scaffold, KE59 is a computational design, and the remainders are library variants. Polar charged positions in 1A53 core are in parenthesis. Likely catalytic polar residues are highlighted; star (*) indicates possible catalytic residues originating from 1A53 scaffold. ND "not detected". Assays were carried out in crude lysates.

Table III. *Comparison of Designed and Random Library Retroaldolases*

	1A53	RA95	RA114	RA117	6F5	8F9
RA, mAU/min	ND	90	45	300	230	4700
9	L			A		
12	V			A		
49	I				L	
(51)	E	V			**K**	
(53)	K	E			A	(*)
56	S		D			
81	S			**K**	A	
83	L	T	S		V	
108	L			A	A	
(110)	K	S	Y	S	V	(*)
112	F			A		
131	L		A	V	I	
157	L				F	
(159)	E	L	I	V	F	L
161					F	V
178	G					V
180	N	M	A	E	A	V
184	L	F	W	W		
187	L	G	G			
(210)	E	**K**	**K**	I		
211	S	L	E	A		
233	G	S				

1A53 is the native scaffold, RA95, RA114, RA117 are computational designs, 6F5 and 8F9 are library variants. Polar charged positions in 1A53 core are in parenthesis. Likely catalytic polar residues are highlighted; star (*) indicates likely catalytic residue originating from 1A53 scaffold. Positions in 1A53 that were computationally designed, but were not diversified in the combinatorial libraries are indicated in gray. ND "not detected". Assays were carried out in crude lysates.

Discussion

The goal of the work described in this article was to investigate how solutions to the enzyme design problem obtained using computational design methods for the KE and retroaldolase reactions compare to the overall solution space. We designed combinatorial libraries encoding variants with overall amino acid composition and mutation sites similar to those in the computationally designed catalysts, but did not specify the exact identity of any amino acid at any of the positions (in the computational designs, in contrast, every amino acid is specified). Screens of 1340 variants from libraries designed for the retroaldolase reaction and 748 variants for the Kemp eliminase reaction identified only one catalyst likely to utilize an introduced charged catalytic residue, and almost all the sequences with activity likely involve optimization of the low intrinsic activity of the scaffold.

For comparison, in the previous computational design experiments, activity was observed for 5 of 70 Kemp designs and 12 of 65 RA designs in our initial study, and at a much higher rate (33 out of 60) in a more recent study.[9] The comparison must take into account, however, that the activity of computationally designed variants was measured using purified proteins, where the detection limit is lower. Of the Kemp designs, made on 1A53 scaffold, 1 of 3 was detectable in the plate assay and of the RA designs, made on the same scaffold, 1 out of 15 (Fig. 3).

Our results raise two complementary questions. First, why did the random combinatorial approach not yield new locations for the catalytic residues? Second, why did the previously tested computational designs not repurpose native scaffold charged residues for catalysis?

The answer to the first question may be that catalytic groups must be positioned and activated by supporting residues, and it is much more likely to generate a catalyst randomly by repurposing a charged residue already buttressed in the native scaffold than by simultaneously introducing new catalytic residues and attendant supporting residues in new positions. Indeed, the charged residues in the 1A53 scaffold are aligned and buttressed by an extensive hydrogen bond network with other polar residues in the scaffold core (Fig. 4). The power of the computational design protocol is that such constellations of catalytic and supporting residues can be introduced simultaneously with apparently much higher than background frequency of generating active catalysts. Creation of such a network in a new location would require many simultaneous modifications and therefore, is a very infrequent event, less accessible to the "randomize-and-screen" approach compared to solutions based on adjustments of preexisting residues—the solutions found

