

Genetically modified proteins: functional improvement and chimeragenesis

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This review focuses on the emerging role of site-specific mutagenesis and chimeragenesis for the functional improvement of proteins in areas where traditional protein engineering methods have been extensively used and practically exhausted. The novel path for the creation of the novel proteins has been created on the farther development of the new structure and sequence optimization algorithms for generating and designing the accurate structure models in result of x-ray crystallography studies of a lot of proteins and their mutant forms. Artificial genetic modifications aim to expand nature's repertoire of biomolecules. One of the most exciting potential results of mutagenesis or chimeragenesis finding could be design of effective diagnostics, bio-therapeutics and biocatalysts. A sampling of recent examples is listed below for the *in vivo* and *in vitro* genetically improvement of various binding protein and enzyme functions, with references for more in-depth study provided for the reader's benefit.

The idea of design and synthesis of proteins with altered properties through genetic modification was suggested by nature itself.^{1–8} Genes contain coded information that leads to the production of proteins. Proteins fold up to form 3D molecular architectures that are essential for biological functions. Although the natural proteins perform wide variety of tasks, they appear to use only a limited number of structural types. Based on perception that protein structures is more conserved than sequences, these are used over and over again, being altered through evolution to generate many different functions under environmental constraints.^{9,10,11,12} Mutations such as amino acid substitution, sequence deletion/insertion or DNA shuffling are selected depending on their impact on the chemical nature of catalytic residues, active site conformation, dynamical flexibility, local packing density, relative solvent accessibility, multimeric complexity, and the protein's ability to fold rapidly and stably.^{13–16} Site-specific evolutionary rates have been found to be mainly determined by side-chain packing.¹⁶ Moreover, the structurally disordered regions often have high conformational flexibility allowing to evolve structure and modulate function in recognition proteins and enzymes.^{11,17–19} The natural selection at the

DNA and RNA level, including splicing and gene duplication or transfer, acting during the protein's production history can also affect protein evolutionary rates and amino acid choice that is as important as expression parameters.^{18,20,21}

The analysis of the natural proteins and their mutants with X-ray crystallography allowed to develop the structure and sequence optimization algorithms for the new protein structure determination, prediction and design generating accurate structure models.^{22–27} Although the design principles used are learnt from natural proteins, some of the designed protein shapes are completely new and have not been observed in nature yet.^{28–32}

Artificial genetic modifications aim to expand nature's repertoire of biomolecules. One of the most exciting potential results of mutagenesis or chimeragenesis finding could be design of effective diagnostics, bio-therapeutics and biocatalysts.^{24,28,33,34} Although certain wild type proteins are very effective pharmaceuticals or industrial agents, they generally suffer from heat/cold stability and activity, undesirable specificity/selectivity, foldability or utility problems.

This review focuses on the emerging role for site-specific mutagenesis and chimeragenesis for the functional improvement of proteins in areas where traditional protein engineering has been extensively used, such as chemical modification, modifying fluorescence, functional studies, biophysical analysis. A sampling of recent examples is listed below for the *in vivo* and *in vitro* genetically improvement of various binding protein and enzyme functions, with references for more in-depth study provided for the reader's benefit. A highlighting of *in silico* or experimentally obtained important structural details of the protein's functional domains demonstrates the power and potential of the protein genetic modifications for their application in pharmacology and industry.

Genetically Modified Proteins in Nature

There is sufficient data to study how proteins of living organism adapt to changes in the environment and how this is connected with cell metabolism and its regulation. Several levels of regulation such as transcription, translation, protein stability, enzyme regulation ensure that the response is optimal.^{35,36} In cells, proteins often occur together with other proteins in protein complexes to fulfill their function. However, at the early stage of evolution the protein's isoforms possessed a broad substrate specificity, high catalytic abilities and/or multifunctionality have

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evolved through mutation, duplication, and horizontal gene transfer from gene families diversified and promiscuous ancestral proteins under specific stress factors or emergence of new substrates.^{7,37,38} Instead of forming a post-genomic multi-enzyme complex, the rat peroxisomal multifunctional enzyme (rpMFE1) has 5 domains with different evolutionary histories connected via flexible linker or tightly associated, 3 catalytic activities and 2 active sites (Table 1).⁶ The N-terminal part of rpMFE1 (domains A and B) catalyzes both a hydratase and an isomerase reaction. The C-terminal part belongs to the 2 tightly associated domain HAD superfamily. The HAD fold consists of an NAD-binding Rossmann-like fold (domain C) plus a dimerization domain (domain D) that extended by one more domain (domain E), whose fold is topologically identical to the domain D. The C-terminal domains (C-E) form the second active site of (3S)-hydroxyacyl-CoA dehydrogenase. The two active sites are separated by a positively charged tunnel possibly facilitating an efficient channeling of substrate from the hydratase/isomerase catalytic site to the dehydrogenase catalytic site. Comparison of the structures of rpMFE1 with the monofunctional analogs such as crotonase and (3S)-hydroxyacyl-CoA dehydrogenase superfamily enzymes, and with the bacterial $\alpha_2\beta_2$ -fatty acid oxidation multienzyme complex, revealed that this tunnel could be important for substrate channeling.⁶

Another example of the increase of metabolic efficiency by chimeragenesis is bifunctional enzyme AgaSK from human intestinal bacterium *Ruminococcus gvanus* E1 coupling α -galactosidase and sucrose kinase activities (Table 1).³ AgaSK is composed of 2 domains: the N-terminal one (residues 1–720) closely related to α -galactosidases of GH36 family enabling hydrolysis of melibiose and raffinose, and the C-terminal domain (residues 721–935) containing a nucleotide-binding motif for specifically phosphorylation of sucrose on the C6 position of glucose in the presence of ATP. The crystal structure of AgaSK highlighted an oligomeric state necessary for efficient substrate binding and suggesting a cross-talk between the galactose and kinase domains.³

For the improvement of catalytic efficiency in the conditions of the membrane topologies and structures, the C-terminus of cyclooxygenase isoform-2 (COX-2) was linked to the N-terminus of microsomal prostaglandin E2 synthase-1 (mPGES-1) through a transmembrane linker to form a hybrid enzyme, COX-2-10aa-mPGES-1 (Table 1).² These inducible enzymes become up-regulated in inflammation and some cancers, and their coupling reaction of converting arachidonic acid (AA) into prostaglandin (PG) E2 (PGE2) is responsible for inflammation and cancers. The engineered hybrid enzyme expressed in HEK293 cells exhibited strong triple-catalytic functions in the continuous conversion of AA into PGG2 (catalytic-step 1), PGH2 (catalytic-step 2) and PGE2 (catalytic-step 3), a proinflammatory mediator. The hybrid enzyme retained similar K_d and V_{max} values to that of the parent enzymes, suggesting that the configuration between COX-2 and mPGES-1 (through the transmembrane domain) could mimic the native conformation and membrane topologies of COX-2 and mPGES-1 in the cells. The results confirmed that the enzymes are localized near each other in a face-to-face

orientation in nature, where the COX-2 C-terminus faces the mPGES-1 N-terminus in the ER membrane.²

