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# Directed evolution methods for overcoming trade-offs between protein activity and stability

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## Abstract

Engineered proteins are being widely developed and employed in applications ranging from enzyme catalysts to therapeutic antibodies. Directed evolution, an iterative experimental process composed of mutagenesis and library screening, is a powerful technique for enhancing existing protein activities and generating entirely new ones not observed in nature. However, the process of accumulating mutations for enhanced protein activity requires chemical and structural changes that are often destabilizing, and low protein stability is a significant barrier to achieving large enhancements in activity during multiple rounds of directed evolution. Here we highlight advances in understanding the origins of protein activity/stability trade-offs for two important classes of proteins (enzymes and antibodies) as well as innovative experimental and computational methods for overcoming such trade-offs. These advances hold great potential for improving the generation of highly active and stable proteins that are needed to address key challenges related to human health, energy and the environment.

**KEYWORDS**
affinity, antibody, catalysis, enzyme, protein design, protein engineering

## 1 | INTRODUCTION

Proteins encode diverse activities through their complex 3D structures that are essential for life. These natural activities range from catalysis of a wide range of chemical reactions (enzymes) to specific and high-affinity recognition of target molecules (antibodies). Many naturally occurring protein functions have been harnessed for technological applications, including specialty chemical production[1-4] and disease-modifying therapeutics.[5-7] However, natural proteins rarely possess the particular biophysical and/or functional properties required for a given application, such as protein activity in extreme conditions (e.g., high temperature or harsh solvent conditions)[8,9] or entirely new protein functions not found in nature.[10,11] Consequently, proteins intended for use in biotechnological applications generally require moderate to extensive engineering and optimization to satisfy these unique constraints.[12-14]

The process of performing directed evolution is relatively simple in concept, as it "only" requires two key steps. The first step is creating protein libraries by introducing mutations into the target protein either in a random or targeted manner. The second step is screening the protein libraries in a manner that enables identification of rare variants with improved protein properties such as catalytic activity. In practice, many studies have shown that the success of directed evolution experiments is strongly influenced by the quality of both the protein libraries and screening methods that are employed. One common challenge in such studies is that "you get what you screen for" and activity screens commonly yield proteins with increased activity but compromised stability (Figure 1).

  

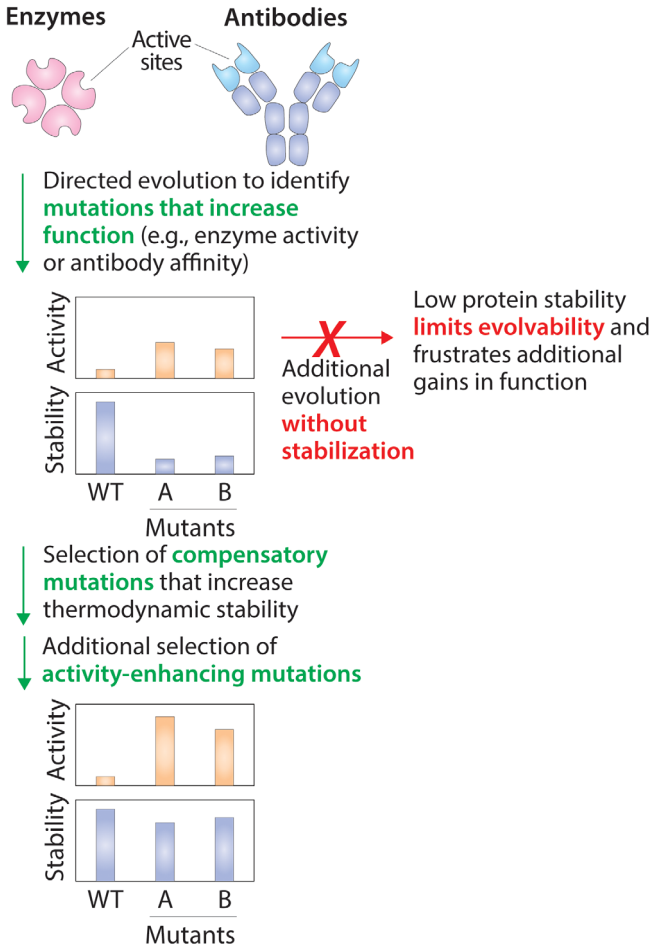


**FIGURE 1** Directed evolution of proteins such as enzymes and antibodies to achieve increased or new activities often results in reduced stability, and low protein stability is a common barrier to protein evolvability. To achieve significant gains in protein activity, it is typically necessary to also select compensatory (stabilizing) mutations that enable the accumulation of activity-enhancing, destabilizing mutations while maintaining protein thermodynamic stability [Color figure can be viewed at wileyonlinelibrary.com]

The origin of problematic protein activity/stability trade-offs during directed evolution is linked to multiple factors. First, proteins tend only to be marginally stable at their physiological conditions,[15] and mutations in proteins carry significant risk for reducing stability.[16-18] Second, mutations that promote gains in protein activity necessarily lead to chemical and structural changes, and these changes are rarely optimal for the existing protein scaffold and have an increased likelihood of reducing protein stability. Indeed, many protein functions gained through directed evolution—especially those that involve multiple rounds of mutation and selection—come at the cost of reduced protein stability.[18-22] Although bottlenecks in directed evolution frequently arise as a result of protein destabilization (Figure 1), new experimental and computational advances are enabling the navigation of protein fitness landscapes in an effective and efficient manner. In honor of Francis Arnold's Nobel Prize in Chemistry for her pioneering work in the area of directed evolution, we review a number of key fundamental studies over the last few decades as well as emerging technologies that are increasing the success of evolving proteins with both high activity and high stability.

## 2 | DIRECTED EVOLUTION METHODS FOR SELECTING PROTEINS WITH HIGH ACTIVITY AND STABILITY

### 2.1 | Cell survival screens for optimizing enzyme activity and stability

The power of directed evolution is only truly realized when robust screens for protein function can be developed that successfully identify improved protein variants. Clonal screening often represents the biggest bottleneck in directed evolution studies because many protein activities are relatively difficult to screen for in a high-throughput manner. The most attractive types of protein activities that have been engineered using directed evolution are those that enable survival of a host (e.g., bacterial or yeast), such as enhanced enzymatic activity against antibiotics that promotes bacterial survival. In such cases, it is possible to screen relatively large libraries ($10^6$–$10^{10}$)—which are typically limited in size by DNA transformation efficiency—for improved protein function by selecting cells that survive in conditions requiring higher activity than observed for the wild-type protein without the need to individually evaluate each mutant.

