**Synthetic biology of modular proteins**

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**ABSTRACT**

The evolution of natural modular proteins and domain swapping by protein engineers have shown the disruptive potential of non-homologous recombination to create proteins with novel functions or traits. Bacteriophage endolysins, cellulosomes and polyketide synthases are 3 examples of natural modular proteins with each module having a dedicated function. These modular architectures have been created by extensive duplication, shuffling of domains and insertion/deletion of new domains. Protein engineers mimic these natural processes *in vitro* to create chimeras with altered properties or novel functions by swapping modules between different parental genes. Most domain swapping efforts are realized with traditional restriction and ligation techniques, which become particularly restrictive when either a large number of variants, or variants of proteins with multiple domains have to be constructed. Recent advances in homology-independent shuffling techniques increasingly address this need, but to realize the full potential of the synthetic biology of modular proteins a complete homology-independent method for both rational and random shuffling of modules from an unlimited number of parental genes is still needed.

Protein engineers mimic natural evolution *in vitro* to create proteins that are better adapted to the application they have envisioned. Natural evolution is featured by selection of the fittest variant that arose either through mutation or exchange of genetic fragments. Whereas the accumulation of mutations represents a gradual, adaptive evolution process, genetic recombination leads to more disruptive evolution and even neofunctionalization. A range of mutagenesis and combinatorial engineering techniques has been developed to create *in vitro* libraries of variants used for directed evolution. Most commonly used combinatorial techniques such as DNA shuffling, *in vivo* homologous recombination, staggered extension process (StEP), random priming recombination, however, all rely on a high degree of homology (at least 70%). In contrast, evolutionary studies suggest that natural evolution is not limited to recombination of highly similar sequences only. Computational analyses and the prevalence of multidomain proteins demonstrate the success of non-homologous recombination to evolve completely novel protein functions in nature (reviewed by Lutz and Benkovic). Nearly half of all proteomes consist of modular or multidomain proteins with each module or domain (both words are often used interchangeably, depending on the specific research field) having a dedicated function, ranging from substrate binding or interaction, transfer of intermediates, structural assembly to catalysis. Studies have shown that the number of known domains has been stagnating for almost a decade with only a few hundred new ones reported every year, while the number of new proteins with rearranged domains is still exponentially increasing. Natural evolutionary mechanisms that result in those new protein architectures include extensive duplication, shuffling of domains and insertion/deletion of a new domain, whereof insertion/deletion is the most frequent event for bacteria. The specific modular architecture and composition determines the function, specificity and performance of each modular enzyme.

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Protein engineers have been inspired by this modularity principle and have been swapping modules between different parental genes to create chimeras with altered properties or even novel functions (neofunctionalization). Indeed, the plasticity of modular proteins offers a large potential to engineer customized proteins that perform optimal under specific conditions, have a tailor-made specificity or synthesize designed products. A prominent example is domain swapping of endolysins. These are peptidoglycan hydrolases encoded by bacterial viruses or bacteriophages and produced at the end of the lytic cycle. They enzymatically degrade the peptidoglycan layer from within the infected bacterial cell. Endolysins are specified proteins that perform optimal under specific conditions, have a tailor-made specificity or synthesize designed products. A prominent example is domain swapping of endolysins. These are peptidoglycan hydrolases encoded by bacterial viruses or bacteriophages and produced at the end of the lytic cycle. They enzymatically degrade the peptidoglycan layer from within the infected bacterial cell. Endolysins from Gram-positive phages mostly display a modular structure, comprising one or more enzymatically active domains (EADs) and one, mostly C-terminal, cell wall binding domain (CBD) (Fig. 1A). Bacteriophages of Gram-negative organisms on the other hand produce primarily globular endolysins with a single EAD. Endolysins have high in vitro and in vivo antimicrobial activity and show a low probability to provoke resistance development. Furthermore, they are specific at the genus, species or even serovar level, leaving beneficial flora unaffected. Horizontal gene transfer between bacteriophages and their host bacterial cells has been suggested based on the evolutionary relationships between bacteriophage endolysins and bacterial lytic enzymes (autolysins). Several natural chimeric endolysins have been described, such as the *Listeria* phage endolysin PlyPSA which consists of an EAD that is related to the amidase domains of *Bacillus* and *Clostridium* phage endolysins and a CBD that is highly similar to those of other *Listeria* phage endolysins. Another example is the lytic enzyme of the pneumococcal phage Dp-1 which comprises an EAD that is related to the murein hydrolase of a *Lactococcus lactis* phage endolysin and a CBD homologous to the pneumococcal lytic system. The discovery of natural chimeras has supported the construction of synthetic chimeric endolysins consisting of modules from different origin. As such, Dong et al. have extended the lytic spectrum of a staphylococcal lysin by fusing its EAD with the CBD from a lysin with lytic spectra across multiple genera. Other researchers demonstrated that switching the CBDs of 2 endolysins results in a swap of their binding and lysis specificity and that combining 2 CBDs results in a binding spectrum comprising the spectra of both original CBDs. Furthermore, an endolysin with an improved thermostability has been made by fusing the N-acetylmuramoyl-L-alanine amidase domain from the endolysin of a thermophilic bacteriophage to the CBD from the *Clostridium perfringens*-specific bacteriophage endolysin. An example of neofunctionalization in endolysin research is the creation of Artilysin®s (Fig. 1B) that fuse modules of unrelated origin to create endolysin-based antibacterials against Gram-negative bacteria. Specifically, Briels et al. have fused the sheep myeloid antimicrobial peptide of 29 amino acids to the N-terminus of the endolysin KZ144. As such, Artilysin®s can pass the protective outer membrane of Gram-negative pathogens from the outside and overcome this limitation of endolysins.

