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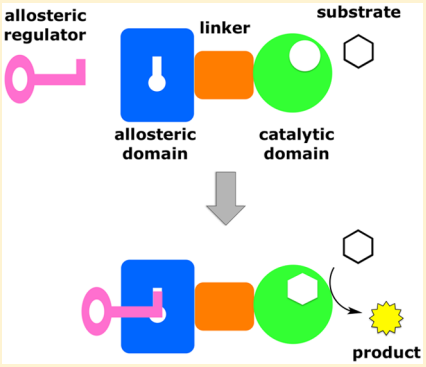
Design of Allosterically Regulated Protein Catalysts

Olga V. Makhlynets, Elizabeth A. Raymond, and Ivan V. Korendovych*

Department of Chemistry, Syracuse University, 111 College Place, Syracuse, New York 13244, United States

ABSTRACT: Activity of allosteric protein catalysts is regulated by an external stimulus, such as protein or small molecule binding, light activation, pH change, etc., at a location away from the active site of the enzyme. Since its original introduction in 1961, the concept of allosteric regulation has undergone substantial expansion, and many, if not most, enzymes have been shown to possess some degree of allosteric regulation. The ability to create new catalysts that can be turned on and off using allosteric interactions would greatly expand the chemical biology toolbox and will allow for detection of environmental pollutants and disease biomarkers and facilitate studies of cellular processes and metal homeostasis. Thus, design of allosterically regulated protein catalysts represents an actively growing area of research. In this paper, we describe various approaches to achieving regulation of catalysis.



Enzymes catalyze a large variety of chemical reactions with remarkable efficiency and selectivity. Moreover, tight control of cellular function requires the enzymatic activity to be regulated through various stimuli such as ligand or protein binding, pH change, electrostatic potential, and light. Many, if not most, enzymes are regulated by conformational changes in their allosteric domain, a stimulus-responsive part of the protein that is distant from its active site.[1−4] The simplest approximation of the principle of allosteric regulation of catalysis is presented in Figure 1. Allosterically regulated proteins can adopt multiple conformations with different properties. An external stimulus, most often the addition of a small molecule, shifts the equilibrium between the different states, resulting in a change in the catalytic properties of the enzyme.

Recent advances in protein engineering and design[5−8] have allowed the development of catalysts with controllable function for a wide variety of practical applications. For example, allosterically regulated protein catalysts can be designed to sense disease biomarkers, environmental pollutants, and explosives, study metal ion homeostasis, or regulate and visualize dynamic cellular processes in response to native or synthetic stimuli.[9−11] Switchable enzymes specifically recognize ligand and translate this event into an easily measurable absorbance or fluorescence signal. Each protein catalyst can greatly amplify the input signal by turning over multiple molecules of substrate to yield a colored or fluorescent product. Allosterically regulated catalysts were developed to modulate metabolic pathways in engineered industrial strains for biocatalysis[12] and to deliver drugs in response to various stimuli *in vivo*.[10,13,14]

The key challenge in designing an allosterically regulated enzyme is addressing how to translate the signal from the input domain into catalytic function of the output domain. This goal could be achieved by engineering the existing allosteric site to respond to a ligand of choice or by grafting ligand-binding domains onto the nonallosteric enzyme. Another route to switchable enzymes is to introduce catalytic function into proteins that undergo significant conformational changes upon exposure to external stimuli. In this review, we present advantages and challenges of various techniques used (1) to couple the effector domain to a functional domain, (2) to design catalytic sites in noncatalytic allosterically regulated proteins, and (3) to design or redesign allosteric sites in enzymes.

■ ALLOSTERICALLY REGULATED CATALYSTS DESIGNED BY CREATION AND/OR MODIFICATION OF THE LINKER DOMAIN

Arguably, the most established approach to creating allosterically regulated catalysts relies on connecting two protein domains with existing ligand-binding and catalytic site(s). The ability to use preexisting functionality is both an advantage and a disadvantage of this method. On one hand, the high binding/catalytic efficiency of natural proteins is desirable; on the other hand, the repertoire of the allosteric ligands and chemical reactions catalyzed by these novel catalysts may be limited. Several general methods for linking protein domains are described below.

