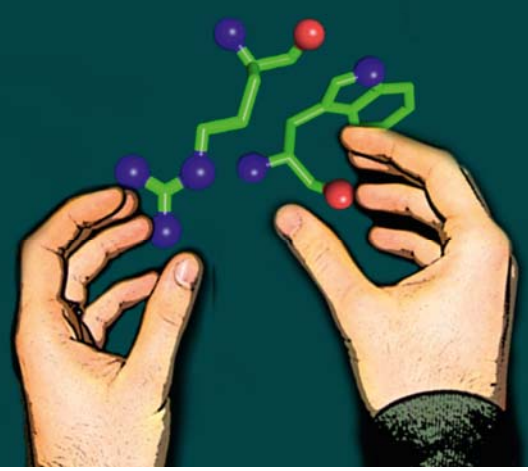
Trends Biotechnol. July 2010 Vol. 28 No. 7, pp. 333–390 ISSN 0167-7799

Trends in Biotechnology

Building enzymes from scratch



Cell PRESS

Review

De novo enzymes: from computational design to mRNA display

Misha V. Golynskiy and Burckhard Seelig

Department of Biochemistry, Molecular Biology and Biophysics & BioTechnology Institute, University of Minnesota, 1479 Gortner Ave., St. Paul, MN 55108, USA

Enzymes offer cheap, environmentally responsible and highly efficient alternatives to chemical catalysts. The past two decades have seen a significant rise in the use of enzymes in industrial settings. Although many natural enzymes have been modified through protein engineering to better suit practical applications, these approaches are often insufficient. A key goal of enzyme engineers is to build enzymes *de novo* – or, 'from scratch'. To date, several technologies have been developed to achieve this goal: namely, computational design, catalytic antibodies and mRNA display. These methods rely on different principles, trading off rational protein design against an entirely combinatorial approach of directed evolution of vast protein libraries. The aim of this article is to review and compare these methods and their potential for generating truly *de novo* biocatalysts.

Enzymes in industry and the case for *de novo* enzymes

The use of enzymes for the production of food, textiles, chemicals, pharmaceuticals and biofuel has been well established [1]. Expanding the applications of enzymes will be instrumental in enabling processes for a more sustainable future and for meeting commercial needs. The vast diversity of naturally occurring enzymes is a valuable source of biocatalysts as they are applied to virtually all areas of biotechnology. Millions of years of evolution have created biocatalysts that excel in their ability to catalyze an enormous variety of chemical reactions with high rate enhancements and excellent chemo-, regio- and stereoselectivities. While the 'survival of the fittest' principle has guided natural selection, the fitness of enzymes in their natural environment does not necessarily translate into efficiency in industrial processes. Common factors that might limit the use of enzymes in industry are low catalytic activity, substrate specificity or protein stability, as well as inhibition by substrate or product and high protein production cost. Therefore, a wide variety of technologies is routinely used to tailor naturally occurring enzymes to specific applications (Box 1).

However, synthetic chemistry has produced a wealth of artificial compounds and novel reactions for which no natural enzymes have been found. To facilitate the use of biocatalysis for these cases enzymes have to be created *de novo*. Here, we define enzymes as being '*de novo*' if they are not based on a related parent protein with regard to substrate or reaction mechanism. Generating enzymes from scratch is one of the major challenges in enzyme engineering. The number of reported examples is limited. The creation of *de novo* enzymes has been accomplished by several different means: (i) entirely knowledge-driven by *in silico* rational design; (ii) partially knowledge-driven by utilizing an understanding of a reaction mechanism and the diversity of the immune system through catalytic antibodies; and (iii) entirely combinatorial by empirically searching vast protein libraries using mRNA display. This review will discuss and compare these individual approaches with an emphasis on the mRNA display technology because computational design and catalytic antibodies have been reviewed more extensively in the past [2–5].

Computational design

Linus Pauling hypothesized that enzyme catalysis relies on the ability of an enzyme to stabilize the transition state of a reaction, thereby lowering the activation energy [6,7]. This principle implies that all proteins capable of binding to the transition state could function as enzymes. Pauling's concept forms the basis of a computational approach that has recently yielded several *de novo* enzymes [2,8,9]. The first step in this approach is the generation of an *in silico* model of the transition state. Next, individual amino acids are positioned around it to create an active site that stabilizes the transition state in a computational process that uses quantum mechanical calculations. Various protein scaffolds are evaluated for their ability to accommodate the *de novo* active site using molecular mechanics modeling software such as RosettaMatch [8–10]. These scaffolds are generated by taking a high resolution structure of different natural proteins and virtually removing the amino acid side chains from the ligand binding pocket. In the final step, the remaining amino acid side chains in the pocket are computationally redesigned for high substrate specificity and tight transition state binding. This methodology has been successfully used to generate *de novo* enzymes capable of a Kemp elimination and a retro-aldol reaction [8,9]. Although the retro-aldolase exhibits low stereoselectivity, in theory, the computational design of highly stereoselective enzymes should be feasible [11].