Indeed, one of the most well-studied enzymes in the field of directed evolution is β-lactamase, which is an attractive enzyme to evolve because its activity (conferring resistance to penicillins) can be easily screened for in bacteria by identifying antibiotic-resistant colonies. Additionally, the structure and function of many β-lactamases have been studied in detail,[23-25] providing a unique opportunity to investigate trade-offs between stability and activity in a well-understood enzyme family. The overall stability of enzymes (and other proteins) results from the organization of a large network of favorable intramolecular interactions, including the stable packing of the hydrophobic core surrounded by solvent-exposed hydrophilic residues and hydrogen bonding networks between elements of secondary structure. However, mutations in and near the active site—which are required for binding ligands and mediating catalysis—have an increased risk of destabilizing enzymes because of their propensity to disrupt the network of intramolecular interactions that collectively govern protein stability.[22,26,27]

An elegant dissection of the molecular determinants of enzyme activity/stability trade-offs has been reported for β-lactamase.[27] The authors sought not to improve β-lactamase activity, but rather to test if its key active site residues are generally destabilizing and if mutating these sites to less active residues would be stabilizing. Notably, the authors found that mutating many active site residues to less active ones resulted in significant increases in stability. For example, a single mutation of a key active site residue (Ser64) to a less active one (Asp64) was sufficient to increase stability by 30%, which is particularly surprising given that the enzyme has >300 residues. Conversely, the investigators found that several other solvent-exposed, nonactive-site mutations had little effect on β-lactamase stability.

Analysis of the structural mechanisms for this and related active-site mutations that also reduced β-lactamase activity revealed several common mechanisms.[27] First, the nature of the active site for wild-type β-lactamase (and for enzymes in general) is such that binding to

the substrate fulfills otherwise unsatisfied intramolecular interactions. Thus, mutating active site residues to less active ones that are larger and/or which contain additional functional groups (e.g., Ser to Asp at position 64) that are able to mimic substrate binding and satisfy key intramolecular interactions increases protein stability. Second, stabilizing mutations in the active site that reduce β-lactamase activity also frequently reduce steric or electrostatic strain (or both) in the enzyme 3D structure. For example, the authors observed large increases in stability (and reduced activity) due to reductions in steric strain via mutation of a key active site residue (Ser64) to a smaller and more flexible one (Gly64). These and other findings[22,28-30] demonstrate that the structural and chemical requirements for high activity and high stability of enzymes are not only different, but often at odds with one another.

A particularly interesting example of enzyme activity/stability trade-offs has been observed for β-lactamase (TEM-1) mutants identified in clinical isolates.[31] While the wild-type enzyme is effective at degrading penicillins, it has little activity against other classes of antibiotics such as cephalosporins. Notably, mutants of β-lactamase observed in clinical isolates gain activity against cephalosporins. These mutants have several intriguing properties. First, the mutations that increase the size of the active site cavity to accommodate cephalosporins are destabilizing. Second, many of the mutants that are active against cephalosporins possess an additional compensatory mutation distal from the active site that compensates for the destabilizing effects of the active-site mutations. These findings further highlight the inherent trade-offs between enzyme stability and activity, and demonstrate how compensatory mutations are required to maintain thermodynamic stability as enzyme activity is enhanced via active-site mutations.

It is also notable that the wild-type and mutant β-lactamase variants in these studies[27,31] have modest thermal stabilities (<60°C). While this level of stability is suitable for physiological enzyme function, it is not ideal for applications that require enzyme function (and therefore enzyme stability) at higher temperatures. To screen for enzyme variants with higher stability using cell survival methods, it is necessary to use cellular hosts that survive at such elevated temperatures. This need has led to the use of thermophiles in directed evolution studies—most commonly thermophilic bacteria—that thrive at elevated temperatures.

Two pioneering studies investigated the use of a thermophilic bacterium (*B. stearothermophilus*) to screen for highly stable variants of kanamycin nucleotidytransferase (KNTase), an enzyme that degrades the antibiotic kanamycin.[32,33] The wild-type enzyme fails to support growth of the thermophile at temperatures above ~55°C in the presence of kanamycin. Therefore, the investigators introduced mutations into the enzyme using several different approaches and screened for rare variants that promoted bacterial growth at elevated temperatures (61–71°C). Encouragingly, using disparate mutagenesis and selection methods, two entirely different research groups identified the same mutations (D80Y and T130 L) that significantly stabilized the wild-type enzyme by >10°C. These and other[34-37] studies have established creative and powerful approaches for using thermophiles to identify highly active, thermostable enzymes.

## 2.2 | Functional (survival-independent) screens for optimizing enzyme activity and stability

Despite the power of performing directed evolution using cell survival screens, this approach is not readily applicable to most enzymes or other types of proteins because it is challenging in general to link protein functions to cell survival. Therefore, most directed evolution studies of enzymes are performed via functional (noncell survival) screens in which the properties of each protein variant are directly evaluated. In such cases, the screens must be conducted on a clone-by-clone basis, which significantly decreases throughput. An outcome of these low-throughput methods is the enzyme library sizes that can be screened are much smaller than what is possible using cell survival selections. For example, traditional functional screens for enzyme libraries performed in microtiter plates typically involve screening ~$10^2$–$10^4$ mutants[38-40] relative to cell survival methods where library size is typically limited by transformation efficiency ($10^6$–$10^{10}$ variants).

However, there are a number of emerging technologies for improving the throughput of functional screens for directed evolution of enzymes and other types of proteins (as summarized in multiple recent reviews[40-42]). A critical element of any enzymatic screen is the spatial isolation (compartmentalization) of each protein variant in the library to maintain the linkage between enzyme function and DNA sequence as well as the linkage between enzyme function and its catalytic products. To accomplish this, several innovative approaches have been reported that shrink the size of compartments from those common for microtiter plates (microliters) to extremely small droplets or wells (nanoliters). These approaches enable the screening of several orders of magnitude more enzyme variants. These important advances—which are still in their infancy and are associated with some additional challenges—will likely continue to mature in the coming years and be invaluable for directed evolution studies. In this review, we focus on studies that have primarily used conventional, lower-throughput screening methods for enzyme engineering.

