Beyond binding: using phage display to select for structure, folding and enzymatic activity in proteins

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Phage display of a wide range of polypeptides has been increasingly used to identify novel molecules with useful binding properties for research, medical and industrial applications. Recent developments include methods for the selection of stabilized variants of a protein, the selection of regulatable enzymes and promising strategies for the selection and evolution of protein catalysts.

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Abbreviations

g3p	gene-3-protein
k_{cat}	catalytic rate constant
K_M	Michaelis–Menten constant
RT	room temperature
scFv	single-chain antibody fragment
SI	suicide inhibitor
SIP	selectively infective phage
TSA	transition-state analog
V_L	light-chain variable domain

Introduction

The ultimate goal for protein engineering is to obtain better or, even, novel molecules with useful functional activities. Despite continuous progress, the purely 'rational' design of proteins with improved complex properties, such as stability or enzymatic activity, is, at least to date, not generally feasible. Even in the presence of extensive structural and mechanistic information, the predictive accuracy of the consequence of sequence changes is, in most cases, insufficient. In contrast, random mutagenesis and directed evolution have proven to be very useful tools for improving proteins with respect to defined parameters or for identifying those with a given function (for recent reviews, see [1–6]).

Successful directed evolution requires several rounds of alternation between generating a library of mutants complex enough to contain rare beneficial mutations, followed by a method of screening for the desired function. Although a number of alternative technologies are now available for *in vitro* or *in vivo* screening [7–11], phage display [10,12–15] has proven to be a powerful method for this purpose and is probably currently the most widely used. Here, we review recent advances in the use of phage display to optimize protein functions other than binding. We discuss improvements in the technology, as well as focus on selecting proteins with improved biophysical properties (i.e. thermodynamic stability and folding) and proteins with novel enzymatic activities.