In another case of computational design, Faiella *et al.* computationally designed not only the active site, but also calculated the scaffold to accommodate it from first principles [12]. To generate a *de novo* metalloenzyme, a metal binding site was installed at the interface of a dimeric

Corresponding author: Seelig, B. (seelig@umn.edu).

 0167-7799/$ – see front matter doi:10.1016/j.tibtech.2010.04.003 Trends in Biotechnology 28 (2010) 340–345

Box 1. Engineering natural enzymes by directed evolution and rational redesign

The most common engineering goals are thermostability, catalytic activity, substrate specificity or stereoselectivity [1,28–30]. Two general strategies are used to optimize natural enzymes: directed evolution and rational redesign. Usually, a combination of both methods yields the highest rate of success [31,32].

Directed evolution

Directed evolution mimics the process of Darwinian evolution. In the initial step, genetic diversity is generated by creating a collection of mutants, which are then subjected to a screening or selection process to isolate the variants with beneficial traits. This process of diversification and selection is repeated until proteins with the desired properties are obtained. A multitude of techniques has been developed to generate genetic diversity. Error-prone PCR amplification can introduce random mutations, and the recombination of genes can generate permutations of mutations [33,34]. The likelihood of finding the desired mutant increases with the number of variants that are tested. When searching for desired properties, assay throughput is usually the bottleneck, which can range from 10^2–10^6 mutants for screening methods to 10^9–10^{13} for selection technologies.

Rational redesign

Rational redesign of proteins is a knowledge-guided process. Therefore, this approach is only used if detailed structural information on the parental enzyme is available. Ideally, a crystal structure of the enzyme in complex with its substrate or reaction product is available in conjunction with a detailed understanding of the reaction mechanism. Under these circumstances, crucial amino acid residues in the enzyme's active site can be targeted by site-directed mutagenesis.

Semi-rational redesign

If the active site is known but detailed understanding of the contribution of individual amino acids is lacking, semi-rational redesign can be a successful alternative. Here, several or all residues that form the active site are varied by saturation mutagenesis. Depending on how many mutants can be screened, mutagenesis of the second shell residues neighboring the active site can also be considered. In this case, the protein engineer quickly encounters the common bottleneck of screening capacity. To alleviate this problem, several clever procedures have been developed to reduce the library size by increasing the probability of beneficial mutants in a given library [35,36].

helix-turn-helix motif *in silico*. Next, the substrate-binding pocket was incorporated into the scaffold. The resulting di-iron metalloenzyme exhibited phenol-oxidase activity [12].

The computational design process typically yields a small number of enzyme candidates that then have to be evaluated experimentally to identify the functional enzymes. In recent work, the *in silico* process narrowed down 10^{18} protein variants to $<10^2$ enzyme candidates for empirical testing [9]. Moreover, computational approaches have also generated *de novo* enzymes that are capable of catalyzing reactions that proceed through multiple transition states, thereby widening the scope of this method [9]. In addition to providing candidate *de novo* enzymes, rational design also allows us to test our understanding of structure–function relations.

Catalytic antibodies

In contrast to the most recent work on *de novo* enzymes by computational design and mRNA display, the first reports of catalytic antibodies (abzymes) date back more than 20 years [13,14]. Pauling's concept of promoting catalysis through stabilization of the transition state also forms the foundation for generating catalytic antibodies. Based on Pauling's idea, Jencks proposed that an antibody capable of binding to, and thus stabilizing, a transition state analog (TSA) could function as an enzyme [6,7,15]. The starting point in this approach is the chemical synthesis of a TSA that closely mimics the transition state for the reaction of interest. Here, the challenge lies in resembling the transition state as closely as possible with an analog that is stable in solution. Antibodies are either raised against the TSA, or TSA-binders are selected *in vitro* from phage-displayed synthetic antibody libraries [5,16]. Catalytic antibodies have also been generated using mechanism-based inhibitors as an alternative to raising TSA binders [17]. Such approaches have resulted in catalytic antibodies suitable for performing a wide range of different chemistries (e.g. acyl transfer, Diels-Alder and cyclization reactions) [5,16].

