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MINI REVIEW

Multidomain chimeric enzymes as a promising alternative for biocatalysts improvement: a minireview

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Abstract

Searching for new and better biocatalysts is an area of study in constant development. In nature, mechanisms generally occurring in evolution, such as genetic duplication, recombination, and natural selection processes, produce various enzymes with different architectures and properties. The recombination of genes that code proteins produces multidomain chimeric enzymes that contain two or more domains that sometimes enhance their catalytic properties. Protein engineering has mimicked this process to enhance catalytic activity and the global stability of enzymes, searching for new and better biocatalysts. Here, we present and discuss examples from both natural and synthetic multidomain chimeric enzymes and how additional domains heighten their stability and catalytic activity. Moreover, we also describe progress in developing new biocatalysts using synthetic fusion enzymes and revise some methodological strategies to improve their biological fitness.

Keywords Chimeric enzymes · Fusion enzymes · Enzyme activity · Enzyme stability · Multidomain enzymes

Introduction

The enzymatic catalysts generate hundreds of compounds of industrial interest; thus, the enhancement of biocatalysts is an area of study in constant development to increase yield in the production process. Biocatalysts with better properties have been obtained through several strategies, such as chemical modification, rational design, and directed evolution, that modify enzyme properties. Researchers aim to get robust biocatalysts displaying essential properties for practical applications such as storage and operational stability, reusability, high catalytic efficiency, and specificity, among others [1].

Genetic recombination, which has occurred in nature over millions of years of evolution, has generated variability of proteins that mix different regions or take regions from other proteins and incorporate them into their sequences [2]. The establishment of multidomain chimeric enzymes results from combining two or more structural or functional domains from different proteins, understanding a protein domain as a structural polypeptide unit having a specific and independent folding and function. The wide distribution of multidomain proteins in nature reflects the success of multidomain combinations. For example, in the kingdoms of archaea and bacteria, 40% of proteins have more than one domain within this group; 20% contain two domains, and another 20% are multidomain proteins with three or more domains. Similarly, in the eukaryotic kingdom, 65% of proteins contain more than one domain: 20% have two domains, while 45% have three or more domains. This large percentage of multidomain proteins in higher organisms suggests that one of the mechanisms for diversification functions could be incorporating domains into these proteins [3].

The diversity of reactions catalyzed by multidomain enzymes and the exciting properties conferred by their additional domains have inspired molecular engineers to turn

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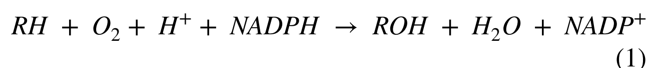
to protein engineering and use those domains as building blocks to create chimeric enzymes in search of ideal biocatalysts. This review shows examples of chimeric enzymes in nature, focusing on how additional domain incorporation modulates their enzyme stability, catalytic activity, and, in some cases, enzyme specificity. It also describes approaches to constructing synthetic chimeric enzymes to improve their properties. Finally, we describe the progress in developing new biocatalysts that generate bifunctional chimeric enzymes and some methodological strategies for constructing and improving their biological fitness.

Multidomain enzymes in nature

There are countless examples of multidomain enzymes belonging to each of the seven enzyme classes in nature: oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, and translocases. We will now describe and discuss an illustrative example of each class of multidomain enzymes in nature (Fig. 1).

Bacillus megaterium cytochrome $P450_{BM-3}$ (EC 1.14.-) is a representative enzyme of a multidomain oxidoreductase [4] containing two domains, Fig. 1. The classical P450 reaction introduces an oxygen atom (from molecular oxygen) to produce hydroxylation at an inactivated carbon on a molecule. The electrons for catalysis derive (usually) from NAD(P)H and are delivered from one or more redox partner enzymes, as shown below:



$$RH + O_2 + H^+ + NADPH \rightarrow ROH + H_2O + NADP^+ \quad (1)$$

In the most frequently characterized P450 redox systems, the electrons are transferred through NADPH-dependent cytochrome P450 reductase (CPR). The flavocytochrome $P450_{BM-3}$ system (CYP102A1) from *B. megaterium* is a fusion between two domains from two enzymes with complementary functions: a P450 and a rat CPR domain. This multidomain architecture allows the enzyme to catalyze the entire monooxygenase reaction of a substrate with the addition of only NADPH and O_2. The natural domain fusion arrangement of the cytochrome $P450_{BM-3}$ produced a very fast enzyme that oxygenates arachidonic acid at ~17,000 min^{-1} with a more efficient inter-cofactor electron transfer and higher catalytic activity than other P450 monooxygenase enzymes [5]. Many researchers have reconstituted functional P450 enzymes by fusion with different reductase proteins, some with improved properties; Sadeghi et al., 2013, have summarized these studies [6].

There is a wide diversity of natural multidomain transferases; some examples of these natural multidomain enzymes are the fructansucrases from lactic acid bacteria. These enzymes catalyze the transfer of the fructosyl unit from sucrose to either a growing fructan polymer chain (transglycosylase activity) or to water (hydrolytic activity). Among this enzyme group, there is the fructansucrase IslA (EC 2.4.1.9), a glycosyl hydrolase synthesized by *Leuconostoc citreum* CW28, able to produce inulin, a fructose polymer joined by β(2 − 1) linkages. This enzyme harbors three domains: the N-terminal domain, which shows identity (40%) with the alternansucrase ASR from *L. mesenteroides* NRRL B-1355; the catalytic domain is similar to single domain fructansucrases from several microorganisms; and the C-terminal, which shows identity (80%) with the