Under the necessity of the regulatory mechanism improvement through chimeragenesis, a bifunctional enzyme of hepatitis C (HCV) protein 3/4A (NS3/4A) comprising 2 separate domains with protease and helicase activities evolved for the more effective viral propagation (Table 1).⁵ The functional analysis of the full-length protein and separately isolated catalytic domains suggested the existence of dynamic coupling through the interdomain communication. The full-length protein was more efficient in RNA unwinding than the isolated protease domain. However, substrate cleavage and DNA unwinding by the disrupted mutants were mostly enhanced compared with the wild-type protein. The HCV NS3/4A crystal structure and molecular dynamic simulations have shown that the hybrid enzyme adopts an “extended” catalytically active conformation, and interface formation acts as a switch to regulate activity modulating the protease and helicase activities.⁵

There are many examples of genetic modification of proteins in nature by recombination of sequences or domains between homologous proteins for obtaining chimeras with improved properties. The mammalian galectin-3 and galectin from the sponge, *Geodia cydonium*, and other members of this subfamily have chimera-type characteristic and sequence polymorphism within their carbohydrate recognition domains (Table 1).¹ Additional sugar-binding amino acid residues and multiple substitutions were identified to involve in a putative extended carbohydrate-binding subsite conferring a galectin-3-like specificity, and the extended groove can accommodate a tetrasaccharide interacting with terminal GalNAc α 1-3 moieties.¹

Single amino acid substitution and sequence insertion/deletion were also found to alter appreciably specific activity/functionality in proteins. An important signaling enzyme, dual-specificity phosphatase (DSP), whose misregulation is linked to cancer, diabetes, inflammation, and Alzheimer's disease, possesses broad substrate specificity and dephosphorylates phosphoprotein monoesters and the β -phosphate of RNA (Table 1).⁸ A conservative mutation of L192 to arginine and noncatalytic C-terminal extension (residues 206–330) affected on enzymatic activity resulting in the appearance of diphosphatase activity in the monospecific ancestral protein phosphatase.⁸

Two α -galactosidases AgaA and AgaB from thermophilic bacterium *Geobacillus stearothermophilus* have 97% identity and differ from each other by only 22 amino acid residues (Table 1).⁴ This has drastically reflected on their different catalytic efficiency and temperature optimum. α -galactosidase AgaA is fully active at 338K against raffinose that can be used for increasing the yield of manufactured sucrose. AgaB has lower affinity for its natural substrates but is more efficient catalyst at 323K for the enzymatic synthesis of disaccharides by transglycosylation. It has been found that the A335E substitution in AgaA caused structural rearrangements resulting in a significant displacement of the invariant Trp336 at catalytic subsite that was not optimal position for ligand stacking. Hence, the active cleft of AgaA is narrowed in comparison with AgaB, and AgaA is more efficient hydrolase than AgaB against its natural substrates.⁴

Table 1. Genetically modified proteins in nature and engineered

Protein	Modification	Structure characteristic	Function	Application
Rat peroxisomal enzyme rpMFE1 ⁶	Quinque-functional hybrid enzyme (Nature)	N-terminal active site: hydratase domain (A) – flexible linker – isomerase domain (B) – positively charged tunnel – C-terminal active site: (3S)-hydroxyacyl-CoA dehydrogenase HAD superfamily domains (C-E) fusion	Δ^3, Δ^2 -Enoyl-CoA isomerase, Δ^2 -enoyl-CoA hydratase-1, (3S)-hydroxyacyl-CoA dehydrogenase activities	Enhancing metabolic efficiency in β -oxidation pathway of fatty acid degradation
Human intestinal bacterium <i>Ruminococcus gvanus</i> E1 α -galactosidase, AgaSK ³	Bifunctional hybrid enzyme (Nature)	N-terminal GH36 α -galactosidase domain (residues 1-720) – C-terminal nucleotide-binding motif (residues 721-935)	α -Galactosidase and sucrose kinase activities	Enhancing metabolic efficiency: melibiose and raffinose hydrolysis and phosphorylation of sucrose on the C6 position of glucose in the presence of ATP
Membrane protein, COX-2-10aa-mPGES-1 ²	Bifunctional hybrid enzyme (Nature)	C-terminus of cyclooxygenase isoform-2 (COX-2) - transmembrane linker – N-terminus of microsomal prostaglandin E2 synthase-1 (mPGES-1)	Cyclooxygenase and prostaglandin E2 synthase-1 activities	Acceleration of inflammation and cancers response: coupling reaction of converting arachidonic acid into prostaglandin E2
Hepatitis C protein 3/4A, HCV NS3/4A ⁵	Bifunctional hybrid enzyme (Nature)	N-terminal chymotrypsin-like serine protease (residues 1-181) – linker residues 184-186 (regulatory switching domain–domain interface) – C-terminal superfamily 2 DExD-box helicase domain	Protease and 3′-5′ helicase activities	Enhancing viral propagation efficiency: duplex DNA and RNA unwinding, viral polyprotein cleavage for viral maturation, the cleavage of host innate immune response antiviral signaling protein (MAVS) and interferon- β (TRIF), autoregulation of the protease and helicase activities
Sponge <i>Geodia cydonium</i> galectin ¹	Chimeric lectin (Nature)	Homologous sequences recombination within carbohydrate recognition domain and extension of galectin-3-like carbohydrate-binding subsite	Carbohydrate-binding activity	Extension of ligand specificity: accommodation to binding tetrasaccharide terminal GalNAc α 1-3 moieties
Human dual-specificity phosphatase, DSP ⁸	Mutant (Nature)	Amino acid substitution L192A and noncatalytic C-terminal extension (residues 206-330)	Protein phosphatase and alkaline phosphatase activities	Enhancing signaling transduction efficiency: dephosphorylation of phosphoprotein monoesters and β -phosphate of RNA for cancer, diabetes, inflammation, and Alzheimer's disease prevention
Thermophilic bacterium <i>G. stearothermophilus</i> α -galactosidases, AgaA ⁴	Mutant (Nature)	Amino acid substitution A335E, displacement of Trp336 at catalytic subsite	α -Galactosidase and transglycosylation activities	Increasing yield of sucrose: raffinose hydrolysis and enzymatic synthesis of disaccharides
Bacteriophage lysine, Ply187N-V12C ⁷⁰	Chimeric protein (Engineered)	Catalytic domain of Ply187 lysin; bacterial cell-binding domain of PlyV12 lysin (residues 146-314)	Lytic activity	Enhancing antimicrobial therapy efficiency: extension of lytic activity against human pathogens (staphylococci, streptococci, enterococci)
<i>Bacillus megaterium</i> steroid hydroxylase, CYP106A2 ⁶⁵	Single mutant (Engineered)	Amino acid substitutions A395L or G397P	Cytochrome P450 activity	Enhancing steroid hydroxylation efficiency: extension of regiospecificity to 3-oxo- Δ^4 -steroids (progesterone, from 15 to 11-position; increase of 11 α -, 9 α - and 6 α -hydroxylation activities up to fourfold
<i>Candida antarctica</i> lipaseB, CalB ⁶⁸	Single, double, triple and quadruple mutants (Engineered)	Amino acid substitutions G39A, T103G, W104F, A141Q, I189Y, L278A	Lipase and amidase activities	Introducing and enhancing amidase activity up to 3-fold wild type activity for the preparation of optically active amines
Mannan-binding holothurian <i>A. japonicus</i> lectin, CmAP/MBL-AJ ⁴⁴	Bifunctional hybrid protein, double mutant lectin (Engineered)	N-terminal alkaline phosphatase domain – flexible linker (G ₄ S) ₃ – C-terminal mannan-binding lectin domain; amino acid substitutions A137N/F159K in the lectin active center	Alkaline phosphatase and carbohydrate-binding activities	Enhancing lectin activity by 25 \pm 5% and utility in enzyme linked lectin assay (ELLA) for early differentiated cervical cancer diagnosis