Unfortunately, natural enzymes routinely outperform abzymes with regard to catalytic efficiency. Furthermore, the antibody protein fold is not suited for industrial processes owing to its relative low stability and high production cost. Therefore, despite the successful generation of abzymes for many different reactions, catalytic antibodies are not utilized for industrial applications. Although abzymes might be unfit for industrial catalysis, they appear promising for *in vivo* therapeutic applications owing to a low propensity to elicit a detrimental immune response. Potential applications of abzymes include antibody-directed abzyme prodrug therapy (ADAPT) and treatment of addiction via abzyme-mediated breakdown of small molecules [4,5].

mRNA display

The two methods discussed above are based on the concept of catalysis through stabilization of the transition state. These methods require a substantial amount of prior mechanistic and structural information and are therefore limited to cases where this information is readily available. In contrast to these knowledge-driven approaches, the mRNA display selection strategy has been recently used to isolate *de novo* enzymes by solely relying on the functional diversity provided by vast libraries of randomized proteins [18].

mRNA display is an *in vitro* selection method that can interrogate highly complex protein libraries [19,20]. This technique has been used in the past to generate proteins that bind to a chosen target (Box 2). The mRNA display method has been extended to the selection of enzymes [18]. The key feature of the selection scheme is linking the substrate of the reaction to the mRNA-displayed proteins. This link is formed by reverse transcription of the mRNA into cDNA with an oligonucleotide primer that is attached to the substrate (Figure 1). The proteins that catalyze the reaction convert their substrate to the product, which is then used to isolate the cDNAs that encode active enzymes. These cDNAs are amplified by PCR and either sequenced directly or used for the next round of selection. Alternatively, the cDNAs are mutagenized to further evolve the enzymes. Because product formation is the only requirement for the enzyme to be selected, understanding the mechanism of the reaction or the protein structure is unnecessary.

Box 2. mRNA display

mRNA display is a technique for the *in vitro* selection and evolution of proteins. This method generates proteins that are covalently attached to their encoding mRNA, effectively rendering each protein directly amplifiable [19,20]. This feature allows for the selection of functional proteins from very large libraries of more than 10^{12} different mutants in a single test tube – well beyond the limits of screening technologies and most *in vivo* and *in vitro* selection methods (e.g. cell surface display or phage display, respectively) [37–39]. Because mRNA display can select from libraries several orders of magnitude larger than most other methods, it could have a particular advantage in the search for *de novo* proteins that are presumably rare in a given library.

In mRNA display, a DNA library, either chemically synthesized or of genomic origin, is transcribed into mRNA [40,41]. The key component in the mRNA display process is the antibiotic puromycin. This molecule mimics a charged tRNA and is therefore incorporated into the growing polypeptide chain by the ribosome. A DNA oligonucleotide containing puromycin is attached to the 3′-end of each mRNA in the library by enzymatic ligation or photo crosslinking [42]. During the subsequent *in vitro* translation, the ribosome synthesizes the protein until it comes to a halt at the RNA–DNA junction. Before the ribosome dissociates from the complex, it covalently attaches the puromycin to the protein chain, thereby generating the mRNA-displayed protein. The resulting mRNA–protein fusions can then be subjected to an appropriate selection scheme to isolate binding proteins or enzymes. Reverse transcription of the selected mRNA portion yields cDNA that is either sequenced directly or used as input for further rounds of selection and evolution.

mRNA display has been extensively used to isolate binding proteins and peptides [21,43–46]. In addition, unnatural amino acids have been readily incorporated into mRNA-displayed peptides, further increasing the chemical library complexity [47,48].