Discrimination between folded and unfolded molecules

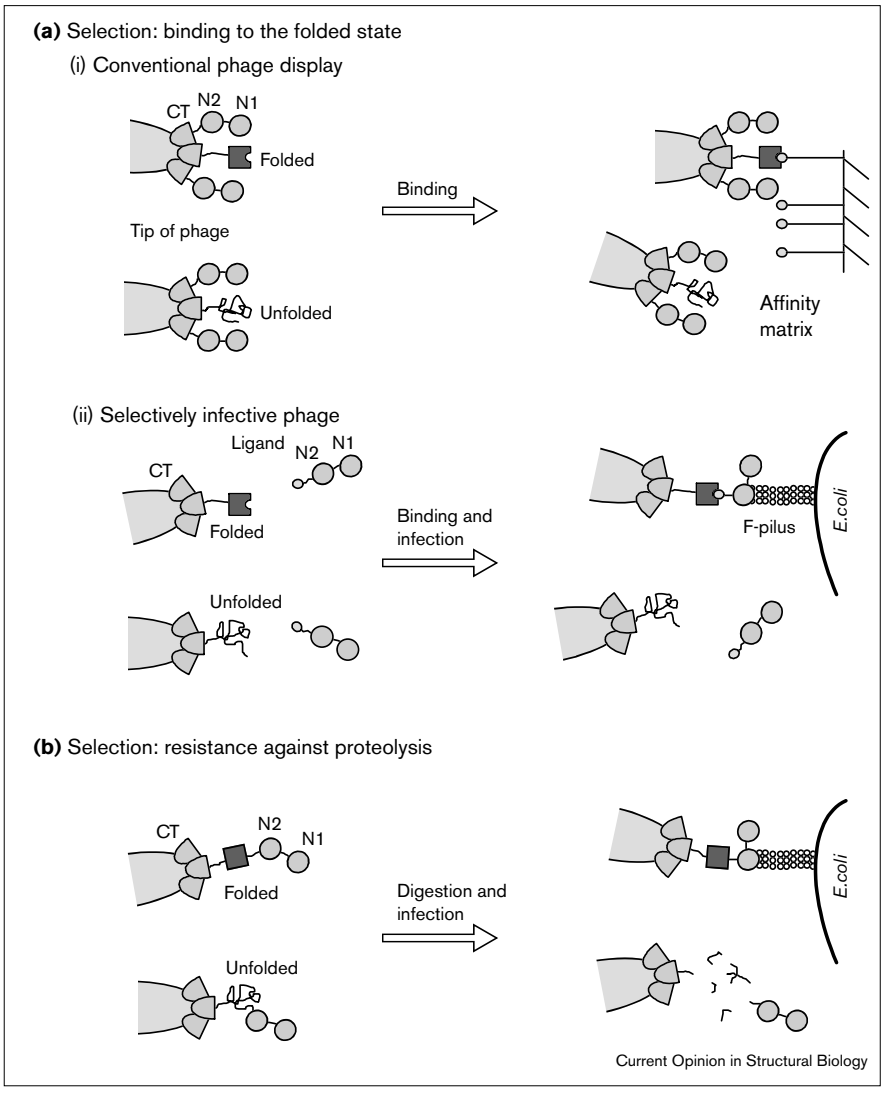
Selection for thermodynamic stability

A recent development has been the use of phage display for the selection of proteins with improved thermodynamic stability. Very often, protein stability is a crucial factor in biotechnological applications, be it at elevated temperatures or even at 37°C for an extended duration in biomedical applications, and it is quite often correlated to protein shelf life. Provided that only the intact molecule with the correct fold and thus the functional binding site can interact with an immobilized ligand, this form becomes selectable by phage display, as long as the nonspecific interactions that are typical of non-native proteins can be efficiently counterselected. Under these conditions and as long as no additional mutations alter the binding site, selection for functional ligand binding favors the polypeptide mutants that are more highly represented on the phage, that is, those for which a higher percentage of the molecules are in the native state. As a consequence, 'normal' selection for binding using phage display always includes an intrinsic selection for correctly folded library members and, thus, a 'composite' selection among acceptable overall properties. Such observations were noted by several investigators [16–18] and were systematically tested and demonstrated to occur in a study [19] in which the best folding and most stable scFv (single-chain antibody fragment) could be selected from a set of scFv with identical binding constants, but with different thermodynamic and folding properties.

It should be noted, however, that even the poor folding variants can be displayed at some level and, in the absence of molecules with more favorable folding properties, they will be selected. Phage display does not, therefore, automatically select for superb stability and folding properties. Moreover, phage display will not discriminate further among library members that all show sufficient stability and folding kinetics under the respective selection conditions [18,20•]. The experimental challenge for phage display experiments intended to improve protein stability is, therefore, to direct the selection pressure towards this property or, alternatively, to high binding affinity, whichever one is desired. In the most extreme case, the majority of the library members do not possess a free energy of folding that favors the native state at room temperature (RT), such that, in the phage display procedure, those library members that sufficiently populate the folded state at equilibrium can be enriched (Figure 1a).

This principle was exploited with a library of mutants derived from the subtilisin prodomain, randomized at four defined positions, whose wild-type sequence is 97%

Figure 1



Strategies for the selection of proteins with improved thermodynamic stability and folding. **(a)** Conventional phage display (i) and SIP (ii) intrinsically select thermodynamically stable and correctly folded members from a protein library on the basis of affinity selection. **(b)** A novel approach for selecting stable variants of the protein links the protease resistance of the protein with the infectivity of a filamentous phage. A protein library is inserted between two parts (N-terminal domains N1 and N2, and the C-terminal domain, CT) of the g3p of filamentous phage. The infectivity of the phage is lost when the two parts are disconnected by the proteolytic cleavage of unstable protein inserts. The selection pressure can be further increased by exposing the phage library to additional stress (e.g. higher temperature) before binding or digestion.

unfolded at RT. From this library, Ruan *et al.* [21•] could select a mutant that is thermodynamically stable at RT using three rounds of phage display. Similarly, a completely disulfide-free scFv stable at RT was selected from a library of mostly unstable point mutants using phage display and DNA shuffling [22]. In this experiment, a statistically improbable combination of beneficial mutations was required. Therefore, a step-wise approach was taken, in which the amino acids replacing the cysteines were selected first, one domain at a time. Only then was DNA shuffling carried out, gently increasing the selection pressure and scFv fragments that are able to fold, but that are devoid of cysteines were thus selected. Restoring the natural disulfide bridges in the stabilized mutants then resulted in an extremely stable scFv [23], demonstrating the additivity of protein stability elements in the cases investigated. Wells and co-workers [24,25] have also used similar approaches in stabilizing a minimized version of protein A.