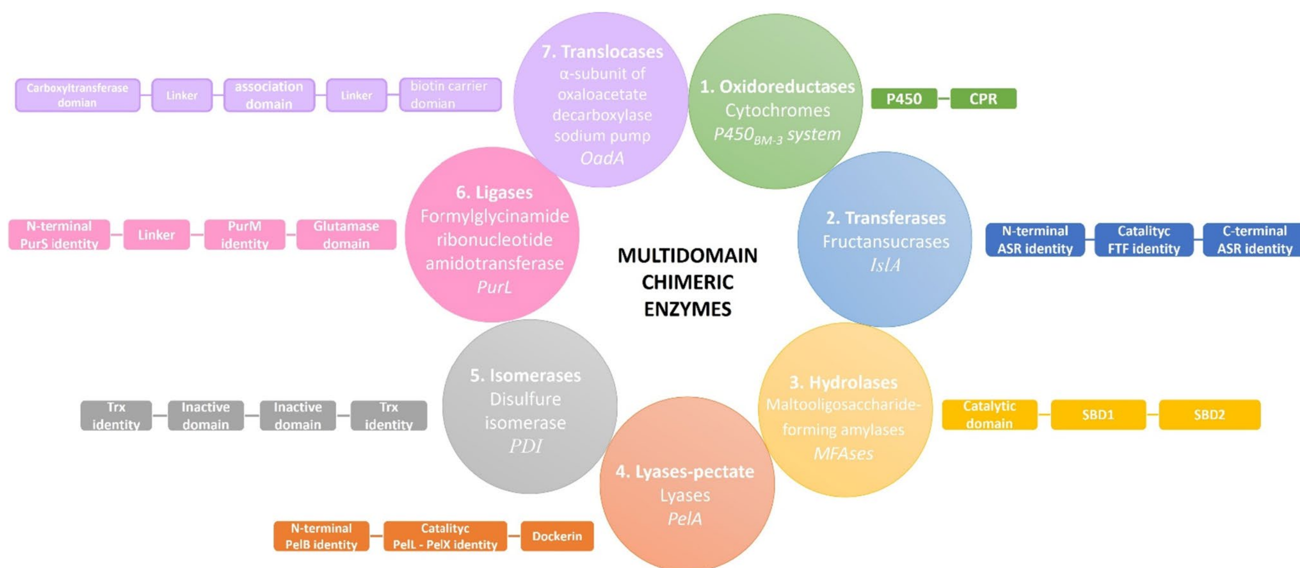


Fig. 1 Multidomain chimeric enzymes in nature. Examples of multidomain enzymes in nature belonging to the seven enzyme classes

C-terminal domain of ASR. The C-terminal domain consists of four related but non-identical tandem repeats of 20 to 30 amino acids, defined by their sequences and capable of binding polysaccharides [7]. IslA was the first natural chimeric glycosyltransferase in nature reported to have fructansucrase activity. Characterization of IslA truncated versions demonstrated that domain acquisition renders fructansucrases more stable and switches the reaction specificity, favoring the transglycosylase reaction over the hydrolytic reaction [8]. Combining domains seems to be a typical process in *Leuconostoc* fructansucrases since three other natural chimeric levansucrases have been reported: LevS in B512F strain as well as LevC and LevL in ATCC 8293 strain from *L. mesenteroides*. All these fructansucrases maintain a similar multidomain architecture to IslA; however, N- and C-terminal domains have identity with the glucansucrase DsrS from *L. mesenteroides* B512F. These additional domains also participate in the stability and reaction specificity of the enzyme, similar to IslA [9–11].

Regarding multidomain hydrolases, there is an extensive repertoire of enzymes; examples of these are maltooligosaccharide-forming amylases (MFAses) (EC 3.2.1.8, Fig. 1), which belong to glycosyl hydrolase family 13 and can hydrolyze starch into maltooligosaccharides, carbohydrates compounds of α-d-glucopyranosyl units linked by α-1,4 glycosidic linkages, usually with a degree polymerization of 2–10. MFAs commonly have a multidomain architecture because they contain starch-binding domains (SBDs). SBDs are structurally independent protein noncatalytic modules but generally enclose substrate binding sites to improve enzymatic performance. The function of SBDs appended to amylases for binding raw starch have been demonstrated through truncation, imaging, and molecular dynamics (MD) simulations [12]. An interesting case is maltooligosaccharide-forming amylase from *Saccharophagus degradans* (SdMFA), which contains a noncatalytic SBD that belongs to the carbohydrate-binding module family 20 and enables modulation of the product specificity. SdMFA exhibited a higher level of exo-action and greater product specificity when reacting with amylopectin than with amylose. Based on analysis of the product profile of truncated versions lacking C-terminal and fusion proteins of this region with MFA from *Bacillus megaterium*, the authors demonstrated that SBD contained in the C-terminal region of the SdMFA is responsible for the production of mainly GP 5 oligosaccharides. These data, plus molecular dynamics simulation, led the author to suggest that SBD could promote the recognition of nonreducing ends of substrates and delivery of the substrate chain to a groove end toward the active site in the catalytic domain [13].

PelA pectate lyase (EC 4.2.2.2) is a multidomain enzyme belonging to the lyase class. This enzyme is made up of an N-terminal domain partially homologous to a putative cellulose-binding domain present at the C-terminus of *Erwinia chrysanthemi* pectate lyase PelB, a catalytic domain homologous to *E. chrysanthemi* pectate lyases PelL and PelX, and a duplicated sequence at the C-terminus that is highly conserved in the enzyme subunits of the cellulosome of *C. cellulovorans*. This enzyme can cleave polygalacturonic acid to digalacturonic acid (G2) and trigalacturonic acid (G3) but cannot act on G2 and G3. Cleavage patterns using substrates of different lengths suggest that PelA is an endo-type enzyme, while its PelX counterpart is an exopolygalacturonate lyase. Therefore, the properties of PelA differ from those of PelL and PelX in the specific activity, substrates, and synthesized products, even though these enzymes belong to the same family of pectate lyases [14]. These differences are probably related to its multidomain structure.

Martinez et al., 2014, analyzed the architectures of 96 isomerases, finding that one-third include more than one domain, and a large majority contain two or three domains [15]. An example of multidomain isomerases is disulfide isomerase (PDI, Fig. 1) (EC 5.3.4.1), an essential folding catalyst and chaperone of the endoplasmic reticulum. This protein introduces disulfide bonds into proteins (oxidase activity) and catalyzes the rearrangement of incorrect disulfide bonds (isomerase activity). The PDI structure contains four domains. The first and fourth domains are homologous to thioredoxin, and both have a respective active site. The second and third are noncatalytic domains similar in sequence [16]. Based on functional studies of linear combinations of PDI domains, Darby et al. (1998) showed that all protein domains of PDI are required for maximum catalytic efficiency. This suggests that PDI has developed its multidomain structure as an adaptation that allows it to catalyze transformations involving unfavorable conformational changes more efficiently [17].