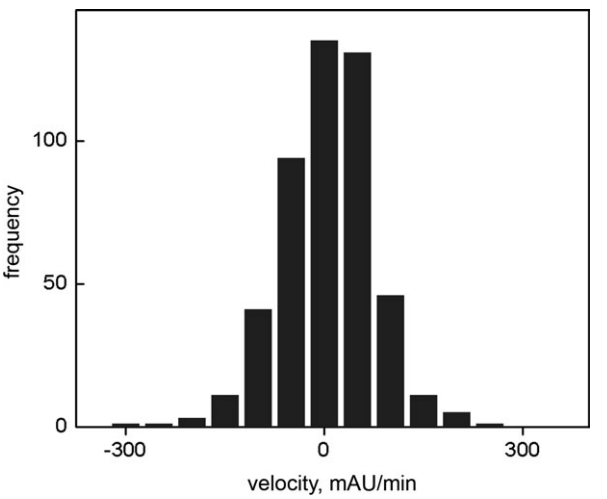

Figure 3. Distribution of apparent retroaldolase activity in the libRA2 library. 696 clones from the 1A53 low mutagenesis libRA2 library were assayed for retroaldolase activity in crude lysate using a 96 well plate assay. The overall Gaussian shape suggests that the signals reflect noise in the assay; this was confirmed by purification of three variants from the rightmost part of distribution with apparent activities $\geq$200 mAU/min — all three were found to be inactive. The activity of the 8F9 variant from libRA2 is several standard deviations above this noise.

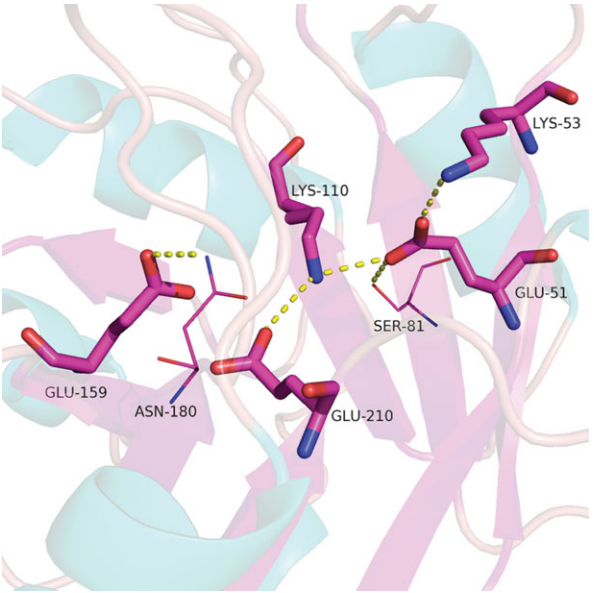

Figure 4. Native 1A53 polar residues form a network of hydrogen bonds, which is not preserved in the computational design calculations.

in the current study. This parallels natural protein evolution, which generally reuses previously existing catalytic motifs to solve newly arising challenges rather than inventing new ones. Computational design, while very far from perfect, is able to introduce many residues simultaneously and can identify active sites with new catalytic residues despite the intrinsic low frequency of such solutions.[10]

To investigate the second question, we carried out computational design calculations for the RA reaction using the previously published theozyme description (Motif IV in[2]). An inability of the computational design protocol to place the theozyme into a prospective active site utilizing native scaffold charged residues would suggest an overly restrictive definition of the active site. However we observed no significant differences in number and ranking of designs utilizing native scaffold charged residues positions as opposed to positions used in first round of RA designs. Comparison of sequence distance distributions (Supporting Information Table II) suggests that the library derived RA variants lie within the sequence space sampled by the design procedure; one of the limitations of the current methodology evidently is not being able to properly rank the large number of potential catalysts identified in the design calculations.

The characterization of the distribution of activities of $\sim 10^3$ variants described here allows assessment of our previous enzyme design results by providing an albeit imperfect negative control. We obtained 8 out of 748 variants for Kemp and 2 out of 1340 for RA. For comparison, 15 and 3 computationally designed variants of 1A53 were experimentally tested for RAse and KEase activity and 4 and 1 catalytically active variants were identified. This suggests a 10-fold or greater enrichment of the active pool using computational design. However, it must be emphasized that the libraries we screened were far from random. First, we chose for the libraries the best overall site and scaffold (the top rungs of the 1A53 scaffold) found in the RosettaMatch active site search calculations in our previous work. Second, the amino acid composition was chosen to match as closely as possible that of the active designs we identified earlier. Both of these choices are likely to significantly alter the frequency of active clones in the libraries. Without direct experimental comparison to more completely random libraries it is not possible to rigorously quantify the resultant changes in active clone frequency, but it is likely that the active clone rates in the biased libraries studied here are significantly higher than in completely unbiased libraries at a randomly selected site in a randomly selected scaffold.

The solutions to the catalyst search problem obtained by the combinatorial approach under almost identical conditions (same scaffold, similar set of modifications allowed) differ from those obtained by design in almost exclusively re-utilizing native charged residues for the key steps in catalysis. This likely reflects the importance of catalytic residue buttressing, and is of practical importance for generating new activities since the number of distinct non-redundant starting points is a determinant of success.