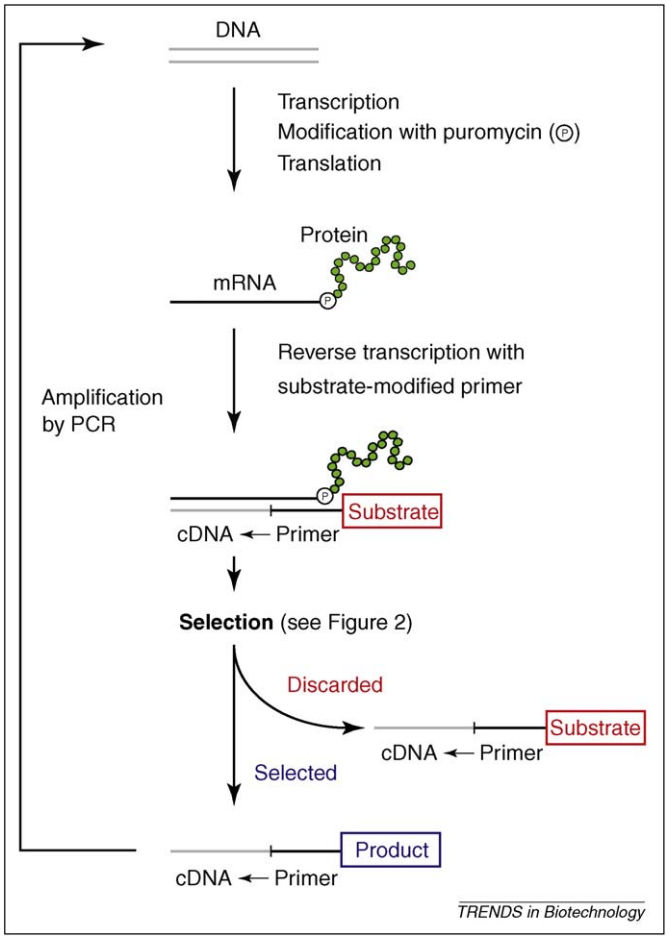


Figure 1. General scheme for enzyme selection by mRNA display. A synthetic DNA library is transcribed into mRNA and modified with puromycin. During the subsequent *in vitro* translation, this modification creates a covalent link between each protein and its encoding mRNA. The library of mRNA-displayed proteins is reverse transcribed with a substrate-modified primer, thereby attaching the substrate to the cDNA/RNA/protein complex. Proteins that catalyze the reaction of the substrate modify their encoding cDNA with the product. Selected cDNA sequences are amplified by PCR and used as input for the next round of selection.

This general selection scheme has recently been used for the direct selection of *de novo* enzymes for bond-forming reactions: [A]+[B]⇒[A–B] (Figure 2a) [18]. Here, substrate [A] is first attached to the DNA/RNA/protein complex by reverse transcription. Then, substrate [B] is added, which carries a selectable anchor group. Those proteins that catalyze the bond formation between [A] and [B] connect the anchor group to their encoding cDNA. The cDNA is isolated by immobilization on a solid support. Using this approach, *de novo* RNA ligase enzymes were isolated from a naïve library of 4×10^{12} proteins. The library was based on a non-catalytic Zn-finger scaffold with two randomized loops of 21 random amino acids [21].

To date, mRNA display has only been used for the isolation of bond-forming enzymes. However, the general selection scheme can easily be adapted to identify enzymes for bond-breaking reactions or other transformations (Figure 2). In each case, the reaction substrate has to be attached to the DNA/RNA/protein complex through an appropriate reverse transcription primer. For example, to select for bond-breaking enzymes, the reverse transcription primer links the cDNA to an anchor group via the substrate. Proteins that cleave the substrate release the anchor group from the cDNA, which remains in solution, whereas cDNA that encodes inactive proteins is immobilized (Figure 2b). Enzymes catalyzing chemical transformations (other than bond forming or bond breaking) can be isolated via a product-specific capture agent (e.g. antibody) (Figure 2c). This approach could prove particularly useful for generating stereoselective enzymes if, for example, an antibody is used that specifically binds to the desired product enantiomer only. In addition, mRNA display is performed *in vitro*, allowing the enzymes to be evolved simultaneously for a range of parameters, such as thermostability, pH and tolerance to solvents, inhibitors or proteases. Although mRNA display is the only *in vitro* selection method that has successfully produced a *de novo* enzyme, alternative methods that can select from equally large protein libraries, such as ribosome display, have been reported [22]. These methods can, in principle, be used in a similar manner.

Comparison of methodologies

Whereas the methods discussed in this review share the common goal of creating enzymes *de novo*, the approaches used by the individual techniques differ significantly (Table 1). One key difference is the amount of prerequisite structural and mechanistic knowledge. The first method, *in silico* enzyme design, requires detailed mechanistic information to build the transition state model and, subsequently, the stabilizing active site. This method also relies on a high-resolution crystal structure to calculate