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Table 1. Genetically modified proteins in nature and engineered (Continued)

Protein	Modification	Structure characteristic	Function	Application
<i>Trametes multicolor</i> pyranose 2-oxidase, TmP2Ox ⁶⁶	Double mutant (Engineered)	Amino acid substitutions V546C/E542K	D-glucose and D-galactose oxidase activity, mediated electron transfer (MET)	Enhancing catalysis efficiency: carbohydrate biotransformations in food applications (ketose sugar) or as the anodic bio-component in sensors and biofuel cells
<i>Paenibacillus macerans</i> cyclodextrin glycosyltransferase ⁶⁹	Triple mutant (Engineered)	Amino acid substitutions Y260R/Q265K/Y195S	Transglycosidase activity	Enhancing maltodextrin specificity by 60% for production of vitamin C nonreducible highly antioxidant derivative AA-2G
<i>Mucor hiemalis</i> endo- β -N-acetylglucosaminidase, Endo-M ⁶⁷	Single mutant (Engineered)	Amino acid substitution N175Q	Glycosid hydrolyse and transglycosidase activities	Enhancing glycosynthase activity with oxazoline and natural N-glycan for syntheses of glycopeptides/glycoproteins
<i>Bacillus subtilis</i> aminopeptidase, BSAP ⁷⁴	Single mutants (Engineered)	Amino acid substitutions I387A, I387C and I387S	Aminopeptidase activities	Changing substrate specificity for ability to hydrolyze phenylalanine derivatives with large side chains
Guanylate kinase (GK) ⁷¹	Single mutant (Engineered)	Amino acid substitution S68P	Phosphotransferase ATP-GMP activity	Changing function from enzymatic to protein-binding (GKdom) of membrane associate guanylate kinases (MAGUK) in mitotic spindle orientation and cell adhesion
Transmembrane zinc metallopeptidase Neprilysin, NEP ⁷⁵	Double mutant (Engineered)	Amino acid substitutions G399V/G714K	Peptidase activity	Changing protein cleavage site specificity for cleavage of amyloid β 1-40 (Ab1-40) at Phe20-Ala21 for the treatment of Alzheimer's disease
Plant <i>Madagascar periwinkle</i> flavin-dependent halogenase, RebH ⁷²	Single mutant (Engineered)	Amino acid substitution Y455W	Tryptophan-specific halogenase activity	Changing substrate specificity to install chlorine preferentially onto tryptamine for production of halogenated alkaloid
Glucose 1-dehydrogenase IV, BmGlcDH-IV ⁷³	Single mutant (Engineered)	Amino acid substitution G259A	D-glucose, D-xylose, D-mannose and D-galactose 1-dehydrogenase activities	Changing substrate specificity to only D-glucose for clinical assay to examine blood glucose levels
<i>Bacillus subtilis</i> phytase ⁸²	Bifunctional hybrid enzyme (Engineered)	N-terminal β -1,4 endoglucanase domain – C-terminal phytase domain	β -1,4 endoglucanase and phytase activities	Enhancing nutrition uptake efficiency as a potential feed additive for monogastric animals
Xylanase Cel5A-XylT ⁸⁴ Glucanase XylT-Cel5A ⁸⁴	Bifunctional hybrid enzymes (Engineered)	N-terminal xylanase domain – C-terminal glucanase domain; N-terminal glucanase domain – C-terminal xylanase domain	Heat-active endoglucanase and endoxylanase activities	Enhancing efficiency of β -glucan and beechwood xylan hydrolysis in biorefineries based on plant waste
α -Glucanohydrolase, mut-dexA ⁸⁵	Bifunctional hybrid enzyme (Engineered)	N-terminal mutanase domain – C-terminal dextranase domain	α -1,3-glucanase and α -1,6-glucanase activity	Enhancing efficiency of α -glucans hydrolysis in the prevention of dental biofilm formation
<i>Bacillus subtilis</i> laccase, CotA-BglS ⁸³	Bifunctional chimeric enzyme (Engineered)	Insertion of β -1,3;1,4-glucanase gene bglS (2–214 bp) into cotA gene of laccase (between 22–24 bp), forming inter-domain contacts in Gly319-Gly320, Asn354-Ala359 and Leu634-Ser638 loops of CotA-BglS	Benzenediol:oxygen oxidoreductase and β -1,3–1,4-glucanases activities	Enhancing efficiency of hydrolysis of plant cell wall β -glucans and oxidation of aromatic compounds for sugar release from milled sugarcane bagasse
<i>Renilla reniformis</i> luciferase, Aluc30 ⁴³	Bifunctional chimeric protein (Engineered)	Insertion of epitop sequences into N-terminal ALuc23 region (between residues Pro20-Val30)	Bioluminescence	Enhancing efficiency of optical intensity and expending utilities of luciferases for the broad use in bioassays <i>in vivo</i> and <i>in vitro</i>

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Table 1. Genetically modified proteins in nature and engineered (*Continued*)

Protein	Modification	Structure characteristic	Function	Application
Multivalent antibody scFv ⁸⁰	Bifunctional hybrid antibody/ ribonuclease (4D5 scFv-Bn) - ribonuclease inhibitor (Bs-UCNP) (Engineered)	Humanized monoclonal antibody 4D5 (scFv) (HER2-targeted module)- flexible linker - bacterial ribonuclease barnase (Bn):cysteine-free C40/82A mutant barnase (Bs) – upconversion nanophosphors (UCNPs)	Ribonuclease, HER2-binding, optical (photoluminescence) activities	Enhancing efficiency of malignant tumor detection deep in the living tissue for accurate molecular diagnosis and therapy of tumor diseases
Human lectin L-FCN/MBL ⁷⁹	Chimeric protein (Engineered)	Lectin MBL carbohydrate recognition domain - L-ficolin (L-FCN) collagenous domain (simplified quaternary structure)	Ligand recognition and effector activities	Enhancing cooperativity between carbohydrate recognition domains and their cognate ligands, complement activation, and calreticulin binding dynamics for reducing infection by wild type Ebola Virus
Tumor-associated antigens MUC1 and HER2 ⁹²	Biepitop hybrid antigen (Engineered)	MUC1 subunit – HER2 subunit	Binding antibodies	Enhancing efficiency of breast cancer detection in ELISA for detection of antibodies against MUC1 or HER2 in human serum
Enterotoxigenic <i>E. coli</i> colonization factor antigens (CFAs) ⁹³	Multi-epitop multifunctional hybrid protein (Engineered)	Seven <i>E. coli</i> CFAs – labile toxin subunit B – binding subunit of heat-labile toxoid (LT) – heat-stable toxoid (ST)	Recognition, binding and toxic activities	Enhancing efficiency of protection against enterotoxigenic <i>E. coli</i> (ETEC) strains in bacterial diarrhea therapy, induction anti-CFA and antitoxin immunity