We select PurL (EC 6.3.5.3) to illustrate multidomain ligases. In Gram-positive bacteria and archaea, PurL is a member of the formylglycinamide ribonucleotide amidotransferase (FGAR-AT) complex constituted by three proteins (PurS, PurL, and PurQ). This complex catalyzes the ATP-dependent conversion of formylglycinamide ribonucleotide (FGAR) and glutamine to formylglycinamide ribonucleotide (FGAM), ADP, P_i, and glutamate in the fourth step of the purine biosynthetic pathway. The structure of PurL reveals four domains: An N-terminal domain structurally homologous to a PurS dimer, a linker region, a FGAM synthetase domain homologous to the PurM dimer of aminoimidazole ribonucleotide synthetase, and a triad glutaminase domain. These domains are intricately linked by interdomain interactions and peptide connectors [18], supporting the hypothesis that all domains are indispensable for correct folding and, thus, to the activity of PurL.

Finally, we use microbial oxaloacetate decarboxylase sodium pump (OAD) to exemplify multidomain translocases (EC 7.2.4.2). This enzyme is present in bacteria and archaea and maintains the sodium gradient, anaerobic citrate fermentation, and pathogenesis [19]. OAD is a membrane-bound multiprotein complex composed of three subunits (α, β, and γ) that catalyze the transfer of the carboxyl group from position 4 of oxaloacetate to the biotin prosthetic group. The carboxy biotin formed is transferred from the carboxyltransferase catalytic site of the α subunit to the decarboxylase site on the transmembrane β subunit, where decarboxylation takes place, liberating the biotin group. This last reaction is Na^+-dependent, where a proton is consumed, and two sodium ions are translocated from the cytoplasm into the periplasm [20]. The α-subunit (OadA) is a cytoplasmatic protein with three domains connected by a flexible linker. The N-terminal domain harbors the carboxyltransferase catalytic site; the C-terminal domain includes the biotin-binding residues; and the third is denominated association domain. The association domain of the β subunit binds tightly to the C-terminal domain of the γ-subunit, taking a critical role in the assembly and stability of the oxaloacetate decarboxylase complex [21].

All these representative examples show that domain acquisition has modified enzyme properties such as substrate binding affinity, catalytic activity, and even enzyme specificity. Also, these domains can favor the correct folding and stability of the enzyme, with a consequent effect on the catalysis. Based on the importance of the additional domains of multidomain chimeric enzymes, we can infer that domain acquisition is an adaptive evolutionary process to expand and improve enzyme properties (Fig. 2). Therefore, it is possible to synthetically design and generate enzymes with higher activity and stability through a domain rearrangement process to produce better biocatalysts.

Construction of chimeric enzymes: improving stability

Enzymes in the industry require that they be stable under process conditions such as high temperatures, extreme pH, and the presence of salts, surfactants, and solvents [22]. One of the biochemical characteristics favored in multidomain proteins is thermostability, which is achieved by stabilizing unstable regions by changing amino acids, placing disulfide bridges, or increasing intermolecular forces. However, some authors have reported the exchange of domains between enzymes to improve their stability, as shown in Table 1. For example, the N-terminal domain of xylanases (EC 3.2.1.8) has been reported as essential for enzyme stability. To improve the thermostability of xylanases, several research groups have performed a partial or total replacement of the N-terminal domain of mesophilic xylanases with a thermophilic counterpart, forming enzymes more thermostable up to 10 °C or more than their parental enzymes [23, 24].

The improvement in the stability can be reflected in more than one characteristic, as in the case of cyanide dehydratase (EC 4.2.1.66) from *Bacillus pumilus*, whose thermostability and pH tolerance were improved when the C-terminal domain was replaced by its homologous C-terminal domain (56 aa) of the thermostable *P. stutzeri*

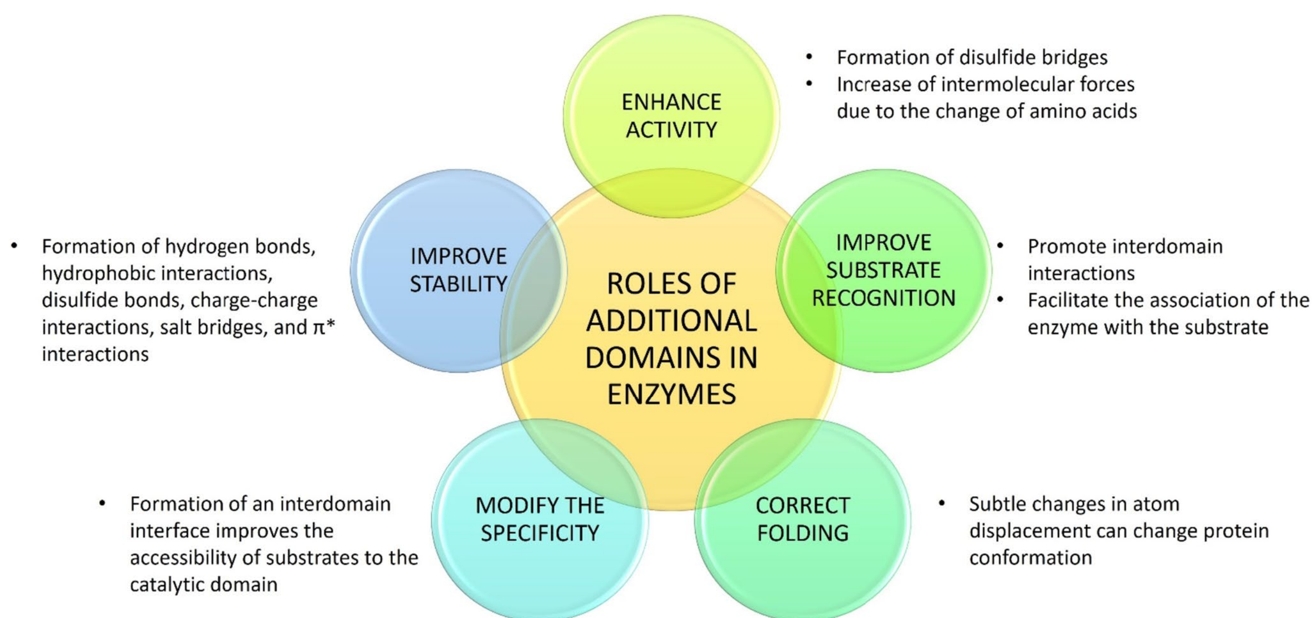


Fig. 2 Functional roles of additional domains in multidomain enzymes

Table 1 Synthetic chimeric enzymes with improved physicochemical properties over native enzymes