Perhaps no class of biotechnologically relevant enzymes has been as extensively studied and engineered in recent years as cytochrome P450s. Thousands of cytochrome P450s, which catalyze mainly oxygenation reactions for a wide variety of organic substrates,[43,44] have been identified from all kingdoms of life and are important for chemoenzymatic synthesis, bioconversion, and bioremediation.[44-46] Cytochrome P450s are hemeproteins that generally function in concert with an NAD(P)H-driven redox partner to facilitate electron transfer to the heme iron during catalysis and/or use hydrogen peroxide to directly generate reactive heme iron-oxo species. Conveniently, some cytochrome P450s are naturally encoded as self-sufficient catalytic fusion proteins containing a reductase domain (e.g., cytochrome $P450_{BM3}$). Others have been engineered for enhanced activity as peroxygenases,[47,48] which eliminates the need for expensive and complicated cofactor regeneration schemes. Their promiscuity enables them to accept a broad range of substrates, which has motivated researchers to engineer cytochrome P450s for oxygenation reactions on novel substrates.[48-52]

However, repurposing enzymes such as cytochrome P450s to accept new substrates and/or catalyze new reactions typically requires significant mutagenesis in and/or near their active sites. The trade-offs between P450 activity and stability have been dissected by Frances Arnold's lab in a particularly clear and detailed manner. An impressive example is highlighted in Figure 2.[19,20,50,53] The Arnold lab asked whether a particular P450 ($P450_{BM3}$)—which hydroxylates long-chain fatty acids ($C_{12}$–$C_{20}$)—could conduct the same reaction for an entirely different substrate (short-chain alkanes, $C_2$–$C_3$). This goal is important because alkanes are both common and relatively inert,[54,55] and converting inert C—H bonds to reactive C—OH bonds enables the use of such upgraded chemicals as feedstocks in many key industrial processes and is also important for bioremediation.[56,57] However, this is generally a challenging problem due to the lack of efficient catalysts for hydroxylating short alkanes (e.g., propane) and in particular for evolving the $P450_{BM3}$ enzyme, which completely lacks activity against short alkanes.

One of the Arnold lab's most influential contributions to the field of directed evolution is its demonstration that greatly improved or entirely new functions can be engineered into proteins if only modest improvements are required in each round of evolution and this process of mutagenesis and selection is repeated many times.[19,50,58,59] Indeed, they used this progressive evolution approach to eventually evolve a variant of the $P450_{BM3}$ enzyme that hydroxylates short-chain alkanes (P450 propane monooxygenase) in a highly efficient, native-like manner. To accomplish this, they made a key initial observation that the wild-type enzyme had weak but detectable activity against long-chain alkanes ($C_8$ and larger). This observation led them to perform selections for mutations in the wild-type enzyme not for activity against short-chain alkanes (their eventual goal) but rather initially against long-chain alkanes ($C_8$). Notably, the most active mutant enzyme identified in the initial screen against long-chain alkanes acquired low levels of catalytic activity against propane. This variant was then subjected to further mutagenesis and selection in the next round of directed evolution against alkanes that were modestly shorter. This process of mutagenesis and selection was repeated multiple times to enhance the rate of propane hydroxylation.

However, at one point in this directed evolution process, it was observed that the mutated P450 enzyme (35E11) could not be further evolved for additional increases in activity and did not meet the target

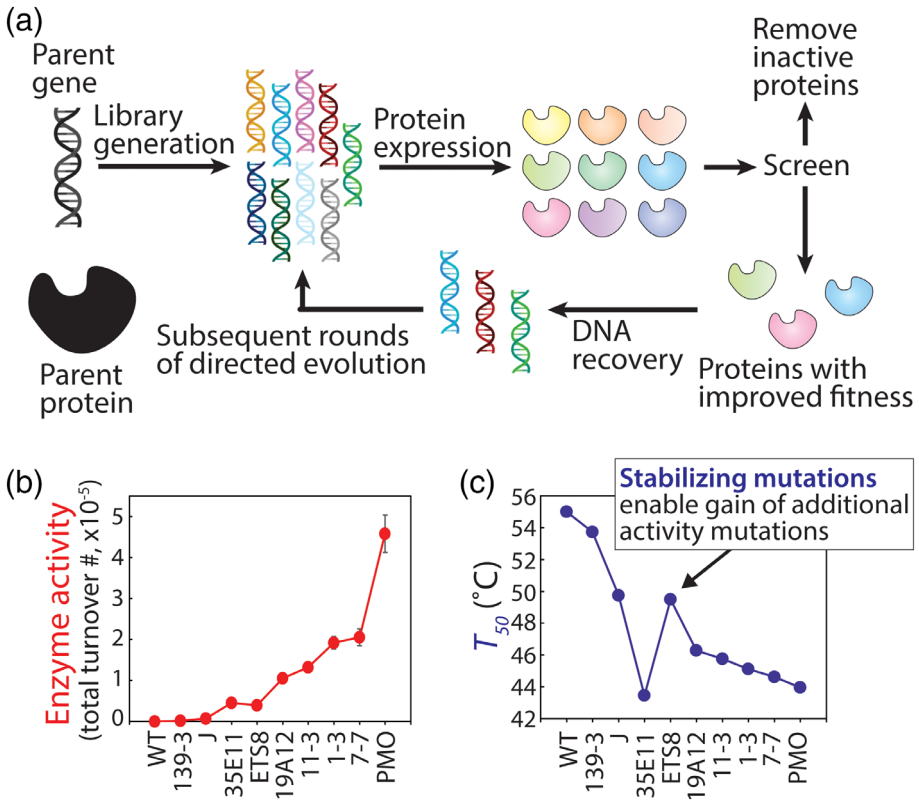


**FIGURE 2** Directed evolution of enzymes reveals trade-offs between activity and stability. (a) Directed evolution experiments are composed of several steps, beginning with mutagenesis of a parent gene and then screening for the desired protein function (e.g., catalytic activity against a desired substrate). Mutants with improved performance are identified and their DNA sequences are used for subsequent rounds of mutagenesis and protein optimization. (b, c) A cytochrome P450 enzyme was engineered for a novel function, namely hydroxylation of a short-chain alkane (propane), and its (b) catalytic activity towards propane (total turnover number) and (c) temperature of half-inactivation ($T_{50}$) are reported. After three rounds of evolution, an optimization plateau was reached as a result of the low stability of the 35E11 mutant enzyme. This plateau was overcome by introducing several stabilizing mutations (leading to the stabilized ETS8 variant), which enabled subsequent rounds of directed evolution to further improve enzyme activity. The data were obtained from previous publications from the Arnold lab,[19,20] and the figure was adapted from a previous one[53] [Color figure can be viewed at wileyonlinelibrary.com]

goal of a highly efficient hydroxylation catalyst against short-chain alkanes (Figure 2).[19,20,50,53] Evaluation of the stability of the evolved enzymes up to the point of failure revealed that enzyme stability progressively decreased as hydroxylation activity increased. For example, the temperature at which the wild-type enzyme was inactivated by 50% ($T_{50}$) was ~55°C, but this was reduced to only 43°C for the evolved enzyme (35E11) after the third round of evolution. This compromised stability—due to accumulation of activity-enhancing mutations—appeared to explain the inability to further evolve P450 hydroxylation activity against short-chain alkanes.