Protein Engineering and Recombinant Production

Protein engineering includes different genomic and post-genomic strategies toward expansion of the functional ability and the synthesis, *in vivo* and *in vitro*, of proteins carrying novel chemical, physical and biological properties to use in many applications in basic research, biotechnology, material sciences and therapy.^{39,40} The strategies of modification proteins through genetic manipulations involve chimeragenesis, site-specific mutagenesis and *de novo* protein engineering. The synthesis of hybrid proteins by domain swapping or the ligation of chemically or biologically synthesized peptides are often used for biological probing.⁴¹⁻⁴⁴ Combinatorial (random insertion, circular permutation, homologous and nonhomologous recombination) and non-combinatorial (rational) methods of chimeragenesis based on the original DNA shuffling or restriction-enzyme-based shuffling are also useful for the study of molecular structure/function at the specific subunit and/or domain levels.^{39,40,45} Directed evolution of DNA is one of the most important advances in biology today. Incorporation of specific amino acids into a target gene by site-directed mutagenesis allows to elucidate relationship between structure-function-biological roles of the natural proteins at the atomic level as well as to obtain the proteins with improved properties such as desired thermoactivity, specificity/selectivity, foldability and utility. The synthesis of proteins carrying a variety of unnatural amino acids are used to the study of receptors and channel proteins.^{30,39,43} Current computational methods of the structure prediction and design based on homology modeling or other structural principles adopted from natural proteins enable to predict or confirm significance either of the introduced protein modifications.²³ Molecular modeling, docking with substrate/inhibitor, dynamic simulation can facilitate adoption of decision

about the protein structure from a set of the mutants that should be realized through recombinant production to obtain the desired properties.^{26,29,44,46,47} Based on the information-driven combinatorial design, the directed evolution experiments consisted of mutation, recombination and screening processes identify interesting mutants. *In vitro* compartmentalization (IVC) is an *in vitro* gene screening system for directed evolution of proteins.^{48,49} Liposome-based IVC is characterized by *in vitro* protein synthesis from a single copy of a gene in a cell-sized unilamellar liposome and quantitative functional evaluation of the synthesized proteins.⁴⁸ Today, monitoring and optimization of the recombinant protein production processes are also advantaged by data-based modeling or by the techniques that physically link individual members of diverse gene libraries to their translated proteins for the production of the water-insoluble proteins.⁴⁹⁻⁵²

However, the recombinant production of the protein that may have the properties of the desired valuable pharmaceutical product will often not have sufficient yield when adapted to the large-scale production due to low expression, solubility or stability.^{45,53} Therefore, there are now many examples of improving the recombinant production results including both genetic modification of the recombinant proteins and their producing cells.

Genetic Modification of Producing Strains

The high efficiency of the valuable proteins is crucial to the productivity of an industrial process. One of the expedients to enhance the protein specific activity or efficiency of interest is an achievement of the natural strain high productivity or expression selectivity. Some of the reports indicated the directed evolution

in vivo by classical mutagenesis of the natural strain-producer with the use of physical/chemical methods or by recombination between the closely related strains.⁵⁴⁻⁵⁷ A random mutation in genetic materials by nucleotide substitution particularly by guanine alkylation improved the alkaline β -keratinase production by *Brevibacillus* sp. mutant strain AS-S10-II and it was successfully applied in the livestock feed formulation from the waste chicken-feathers.⁵⁵ Psychrophilic *Pseudoalteromonas haloplanktis* TAC125 was engineered for the production of the aromatic oxidative activity encoded by toluene-oxylen monooxygenase from the mesophilic bacterium *Pseudomonas* sp. OX1 for its effective application in the decontamination of cold environments.⁵⁴ The recombination of *P. haloplanktis* TAC125 genome was performed by insertional mutagenesis strategy, avoiding the introduction of any antibiotic resistance encoding gene followed by conversion of the inducible gene *PhcopA* into a constitutive one, substituting the copper inducible promoter located upstream the *PhcopABCD* cluster.⁵⁴

However, the heterologous expressions in *Escherichia coli* and the yeast strains such as *Saccharomyces cerevisiae*, *Pichia pastoris* are most often used for *in vivo* protein engineering.^{51,58-60} The commercially available DNA repair-deficient strains *E. coli* XL1-Red deficient in *mutD*, *mutS*, and *mutT* genes are transformed with a plasmid encoding the target DNA, and random mutations are accumulated at each round of DNA replication.⁵⁵ Recently several new approaches for the genome-scale engineering of *E. coli* to enhance recombinant protein expression, including improvement of mRNA stability and translational efficiency, protein folding by chaperone co-expression, expression of

disulfide-bonded proteins, acetylated and glycosylated protein production have been developed.^{51,61,62} Strains that confer improved protein expression can be engineered by screening libraries of chromosomal mutants as well as plasmid-encoded expression libraries of heterologous or native genes.^{51,58-60} The directed evolution based approach to increase protein production levels by the vector backbone optimization has also been reported.⁶³ Randomly introducing mutations in the pET28a(+), pET22b(+), and pHY300PLK vector backbones allowed to increase cellulase, lipase and protease production from two- to fourfold, respectively.

Enhancing Specific Activity

To assess a potential role of each structural element in protein, the site-specific mutagenesis experiments under controlled laboratory conditions can apparent the relationship “structure-function” at the atomic level using X-ray crystallography or molecular modeling approaches.²²⁻²⁷ Generally, the significance of amino acid residue(s) for the exhibition of the protein specific activity is determined by disruption of inter- or intramolecular bonds that are formed by the target residue(s).^{47,64} This is feasible by the substitution of the binding residue for Gly or Ala. The lost or decrease of a specific activity in the mutant variant allows to conclude about the level of participation of the substituted residues in the protein functionality. Thus, the point mutations of the residues Asp12, Asp273, Asp315, His316, Thr118, Glu268, and Trp274 to Gly in the marine bacterium alkaline phosphatase

CmAP were predicted by *in silico* mutagenesis to bind ion Mg^{2+} maintaining the correct structure of the catalytic bimetal core.⁴⁷ When the mutation interrupted a direct or indirect binding of the target residue with Mg^{2+} , the enzymatic activity was completely lost, although the integrity of the molecular structure remained and only one or 2 hydrogen bonds were broken in the entire *CmAP* hydrogen bond network (Fig. 1). Moreover, it was predicted the key role of the substrate-binding residue Arg129 and a side residue Tyr441 protruded above the active site entrance for exhibiting the unexampled high specific activity among other known alkaline phosphatases by the computational pKa calculations at the high pH ≥ 10.0 .⁴⁷

By the steric effect of some amino acid residue substitutions, the significance of decrease in a specific activity of protein can be also explained. For example, the single substitution of Cys 170 in the conserved Cys-His-Asn catalytic triad for Arg or Ser decreased the ability of the chalcone synthase PpCHS to utilize