Enzyme	EC number	Organism	Domain Added	Domain origin	Improved Property	Refs.
Laccase	EC 1.10.3.2	*Pleurotus ostreatus*	Complete class I hydrophobin Vmh2	*Pleurotus ostreatus*	• Increased immobilization yield	[25]
Glicosylhidroolase	EC 3.2.1	*Thermotoga maritima*	Dockerin domain	*Piromyces finnis*	• Thermostability	[26]
Glucoamylase GATE	EC 3.2.1.3	*Talaromyces emersonii* Ld418 TE	Starch-Binding Domain of glucoamylase GAA1	*Apergillus niger* Ld418A1	• Enzyme activity • pH stability	[27]
Xylanase	EC 3.2.1.8	*Thermobacillus xylanilyticus*	N-terminal of GH11 xylanase	*Neocallimastix patriciarum*	• Wider substrate specificity	[28]
Xylanase A AnxA	EC 3.2.1.8	*Aspergillus niger*	N-terminal of xylanase A TfxA	*Thermomonospora fusca*	• Thermostability • Catalytic activity	[23]
β-xylanase	EC 3.2.1.8	*B. subtilis*	A complete β-xylosidase	*B. subtilis*	• Substrate cleavage rate • Optimum temperature • Thermostability	[29]
Cyanide dehydratase	EC 4.2.1.66	*B. pumilus*	C-terminal of cyanide dehydratase	*P. stutzeri*	• Thermostability • Optimum pH • Operational pH range	[30]
Nitrile hydratase	EC 4.2.1.84	*P. putida* NRRL-18,668	N-terminal of nitrile hydratase C-terminal of nitrile hydratase	*Comamonas testeroni* 5-MGAM-4D *P. thermofila* JCM3095	• Enhancement in thermostability • More tolerant to high-concentration product • Increase in activity	[31]

CMB Carbohydrate binding module *GH* Glycosyl hydrolase

cyanide dehydratase. The half-life of the chimeric enzyme was increased 17-fold, and the optimum pH was 8–9. At this pH, wild-type cyanide dehydratase showed no activity; however, the chimeric enzyme showed an enhanced affinity for the substrate. This study suggests that oligomerization stimulated by the C-terminal domain was responsible for the longer half-life of the chimeric enzyme [30].

Proteins are stabilized by intermolecular forces such as hydrogen bonds, hydrophobic interactions, disulfide bonds, charge-charge interactions, salt bridges, and π* interactions. Although hydrogen bonds are the main force involved in protein structure formation, the hydrophobic patches are energetically the most stable. Some authors mention that the interior of a protein is very compact; therefore, its molecules are twice as close as in a water drop. Therefore, subtle changes in atom displacement can change protein conformation, increasing or decreasing its stability [32]. As seen in Table 1, chimerization can modify the pH and temperature stability at which enzymes remain stable. However, few studies used this strategy to improve enzyme stability in salts, ionic surfactant liquids, and organic or eutectic solvents. Due to thermal and pH stability determinants being similar to those involved in enzyme stabilization in salts, surfactants, or solvents, we consider this an opportunity for protein engineering to explore in the future.

Construction of chimeric enzymes: improving activity

An optimum biocatalyst must have a high turnover number (k_{cat}) or the maximum specificity constant (k_{cat}/Km) in an efficient bioconversion reaction. Some strategies used to improve enzymatic activity during the last years are immobilization, organic solvents, and directed evolution. However, the design of chimeric enzymes is an exciting alternative to generate more efficient biocatalysts. Chimeric enzymes can be designed to synthesize new products by combining two or more catalytic features in a single molecule. These combinations sometimes convey an increase in catalytic activity and improve the effectiveness of these biocatalysts in developing bioprocess. Below are some examples to describe activity improvement by constructing chimeric enzymes.

Branchini et al. (2014) increased the bioluminescence properties of luciferase by constructing a chimeric enzyme (PpyLit) that contains the N-terminal domain of *Photinus pyralis* luciferase (EC 1.13.12.7) linked to the C-terminal domain of *Luciola italica* luciferase. PpyLit chimera exhibited 1.8-fold enhanced flash-height specific activity and a 2.9-fold improved catalytic efficiency (kcat/Km) compared to the *P. pyralis* enzyme. The enzyme conformation, where the N- and C-terminal domains interact, provided a favorable environment for an electronically excited state of

oxyluciferin. Suggesting that the interactions between these domains generated an auxiliary microenvironment for the reaction mechanism [33].

Interdomain interactions also promote access of substrate molecules to the active site by increasing enzymatic activity. In 2012, the construction of three chimeric levansucrases from SacB, a single domain levansucrase produced by *B. subtilis*, was reported. These chimeric enzymes were formed by adding the transition region or entire C-terminal domain of the inulosucrase IslA (EC 2.4.1.9) from *L. citreum* CW28, the levansucrase LevC (EC 2.4.1.10) from *L. mesenteroides* ATCC 8293, and the glucansucrase DsrP (EC 2.4.1.5) from *L. mesenteroides* IBT-PQ. Adding the C-terminal domain of IslA and LevC increased transglycosylase activity by up to 90% compared to the wild-type SacB enzyme, similar to the transglycosylation activity of IslA. The authors suggest that the additional domains favor a fit in the catalytic domain that increases the affinity for the acceptor molecule. The addition of the C-terminal domain of DsrP did not affect the properties of SacB, indicating that there are molecular determinants that favor these changes. The stability of these chimeric enzymes was not affected, as it remained the same as that of SacB [34].