To address the problem of poor stability of the P450 enzyme mutant (35E11), the Arnold lab performed a round of mutagenesis and selection aimed at increasing enzyme stability without significantly reducing enzyme activity (Figure 2).[19,20,53] Notably, they incorporated mutations previously shown to stabilize a related P450 enzyme that they found also significantly stabilized the monooxygenase (increase of $T_{50}$ of >5°C), and this resulted in only modest decreases in enzyme activity. At this point, it was not obvious that the stabilized enzyme could be further evolved for additional increases in activity without simply being limited again by poor stability. However, the Arnold lab used their incremental approach of directed evolution to eventually achieve a P450 that hydroxylates propane with a catalytic efficiency similar to the efficiencies of natural P450s against their preferred substrates. Notably, the stability of the highly evolved propane monooxygenase variant (PMO) was reduced almost to the point of the variant that could not be further evolved for increased activity (35E11), suggesting that even greater improvements in enzyme activity would likely require additional stabilizing mutations.

What do we learn from these exciting studies? First, very large changes in enzyme function can be achieved gradually by requiring only modest activity improvements in any single round of directed evolution. Given that directed evolution studies commonly fail to meet all of their objectives, it is likely that such failures are due (at least in part) to using screening methods that require too large of an activity improvement in any given round of directed evolution. Second, these studies suggest that trade-offs between enzyme activity and stability are the norm, not the exception. The extensive mutagenesis of the P450 enzymes required to achieve large improvements in activity against short-chain alkanes necessarily resulted in reduced stability, and this trend was observed even after the P450 enzymes were stabilized at an intermediate step in the evolutionary process (Figure 2c).[19,20,53] These and related findings in the Arnold lab[60-64] have greatly advanced the field of directed evolution and provided valuable approaches for overcoming inherent trade-offs between enzyme activity and stability.

Another important application of functional (noncell survival) screens is the evolution of enzymes with high stability at elevated temperatures that are required for diverse biotechnology applications. For cases in which extremely high stability is required, it can be challenging to identify stabilizing mutations for enzymes that are already moderately to highly stable. One powerful approach to address this challenge is to first introduce destabilizing mutations into enzymes at the beginning of the directed evolution process to reduce stability in order to simplify the identification of stabilizing mutations at other sites. It was hypothesized that introducing newly discovered stabilizing mutations into the more stable wild-type enzyme will yield mutant enzymes with extremely high stability.

This approach has been reported for evolving α-amylase to be extremely stable at high temperatures.[65] This is significant because α-amylase is a key enzyme in the breakdown of starch, an important industrial process that is performed at high temperatures (e.g., 90°C). The investigators initially introduced a pair of destabilizing mutations into the wild-type enzyme and then mutagenized the destabilized variant to identify compensatory mutations that restored stability. They identified a single mutation (A209V) that largely restored stability and, by introducing this and related mutations[66] into the wild-type enzyme, the resulting α-amylase variant displayed a 10-fold increase in its half-life at 90°C.[65] These and related studies[67-69] demonstrate the power of functional screens to identify rare mutations that result in extremely high enzyme stability in addition to high activity.

It is also important to consider that the utility of enzymes is not only linked to their folding stability, but also to their solubility. One particularly compelling study[70] evaluated the impact of nearly every single mutation on enzyme expression properties—namely enzyme expression on the surface of yeast cells and enzyme secretion via a bacterial folding pathway specific for soluble and folded proteins—that serve as surrogates for protein solubility.[71-75] The investigators screened mutant libraries of a β-lactamase enzyme to identify mutations that promote high expression levels and high expected solubilities.[70] Deep sequencing of the enzyme libraries before and after selection enabled the evaluation of the impact of nearly every mutation on solubility-like properties. By comparing these vast data sets with previous data sets that quantify the impact of nearly every single mutation on enzyme fitness (which is correlated with activity),[76] they found that a significant fraction (~40%) of mutations that increase solubility are detrimental to activity. This striking observation could be explained in part by the proximity of the solubilizing mutations to the enzyme active site (mutations close to the active site are more likely to be detrimental), contact number (mutations at sites with more contacts are more likely to be detrimental), and evolutionary conservation (mutations at more conserved sites are more likely to be detrimental).[70] Moreover, they also found that mutations beneficial for both activity and solubility are extremely rare (~0.05–0.15%), which appears to explain previous observations of trade-offs between these and related properties such as stability.[22,31] This and other deep sequencing studies[77,78] are enabling unprecedented analysis of trade-offs between enzyme activity, stability and solubility, which is improving the systematic and reliable generation of enzymes with optimized properties.

## 2.3 | Directed evolution of antibody affinity and stability

The active sites of antibodies, like enzymes, are commonly engineered to increase their function, and trade-offs between activity and stability are also commonplace for antibodies.[79-81] In some ways, directed

evolution of antibodies is simpler than for enzymes because it is easier to screen for antibody function (binding to target antigens) than it is to screen for enzyme activity. This is largely because the catalytic product does not remain stably bound to the enzyme. This significant difference means that it is only necessary to maintain the linkage between protein function and DNA sequence for antibody screens, which eliminates the need for spatial compartmentalization of each protein variant. The linkage between antibody function and DNA sequence can be readily accomplished in the laboratory using methods such as phage, bacterial, and yeast surface display.[82-84] This results in the ability to screen large antibody libraries ($10^6$–$10^{10}$) that are only limited by DNA transformation efficiency of the host cells.