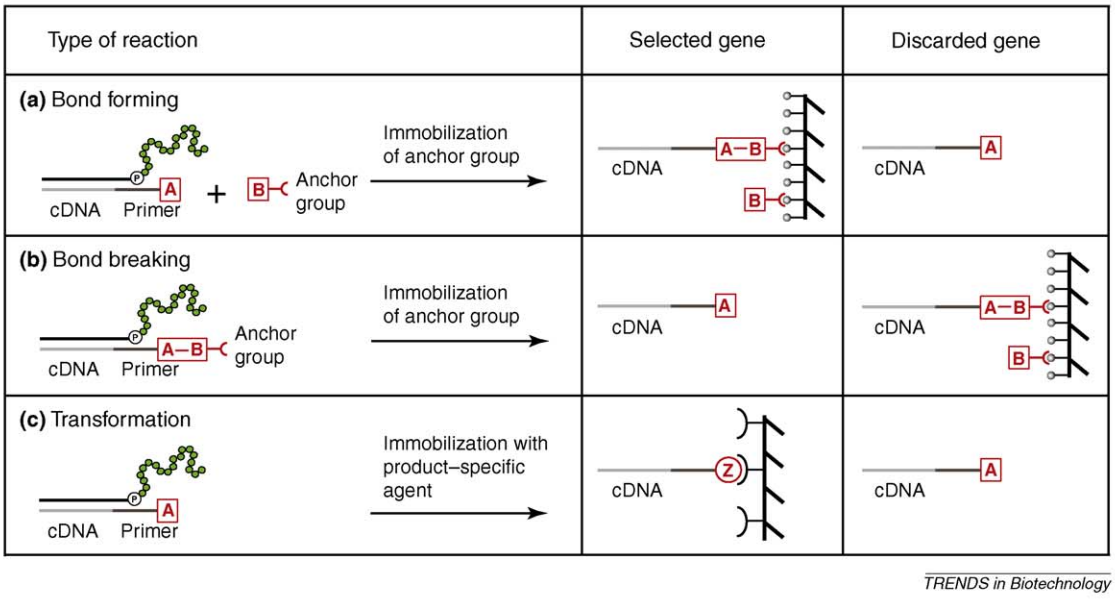


Figure 2. Selection strategies to isolate enzymes by mRNA display. **(a)** Bond formation reaction. Substrate [A] is attached to the complex of cDNA and mRNA-displayed protein via the reverse transcription primer. Substrate [B] carries an anchor group, allowing for the immobilization of cDNAs that encode active enzymes. **(b)** Bond breaking reaction. The cDNA is modified with the anchor group via the substrate to be cleaved [A–B]. Genes encoding active enzymes cleave off their anchor group and remain in solution whereas inactive genes are removed by immobilization **(c)** Transformation reaction. An agent (e.g. antibody) that specifically binds to the product (Z) is used to isolate genes encoding active enzymes.

a set of protein scaffolds that can accommodate the active site model. The generation of abzymes also necessitates a thorough understanding of the reaction mechanism to devise the ideal TSA.

In contrast to these knowledge-driven methods, mRNA display does not need any mechanistic information. The only requirement for mRNA display is an appropriate selection scheme for the reaction of interest (Figure 2). In such a scheme, product formation is the only selection criterion regardless of the particular reaction mechanism or protein structure. Although mRNA display can take advantage of structural knowledge for the initial library design, this information is dispensable for a selection. For example, an entirely unstructured random library of mRNA-displayed proteins was used to isolate several artificial ATP-binding proteins [23]. This unique case demonstrates the power of mRNA display to explore the uncharted regions of protein sequence space. Currently, research is underway to isolate enzymes from the same randomized library to investigate the possible origin of protein enzymes from random peptides.

Library size, or the number of proteins that need to be experimentally interrogated, is another significant difference between the methods presented in this review. There is an inverse correlation between library size and amount of prerequisite knowledge required to successfully generate a *de novo* enzyme. For example, computational design evaluates a very large number of possible candidates *in silico* and therefore only a small number of the best candidates ($<10^2$) are assessed in the laboratory experimentally. Because these proteins are typically expressed and tested individually, catalytic efficiency can be investigated

Table 1. Comparison of methods for *de novo* enzyme generation

	 **Computational design**	 **Catalytic antibodies**	 **mRNA display**
Principle	Transition state stabilization	Transition state stabilization	Product formation
Prerequisite	• Reaction mechanism • Crystal structure of scaffolds	• Reaction mechanism • Transition state analogue (TSA)	• Selection scheme
Key steps	• Model transition state and active site • Fit active site into scaffold • Test candidates experimentally	• Synthesize TSA • Raise antibodies against TSA • Screen antibodies for catalysis	• Prepare DNA library • Devise selection scheme • Select enzymes
Experimental library size	$<10^2$	10^7–10^9	$>10^{12}$

Table 2. Catalytic parameters of enzymes compared by their origin

	Computational design[a]	Catalytic antibodies	mRNA display[b]	Nature
Turnover, k_{cat} (min^{-1})	1.7×10^{1} (10^{-2}–10^{1})	2.2×10^{2} (10^{-2}–10^{2})	1.1×10^{-2}	5×10^{6} (10^{3}–10^{4})
Catalytic efficiency, k_{cat}/K_M ($M^{-1}s^{-1}$)	1.6×10^{2} (10^{0}–10^{2})	4.5×10^{5} (10^{2}–10^{4})	Not available	7×10^{9} (10^{6}–10^{8})
Rate enhancement, k_{cat}/k_{uncat}	2.5×10^{5} (10^{4}–10^{5})	2.3×10^{8} (10^{3}–10^{5})	$>10^{6}$	7×10^{19} (10^{6}–10^{12})
Refs.	[8,9,12]	[16,49–51]	[18]	[1,16,50,52,53]