Intrinsic stability turned out to be the selection criterion in a study designed to characterize and optimize a distinct folding motif in the first framework region of the V_L (light-chain variable domain) of an antibody in the context of an scFv fragment [26•]. In this framework region, the β strand forms a kink, as it needs to switch from one face of the β sandwich to the other. This kink occurs with length variation and different amino acid sequences in V_κ- and V_λ-type sequences. The selection was performed using the selectively infective phage (SIP) technology (Figure 1aii) [10,27]. The selected sequences were the most stable of all the sequences tested from the initial library and they are also the most abundant natural sequences.

This basic principle of phage display selection for intrinsic stability demonstrated in these examples should prove useful for rescuing molecules at the edge of stability with beneficial point mutations. Both artificially designed proteins and individual protein domains taken out of their

natural assembly may frequently require fine tuning, which will be difficult to provide by rational means at this stage. The SIP selection technology seems to be an especially powerful tool for discriminating among molecules with subtle stability differences within large libraries. For the selection of the most stable V_L kink sequence, only three selection rounds were required to separate it from species with only slightly reduced stabilities [26•] and, usually, only one selection round is required to separate functional from nonfunctional molecules [28,29].

Another strategic option in selecting for increased protein stability is to take advantage of the fact that unfolded proteins are more sensitive towards proteolytic cleavage when compared with folded proteins. It has been possible to select mutants of small, one-domain proteins, such as ribonuclease T1 [30••], barnase or villin [31••], for higher thermodynamic stability using protease cleavage sites that are not or are only poorly accessible to proteases in the folded state (Figure 1b). Sieber *et al.* [30••] termed this methodology 'protein stability increased by directed evolution', in short 'Proside'. In principle, the selection step following the destruction of the unfolded protein by proteases might be carried out by conventional ligand-binding capture. The two studies present a novel selection concept, however, by linking protease resistance directly to infectivity, thus rendering an affinity-selection step unnecessary, thereby making stability selection applicable to a broader range of proteins — those without any known ligand-binding properties.

For this purpose, the phage display system must have two special features. First, the displayed protein has to be inserted within the full-size gene-3-protein (g3p). In g3p, the infectivity-mediating N-terminal domains (N1 and N2) are linked to the phage-bound C-terminal domain of g3p (CT), normally by a glycine-rich linker, and the protein of interest can be inserted here. The recently solved structure of the N1–N2 fragment further defines the structural requirements for such fusions [32]. Second, no wild-type g3p, which would convey infectivity independent of protease resistance, must be present. Kristensen and Winter [31••] elegantly solved this problem by engineering a helper phage with protease-sensitive wild-type g3p for use in their phagemid system. In contrast, Sieber *et al.* [30••] directly used a phage system without any wild-type g3p which was derived from the SIP phages. In all of these systems, the stability range that can be selected can be extended by increasing the selection pressure using temperature or chemical denaturants (S Jung, A Plückthun, unpublished data) to which filamentous phages are relatively resistant. With these various tools at hand, it should be possible, in the future, to tailor the selection pressure directly to the protein whose stability is to be improved.