Domain Insertion. The domain insertion approach aims to fuse two individual proteins to combine their properties. Early examples of protein fusions demonstrated that protein structures could tolerate large insertions into their surface loops and, in some cases, even into sites with secondary structure and still retain their original functions.[15−19] The feasibility of the domain insertion allowed for the development of fusion proteins where catalytic function of one protein was linked to a binding event at another. Lee et al. identified

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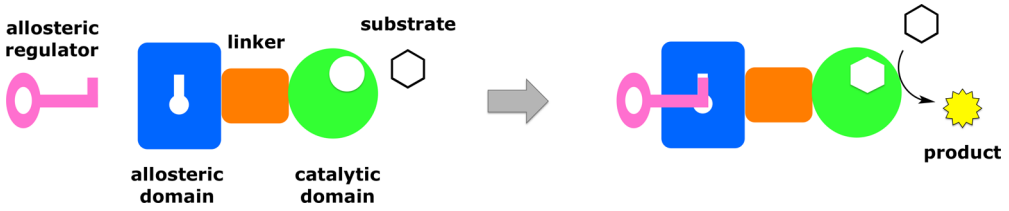

Figure 1. Key features of an allosterically regulated enzyme. When an allosteric regulator (purple) binds to the allosteric domain (blue), the resulting structural changes are translated through a linker (orange) to the catalytic domain (green), allowing catalysis. The color code is preserved throughout this paper.

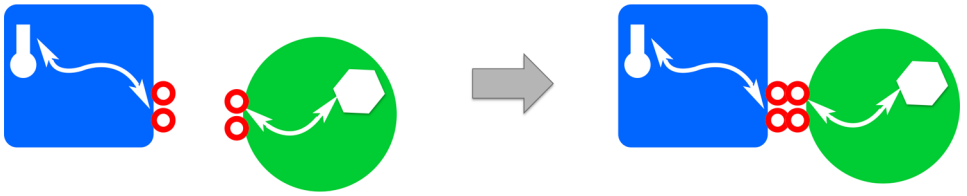

Figure 2. Rational design of a fusion site between two proteins using statistical coupling analysis. The network of co-evolved residues (white arrows) connects the functional site with distant positions (red circles) on the surface. Linking two proteins at the predicted allosteric sites (red circles) resulted in coupling between ligand binding in one protein and enzymatic function in another.

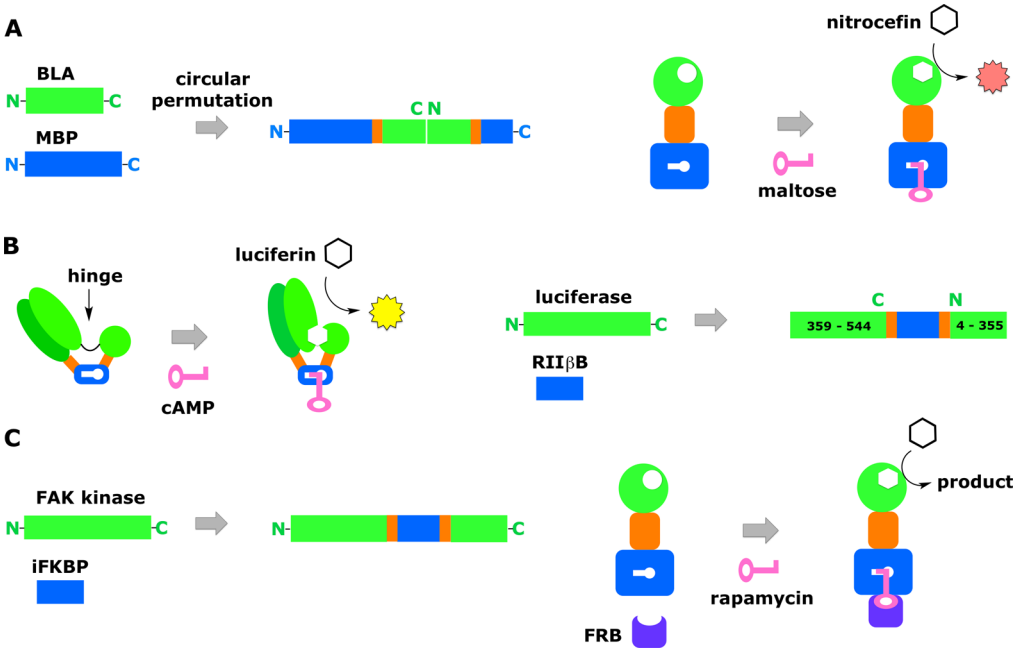

Figure 3. Insertion of one protein into another. (A) β-Lactamase (BLA) was first permuted and then inserted into a maltose-binding protein (MBP). Binding of maltose to the MBP domain induces conformational changes at the BLA domain that trigger hydrolysis of the nitrocefin substrate into a red product. (B) Binding of cAMP to the RIIβB domain of engineered firefly luciferase promotes a conformational change that turns on catalytic formation of the bioluminescent product. (C) Truncated FKBP (iFKBP) was inserted into the catalytic domain of FAK kinase using linkers. Rapamycin-mediated binding of FRB to iFKBP stabilizes the catalytic domain of the kinase (FAK) and allows for product formation.