However, in other ways, it is more challenging to evolve antibody function (relative to enzyme function) and stability using directed evolution methods. First, identification of antibodies that bind to the desired target antigen is often complicated by co-selection of antibodies with nonspecific binding activity (e.g., off-target binding). Second, it is challenging to screen for both antibody activity and stability at the same time because most cells (e.g., bacteria), viral particles (e.g., phage), and antigens used for evolving antibodies are not stable at high temperature and/or other harsh conditions required for evolving protein stability. These and other challenges have motivated researchers to develop innovative approaches for selecting and engineering antibody variants to minimize trade-offs between antibody activity and stability.

One example of activity/stability trade-offs for antibodies has been reported for single-domain antibodies specific for the Alzheimer's Aβ peptide.[79,80] The investigators generated a mutant library (>$10^6$ variants) based on a stable, human $V_H$ domain and screened for mutants with increased affinity for the Aβ peptide using yeast surface display and fluorescence-activated cell sorting (FACS).[79] The best variant isolated in the first round of mutation and selection was further mutagenized and screened for additional increases in affinity. This process of mutation and selection was performed four times. Evaluation of the best clone identified at the end of each round revealed several notable findings. First, the stabilities of the antibody domains were progressively reduced as the number of mutations increased, and the highly evolved variants were greatly destabilized and unfolded when produced as soluble antibody domains. Second, significant reductions in antibody stability were observed even after the first round of mutagenesis, as the apparent melting temperature was reduced from 75°C for wild-type to 57°C for the best variant isolated after the first round. Third, there was a strong pressure for selection of destabilizing mutations even when mutations were targeted to the complementarity-determining regions (CDRs) to avoid non-CDR sites that were known to be important for stability. For example, antibody domains are typically stabilized by one highly conserved, intramolecular disulfide bond, and mutations that eliminate this covalent bond are expected to be highly destabilizing. Surprisingly, even in cases in which the investigators targeted mutations to the CDRs in order to avoid mutations that disrupt the stabilizing disulfide bond, they still obtained variants with cysteine knockout mutations due to low frequency mutations (introduced due to PCR errors) that were strongly favored for antigen binding.

To overcome this problem, the investigators sought to repeat the screening process by co-selecting for both antibody affinity and stability at the same time to minimize trade-offs between these two properties.[79] While a variety of methods for evolving affinity and stability had been reported, there were no previous methods for simultaneously selecting for both antibody affinity and stability during directed evolution studies. Given that it is not possible to perform antibody selections directly at high temperature due to antigen instability and poor yeast viability, the investigators evaluated the use of conformational probes that are specific for stably folded antibody domains and which could be monitored during FACS to identify antibody variants with both high affinity and stability. Notably, the antibody domains—which belong to a particular human antibody subclass ($V_H3$)—are recognized by Protein A via a conformational epitope on their frameworks.[85,86] Although the Protein A binding site is close to the antigen binding site, the investigators reasoned that both antigen and Protein A binding could be evaluated at the same time without interfering with each other.[79] Thus, the investigators repeated four rounds of directed evolution by selecting for both antigen binding and stability (as judged by Protein A binding), and identified progressively evolved variants with significant increases in affinity while maintaining high stability. The variants, which are highlighted in Figure 3, had increasing numbers of mutations after each round of evolution, including 4 mutations after round 1 (P1 variant), 7 after round 2 (P2 variant), 9 after round 3 (P3 variant), and 12 after round 4 (P4 variant).

To understand how the evolved antibody domains minimized trade-offs between affinity and stability, the investigators performed reversion mutational analysis to evaluate how each mutation contributed to both properties (Figure 3b,c).[80] Mutations that improve antibody affinity or stability are expected to result in reductions in either property when reverted to wild-type. The directed evolution process resulted in selection of several mutations that improved affinity (as evidenced by reduced equilibrium association constants when reverted to wild-type), such as R62, N72, and R50 (Figure 3b), that were detrimental to stability (as evidenced by increased melting temperatures when reverted to wild-type; Figure 3c). Such destabilizing mutations are expected to compromise stability significantly and prevent evolution of stable and high-affinity antibody domains, as the investigators had observed when performing selections for antibody affinity without selecting for stability.[79] However, the mutational analysis revealed that two key stabilizing mutations were co-selected, namely K45 and K98, and these mutations appear to compensate for the destabilizing effects of several affinity-enhancing mutations.[80] These results demonstrate that affinity-enhancing mutations can be destabilizing, and minimizing trade-offs between antibody affinity and stability requires selection of compensatory mutations to maintain thermodynamic stability.

It is also notable that these observations related to affinity/stability trade-offs are not specific to antibodies but have also been observed for other affinity (nonimmunoglobin) proteins that have been engineered using directed evolution methods. For example, a nonimmunoglobin protein scaffold (DARPin) was subjected to multiple rounds of mutation and selection for high-affinity binding to a cancer-associated antigen