Another approach to enhance enzyme activity by chimeric enzymes is to add specific modules to single-domain enzymes. Such is the case for carbohydrate-binding modules (CBMs), which have a high binding affinity for polysaccharides. Although these modules are not domains, they have a specific fold and enhance enzymatic activity. CBMs can be found in the N- or C-terminal domain of glycosyl hydrolases (GH) and are connected to the catalytic domain by a repeat linker of threonine-proline residues [35]. CBMs increase the activity of enzymes by facilitating the association of the enzyme with the substrate; therefore, the local substrate concentration at the active site increases (Fig. 3) [36]. CBMs have different folds, for example, OB-fold, β-trefoil, lectin-like, and β-sandwich, the latter being the most common. However, these folds are not predictive of their function [37].

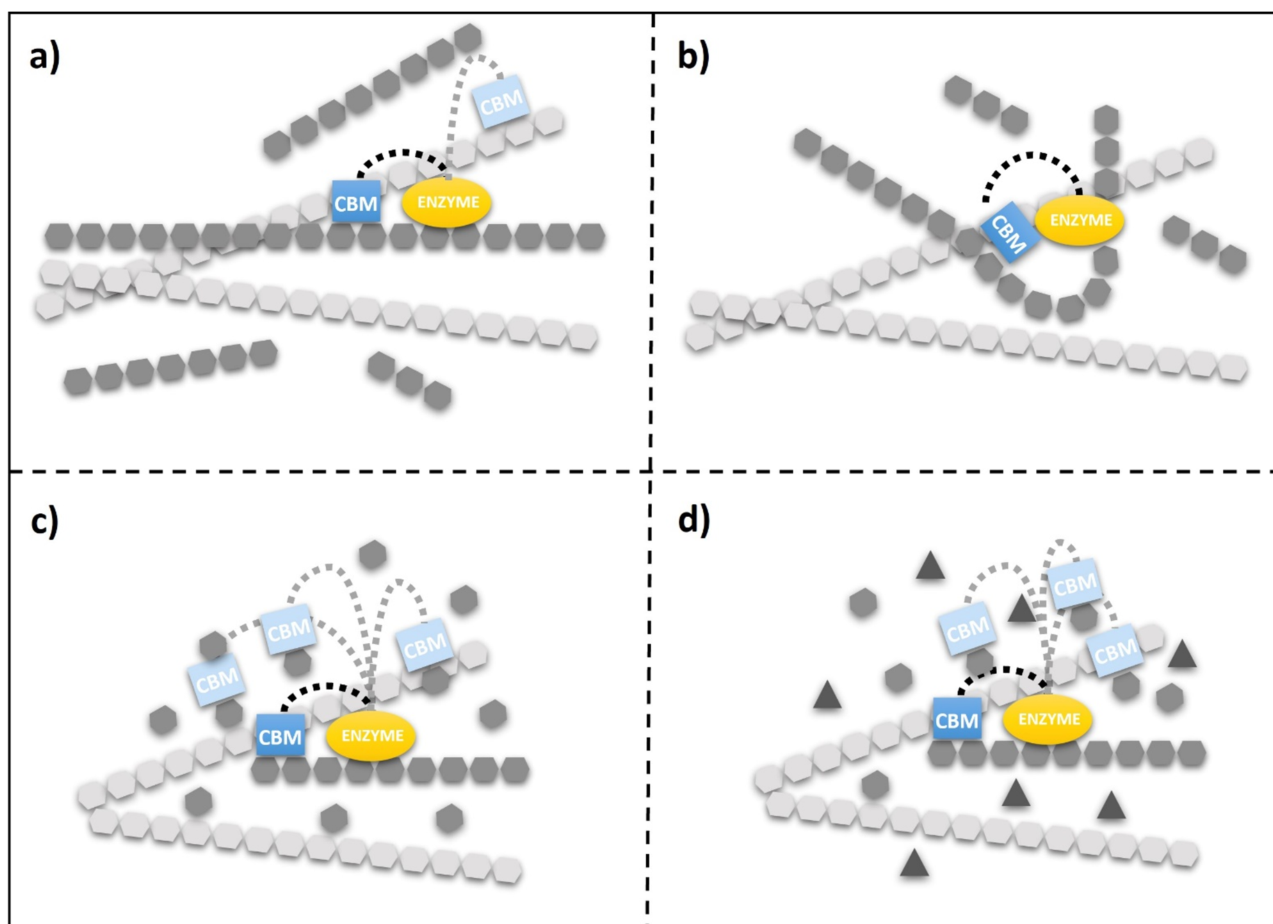


Fig. 3 Functional significance of carbohydrate binding modules (CBMs). A schematic description of the putative functions of CBMs is presented: **a** targeting of the enzyme towards its substrate, **b** guidance of the substrate towards the active site groove, **c** improving processivity, **d** specificity specificity

Furtado et al. (2015) fused the xyloglucan-specific CBM44 from *C. thermocellum* with the GH12 XEGA from *Aspergillus niveus* (EC 3.2.1.151). This chimeric enzyme (CBM44-XEGA) increased xyloglucan hydrolysis by 30%, and the catalytic efficiency showed a kcat 1.25 times higher than the parent enzyme XEGA. The presence of the CBM did not modify the hydrolysis product profile; therefore, this module does not affect the enzyme catalytic mechanism [38]. The chimeric XynB-CBM2b xylanase was formed by fusing a *Streptomyces thermoviolaceus* STX-II family 2b carbohydrate-binding module to the carboxyl terminus of XynB, a thermostable single-domain xylanase from *Thermotoga maritima* family 10. XynB-CBM2b showed a 1.7-fold higher kcat against soluble birchwood xylan than the wild-type enzyme [39]. In another example, the chimeric enzyme XYN-TmCBM9-1_2 is a fusion of TmCBM9-1_2 (CBM from *T. maritima*) and the xylanase XYN from *A. niger*. This chimeric enzyme resulted in a 4.2-fold increase in xylanase activity on insoluble oat-spelled xylan compared to soluble birchwood xylan [40].