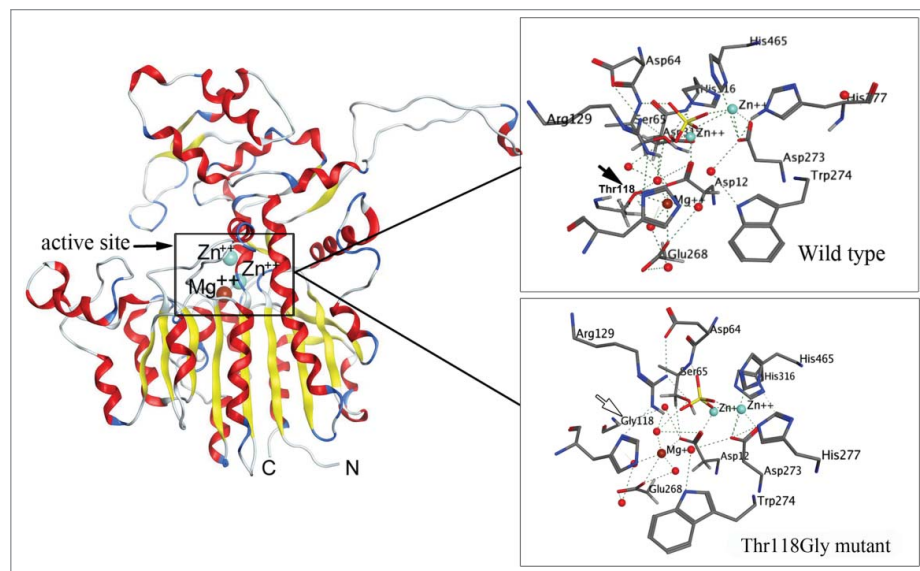


Figure 1. Molecular model of the alkaline phosphatase *CmAP* (left) and atomic structure of the wild type *CmAP* active center (above) and its mutant Tre118Gly (below) *in silico* generated with program MOE 2012.10.⁴⁷ The coordinates of the metal ions produced by superposition model *CmAP* with crystal structure of VAP (PDB: 3E2D). Water is shown as red sphere. Metal ions Zn^{2+} and Mg^{2+} were shown as blue and brown spheres, respectively. Hydrogen bonds were shown as discrete lines. The residues Tre118 (above) and Gly118 (below) were shown as full and empty cursor, respectively.

hexanoyl-CoA as a starter molecule due to the smaller cavity volume of the mutant's active site.⁶⁴

The Thr118Gly mutation resulted to the complete loss of activity due to the loss of hydrogen bond Thr118.OG1-Glu268.OE2 and hydrogen bonds with 3 water molecules in the wild type *CmAP* active site.⁴⁷

At the same time, it is a challenging goal to change the recombinant protein specific activity or narrow/broad substrate specificity toward an increase their efficiency in any biotechnology or pharmacology process. The influence of the mutations on the catalytic or binding activity in many recombinant proteins of interest was theoretically and experimentally investigated to find the essential amino acid residues participated in the active site formation for designing of their more functionally usable mutants. In many pharmaceutically relevant enzymatic processes, it is desired to utilize the enzymes with enhanced specific activity and/or extended substrate specificity/selectivity.⁶⁵⁻⁷⁰

The bacteriophage lysin Ply187N-V12C was constructed by fusing the catalytic domain of the lysin Ply187 with the bacterial cell-binding domain (from 146 to 314 amino acid residues) of the lysin PlyV12 of another bacterial selectivity for extending the lytic activity spectrum toward pathogens from staphylococcal strains to streptococci and enterococci (Table 1).⁷⁰

Five mutants in the substrate recognition site 6 (S394I, A395L, T396R, G397P and Q398S) of the bacterium *Bacillus megaterium* steroid hydroxylase (CYP106A2) related to cytochromes P450 were selected with the use of molecular docking calculations for the progesterone (P) as a substrate for the following mutagenesis studies to create mutants with the altered regio-specificity of 3-oxo-D4-steroids hydroxylation from the 15 to the 11-position (Table 1).⁶⁵ Despite a low homology model of CYP106A2, its predictions about the haem charge neutralization, catalysis and redox-partner interaction correlated well with the features of other cytochrome P450 structures. Supported by the results of molecular docking, the broader regioselectivity and the increase of 11 α -, 9 α - and 6 α -hydroxylation activities up to four-fold for the mutants A395L and G397P were found in comparison with the wild type of the enzyme.⁶⁵

The computational method was used to introduce amidase activity and estimate its reaction barriers in *Candida antarctica* lipase B (CalB) mutants (Table 1).⁶⁸ The qualitative activity of 15 out of 22 mutants was correctly predicted to obtain the enzymes with 0.5-3-fold wild type activity. The point mutations are selected based on different design principles: introduction of structural rearrangements in the active site to change the binding site properties of the active site (residues P38, G39,

G41, T42, T103); introduction of space to accommodate the substrate (W104, L278, A282, I285, V286); introduction of dipolar interactions between the enzyme and the substrate (A132, A141, I189); reduction of polarity in the active site (D223). For the analysis, the reaction barrier was defined by the difference between the highest energy point on the reaction profile and the energy corresponding to the enzyme substrate complex. Six residues G39, T103, W104, A141, I189, L278 was assumed to contribute strongest to increased the activity and the preferred residues for their substitutions were defined at each position. Remarkably, the single mutants at the position 104 were predicted to have low activity and to distinguish by very high activity in the combination with other mutation at the position 189.⁶⁸

The same effect was observed in the double mutant of the recombinant hybrid mannan-binding holothurian lectin *CmAP/MBL-AJ* (Table 1).⁴⁴ It has been experimentally confirmed that the double mutation A137N/F159K has synergy effect on the lectin-binding activity of *CmAP/MBL-AJ* enhancing its activity by 25 \pm 5%, 40 \pm 2% and 28 \pm 3% in comparison with the wild type lectin and the single mutants A137N and F159K, respectively. *In silico* mutagenesis calculations and molecular docking with the model oligosaccharide have revealed that the double mutation A137N/F159K lead to the significant rearrangement of the amino acid residues around the lectin binding site, where the residue Leu in the mutant position F159K becomes able to react with the ligand forming the additional 2 ionic and one hydrogen bonds (Fig. 2)

The double mutation A137N/F159K in the lectin *MBL-AJ* resulted to synergistic positive effect increasing its binding activity by 25 \pm 5%, 40 \pm 2% and 28 \pm 3% in comparison with the wild type *MBL-AJ*, and single A137N and F159K mutants, respectively, due to rearrangement of the amino acid residues around the lectin binding site leading to hydrogen bonding of Lys159 with OH-groups of the ligand mannose residues.⁴⁴

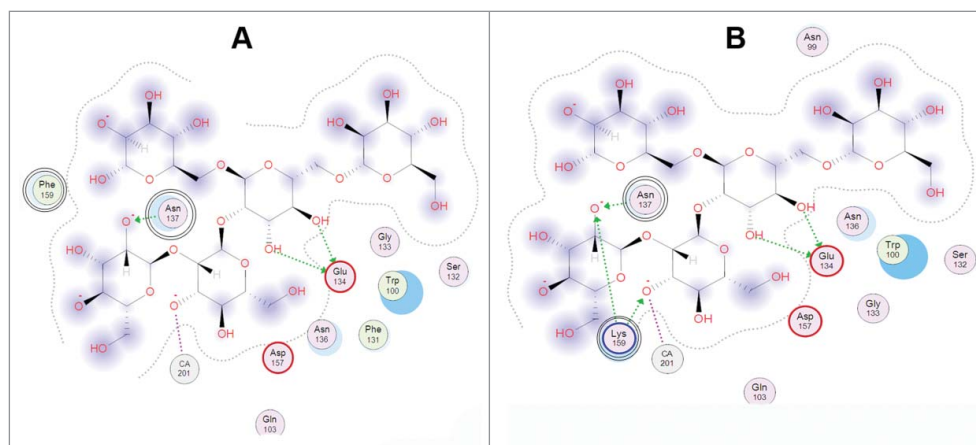


Figure 2. 2D-diagram of the model oligosaccharide binding with the single mutant A137N of the Far Eastern holothurian mannan-binding lectin *MBL-AJ* and its contacts with the oligosaccharide (left). 2D-diagram of the model oligosaccharide binding with the *MBL-AJ* double mutant F159K and its contacts with the oligosaccharide (right). The residue substitution positions 137 and 159 were indicated by double circles.