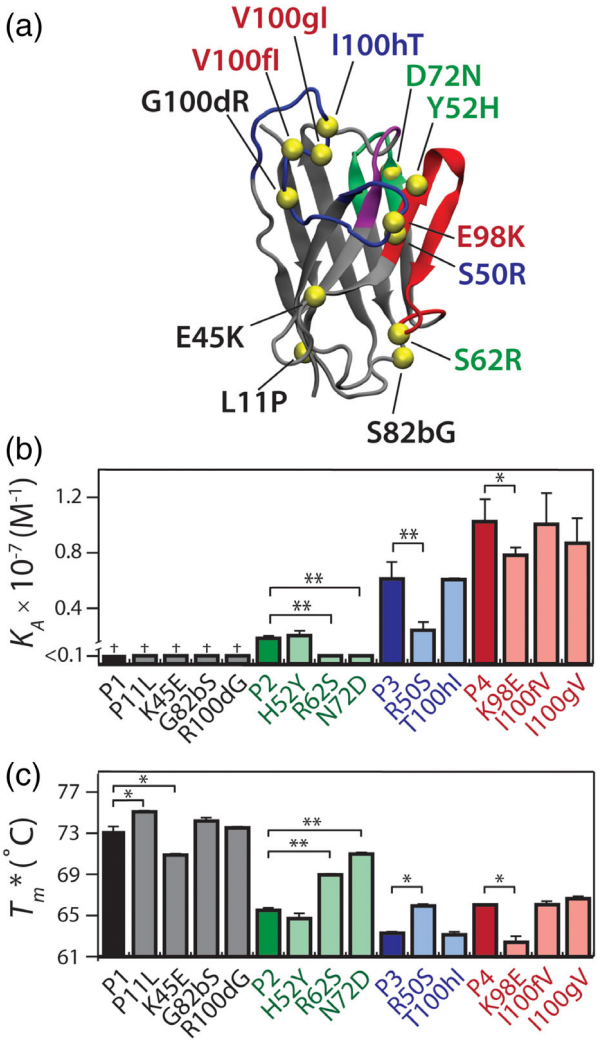


**FIGURE 3** Antibody directed evolution reveals trade-offs between affinity and stability. (a) Structural model of an evolved human antibody ($V_H$) domain, which is referred to as P4. This antibody domain was identified following four rounds of mutagenesis and co-selection for affinity (binding to the Alzheimer's Aβ42 peptide) and stability [binding to a conformational probe (Protein A) that is linked to stability]. (b, c) Reversion mutational analysis reveals how each mutation selected in rounds 1–4 of mutagenesis and selection contribute to antibody (b) affinity and (c) stability. Antibody domain P1 contains four mutations, P2 contains seven mutations (including the same ones as P1), P3 contains nine mutations (including the same ones as P1 and P2) and P4 contains 12 mutations (including the same ones as P1, P2, and P3). Each mutation was reverted to wild-type, and its impact on affinity and stability was evaluated. Mutations that increase affinity or stability are expected to reduce the corresponding property when reverted to wild-type, and vice versa for mutations that reduce affinity or stability. In (b), the equilibrium association constant ($K_A$) is reported, and increased values of $K_A$ reflect increased affinity. In (c), the apparent melting temperature ($T_m$*) is reported, and increased values of $T_m$* reflect increased stability. Statistical significance [*p*-values < .05 (*) or .01 (**)] was judged by a two-tailed Student's *t* test. The data were obtained from a previous publication,[80] and the figure was adapted from figures in the same previous publication [Color figure can be viewed at wileyonlinelibrary.com]

(Her2).[87] The investigators screened their DARPin libraries using phage display, which requires antigen immobilization and selection for only one property at a time (e.g., affinity) and generally does not permit simultaneous co-selection for both affinity and stability. This appears to explain the fact that the progressively evolved variants with increased affinity resulted in significant reductions in stability. Interestingly, the most highly evolved variant with high affinity ($K_D$ of 0.4 nM) had a much lower stability (melting temperature of 57°C) relative to wild-type (melting temperature of 86°C). Mutational analysis revealed that the highest affinity DARPin had accumulated several affinity-enhancing mutations that were strongly destabilizing and, despite its modest stability, also accumulated multiple mutations that primarily contributed to maintaining thermodynamic stability. This and other findings[88-90] highlight that directed evolution of antibodies and related affinity proteins commonly involves activity/stability trade-offs, and selection of compensatory mutations that maintain thermodynamic stability is key to the success of these efforts.

## 3 | DESIGN METHODS FOR IMPROVING DIRECTED EVOLUTION OF PROTEIN ACTIVITY AND STABILITY

Although directed evolution methods have been modified with great success for the identification of stabilized enzymes and antibodies, rational design methods hold significant potential to improve the likelihood of success for directed evolution experiments. Indeed, a significant fraction (~30–50%)[91-93] of individual mutations in proteins are destabilizing, and many gain-of-function mutations have destabilizing effects on proteins.[17,22,27,29,31] A common paradigm of protein library design methods is to identify "consensus" (ancestral) protein sequences or stable structural elements that can be sampled during mutagenesis. In this section, we highlight examples of such design methodologies and describe instances where these approaches have been successful for engineering highly active proteins while minimizing activity/stability trade-offs.

### 3.1 | Computational methods for identifying activity-enhancing and stabilizing mutations in enzymes

Consensus-based evolutionary strategies rely on sequence-alignment of homologous proteins in order to identify residues where the sequence of the protein of interest has deviated from the ancestral protein sequence. The consensus design paradigm reasons that common, naturally occurring residues at a given position in related proteins are more likely to be active and/or stabilizing than a random mutation because they are frequently found in functional homologs and are likely present in the ancestral protein. For example, sampling a contiguous stretch of residues from homologs within flexible regions of L-arginase, an enzyme with applications in cancer treatment,[94] was recently shown to greatly stabilize and increase enzyme activity.[95]

This interesting study—which demonstrates that both activity and stability can be simultaneously increased if mutations are judiciously selected—involved optimizing L-arginase through exchanging homologous sequences in a flexible loop adjacent to the enzyme active site. This resulted in a doubling of the specific activity of the enzyme, and the authors observed that enzyme activity appeared inversely related to flexibility of this loop. Additional improvements in activity (>threefold) and half-life at 65°C (>sevenfold) were achieved via additional mutagenesis within the exchanged loop. These findings demonstrate the power of using limited sequence diversity informed by related, functional proteins during directed evolution.

Another important example of rational design methods informed by protein structure is related to the B-Factor Iterative Test (B-FIT), which identifies flexible sites in a protein crystal structure based on B-Factors (a measure of the flexibility of a protein at a given residue). It is expected that these flexible sites can be rigidified to increase protein stability,[96] although notable exceptions have been reported.[97,98] Indeed, the stability (half-inactivation temperature) of an enzyme (lipase) was improved by >40°C through iterative saturation mutagenesis at residues identified based on B-Factors,[96] and subsequent characterization of the enzyme mutants also revealed much higher tolerance to three organic solvents (acetonitrile, DMSO, and DMF).[99] The broader utility of using B-Factors to inform mutagenic regions during stability engineering has been demonstrated through increasing the melting temperature of an esterase by 9°C with little change to its activity.[100]

The importance of rationally designing enzyme libraries with amino acid diversity in specific regions has also been recently demonstrated in a variety of examples that involve optimization of substrate access tunnels of enzyme active sites to improve activity and stability.[101-109] Several computational tools have been generated for identifying and engineering substrate access tunnels for diverse applications in enzyme engineering, including altering substrate selectivity (see references within a recent review[110]). A notable example of improving the stability of enzymes through access tunnel engineering involved increasing the organic solvent tolerance of a haloalkane dehalogenase (DhaA).[101] High concentrations of DMSO reduce the activity of the enzyme by approximately an order of magnitude, but one stabilized variant identified after mutagenesis and selection contained a mutation within the access tunnel that was entirely responsible for its improved tolerance to the organic solvent. The mechanism of structural stabilization in DMSO appears to be due to improved packing of the hydrophobic core, which prevents DMSO access to the folded protein core.[101] Moreover, the apparent melting temperature of the enzyme mutant was improved by 19°C following saturation mutagenesis at another position within its access tunnel. Interestingly, to demonstrate the generality of stabilization via access tunnel engineering, the authors also used the FoldX[111] computational algorithm to assess the stability changes for all possible point mutations in a panel of 26 different enzymes and found that mutations predicted to be highly stabilizing were preferentially located within the access tunnels.[101] Access tunnel engineering has now also been used for improving the stability of a lipase in methanol,[103] demonstrating the utility of this approach.