A similar effect was reported when fusing CBM (type A and B) to the end of the N- or C-terminal domain of the gluco-oligosaccharide oxidase (EC 1.1.99) from *Sarocladium strictum* CBS 346.70 (GOOX). All enzymes with CBM at the N-terminus and one at the C-terminus showed higher catalytic activity on the oligosaccharides tested than on wild-type GOOX, suggesting a positive conformational change. Unchanged Km values confirmed that the fused CBMs did not compete with GOOX subsites for oligosaccharide binding. The addition of type-B CBM increased the affinity of GOOX towards soluble polysaccharides, including β-glucan, glucomannan, and xyloglucan. These chimeric enzymes increased the catalytic activity (kcat) on cellotetraose by 50%, glucomannan by 30%, and amorphous (insoluble crystal) cellulose by more than 50%. The addition of CBM at the end of the N- or C-terminal domain did not affect the catalytic activity [41].

Chimeric enzymes using CBMs have also allowed modification of the enzyme's substrate specificity. Han et al. (2013) constructed a chimeric enzyme formed by the cyclodextrin glycosyltransferase (EC 2.4.1.19) from *Paenibacillus macerans* (CGTase) and the CBM of α-amylase from *Alkalimonas amylolytica* (CBMAmy). The chimeric enzyme synthesized 2-OD-glucopyranosyl-L-ascorbic acid (AA-2G), an ascorbic acid derivative produced from soluble starch as a cheap and easily soluble glucosyl donor. Under optimal conditions, the yield of AA-2G produced with CGT-CBMAmy was 5.94-fold higher than the yield obtained with wild-type CGTase. The authors suggest that the enhancement of soluble starch specificity may be related to changes in substrate binding capacity and substrate binding sites between the CBM and the starch granule. A kinetic reaction model carried out by the authors confirmed that this effect is due to the increased specificity of the soluble starch provided by CBMAmy [42]. These examples demonstrate that generating CBM-linked chimeric enzymes improves the catalytic activity. These and other recent and promising examples are summarized in Table 2.

Construction of chimeric enzymes: combining and modifying the specificity

Generating new biocatalysts is an imperative issue that innovative areas need to obtain new compounds and improve bioprocesses. An alternative to achieve this goal is to design biocatalysts by modifying or combining the specificity of the enzymes by chimerization.

Domain combinations may modify the specificity of enzymes and even confer novel biosynthetic activities, such as in the case of the chimeric 6-methyl salicylic acid synthase (6MSAS) from *A. terreus*. 6MSAS catalyzes the synthesis of 6-methyl salicylic acid (6MSA); it is a multidomain enzyme consisting of a keto synthase (KS), acyltransferase (AT), dehydratase (DH), ketoreductase (KR) and acyl carrier protein (ACP) domains. The construction of a chimeric 6MSAS enzyme replacing the ACP and methyltransferase (MT) domains by those of the polyketide synthase (PKS) from *Pseudallescheria boydii*, resulted in a specificity change, synthesizing an entirely different compound, the 2-hydroxy-2-(propane-2-yl) cyclobutane-1,3-dione. Although the exact role of the fused domains remains to be demonstrated, this example highlights the potential of the chimerization strategy to modify enzyme specificity [52].

Sometimes, combining activities changes the enzyme activity or specificity and improves other properties. The union of a β-xylanase (26 kDa) from *B. subtilis* to the catalytic domain of a β-xylosidase (60 kDa) from *B. subtilis* by a linker of 26 residues from *Thermotoga petrophila* resulted in a bifunctional chimera with improved thermostability. The optimum temperature for β-xylosidase activity was increased by 5.3 °C and retained 80% activity after 45 h-incubation at 45 °C, while the wild-type enzyme retained only 30%. Further, the chimera catalyzed the production of 3-fold more xylose than parental enzymes. These changes are due to the oligomerization of the chimeric enzyme [29]. For another example, Adlakha et al. (2011) constructed four chimeric enzymes with β-1,4-endoglucanase (Endo5A) and β-1,4-endoxylanase (Xyl11D) from the *Paenibacillus* sp. ICGEB2008. These chimeric enzymes were built by changing the order of the enzymes and using a glycine-serine linker. The chimeric enzymes showed 0.5- to 1.6-fold and 1.3- to 2.3-fold higher enzyme activity than Xyl11D and Endo5A, respectively. One of these chimeras showed the highest endoglucanase (1070 U μmol^{-1}) and xylanase (899 U μmol^{-1}) activities. The authors mention that the

Table 2 Chimeric enzymes with improved catalytic properties by addition of a CBM.

Enzyme	EC number	Organism	CBM added	CBM Origin	Enhanced activity properties	Refs.
Gluco-oligosaccharide oxidase GOOX	EC 1.1.3	*Sarocladium strictum* CBS 346.70	Type-A CtCBM3 Type-B CtCBM11 Type-B CtCBM44	*Clostridium thermocellum* CipA *C. thermocellum* Lic26A-Cel5E *C. thermocellum* Cel9D-Cel44A	1.2–1.4-fold enhanced activity on linear β-glucans. Increase in kcat over cellotetraose (50%), glucomannan (30%), and amorphous cellulose (50%).	[41]
Gluco-oligosaccharide oxidase GOOX-VN	EC 1.1.3	*Sarocladium strictum* CBS 346.70	CBM22-2	*Clostridium thermocellum*	CBM22-2 at the N-terminal of GOOX-VN increased catalytic activity on mono- and oligo-saccharides by 2–3 fold.	[43]
Cyclodextrin glycosyltransferase CGTase	EC 2.4.1.19	*Paenibacillus macerans*	CBMAmy	*Alkalimonas amylolytica* α-amylase	CGT-CBMAmy yield was 5.94-fold compared to wild-type CGTase when using soluble starch as substrate.	[42]
Cutinase	EC 3.1.1.74	*Thermobifida fusca*	CBMCel6A CBMCenA	*T. fusca* cellulase Cel6A *Cellulomonas fimi* cellulase CenA	Binding activity on cotton fiber was increased by 2% for cutinase-CBMCel6A and 28% for cutinase-CBMCenA compared to wild-type cutinase.	[44]
Cellobiohydrolases Cel7A	EC 3.2.1.	*Talaromyces emersonii*	CBM1 CBM2 CBM3 cellulosomal-scaffolding protein (1NBC)	*T. reesei* Cel7A *C. fimi* *Clostridium thermocellum*	Purified chimeric enzymes bound to cellulose better than the catalytic module alone. They proved to have high thermal stability, with temperatures between 72 and 77 °C.	[45]
Chitinase D SpChiD	EC 3.2.1.14	*Serratia proteamaculans*	Polycystic kidney disease (PKD) domain Chitin binding protein 21 (CBP21)	ND	ChiD + PKD and ChiD + CBP have a larger catalytic efficiency, 1.9 and 1.3-fold respectively than wild type enzyme.	[46]
Endoglucanase Cel5A	EC:3.2.1.4	Derived from the metagenomic library of vermicompost.	CBM6	*Saccharophagus degradans* endoglucanase Cel5H	Cel5A_2R2-CBM6 showed 7-fold higher activity and a significantly higher binding affinity towards avicel than the parent Cel5A_2R2.	[47]
Thermophilic endoglucanases Cel9A Cel5A	EC 3.2.1.4	*Alicyclobacillus acidocaldarius* *Thermotoga maritima*	CBM2a	*Thermomonospora fusca*. thermophilic exoglucanase E3	Increases cellulase activity by up to 3-fold on insoluble cellulosic substrates as compared to wild type.	[48]