A natural exponent of protein modularity that has been exploited by protein engineers are cellulosomes that degrade lignocellulose. Lignocellulosic biomass is the most abundant renewable resource and the most attractive substrate for biorefinery strategies to produce bio-based products such as biofuels, bioplastics or enzymes. Full enzymatic breakdown of lignocellulose requires the simultaneous action of several synergistic catalytic activities (e.g. cellulases, hemicellulases, lytic polysaccharide monoxygenases and pectinases). In nature, anaerobic bacteria have developed complex modular systems called cellulosomes (Fig. 1D) comprising a modular non-catalytic scaffold which can be linear or in most mesophilic *Clostridia*, or hierarchically branched as for *Clostridium thermocellum*. Each subunit of the scaffold can strongly interact with a complementary dockerin subunit, borne by enzymes with diverse catalytic activities. These multi-enzyme complexes give an advantage to aerobic cellulolytic bacteria that secrete free (hemi)cellulases because of the enhanced synergism among the catalytic units and close association between the cell-bound cellulose and the substrate. The modular nature of the cellulose complex and its beneficial properties led to the concept of designer cellulosomes (also called minicellulosomes) by incorporating recombinant (hemi)cellulose-degrading enzymes from diverse origin into the complex using the specific affinity of cognate cohesin-dockerin pairs. You et al. obtained an enhanced hydrolysis rate on Avicel and regenerated amorphous cellulose (RAC) using a four-component designer mini-cellulosome containing endoglucanases and a cellbiohydrolase from 3 different organisms compared to the non-complexed
Figure 1. Natural modular proteins and 2 neofunctionalized chimeric enzymes. (A) Endolysin (adapted from Roach and Donovan); (B) Artilysin; (C) Scaffolded enzyme cascade; (D) Simplified natural cellulosome with a linear scaffoldin; (E) Two parts of the 6-deoxyerythronolide B synthase (DEBS) polyketide synthase (adapted from Weissman). EAD = Enzymatically active domain; CBD = Cell wall binding domain; OMP = Outer membrane permeabilizing peptide; CO = Cohesin; DO = Dockerin; ADH = Alcohol dehydrogenase; FAL = Formaldehyde dehydrogenase; FDH = Formate dehydrogenase; SLH = S-layer homology module; CM = Catalytic module (each number refers to a different module); CBM = Carbohydrate binding module; AT = Acyl transferase; ACP = Acyl carrier protein; KS = Ketosynthase; KR = Ketoreductase; DH = Dehydratase; ER = Enoyl reductase.
cellulose mixture. The addition of an expansin-like protein, i.e. a protein which disrupts hydrogen bonding between cellulose microfibrils and other cell wall polysaccharides without hydrolytic activity, to a bifunctional designer cellulosome of cellulases, enhanced the reducing sugar yield by 2-11%. Other noncellulosomal enzymes have also been added to the designer cellulosome for synergistic effects. For example, Arfi et al.21 have developed a designer cellulosome consisting of a lytic polysaccharide monooxygenase of Thermobifida fusca and 2 cellulases resulting in a 63-68% improvement in soluble sugars release. The cohesin-dockerin scaffold has also served to create other enzyme cascades. Liu et al.22 have created a protein scaffold with 3 enzymes docked to cohesins, namely 3 dehydrogenases responsible for sequential conversion of methanol to carbon dioxide (Fig. 1C), while You et al.23 used a similar assembled structure to convert glyceraldehyde-3-phosphate to fructose-6-phosphate.