Although de novo computational protein design is still in its infancy, the field holds promise for the generation of enzymes catalyzing novel chemical transformations. Many successfully designed enzymes are minimally active on the desired substrate, but low levels of catalytic activity can be a valuable starting point for significant gains of function obtained via directed evolution.[19,20,112] Until recently, no natural enzymes had been identified that efficiently catalyze the Kemp eliminase reaction, which is an important high-energy barrier model reaction involving proton transfer from carbon. However, several Kemp eliminases (i.e., enzymes that catalyze this reaction) with low levels of activity have been computationally designed.[113] While the activities of two of the designed enzymes could be subsequently improved (~200- to 400-fold) via directed evolution,[114,115] the engineered enzymes failed to meet the design goals. Furthermore, the most active computationally designed Kemp eliminase (KE59) was not amenable to evolutionary optimization due to its low stability. By sampling consensus mutations that were predicted to be stabilizing, the catalytic activity of the enzyme (KE59) was improved ~2,000-fold via directed evolution.[116]

Additional computational tools have been created that are informed by consensus-based sequence information. For instance, the PRotein One Stop Shop (PROSS) algorithm was developed for stabilization and high-level expression of proteins with solved molecular structures.[117] PROSS analyzes homologous protein sequences and locates mutations that are most frequently observed at each position within the protein family. Importantly, the algorithm can be instructed to avoid active-site residues or other positions known to be important for function. Using the molecular structure of the protein, Rosetta simulations then identify individual mutations that are predicted to stabilize the protein structure. Next, protein mutants containing combinations of these mutations are then designed using Rosetta combinatorial sequence optimization to identify variants with improved native-state energy. Such an approach was employed during directed evolution to convert bacterial phosphotriesterases into efficient nerve agent bioscavengers with high activity against both VX and Russian VX.[118] Prior efforts to engineer these phosphotriesterases had reached an optimization plateau short of the catalytic design goals. The PROSS design tool was used to guide library design for stabilization of the phosphotriesterases, and this enabled additional productive rounds of directed evolution and incorporation of previously identified beneficial mutations that were not well tolerated when introduced into the less stable phosphotriesterases.[118] PROSS has also been used to stabilize the malaria invasion protein PfRH5, a promising malaria vaccine candidate, without disrupting its immunogenicity or ligand binding.[119] This stabilized version of the protein could enhance its utility when deployed in challenging climates that are common to malaria infections.

Consensus-based protein sequence analysis is also helpful for identifying stabilizing, global suppressor mutations. However, primary sequence alignments do not typically consider the disruption of secondary and tertiary structures that can arise from mutating sites in proteins to consensus residues. Therefore, many efforts have been made to create novel chimeric proteins, wherein domains from different proteins

are exchanged or recombined to improve or gain new functions. However, determining the optimal locations for such recombination events is challenging. A significant development for addressing this problem—namely a structure-based algorithm (referred to as SCHEMA)—was developed in Frances Arnold's laboratory. This approach is based on calculating disruptions of residue-residue contacts upon exchanging one domain (or schema) of a protein with the corresponding domain from another homologous protein.[120] For example, random chimeric libraries analyzed in silico (containing 8-domain chimeras assembled from three related P450 enzymes) revealed that 14 recombination sites were dominant in libraries predicted to contain a high fraction of properly folded chimeras.[121] A diverse library designed to use seven of these 14 crossover sites was constructed and used to identify an enzyme variant whose melting temperature was increased by 7°C relative to any of the parent enzymes. Notably, the SCHEMA-guided design can improve the fraction of properly folded enzyme variants in libraries by more than three orders of magnitude compared to traditional enzyme libraries containing similar amounts of sequence diversity.[51]

### 3.2 | Computational methods for identifying affinity-enhancing and stabilizing mutations in antibodies

Computational antibody engineering is an attractive complementary method that can be used in conjunction with traditional directed evolution methods to enable in silico screening of much larger numbers of mutants than would be possible experimentally. We highlight in this review selected examples in which computational methods have been used successfully for optimizing antibody properties, and additional references can be found in a more comprehensive review.[122] Computational methods have been primarily used to improve either antibody activity (affinity and specificity) or stability. Many such approaches rely on calculating energy differences between a given antibody mutant of interest relative to wild-type, which are typically performed using software programs such as Rosetta,[123] CHARMM[124] and others.[125,126]

Several research groups have demonstrated that these computational approaches can be successfully used for antibody affinity maturation or stabilization.[127-139] For example, one study of multiple