Table 2 (continued)

Enzyme	EC number	Organism	CBM added	CBM Origin	Enhanced activity properties	Refs.
Hybrid microbial cellulase CEL-HYB1	EC 3.2.1.4	*Hordeum vulgare* L. cv. Golden Promise	CBM of the tomato	*Solanum lycopersicum* SlCel9C1 cellulase	CEL-HYB1-CBM demonstrated a 4-fold higher exo-glucanase activity and 6-fold higher catalytic efficiency for the hydrolysis of crystalline cellulose than the wild-type CEL-HYB1.	[49]
β-mannanase AuMan5A	EC 3.2.1.78	*Aspergillus usamii* YL-01-78	CBM1	*Trichoderma reesei* cellobiohydrolase I (TrCBH I)	The cellulose-binding capacity of the reAuMan5A-CBM was up to 92.3%, whereas reAuMan5A was unable to bind CBM, and Km values were 0.66 mg/mL and 1.36 mg/mL, respectively.	[50]
D-xylose isomerase	EC 5.3.1.5	*Thermotoga neapolitana* 5068 (TNXI)	Chitin-binding domain (CBD)	*Pyrococcus furiosus* chitinase (PF1233)	kcat for CBD-TNXI increased 4-fold compared to wild-type TNXI.	[51]

ND Not described

orientation and the linker are important for the optimal activity of both enzymes, suggesting that the reaction yield was increased by the proximity of the catalytic centers in the chimeric enzyme [53]. Although domain combining is a promising strategy to increase enzyme activity, enzyme activity is sometimes negatively affected [54] or does not significantly increase compared to parental enzymes [55].

Until now, multifunctional chimeric biocatalysts have been used in bioprocesses for the bioconversion of agricultural by-products, the biotransformation of xenobiotics, and the production of food ingredients [56]. For example, Furtado et al. (2013) developed a chimeric enzyme with laccase and glucanase activities. The chimeric enzyme consisted of *B. subtilis* CotA laccase (EC 1.10.3.2) and *B. subtilis* β-1,3–1,4-glucanase BglS (EC 3.2.1.73). Laccase breaks down lignin by catalyzing the oxidation of aromatic compounds with a simultaneous reduction of oxygen to water. On the other hand, glucanase hydrolyzes the β-glucans of hemicellulose. The hydrolytic activity of the chimeric enzyme was 20% higher in sugarcane bagasse compared to equimolar mixtures of wild-type enzymes. The authors associated this increased hydrolytic activity with the proximity between domains in the chimera, resulting in higher reaction rates in sequential reactions and improved substrate binding. Molecular dynamics simulations determined that forming an interface between domains enhanced the accessibility of substrates to the catalytic domain [57].

Chimeric enzymes can be constructed by two enzymes catalyzing consecutive reactions. In this case, one enzyme converts a substrate to a product, which the second enzyme uses as a substrate to synthesize a final product. One example is the chimeric enzyme DXSR, which was constructed to develop a one-step process for producing isomalto-oligosaccharides (IMO). DXSR is a chimeric enzyme that contains an endo-dextranase from *Arthrobacter oxydans* and an α- [1–6] dextransucrase from *L. mesenteroides* B-1299CB4. DXSR had a 150% increase in endo-dextranase activity and a 98% decrease in dextransucrase activity compared to the individual enzymes. However, DXSR catalyzed a 30-fold higher production of IMO than an equal activity mixture of the wild-type enzymes. DXSR can be applied to produce IMO from sucrose by a one-step reaction, and the size of IMOs can be controlled by modulating sucrose concentration and overall reaction time [58].

Constructing chimeric enzymes is a strategy that can be used to evaluate other activities in preparing metabolites and other compounds of interest for medicinal chemistry. To identify activities for the transformation of drug molecules, Kulig et al. (2015) constructed fifteen chimeric enzymes which comprised several *Rhodococcus jostii* cytochrome P450 heme domain with the *Rhodococcus sp* P450 reductase domain (RhfRED) of cytochrome P450Rhf. Strains expressing chimeric RhfRED enzymes were exposed to

commercially available drugs and revealed different activities commensurate with P450-catalyzed hydroxylation and demethylation reactions. One of these chimeric enzymes catalyzed the N-demethylation of diltiazem and imipramine. Characterization of this enzyme revealed a 63% conversion of imipramine to the N-demethylated product [59].

Another strategy used to improve biocatalysts is the development of libraries to bio-convert hydrophobic and xenobiotic compounds. An example of the combination of activities by domain binding is the work reported by Corrado et al. (2018). The authors generated a chimeric styrene monooxygenase (Fus-SMO) by fusing, through a flexible linker, the enzymes StyA and StyB that belong to the styrene monooxygenase (SMO) system from *Pseudomonas sp*. StyB catalyzes the reduction of FAD to $FADH_2$ at the expense of NADH, while StyA utilizes $FADH_2$ and O_2 to generate FAD-OOH, the epoxidizing agent. In this case, the epoxidation activity of Fus-SMO was up to three times higher than the two-component StyA/StyB (1:1, molar ratio) system.