Selection for *in vivo* folding

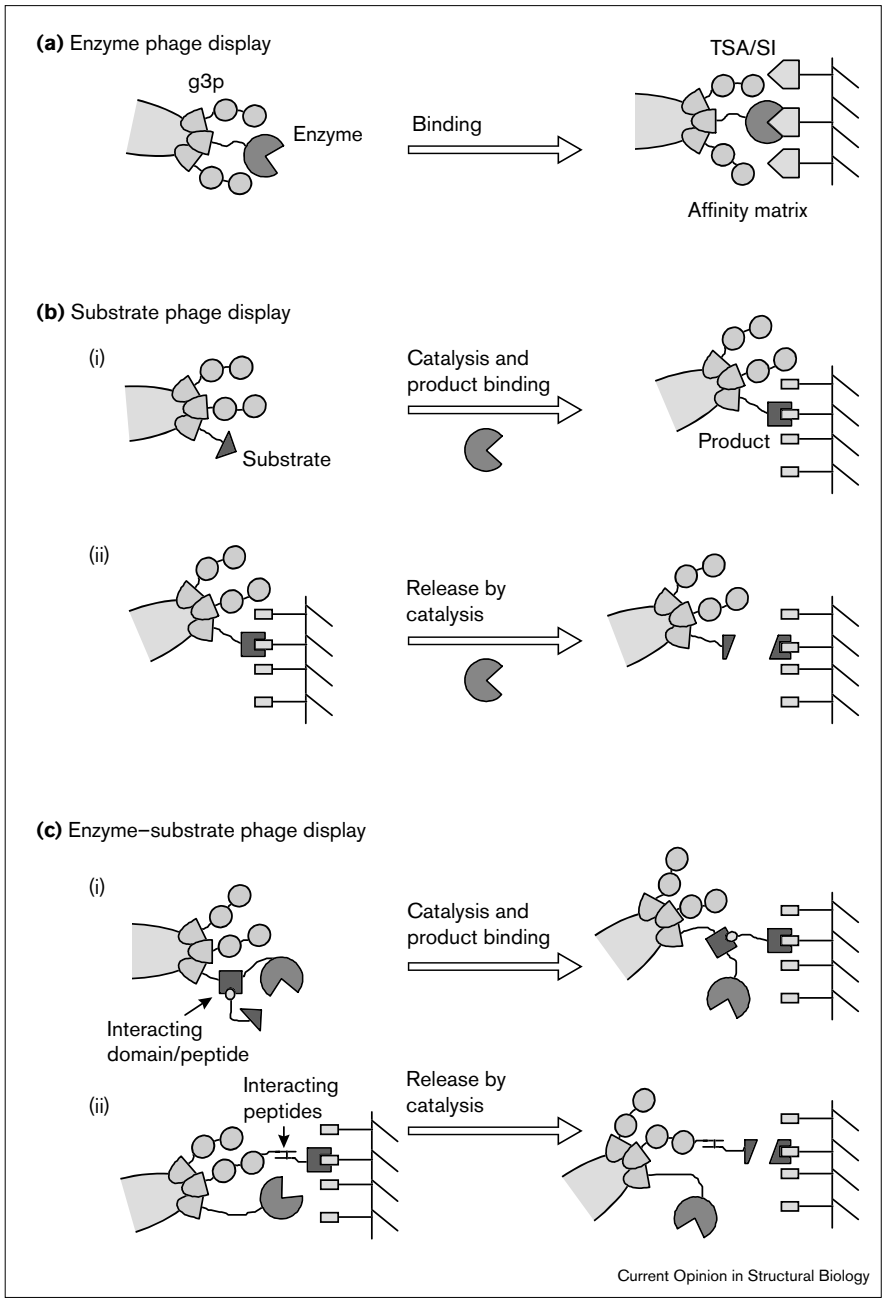
The functional display of proteins is limited not only by the thermodynamic stability, but also by the yield of the fusion protein, correctly folded during phage morphogenesis and, thus, is dependent on the extent of the off-pathway aggregation of folding intermediates. As the last step, the phage extruding from the bacterial cell is capped with a complex of the coat proteins g3p and g6p. g3p is, prior to incorporation into the phage coat, exported to the bacterial periplasm, but remains anchored to the inner membrane by a C-terminal hydrophobic helix [33]. Thus, only those g3p-fused molecules that have correctly folded in the periplasm, in the membrane-anchored form without aggregation, can be incorporated into the phage coat. In a phagemid/helper phage system, in which wild-type g3p is supplied by the helper phage, a poorly folding g3p–protein fusion will be incorporated only at low levels. Consequently, phage display can be used to select for well-expressed proteins [16,17,19].

Bothmann and Plückthun [34••] took advantage of this circumstance for the selection of periplasmic chaperones from a genomic bacterial library using phage display of a poorly folding scFv. Note that, in this approach, the displayed protein is always the same — the 'library' is not displayed, but merely co-expressed. Those cells that co-express a useful factor make progeny phage with slightly more functional scFv and become enriched. The periplasmic factor Skp was enriched by this method and the chaperone effect of Skp turned out to be not limited to the phage display system, but was also beneficial for soluble, periplasmic recombinant scFv proteins. Consequently, phage display is not only a means of selecting for the affinity and other biophysical properties of the displayed molecules, but can also be used indirectly to select for chaperones or even for other factors improving the process.