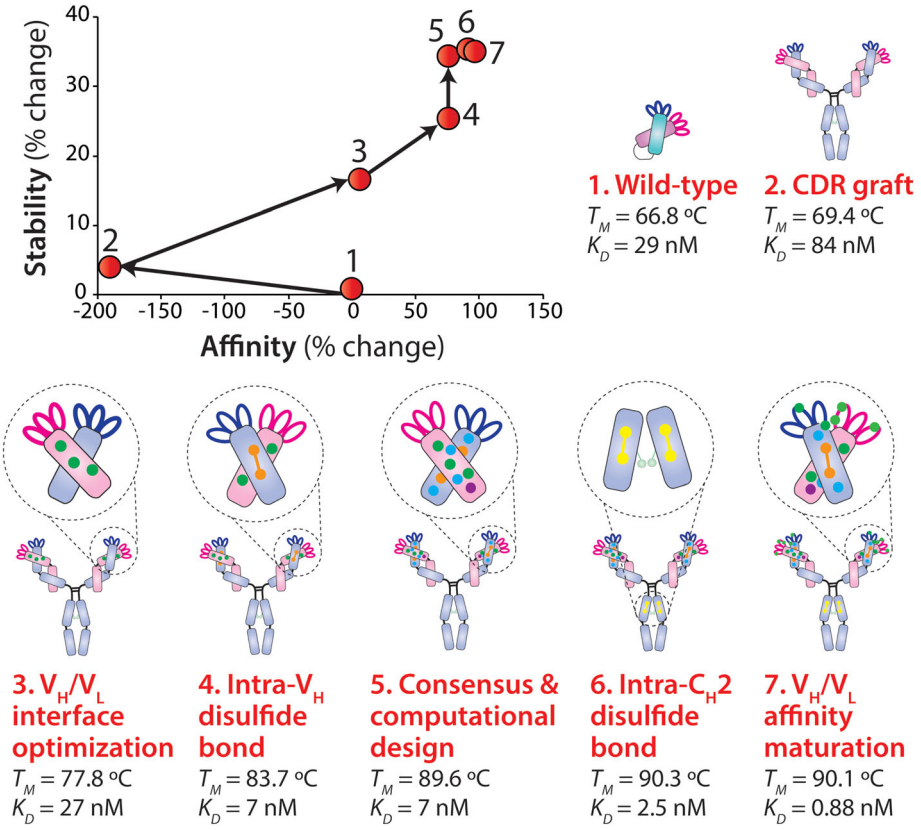


**FIGURE 4** Combined computational design and directed evolution for optimizing antibody affinity and stability. The evolutionary path of a single-chain variable fragment (scFv, specific for the MS2 phage protein) to a stabilized, affinity-matured full-length (IgG) antibody is illustrated by tracking its progression through the affinity/stability landscape. (1) The initial scFv used for CDR grafting was only modestly stable. (2) Grafting the CDRs of the scFv onto a full-length antibody improved stability, but decreased affinity. (3) Computational optimization of the interface between the variable ($V_H$ and $V_L$) domains resulted in improved stability and affinity. (4) Incorporation of a computationally designed intramolecular disulfide bond into the variable heavy ($V_H$) domain further increased both stability and affinity. (5) Additional mutations predicted based on consensus and computational design further increased stability without compromising affinity. (6) Introduction of a designed intramolecular disulfide bond in the second constant region of the heavy chain ($C_H2$) modestly improved both antibody stability and affinity. (7) Finally, the CDRs were affinity matured in the context of a suboptimal antibody and then grafted onto the stabilized framework to yield an optimized antibody with both high stability (melting temperature > 90°C) and high affinity (equilibrium dissociation constant of <1 nM). The data were obtained from a previous publication,[142] and the figure was adapted from one in the same previous publication [Color figure can be viewed at wileyonlinelibrary.com]

antibodies demonstrated that calculations of electrostatic interactions gave better predictions of affinity improvements relative to the full energy function, which includes contributions from nonelectrostatic interactions.[127] Similar results have been reported by several other groups, where mutations that improved antibody/antigen electrostatic interactions resulted in dramatic improvements in affinity.[128-130] For improving antibody stability, a relatively simple approach is to graft the CDRs with desired antigen specificity onto a known stable antibody framework that is predicted to be compatible with the CDRs.[133,139] However, unexpected incompatibilities between the CDRs and the desired framework can alter loop conformations and thereby reduce affinity.[140,141] Another promising method is to use consensus design to incorporate mutations into the destabilized antibody that are commonly observed in stable antibodies.[134] Finally, the introduction of non-native inter- and intra-domain disulfide bonds into antibody variable regions has been shown to significantly improve stability,[137,138] albeit at the expense of increasing the complexity of the molecule.

Nevertheless, given that affinity-enhancing mutations have an increased risk of being destabilizing and stabilizing mutations may compromise affinity (depending on how they influence the active site), it remains challenging to predict the interplay between affinity-enhancing and stabilizing mutations. Therefore, it is important to integrate computational approaches into the directed evolution process that are capable of predicting either affinity-enhancing or stabilizing mutations in order to minimize the trade-offs between the two properties.

A strong example of combining computational design methods and experimental (directed evolution) methods was reported for optimizing the affinity and stability of an antibody specific for a bacterial phage protein (Figure 4).[142] The investigators first stabilized a single-chain antibody by grafting the CDRs onto a stable IgG framework. While the resulting antibody was more stable ($T_m$ increased from 67 to 69°C), its affinity was reduced approximately threefold. Nevertheless, the researchers further optimized antibody stability by engineering the interface between the variable regions [variable heavy ($V_H$) and variable light ($V_L$)] by mutating multiple residues of the stable framework (identified using previously described methods[140]) back to those in the parental antibody. The stabilization of the $V_H/V_L$ interface increased the melting temperature by >8°C and increased the affinity back to levels similar to wild-type. The stability was also increased further by computationally designing an additional intramolecular disulfide bond in the $V_H$ domain.[137,138] RosettaDesign[123] software predicted that the disulfide bond would stabilize the antibody, which was also verified experimentally.[142] Interestingly, this additional disulfide bond not only increased antibody stability ($T_m$ of 84°C), but also improved its affinity. These and additional stabilization steps, including consensus and computational design, resulted in an extremely stable antibody ($T_m$ of 90.3°C) with improved affinity (equilibrium dissociation constant [$K_D$] of 2.5 nM).

Finally, it was unclear if the highly stable antibody could be further affinity matured while retaining its extremely high stability (Figure 4).[142] Therefore, the investigators performed directed evolution to identify affinity-enhancing mutations in the CDRs using in vitro somatic hypermutation and mammalian cell display,[142,143] and identified several CDR mutations that (when combined) led to a >27-fold improvement in affinity. Notably, the resulting antibody maintained its stability (90.1°C for the affinity-matured variant relative to 90.3°C for the parental antibody). This and related studies[144,145] demonstrate how integrating computational and experimental approaches can enable the optimization of both antibody affinity and stability while minimizing trade-offs between these properties.

## 4 | CONCLUSIONS

The success of directed evolution for generating highly active proteins requires methods for addressing and minimizing activity/stability trade-offs. The studies reviewed here—including pioneering studies from Frances Arnold's lab—demonstrate that activity-enhancing mutations are commonly destabilizing, and compensatory mutations are required to maintain thermodynamic stability. Given the unimaginably large chemical diversity that is possible for all combinations of mutations within a single protein, an important future direction will be to further improve library design methods to focus amino acid diversity in protein libraries on the small fraction of sequence space that is likely to yield both highly active and stable proteins. This ambitious goal—which is starting to be realized[70,77,78]—will require improvements in the prediction of both activity-enhancing and stabilizing mutations, and assessment of the complex interplay between these two properties. Combining such novel library design approaches with emerging screening methods that enable unprecedented throughput are expected to lead to successful generation of proteins with levels and types of activities that have previously been unattainable to protein engineers. Such advances are critically important for solving many of the most pressing challenges related to energy, the environment and human health.

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