Furthermore, the solubility of Fus-SMO was higher than that of heterologous StyB expressed in *E. coli*. This work shows that combining activities could improve the expression levels of the reductase and epoxidase units [60]. The examples mentioned above illustrate the possibility of improving the catalytic activity of enzymes and generating new catalytic activities using chimeric enzymes. Table 3 shows more examples of the benefits the chimerization strategy of combining activities can confer to obtain better biocatalysts.

Design and methods for constructing multidomain enzymes

The molecular biology methods established to manufacture end-to-end fusion enzymes allow the linkage of two or more domains at the N- or C-terminus. These methods include overlapping extension polymerase chain reaction (OE-PCR), restriction cloning, and recombination approaches. OE-PCR is now the mainstay of gene fusions, allowing exact manipulation of DNA and modification of the ends of DNA fragments for downstream processing. This method generally requires a pair of primers for each gene. The forward primer encloses a restriction enzyme cleavage site necessary for further ligation into an expression vector. The reverse primer presents a region complementary to the second gene of the construct. The primers of the second gene are generated oppositely. OE-PCR allows the two PCR products obtained above to bind freely, regardless of restriction sites. Like OE-PCR, restriction cloning, homologous recombination approaches, and Golden Gate cloning help combine multiple genes into a single vector system [67–72].

Table 3 Synthetic chimeric enzymes that merged two activities

Fused Enzymes	EC numbers	Organism	Improved Property	Refs.
Geranylgeranyl diphosphate synthase (GPPS) and phytoene synthase (PSY)	2.5.1.29 and 2.5.1.32	*Arabidopsis thaliana*	Removed enzymatic competition for geranylgeranyl diphosphate and increased the carotenoid content by 50%.	[61]
Polyethylene terephthalate hydrolase (PETase) and mono(2-hydroxyethyl) terephthalate hydrolase (MHETase)	3.1.1.101 and 3.1.1.102	*Ideonella sakaiensis*	Improved MHETase and PETase activities increased PET degradation and MHET hydrolysis rates.	[62]
Lipase (Lip) and cutinase (Cut)	3.1.1.3 and 3.1.1.74	*Thermomyces lanuginosus* and *Thielavia terrestris*	Enhanced lipase and cutinase activities by 127% and 210% than their parental enzymes.	[63]
Endoglucanase (CtGH5) mutant F194A and β-1,4-glucosidase (CtGH1)	3.2.1.4 and 3.2.1.21	*Clostridium thermocellum*	Enhanced enzyme activity, thermostability and structural integrity of both enzymes.	[64]
Mutanase and dextranase	3.2.1.59 and 3.2.1.11	*Paenibacillus humicus* NA1123 and *Streptococcus mutans* ATCC 25,175	4.1 times more effective at glucan inhibition of biofilm formation than a mixture of dextranase and mutanase.	[65]
Glucuronan lyase (TrGL) and a chitinase (ThCHIT42)	4.2.2.14 and 3.2.1.14	*Trichoderma sp.*	Increased the velocity of glucuronan lyase	[66]

In contrast to the above techniques, the LE (*LguI/Eco81I*)-cloning system has been specially designed to generate end-to-end multi-fusion enzymes in a continuously growing vector system, facilitating their arrangement of different domain combinations to establish which specific grouping of domains exhibits an improved activity [73].

Occasionally, the proximity of two domains can result in unfavorable folding, resulting in the loss of activity of one or both catalytic domains. In these cases, adding linker sequences (subdivided into three classes: flexible, rigid, and in vivo cleavable linkers) may allow for better conformation, stability, and autonomous actions of each functional domain in a fusion protein [74].

Linkers could provide mobility and flexibility between domains, factors essential to the efficient biocatalytic function of a chimeric enzyme. Xue et al. (2009) constructed a trifunctional enzyme to degrade agricultural by-products effectively. This chimeric enzyme was composed of xylosidase-arabinosidase (Xar, EC 3.2.1.55) from *Thermoanaerobacter ethanolicus* and xylanase (XynA, EC 3.2.1.8) from *Thermomyces lanuginosu*. The chimeric enzyme decreased arabinosidase-xylosidase activity despite increased xylanase activity [75]. However, the chimeric enzyme was improved by adding a glycine-rich linker [76]. In the same way, Lu et al. (2006) reported the construction of a chimeric enzyme with xylanase and β-1,3 – 1,4-glucanase from *B. amyloliquefaciens* by end-to-end gene fusion. Although this chimeric enzyme decreased xylanase activity, adding a glycine-rich linker between the subunits enhanced xylanase and glucanase activity [77, 78].

Concluding remarks

In nature, domain acquisition is an adaptive evolutionary process that expands and modulates enzyme properties. This process has been replicated in all types of enzymes so that chimeric enzymes are present in the seven enzyme classes-: oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, and translocases. In some instances, domain acquisition is responsible for the modulation of essential properties from the biocatalysis point of view, such as catalytic activity and stability. It may also affect substrate recognition and specificity. Thus, creating multidomain chimeric enzymes may be another strategy for biocatalysis improvement, which could potentiate the local effects of single-point mutation obtained through rational or directed evolution, expanding the toolbox of protein engineering.

However, at the moment, the role of domain-domain interactions in enzyme catalysis still needs to be understood entirely. Hence, creating improved enzymes through this strategy is an attractive yet undeveloped alternative. Successful examples are based on reasonable expectations, such as replacing one mesophile domain for an extremophile domain, as in the case of thermostable multidomain chimeric enzymes. More subtle properties, such as substrate specificity, are more difficult to rationalize, manipulate, and predict for multidomain chimeric enzymes. Recent advances *in silico* approaches, such as computational simulation of proteins and the inclusion of powerful tools such as artificial intelligence for the structural study of proteins, may provide a massive leap in our comprehension of complex protein interactions in a short time.

Author contributions All authors contributed to the conception and design of the study. The article idea was proposed by Olvera-Carranza. The literature research and the preparation of the material were carried out by García-Paz, Del Moral, Morales-Arrieta, Treviño-Quintanilla, and Olvera-Carranza. Data analysis was carried out by García-Paz, Ayala-Aceves, and Olvera-Carranza. The work was critically revised by Ayala. The first draft of the manuscript was written by García-Paz, and all authors commented on earlier versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval Not applicable.

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