Numbers represent maximum values reported. Numbers in parentheses represent ranges of values that are commonly observed.
[a]Ranges are based on the best enzyme from each of the three computational design studies discussed in this review, but do not include variants that were subsequently optimized by directed evolution.
[b]Values represent the only *de novo* enzyme created by mRNA display to date.

simultaneously along with other parameters. In contrast to the individual screening of enzymes designed *in silico*, abzymes and *in vitro*-displayed enzymes are isolated via a selection protocol, which is necessitated by the large library sizes that these methods use. For example, today's biotechnologist routinely isolates target-binding antibodies from libraries of 10^{7}–10^{9} variants. mRNA display selections sample even larger libraries ($>10^{12}$) to increase the odds of finding a rare functional protein in the naïve starting library. Searching large libraries substitutes the need for structural and mechanistic information input.

Lessons learned

Several laboratories have now demonstrated the ability to generate *de novo* catalysts. This success is regarded as a major achievement in the field of enzyme engineering. However, the comparison of rate enhancements reveals that natural enzymes generally outperform *de novo* enzymes (Table 2).

The catalytic activity of an enzyme is dependent on multiple parameters such as substrate affinity, product release and turnover. Furthermore, properties such as stability, structural dynamics and accessibility of the active site to substrate and solvent all affect enzyme performance. Unfortunately, the methods for generating *de novo* enzymes are generally limited to evaluating an enzyme by a small subset of parameters. This limitation imposes a method-specific bias onto the enzyme. For example, *in silico* design relies on the ability of an active site model to stabilize the appropriate transition state; this computation does not focus on either the substrate or product affinities. Furthermore, the sampling of potentially beneficial mutations outside the active site is clearly important for catalytic performance [24,25], protein dynamics ('breathing') and enzyme stability. Yet, this task is still largely beyond the capabilities of existing computational methods. The only parameter that dominates the generation of catalytic antibodies is the binding to the TSA. This process can result in antibodies that bind – but do not catalyze – the reaction. Alternatively, abzymes might exhibit slow product release if the transition state is similar to the reaction product. In addition, as illustrated with computational design, many diverse scaffolds are capable of supporting identical active sites and some might prove better suited than others [8,9]. Because abzymes are classically limited to a single scaffold, they could possess some inherently inferior features relative to natural enzymes. Finally, in the case of mRNA display, each new reaction requires the respective substrate to be chemically linked to the reverse transcription primer, which might be challenging for particularly small substrates. During catalysis, this linkage might interfere with some potential enzyme–substrate interactions. In addition, the attachment of the substrate to the mRNA-displayed protein translates into a high local substrate concentration. The enzymes are therefore not optimized for high substrate affinity. Furthermore, because a single product formation event leads the selection of a protein, the enzymes are not subjected to selective pressure for multiple turnover. Nevertheless, the enzyme selected by Seelig and Szostak did show multiple turnover [18].

Despite these limitations, all of the methods for *de novo* enzyme creation are capable of producing useful starting points for further optimization. Although current *de novo* enzymes are inferior to natural enzymes (Table 2), their catalytic efficiencies can be significantly improved via additional rounds of directed evolution. For example, Rothlisberger *et al.* used seven rounds of random mutagenesis and shuffling to improve a computationally designed enzyme more than 200-fold [8,26]. Using a combination of methods, they successfully optimized amino acid residues outside of the enzyme's active site and thus overcame an inherent limitation of their method. A similar approach of combining complementary methods is likely to improve enzymes generated by mRNA display. For example, directed evolution using *in vitro* compartmentalization (IVC) could further optimize a *de novo* enzyme [27]. This technique can directly select for properties such as substrate affinity and turnover. Although IVC is limited to protein libraries several orders of magnitude smaller than those used by mRNA display, the optimization of an existing enzymatic activity is much simpler than the creation of a novel activity.

De novo enzymes reported to date have undergone 20 selection cycles at most – a number that pales in comparison to the evolutionary process of natural enzymes. Taken into account that bacterial species can double every 20 minutes, one half of a day of bacterial growth under selective pressure would be equivalent to 36 selection cycles. Considering the trajectory of random mutagenesis and DNA recombination used by nature to improve enzymes, *de novo* enzymes could achieve similar catalytic efficiencies if they are subjected to a sufficient number of additional cycles of selective pressure.

Conclusions and perspective

The *de novo* enzymes discussed above show that we have reached a significant milestone in creating tailored catalysts. Future efforts will focus on developing *de novo* enzymes with higher catalytic efficiencies by improving current methods and combining several existing methods.