Generally, site saturation mutagenesis is very useful tool for enhancing drastically specific activity in recombinant proteins.^{66,69} By combining V546C variants with other amino acid replacements in the *Trametes multicolor* pyranose 2-oxidase (P2Ox) for carbohydrate biotransformations in food applications or as the anodic bio-component in biofuel cells, it was found that the mutant V546C/E542K had 4.4- and 17-fold increased k_{cat} for 1,4-benzoquinone (BQ) compared to the wild-type enzyme when D-glucose and D-galactose, respectively, and were the saturating substrates. While the V546C/T169G mutant showed approximately 50-fold higher k_{cat} for BQ with D-galactose in excess (Table 1).⁶⁶

The highest activity in the cyclodextrin glycosyltransferase (CGTase) from *Paenibacillus macerans* for the synthesis of vitamin C derivatives was achieved by the site saturation mutagenesis of Tyr 195, Tyr 260 and Glu 265 resulted in the triple mutant Y260R/Q265K/Y195S finding (Table 1).⁶⁹ The triple mutation enhanced maltodextrin specificity by 60% in comparison with the wild type enzyme, altering the hydrogen bonding interactions between the side chain of the residues at these positions and the substrate sugars.⁶⁹

The significance of the amide side chain in Asp 175 of the endo- β -N-acetylglucosaminidase from *Mucor hiemalis* (Endo-M) for promoting oxazoline transglycosylation in the second step of the catalysis was confirmed, when the N175Q mutant was found to possess dramatically enhanced glycosynthase-like activity with sugar oxazoline in comparison with the N175A and a transglycosidase-like activity with nature N-glycan as well (Table 1).⁶⁷

Changing Specificity

The interaction between the substrate and the enzyme is so complex that only few researches on modifying the substrate specificity of enzymes have been reported.⁷¹⁻⁷⁵

Three mutants (I387A, I387C and I387S) of aminopeptidase from *Bacillus subtilis* (BSAP) with a broad substrate specificity toward *p*-nitroanilides (*p*NA) derivatives of Leu, Arg and Lys hydrolyzed Phe-*p*NA, which was undetectable in wild-type BSAP (Table 1).⁷⁴ In docking simulation, bestatin was docked into the predicted structure of BSAP as the substrate analog. The result showed that there were several residues in direct contact with the side chain of the substrate. It has been suggested that the change in the environment of the substrate-binding region by this mutation leads to a subtle orientation shift of the bound substrate. This shift changed the distance between the substrate and the catalytic residues, reducing the side chain of the residue. The Phe residue with the large side chains does not suit the substrate-binding pocket due to its large side chain. Therefore, the replacement of Ile 387 by a small residue can expand the substrate-binding pocket of BSAP. However, the I387A mutation weakened the hydrophobic interaction in substrate binding region, which led to the poorer thermostability.⁷⁴

The entirely new functions have been found to arise even from the single mutations altering protein dynamics. The S68P mutation converted the guanylate kinase enzyme (GKenZ), which

catalyzes phosphotransfer from ATP to GMP, into a protein-binding domain (GKdom) found in membrane associate guanylate kinases (MAGUK) that function in mitotic spindle orientation and cell adhesion (Table 1).⁷¹ A dramatic functional change suggested that the MAGUK family proteins with a defining protein-binding feature have diverged from the enzymes GKenZ, which is broadly distributed throughout evolution. With respect to the all known GKenZ sequences, Ser is conserved at this position. Although the Pro mutation abrogated catalytic activity in GKenZ as revealed by crystalization, it retained the ability to bind ATP and altered the protein's conformational response to ligand binding.⁷¹

The mutations introduced into neprilysin (NEP), a transmembrane zinc metallopeptidase that degrades a wide range of peptides, have fundamentally altered the cleavage site preference of the enzyme (Table 1).⁷⁵ Cleavage of amyloid β 1-40 (Ab1-40) at Phe20-Ala21 by double mutant G399V/G714K has not been previously observed in NEP that was largely driven by a decrease in K_M , which may result from the substrate being able to adopt an altered binding conformation at the active site. The alteration was in the shape and size of the pocket containing the active site compared with the wild-type enzyme. The new variant of NEP preferable to increase its activity 20-fold at the desired substrate Ab1-40, and reduced activity at peptides where the potential for unwanted side effects exist, can be efficient in the degradation of amyloid β *in vivo* as a therapeutic for the treatment of Alzheimer's disease.⁷⁵

Overall, the narrowing of substrate specificity is more derivable result in the changing of protein specificity. The flavin-dependent tryptamine-specific mutant Y455W of the halogenase RebH was engineered to install chlorine preferentially onto tryptamine rather than the native substrate tryptophan (Table 1).⁷² The mutant gene was transformed into the alkaloid-producing plant *Madagascar periwinkle* (*Catharanthus roseus*) for *de novo* production of the halogenated alkaloid 12-chloro-19,20-dihydroakuaammicine. The resulting tissue cultures did not accumulate 7-chlorotryptophan due to the changing of the enzyme RebH substrate specificity from tryptophan to tryptamine.⁷²

The disadvantage of the glucose 1-dehydrogenase IV (BmGlcDH-IV) used for a clinical assay to examine blood glucose levels is its broad substrate specificity. The G259A variant of BmGlcDH-IV exhibited the narrowest substrate specificity toward only D-glucose, while retaining comparable catalytic activity and thermostability to the wild-type enzyme (Table 1).⁷³ Its activity with D-xylose, D-mannose and D-galactose was not detected at pH 8.0. To understand the structural basis for the broadened substrate specificity in the mutant, crystal structures of G259A was determined. The electron density for the C-terminal residues 259-261 (chain A) and 260-261 (chain B) was completely absent, suggesting that these residues at the C-terminal region are protruded into the solvent region, and that the conformation of the C-terminal region is disordered. The residues of α -helix in the structure of the G259A mutant had relatively high B-factor values (60 \AA^2) and moved slightly away from the active site, probably due to the lack of electrostatic interactions between Arg 257 and Asp 208. The side chain amino group

of Lys 199 derived from the α -helix formed hydrogen bonds with both O4 of D-glucose and the C-terminal carboxyl, and also moved away from the active site. The observation implies that the mobility of the C-terminal region and the α -helix might be related to sugar-binding selectivity of the enzyme.⁷³