Another striking example of modular enzymes subjected to swapping of non-homologous domains are the gigantic multi-enzyme type I polyketide synthases (PKSs). The biosynthesis of large numbers of structurally and stereochemically complex polyketides from a relatively small pool of simple precursors is achieved by cascades of individual enzymes present in the multi-enzyme complex.24 Structurally, type I PKSs consist of independently folded domains which are grouped into working units called modules for the introduction and functional tailoring of one building block into the growing chain (Fig. 1E). Two distinct groups of modular enzymes can be distinguished: the cis-acyl transferase (AT) PKS and the trans-AT PKS. The first one arose from repeated rounds of gene duplication coupled with domain diversification by homologous recombination, while the second one evolved through horizontal transfer of substrate-specific ketosynthase domains.25,26 These distinct modes of evolution resulted in structurally diverse classes of modular enzymes. The cis-AT PKS minimally contains one AT domain in each module, whereas AT is an isolated protein which acts iteratively in trans to deliver an extender unit to all of the modules in trans-AT PKS. Furthermore, extension modules of the cis-AT PKS contain a ketosynthase (KS), an acyl carrier protein (ACP), or some domains for optional processing activities, while the trans-AT PKS class consists of duplicated domains (e.g., doublets or triplets of ACPs), inactive domains with a cryptic function such as epimerase activity, and modules of nonribosomal peptide synthetase (NRPS) permitting the activation and incorporation of amino acids into the chain-extension intermediates.24,27 To date, several strategies have been applied to engineer PKSs with novel properties ranging from inactivation of specific reductive domains, over site-directed mutagenesis to domain and module swapping. Kushnir et al.28 have disabled ketoreductase, dehydratase and enoyl reductase domains to produce a library of 22 oxidized derivatives of premonensin, a shunt metabolite which structurally resembles an anti-cancer polyketide, while Kong et al.29 have introduced modifications within the polyene class of antifungal polyketides, leading to improved solubility and reduced hemolytic activity of the polyketide. Hagen et al.30 have produced adipic acid using a neofunctionalized, chimeric PKS consisting of heterologous reductive domains from various PKS clusters into the borrelidin PKS’ first extension module. However, most domain and module swapping approaches were less successful. Menzella et al.31 for example have interchanged 14 modules from 8 PKS clusters resulting in 154 bimodular combinations. Only half of the combinations gave the expected triketide lactone product due to the introduction of unnatural intermodular junctions.

As noted from the examples above, natural protein modularity and domain swapping efforts demonstrate the disruptive potential of non-homologous domain shuffling. However, the majority of protein engineering studies has been based on a rational approach using classical restriction and ligation protocols, which becomes particularly cumbersome when a large collection of variants or variants of multidomain proteins (e.g. PKSs) have to be constructed. More diverse libraries of shuffled endolysins, designer cellulosomes and PKSs would allow to explore sequence space to a larger extent. Methods that allow full combinatorial, complete homology-independent shuffling from an unlimited number of parental genes would therefore significantly enhance the synthetic biology of modular proteins. Recent advances in the field such as the development of SHIPREC, ITCHY, SCRATCHY, SCOPE, SISDC and USERe32-37 increasingly respond to this unmet need with each method having its benefits and drawbacks. The strength of SHIPREC, ITCHY and SCRATCHY is the creation of non-biased and almost non-homologous recombination. Unfortunately, they are all limited to the shuffling of 2 parental genes. SISDC already allows for the facile
recombination of 2 to 4 distantly related (or unrelated) proteins at multiple discrete sites. Moreover, this technique makes use of type IIb restriction-enzymes which cut outside the recognition site resulting in a scarless hybrid. The use of these restriction enzymes are actually also a disadvantage of the method as their introduction is laborious covering multiple PCRs. Furthermore, due to frameshifts a large number of non-functional sequences are obtained with the former methods.36,37 The most recently developed method, USERec, allows to shuffle several completely unrelated modules in a scarless manner, but a random combinatorial approach of unrelated fragments is complicated because specifically designed primers are required for each junction. It is expected that completely random shuffling of non-related fragments will – similar to random mutagenesis – generally yield many unexpectedly novel, improved chimeric proteins that combine fragments that could not be rationally envisioned beforehand. As such, unlimited, random shuffling of a high number of non-related gene fragments from many different sources remains the next big leap in the field of synthetic biology of modular proteins.

**Abbreviations**

| Acronym | Description |
|---------|-------------|
| ACP     | acyl carrier protein |
| AT      | acyl transferase |
| CBD     | cell wall binding domain |
| EAD     | enzymatically active domain |
| ITCHY   | incremental truncation for the creation of hybrid enzymes |
| KS      | ketosynthase |
| PKS     | polyketide synthase |
| SCOPE   | structure-based combinatorial protein engineering |
| SHIPREC | sequence homology-independent protein recombination |
| SISDC   | sequence-independent site-directed chimeragenesis |
| StEP    | staggered extension process |
| USERec  | uSER friendly DNA recombination |

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