Although computational methods will always rely on *a priori* knowledge, these methods will improve with increasing processing power and the use of more sophisticated algorithms that include multiple aspects of catalysis such as substrate affinity, product inhibition and long-range interactions of amino acid residues. By contrast, mRNA display requires no *a priori* mechanistic or structural information. With little adaptation, this method can be expanded from bond-forming reactions to bond-breaking and other modification reactions. The new enzymes selected by mRNA display can be further optimized with established complementary methods of directed evolution. In summary, combining these methods of directed evolution and rational design holds great potential for the enzyme engineers of tomorrow.

Acknowledgments

We thank Zohar Sachs, Romas J. Kazlauskas, Claudia Schmidt-Dannert and Ethan T. Johnson for the critical reading of the manuscript. B.S. is supported in part by the NASA Astrobiology Institute.

References

1 Aehle, W. (2007) *Enzymes in Industry*, Wiley-VCH Verlag GmbH & Co. KGaA
2 Damborsky, J. and Brezovsky, J. (2009) Computational tools for designing and engineering biocatalysts. *Curr. Opin. Chem. Biol.* 13, 26–34
3 Marti, S. *et al.* (2008) Computational design of biological catalysts. *Chem. Soc. Rev.* 37, 2634–2643
4 Alexey, B., Jr *et al.* (2009) Catalytic antibodies: balancing between Dr. Jekyll and Mr. Hyde. *BioEssays* 31, 1161–1171
5 Wójcik, T. and Kiec-Kononowicz, K. (2008) Catalytic activity of certain antibodies as a potential tool for drug synthesis and for directed prodrug therapies. *Curr. Med. Chem.* 15, 1606–1615
6 Pauling, L. (1948) Nature of forces between large molecules of biological interest. *Nature* 161, 707–709
7 Pauling, L. (1946) Molecular architecture and biological reactions. *Chem. Eng. News* 24, 1375–1377
8 Rothlisberger, D. *et al.* (2008) Kemp elimination catalysts by computational enzyme design. *Nature* 453, 190–195
9 Jiang, L. *et al.* (2008) *De novo* computational design of retro-aldol enzymes. *Science* 319, 1387–1391
10 Zanghellini, A. *et al.* (2006) New algorithms and an in silico benchmark for computational enzyme design. *Protein Sci.* 15, 2785–2794
11 Lassila, J. *et al.* (2010) Origins of catalysis by computationally designed retroaldolase enzymes. *Proc. Natl. Acad. Sci. U. S. A.* 107, 4937–4942
12 Faiella, M. *et al.* (2009) An artificial di-iron oxo-protein with phenol oxidase activity. *Nat. Chem. Biol.* 5, 882–884
13 Tramontano, A. *et al.* (1986) Catalytic antibodies. *Science* 234, 1566–1570
14 Pollack, S.J. *et al.* (1986) Selective chemical catalysis by an antibody. *Science* 234, 1570–1573
15 Jencks, W.P. (1969) *Catalysis in chemistry and enzymology*, McGraw-Hill
16 Xu, Y. *et al.* (2004) Catalytic antibodies: hapten design strategies and screening methods. *Bioorg. Med. Chem.* 12, 5247–5268
17 Cesaro-Tadic, S. *et al.* (2003) Turnover-based *in vitro* selection and evolution of biocatalysts from a fully synthetic antibody library. *Nat. Biotechnol.* 21, 679–685
18 Seelig, B. and Szostak, J.W. (2007) Selection and evolution of enzymes from a partially randomized non-catalytic scaffold. *Nature* 448, 828–831
19 Roberts, R.W. and Szostak, J.W. (1997) RNA-peptide fusions for the *in vitro* selection of peptides and proteins. *Proc. Natl. Acad. Sci. U. S. A.* 94, 12297–12302
20 Nemoto, N. *et al.* (1997) *In vitro* virus: bonding of mRNA bearing puromycin at the 3′-terminal end to the C-terminal end of its encoded protein on the ribosome *in vitro*. *FEBS Lett.* 414, 405–408
21 Cho, G.S. and Szostak, J.W. (2006) Directed evolution of ATP binding proteins from a zinc finger domain by using mRNA display. *Chem. Biol.* 13, 139–147
22 Zahnd, C. *et al.* (2007) Ribosome display: selecting and evolving proteins *in vitro* that specifically bind to a target. *Nat. Methods* 4, 269–279
23 Keefe, A.D. and Szostak, J.W. (2001) Functional proteins from a random-sequence library. *Nature* 410, 715–718
24 Kazlauskas, R.J. and Bornscheuer, U.T. (2009) Finding better protein engineering strategies. *Nat. Chem. Biol.* 5, 526–529