It is evident that the effects of point mutations relate to electronic structure and protein dynamics.^{76,77} Changes in geometrical parameters introduced by a mutation are usually limited to the local mutational site. However, this local structural modification could affect the global protein dynamics through correlated motions of particular amino acid residues even far from the mutation site.^{47,76} Transition state stabilization is a result of conformational modifications and reorganization around the active site. As for the electrostatic effect created by the polar protein environment and electrostatic media for optimum catalysis, the effect of global polarization in the electronic structure was found to be a small catalytic element during the process, and the proteins structures are generally robust against external electrostatic perturbations.⁷⁶ The protein structures have a certain flexibility, which allows them to slightly modulate their conformations to maximize the transition state stabilization in response to the steric perturbations induced by mutations. Therefore, the enzyme must be effectively designed to stabilize the transition state of the reactive substrate, including algorithms based on evolutionary principles.^{76,77}

Hybrid Proteins and Multifunctionality

Hybrid protein can be defined as a chimeric multifunctional polypeptide originated from the modified DNA sequence combining 2 or more unrelated parent sequences. The synthesis of hybrid proteins by domain fusion or the ligation of chemically or biologically synthesized peptides improves apparently a functional unit utility and is generally used for biological probing approach such as biosensors and bioassays, molecular imaging, targeted cancer or anti-inflammatory therapy, regulated drug delivery.^{41-44,78-81} Hybrid proteins may possess 2 or more functional activities realized by different modes of action indicating that the molecules of hybrid act as several distinct pharmacophores. Many pharmaceutically or industrially relevant enzymatic processes can also utilize hybrid enzymes with multiple domains.⁸²⁻⁸⁵ There are terminal-tagging schemes of a hybrid protein production, when one functional domain is tagged at the N- or C-terminal ends of other functional domain through a covalently linked short amino acid sequence (linker or spacer).⁸⁶ Linker can be rigid (Pro-rich) to prohibit undesired interactions or steric effects between the discrete domains, and flexible (Gly-rich) to connect domains without interfering with the function of each domain in a single protein. It improves the recombinant protein's properties such as foldability, solubility and functionality.^{44,82,86} The decision of whose domain should follow downstream of C-terminal end depends on the many factors, particularly the possibility of quick folding during the recombinant production or the involvement of the N- or C-terminal ends in the active site formation or oligomerization.

Thus the *Bacillus* phytase, when expressed in *E. coli*, was found in inclusion bodies, whereas its endoglucanase was found in the active soluble form (Table 1).⁸² A chimeric gene construct coding for these fused enzymes allowed their production in soluble form. The hybrid enzyme exhibited both endoglucanase and phytase activities across broad pH (4.0–8.0) and temperature (25–75 °C) ranges, and it might have served as a potential feed additive for enhanced nutrition uptake in monogastric animals.⁸²

An improvement in endoglucanase (Cel5A) and endoxylanase (XylIT) catalytic activities was observed in the bifunctional enzymes generated by the fusion of 2 genes encoding these heat-active enzymes (Table 1).⁸⁴ The N-end to C-end fused xylanase Cel5A–XylIT and glucanase XylIT–Cel5A were active on both β -glucan and beechwood xylan. The specific activity of the hybrid enzymes toward xylan was significantly raised when compared to the nature xylanase XylIT. The fusion constructs were found to be active from 40 to 100 °C for endoglucanase and from 40 to 90 °C for endoxylanase. However, the temperature optimums were lowered from 90 to 80 °C for the endoglucanase and from 80 to 70 °C for the endoxylanase, underlining the relationship between the catalytic domain residues surroundings and their function. XylIT in the hybrid XylIT–Cel5A was less stable at higher temperatures compared to Cel5A–XylIT.⁸⁴

For the improvement of the glucanohydrolases utility in the prevention of dental biofilm formation, the mutanase (α -1,3-glucanase) of *Paenibacillus humicus* and dextranase (α -1,6-glucanase) of *Streptococcus mutans* were fused, synthesized in *E. coli* and partially purified to hydrolyze water-insoluble glucans (WIGs) produced by cariogenic pathogens on the tooth surfaces (Table 1).⁸⁵ The chimeric glucanase reduced the formation of the total amount of WIGs in a dose-dependent manner, and the significant WIGs reduction in the adherent fraction were observed. Moreover, the chimeric glucanase was able to decompose biofilm, being 4.1 times more effective toward glucan inhibition of biofilm formation than a mixture of dextranase and mutanase.⁸⁵

In contrast to the end-to-end fusion, there are only a few studies showing the use of insertional fusion to create multifunctional hybrid proteins.^{43,83} For-example, the *Bacillus subtilis* chimeric bifunctional enzyme laccase/ β -1,3–1,4-glucanase that catalyzes the hydrolysis of both plant cell wall β -glucans and oxidation of aromatic compounds was created by an insertion fusion of the *bglS* and *cotA* genes (Table 1).⁸³ The hydrolytic activity of the chimera was 20% higher against natural milled sugarcane bagasse as compared with equimolar mixtures of the separate parental enzymes. Molecular dynamics simulations indicated approximation of the 2 catalytic domains in the chimeric enzyme, and the formation of an inter-domain interface may underlie the improved catalytic function. Contacts were observed in the chimeric laccase loops Gly319–Gly320, Asn354–Ala359 and Leu634–Ser638 between side chain atoms of one domain and main chain polar groups of other domain, and main chain interactions between the 2 domains. It was also observed that the conformation of the loops in the laccase domain altered because of the formation of the CotA–BglS domain interface, increasing in the channel diameter for the water product exit.⁸³

A set of methods was developed for subcloning, expression, purification and application of chimeric proteins containing a protein domains of interest such as cytotoxic and imaging agents fused to C-terminal moiety derived from the antigen recognition domains (Fc region) of immunoglobulins in the studies of protein-ligand interactions.⁸⁷⁻⁹⁰ However, the C-terminal end of luciferases is a part of the active site and thus tagging an epitope to it invades the optical intensities for the broad use in bioassays. The problem of the limited functionality of the hybrid epitope was solved by creating a copepod luciferase Aluc30 of *Renilla reniformis*, embedding epitopes in the middle of the N-terminal region with a high optical intensity (Table 1).⁴³ Molecular modeling with specific softwares the molecular structure of ALuc30 was optimized by a molecular mechanics (MM) calculation based on a Polak–Ribiere algorithm template-based modeling (TBM) is the only reliable method for high-resolution structure prediction. The supersecondary structure revealed EF hand-like motifs between helices 5 and 6, and helices 7 and 8. Interestingly, the deepest end of the cave provided a large hole for iodide, exerting the excellent substrate specificity to coelenterazine derivative (CTZi) reported as the poorest substrate for the existing marine luciferases and photoproteins. This drastic contrast was explainable by a unique size selection effect of the Aluc30 pocket to the residues of CTZ.⁴³

The targeting Fc-based hybrid proteins have been recently shown to be advantaged by organic or inorganic nanoparticles. For example, nanoconstructs consisting of targeting biopolymer molecules and inorganic photoluminescent nanocrystals were suggested to serve as promising carriers for the targeted delivery and detection deep in the living tissue of a wide variety of cytotoxic and imaging agents, which determines their particular potential for personalized optical diagnosis of malignant tumors (Table 1).⁸⁰

There are non-Ig scaffolds for the engineering of the hybrid proteins, which constitute a distinct family of proteins with functions in ligand binding. Lectins with the databased or experimentally prescribed target specificities can be easily generated. Such molecules with a narrow specificity, which are able to bind selectively to carbohydrates, have also a key importance in the development of research related with mechanism of cancerogenesis or inflammation at the molecular level as well as for designing drugs targeted to a relevant molecule.^{44,78,79,81} To develop a less complex chimeric fusion protein then the native human lectin MBL with similar ligand recognition and enhanced effector functions, the MBL carbohydrate recognition domain and L-ficolin (L-FCN) collagenous domain were fused and applied to reduce more significantly infection by wild type Ebola virus (Table 1).⁷⁹ It has been proposed that alterations in the quaternary structure of the hybrid lectin L-FCN/MBL76 resulted in greater flexibility in the collagenous region, enhancing cooperativity between the carbohydrate recognition domains and their cognate ligands, complement activation, and calreticulin binding dynamics. L-FCN/MBL chimeric proteins should be considered as potential novel therapeutics.