Engineering of proteins with new enzymatic activities

The generation of active catalysts and their evolutionary improvement has been studied using a wide range of approaches (reviewed in [1,5,6,35]). Various *in vivo* procedures, such as auxotrophic complementation (e.g. [3]) or, in the case of catalytic antibodies, immunization with transition-state analogs (TSAs) [36] and reactive immunization with a suicide inhibitor (SI) [37], demonstrated that catalysts with improved or novel activities could be generated. In contrast to these cell-based selection systems or even whole-animal experiments, as in most catalytic antibody studies, *in vitro* selection systems are potentially applicable in a much broader way [1]. Although phage display does not take place entirely *in vitro*, the selection step does and it has been used for the selection and evolution of protein catalysts, such as β-lactamase [38], glutathione-*S*-transferase [39] and catalytic antibodies [40•,41–43]. The selection systems described function either by covalent trapping of active catalysts with a SI (phage trapping) [38,42,43] or by selection for TSA binding [39,40•,41] (Figure 2a). These procedures have the main limitation, however, that appropriate SIs or TSAs are not available for most enzymatic reactions or, at least, constitute a considerable synthetic effort. In addition, screening is indirect, as turnover is not directly selected for. Selected candidate clones have to be

Figure 2



Strategies for the selection of proteins, catalysts or polypeptide substrates. **(a)** Protein libraries are displayed on the phage surface and are selected by binding to a TSA or by covalent trapping with a SI. **(b)** A phage-displayed polypeptide library is incubated with the enzyme of interest, followed by selection upon product formation – either by product binding (i) or by substrate cleavage (ii). In contrast to (c), this technique is limited to polypeptide substrates. **(c)** Enzyme–substrate phage display is a combination of (a) and (b). Each phage of the library displays simultaneously a protein variant and an appropriate substrate in close proximity. After incubation of the library under the desired reaction conditions, phages displaying active enzymes can be physically isolated using specific antiproduct affinity reagents (i) or they can be released by substrate cleavage (ii).

screened subsequently *in vitro* for efficient catalysis and interesting catalysts might have been missed in the steps before. To circumvent these problems, the most direct and elegant approach would be to isolate the catalysts from a mixture by selection for catalytic turnover.

Regulatable enzymes

Legendre *et al.* [44••] have now reported a new variation of directed enzyme evolution using phage display — the engineering of a regulatable TEM-1 β-lactamase. They introduced random peptide sequences near the active site of β-lactamase and used the antibiotic resistance provided by this enzyme to preselect, *in vivo*, the clones encoding active proteins. Biopanning of the active library on immobilized monoclonal antibodies or streptavidin resulted in hybrid enzymes whose activity was reduced, in most cases, upon binding to the new epitope, as the antibodies or streptavidin may block the entrance to the active site. Such artificially regulatable enzymes might have potential as biosensors. One limitation of this system, however, appears to be the *in vivo* preselection of catalytic activity, which will not be feasible for most enzymes. Thus, this strategy would be more generally applicable if the preselection of activity could also be done *in vitro*.

Substrate phage display

Phage display is highly useful not only for the selection and evolution of enzymes, but also for the selection and evolution of polypeptide substrates (substrate phage display) [13,45–47] (Figure 2b). Matthews and Wells [46] first reported such an experiment for identifying good protease substrates among a large, random peptide library displayed on filamentous phage. In this particular case, the desired phage can be liberated by the protease. In a more general way, the product-containing phage would be bound, for example, by an antibody, similar to the catELISA approach, in which the occurrence of an enzymatic reaction is detected by an antibody specific for the product [48]. In a similar fashion, Stolz *et al.* [49•] recently used bacteriophage Lambda for the display of random polypeptide fragments derived from a biotin-accepting protein domain. Phages displaying a functional domain, which can be biotinylated in the cytoplasm of *Escherichia coli*, were identified after affinity selection with avidin. In this way, a minimal polypeptide of 66 amino acids required for *in vivo* biotinylation was determined. This work shows that it is feasible to select for the occurrence of an enzymatic reaction by product binding. In addition, their work underscores the use of Lambda phage display [50,51] as an alternative to the widely used filamentous phage display. Peptides that can be enzymatically biotinylated *in vivo* had previously been identified using the peptide-on-plasmid technology [7].

Functional cloning of enzymes

In independent studies, Pedersen *et al.* [52••] and Demartis *et al.* [53••] recently developed a conceptually similar phage display technique for the functional cloning and directed evolution of protein catalysts (Figure 2c). It consists of a novel combination of phage-displayed enzymes and substrate phage display. In contrast to substrate phage display, however, this approach is not limited to the use of polypeptide substrates. The enzyme and an appropriate substrate have to be anchored to the same single phage particle and the link between catalysis and phage replication is thereby established. This technique directly selects for the occurrence of a single catalytic turnover, as the reaction product is captured in the selection step. Thus, this methodology requires neither a TSA nor a SI for the reaction in question, but it does need a product-specific affinity matrix.