25 Morley, K.L. and Kazlauskas, R.J. (2005) *Improving enzyme properties: when are closer mutations better? Trends Biotechnol.* 23, 231–237
26 Khersonsky, O. *et al.* (2010) Evolutionary optimization of computationally designed enzymes: Kemp eliminases of the KE07 series. *J. Mol. Biol.* 396, 1025–1042
27 Miller, O.J. *et al.* (2006) Directed evolution by *in vitro* compartmentalization. *Nat. Methods* 3, 561–570
28 Otten, L.G. *et al.* (2010) Enzyme engineering for enantioselectivity: from trial-and-error to rational design? *Trends Biotechnol.* 28, 46–54
29 Iyer, P.V. and Ananthanarayan, L. (2008) Enzyme stability and stabilization – aqueous and non-aqueous environment. *Process Biochem.* 43, 1019–1032
30 Rubin-Pitel, S.B. and Zhao, H.M. (2006) Recent advances in biocatalysis by directed enzyme evolution. *Comb. Chem. High Throughput Screen.* 9, 247–257
31 Turner, N.J. (2009) Directed evolution drives the next generation of biocatalysts. *Nat. Chem. Biol.* 5, 567–573
32 Brakmann, S. and Johnsson, K. (2002) *Directed Molecular Evolution of Proteins*, Wiley-VCH Verlag GmbH
33 Bloom, J.D. *et al.* (2005) Evolving strategies for enzyme engineering. *Curr. Opin. Struct. Biol.* 15, 447–452
34 Wang, T-W. *et al.* (2006) Mutant library construction in directed molecular evolution. *Mol. Biotechnol.* 34, 55–68
35 Reetz, M.T. and Carballeira, J.D. (2007) Iterative saturation mutagenesis (ISM) for rapid directed evolution of functional enzymes. *Nat. Protocols* 2, 891–903
36 Fox, R.J. *et al.* (2007) Improving catalytic function by ProSAR-driven enzyme evolution. *Nat. Biotechnol.* 25, 338–344
37 Daugherty, P.S. (2007) Protein engineering with bacterial display. *Curr. Opin. Struct. Biol.* 17, 474–480
38 Gai, S.A. and Wittrup, K.D. (2007) Yeast surface display for protein engineering and characterization. *Curr. Opin. Struct. Biol.* 17, 467–473
39 Paschke, M. (2006) Phage display systems and their applications. *Appl. Microbiol. Biotechnol.* 70, 2–11
40 Huang, B.C. and Liu, R. (2007) Comparison of mRNA-display-based selections using synthetic peptide and natural protein libraries. *Biochemistry* 46, 10102–10112
41 Cho, G. *et al.* (2000) Constructing high complexity synthetic libraries of long ORFs using *in vitro* selection. *J. Mol. Biol.* 297, 309–319
42 Kurz, M. *et al.* (2000) Psoralen photo-crosslinked mRNA-puromycin conjugates: a novel template for the rapid and facile preparation of mRNA-protein fusions. *Nucleic Acids Res.* 28, e83
43 Tabata, N. *et al.* (2009) Rapid antibody selection by mRNA display on a microfluidic chip. *Nucleic Acids Res.* 37, e64
44 Liao, H.I. *et al.* (2009) mRNA display design of fibronectin-based intrabodies that detect and inhibit severe acute respiratory syndrome coronavirus nucleocapsid protein. *J. Biol. Chem.* 284, 17512–17520
45 Millward, S.W. *et al.* (2007) Design of cyclic peptides that bind protein surfaces with antibody-like affinity. *ACS Chem. Biol.* 2, 625–634
46 Takahashi, T.T. *et al.* (2003) mRNA display: ligand discovery, interaction analysis and beyond. *Trends Biochem. Sci.* 28, 159–165
47 Josephson, K. *et al.* (2005) Ribosomal synthesis of unnatural peptides. *J. Am. Chem. Soc.* 127, 11727–11735
48 Frankel, A. *et al.* (2003) Encodamers: unnatural peptide oligomers encoded in RNA. *Chem. Biol.* 10, 1043–1050
49 Wentworth, P. and Janda, K. (2001) Catalytic antibodies, structure and function. *Cell Biochem. Biophys.* 35, 63–87
50 Hilvert, D. (2000) Crictical analysis of antibody catalysis. *Annu. Rev. Biochem.* 69, 751–793
51 Guofu, Z. *et al.* (1999) Broadening the aldolase catalytic antibody repertoire by combining reactive immunization and transition state theory: new enantio- and diastereoselectivities. *Angew. Chem. Int. Ed.* 38, 3738–3741
52 Miller, B.G. and Wolfenden, R. (2002) Catalytic proficiency: the unusual case of OMP decarboxylase. *Annu. Rev. Biochem.* 71, 847–885
53 Snider, M.J. and Wolfenden, R. (2000) The rate of spontaneous decarboxylation of amino acids. *J. Am. Chem. Soc.* 122, 11507–11508