The human lipocalins derivations named anticalins with picomolar affinities have been developed for 3 classes of ligands and already reached the clinical trial stage.⁸¹ Due to their very small

size and simple composition of a single polypeptide chain, which also facilitates the construction of bifunctional fusion proteins, anticalins promise benefits as a next class of biopharmaceuticals.

A method for producing a highly carbohydrate-specific bifunctional hybrid holothurian lectin MBL-*AJ*, whose expression and purification were monitored by the alkaline phosphatase *CmAP* activity, has been developed for improving the enzyme-linked MBL-*AJ*-based assay (ELLA) for cervical cancer diagnosis (Table 1).⁴⁴ The hybrid protein *CmAP*/MBL-*AJ* was produced in *E. coli* cells as a fully soluble and bifunctional homodimer, whose one subunit consisted of the mature proteins of the highly active alkaline phosphatase *CmAP* (55 kDa) and holothurian lectin MBL-*AJ* (17 kDa) connected through a flexible peptide linker ($(G_4S)_3$) (Fig. 3). The molecular modeling has shown that

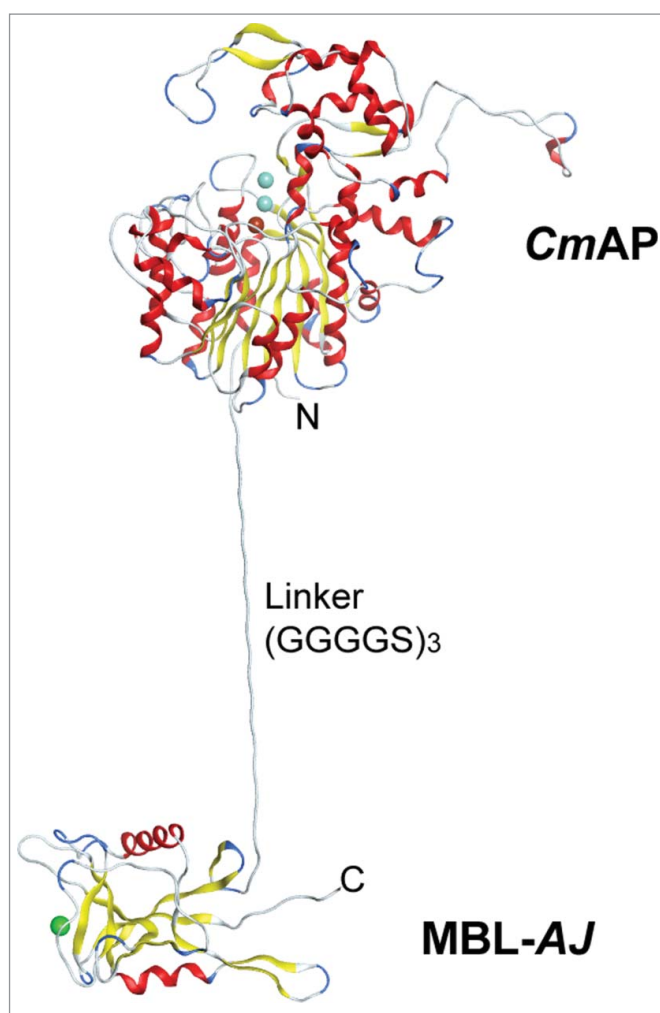


Figure 3. The structural model of the Far Eastern holothurian mannan-binding hybrid lectin MBL-*AJ* monomer with the alkaline phosphatase *CmAP* (*CmAP*/MBL-*AJ*). C-terminal end of the *CmAP* subunit is linked via flexible linker $(G_4S)_3$ with N-terminal end of the lectin MBL-*AJ* subunit that allow to function domains independently from each other.⁴⁴ Three spheres in *CmAP* module indicate 2 ions Zn^{2+} and one ion Mg^{2+} ; one sphere in MBL-*AJ* indicates ion Ca^{2+} . α -Helices and β -strands are shown as ribbons. Unordered structures are shown as threads.

CmAP/MBL-AJ dimer consists of the 2-subunit lectin part associated with the 2 molecules of alkaline phosphatase functioning independently from each other.⁴⁴ An attempt to fuse *CmAP* and *MBL-AJ* through a rigid Pro-reach linker led to decrease significantly in alkaline phosphatase activity in the hybrid protein *CmAP/MBL-AJ* (unpublished data). Probably, the psychrophilic nature of the movable marine alkaline phosphatase requires more degree of freedom for the fulfillment of its function.⁴⁷

There are hybrid enzymes possessing not only protein-protein interaction activity, but also other specific binding activity. TAL nucleases (TALNs) produced by fusion of the restriction enzyme FokI endonuclease domain with the highly specific DNA-binding domains (transcription activator-like (TAL) effectors) were created for targeted genome editing.⁹¹ When expressed in yeast, TALNs promoted DNA homologous recombination of a LacZ gene containing paired AvrXa7 or asymmetric AvrXa7/PthXo1 target sequences. The results demonstrate the feasibility of creating a tool box of novel TALNs with potential for targeted genome modification in organisms lacking facile mechanisms for targeted gene knockout and homologous recombination.

The hybrid proteins consisted of immunogenic sequences such as multiepitope fusion antigens and/or toxoids have been designed and tested as potential vaccines or inflammation diagnosis tools.^{92,93} The bioinformatics analysis of immunogenic sequences of the tumor-associated antigens MUC1 and HER2 allowed combining it in the hybrid protein for using as the breast cancer antigens in ELISA for detection of antibodies against MUC1 or HER2 in human serum (Table 1).⁹² The optimized chimeric gene composed of the structural subunits of

colonization factor antigens (CFA), labile toxin subunit B and the binding subunit of heat-labile and heat-stable toxoid, was designed to provide broad-spectrum protection against 7 enterotoxigenic *E. coli* (ETEC) strains, the most common cause of bacterial diarrhea, inducing anti-CFA and antitoxin immunity (Table 1).⁹³

The above will show that alterations in protein's thermoactivity, stability, pH-dependence and foldability are generally special cases for the rational improvement of the valuable protein function. Beneficial mutations that lead to increased thermostability or decreased thermoactivity, improved pH-optimum or solubility can be involved in many different mechanisms determined by the laws of physics and chemistry including solvent interactions, structural support, and electrostatic balance.^{59,94-100} Consequently, the search for variants with improved function is best treated as a combinatorial optimization problem, in which a number of parameters must be optimized simultaneously to achieve a successful outcome imitating the nature's manner.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

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