The ultimate goal of computational enzyme design is to be able to generate highly active catalysts at very high frequency. The current state of the art is still far from this goal: as highlighted by the results reported here, protein design is still a science of probability. Increasing the rate of successful designs on less activated substrates is a primary goal for computational methods development.

Materials and Methods

Library construction

The indole-glycerol phosphate synthase (IGPS) gene corresponding to the sequence of 1A53[11] was synthesized by Genescript and cloned into the NdeI/XhoI sites of a modified pET29b vector containing sequence coding for a N-terminal FLAG tag and a C-terminal His-tag. The resulting plasmid, called pET29-FLAG-1A53, was used to prepare single stranded template for library construction by modified Kunkel mutagenesis protocol. Mutagenic and wild-type oligos were obtained from IDT. Four types of oligos were synthesized for each of the target locations corresponding to eight β-strands of IGPS. Diversified positions, their identities in IGPS, and

the variability introduced at each position by each of four types of oligos are summarized in the Table I.

The library was assembled by annealing the template DNA with a mixture of 5′ phosphorylated mutagenic and wild-type oligos. The frequency of mutations was controlled by changing the ratio between mutagenic and wild-type oligos annealing to the same fragment of the gene. Similarly, the frequency of obtaining Lys or Asp/Glu at desired positions was controlled by changing the ratio between oligos of Type 3 and Types 1 and 2. Two sets of libraries were generated for both Kemp eliminases and retroaldolases. In the first set (libKE1, libRA1), the ratio of type 1:2:3:4 oligos was 1:1:1:0.2, and in the second set (libKE2, libRA2), the ratio was 1:1:1:2. Additional libraries (libRA3, libRA4) with ratios 1:1:1:1 and 3:0.5:0.5:1 were made for retroaldolases only. Constructed libraries were characterized by sequencing of randomly selected clones.

Solubility characterization by dot-blot

The libraries were transformed into BL21 (DE3)* cells and plated on LB agar plates supplemented with 50 μg/mL of kanamycin. Individual colonies were picked and grown overnight at 37°C with vigorous shaking in 0.5 mL LB liquid medium containing antibiotic in 96 deep-well plates. The starter cultures were diluted 25-fold into 0.5 mL of fresh LB medium with antibiotic, grown for 2 h at 37°C with vigorous shaking, induced with 1 m*M* IPTG and grown for additional 20 h at 18°C. Harvested cells were lysed by several cycles of freezing and thawing and resuspended in 25 m*M* TrisHCl buffer pH 7.25 containing 100 m*M* NaCl. Insoluble material was removed by centrifugation at 3000*g* for 15 min and soluble extract was blotted onto nitrocellulose membrane using Minifold I dot-blot apparatus (Whatman). The amount of soluble FLAG tagged protein was estimated using monoclonal AntiFLAG M2-peroxidase (HRP) antibody (Sigma, A8592) and Novex HRP Chromogenic substrate (Invitrogen, WP20004). The reliability of the dot-blot assay was tested by purifying soluble proteins from selected clones using His-tag affinity column and running SDS-PAGE.

Kemp elimination and retroaldolase activity assay

The KE or RA activity of individual clones was measured in lysate or after purification of selected variants on His-tag affinity column. Enzymatic activity of the purified proteins was measured in a 25 m*M* HEPES buffer pH 7.25 containing 100 m*M* NaCl. KE activity was measured at 250 μ*M* of 5-nitro benzisoxazole substrate by monitoring change in optical density at 380 nm.[3] RA activity was measured at 100 μ*M* of Methodol substrate by monitoring change in fluorescence at Ex 330 nm/Em 452 nm.[2,12] For crude lysate assays, pellets of cells from 0.5 mL cultures were subject to several cycles of freezing and thawing, then pellets were resuspended in 250 μL of the assay buffer and 100 μL of the clarified lysate was mixed with 100 μL of appropriate substrate solution to record enzymatic activity.

Computational design using Motif IV retroaldolase theozyme

Previously published parameters for Motif IV retroaldolase theozyme[2] were used to set up computational design calculations. Set of positions for catalytic Lys placement was limited to positions observed in RA active variants from combinatorial library. Sequences of 1A53 scaffold, library variants 6F5 and 8F9, published designs, newly produced designs were aligned and distances calculated using Jukes-Cantor genetic distance model using Geneious software package.[13] Full sequences of previously characterized designs and variants from combinatorial library aligned using sequence of 1A53 scaffold as a reference are given in Supporting Information Table I.

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