Pedersen *et al.* [52••] developed this technique using staphylococcal nuclease as a model enzyme. The reaction substrate was covalently and site-specifically attached to one or more copies of g3p in the filamentous phage capsid, whereas the protein catalyst was attached to another copy of g3p on the same phage particle. Thereby, the required proximity of enzyme and substrate is achieved. The authors showed that phages displaying this nuclease could be enriched 100-fold in a single step from a library-like ensemble of phage displaying noncatalytic proteins. In contrast to Pedersen *et al.* [52••], Demartis *et al.* [53••] tethered the enzyme and the reaction substrate to the same copy of g3p using enzyme–calmodulin fusion proteins and a substrate that is linked to a calmodulin-binding peptide. Thus, upon the binding of the calmodulin-binding peptide to calmodulin, the desired proximity between enzyme and substrate is achieved. After product binding, the selected phages may be simply eluted by breaking the calmodulin-binding peptide–calmodulin interaction using calcium chelators. This scheme might also be transferable to other phage display systems, because only a single fusion to a coat protein is needed. Demartis *et al.* [53••] used glutathione-*S*-transferase and an endopeptidase in a model selection experiment and obtained enrichment factors on the order of 50 per single round of selection. No actual library experiments have been reported yet by either group, and it will be interesting to see whether these enrichment factors, obtained against zero-activity background, are powerful enough to favor those variants with a quantitative advantage, which will be the crucial requirement for any directed evolution experiment.

The high local substrate concentration achieved by coupling the substrate in the vicinity of the enzyme, together with a low overall substrate concentration, elegantly ensures that only the reaction involving the substrate and catalyst from the same phage particle will occur; however, this high local substrate concentration may, at the same time, disfavor selection for improved Michaelis–Menten constants (K_Ms) above a certain minimal level. As only a single turnover has to occur per phage particle in order to be selectable and because of the slow phage handling, it may also be difficult to select for high k_{cat} (catalytic rate constant) values. Moreover, the need for a covalent linker to anchor the substrate on the phage might be a limitation for some reactions, as it prevents the substrate from being completely buried by the protein catalyst, a step that is inherent in the mechanism of many natural enzymes.

Conclusions

During the past decade, phage display has proven to be highly successful for affinity selection of novel binders against innumerable targets [12,13,15,54]. It is now recognized that, in addition, phage display intrinsically selects reasonably thermodynamically stable and folded members from a protein library, provided, of course, that such members exist in the library, because of the interdependence of functional display and affinity selection [21•,22,26•]. A new selection concept for the isolation of proteins with increased stability links the protease resistance of folded species directly to infectivity [30••,31••]. This method has the great advantage that it is broadly applicable to all polypeptides without the need for a binding ligand, yet at the price of not maintaining the selection pressure for function.

On another front, new techniques for the identification and enrichment of proteins with enzymatic activity using phage display have been developed [52••,53••]. The main value of this strategy appears to lie in the identification of novel protein catalysts from repertoires on phage, such as, for example, the selection of a specific enzyme out of a

cDNA library or a catalytic antibody out of an appropriate antibody library. In this case, the proximity of the substrate and catalyst on the phage particle has the potential advantage that poor catalysts are also selectable. It is much less clear whether this technique may be suitable for improving protein catalysts, because, in this single turnover high-substrate experiment, it may be difficult to select K_M and k_{cat} values above a certain minimal level.

Despite the great potential of filamentous phage, inherent limitations imposed by its assembly mechanism limit the scope of proteins that can be displayed with this phage class. Display with lytic phage, such as bacteriophage Lambda [13,14,50,51,55], may complement the filamentous phage system, especially for the functional cloning of intracellular enzymes.

The ultimate aim driving protein engineering is to modify or enhance the stability, binding or catalytic properties of a protein. The new phage-display techniques presented here are very promising further steps in this direction. They show the great potential of this selection system, which needs to be developed further and fine tuned for the selection of any desired selectable property of a protein beyond binding, be it catalysis, stability or even an external factor acting as a chaperone. Nonetheless, phage display is only one of several options for powerful selection systems [7–11] and future work will have to define which systems perform best in which setting. The major scientific challenge for the future, however, is to define the selection pressure to truly direct evolution in the desired selection. "You get what you screen for" (YGWYSF) [4], and it will be essential to learn how to fully control this. This is where directed evolution and rational engineering will converge.

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