networks of residues conserved within a protein family using statistical coupling analysis (SCA).[20] They hypothesized that these networks link the main functional site of a protein with its distant surface sites; thus, joining two proteins at their statistically important surface sites would create an efficient linker between various functionalities (Figure 2). This idea was tested by fusing the light-sensing *Avena sativa* LOV domain from the Per-Arnt-Sim (PAS) family to the *Escherichia coli* dihydrofolate reductase (DHFR). SCA of 418 members of the DHFR family identified two sites in distant surface-exposed loops that co-evolved with the enzyme's catalytic site. Similar analysis of 1104 PAS domains showed that the light-sensing flavin mononucleotide (FMN) cofactor is functionally linked to the surface-exposed residues of the protein's N- and C-termini. On the basis of this information, Lee et al. predicted that inserting the LOV through its termini into two surface-exposed loops of DHFR identified by SCA would couple the light-sensing structural rearrangement in the LOV to the catalytic function of DHFR. One of the chimeras showed a 2-fold increase in catalytic activity in the presence of light. Despite the low degree of allosteric regulation, this work proves the concept that conserved networks can be used as connection points when engineering multidomain proteins with coupled functionalities. To confirm the functional relationship between allosteric

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regulation and the sites identified using SCA, a library of 70 mutants was created by insertion of the LOV domain at every possible surface-exposed site of DHFR, and the mutants were assayed for light-dependent DHFR activity.[21] All identified insertion sites that led to allosteric regulation belonged to a network of residues that co-evolved with the active site of the protein, validating the overall approach. Interestingly, even complete screening of all possible sites did not improve the levels of allosteric regulations; the best mutants still had only 2-fold differences in activity.

Engineering of allosterically regulated proteins by rational design of domain insertion has had limited success because it is still difficult to identify sites that will both tolerate domain insertion and result in allosteric regulation.[16,17,20] A more efficient design strategy is based on generation of large libraries by random insertion of one protein into another using various linkers followed by testing for efficient allosteric regulation. The approach is best exemplified by the work of Ostermeier et al., who designed a series of allosteric enzymes by fusing a β-lactamase (BLA) to maltose-binding protein (MBP).[22,23] The use of β-lactamase as a signal output connects protein−ligand interaction at the allosteric domain with antibiotic resistance, a growth phenotype that allows for screening large libraries of mutants. Simple insertion of the *bla* gene at random positions within the *mbp* gene transformed BLA into a maltose-dependent enzyme with an only modest 1.6-fold increase in activity.[24] To improve this modest level of regulation, BLA was subjected to circular permutation[25] to change its topology (Figure 3A). The strategy of circular permutation requires that original N- and C-termini of the inset are connected and new termini created at a different site. This approach allows sampling of numerous combinations without significantly perturbing the structure. Approximately half of the single-domain proteins in the Protein Data Bank have their N- and C-termini positioned close in space, and therefore, circular permutation can be applied to many more designs.[26] A library of genes encoding circularly permutated *bla* obtained by joining the original C- and N-termini with a linker and cutting the sequence in various sites was randomly inserted into the *mbp* gene using an endonuclease DNase I.[27] The library was transformed into *E. coli*, and the colonies were screened using a colorimetric assay for nitrocefin hydrolysis to identify mutants whose β-lactamase activity depended on maltose (Figure 3A). This approach identified a protein, RG13, with a 25-fold higher β-lactam hydrolysis activity in the presence of maltose. In addition to allosteric regulation, RG13 demonstrated selectivity (no response to other carbohydrates such as sucrose, lactose, and galactose) and complete reversibility upon removal of maltose. The sensitivity of RG13 to maltose could be further improved by introducing mutations known to increase the affinity of MBP for maltose. In a follow-up study, Guntas et al. created several libraries, in which randomly circularly permutated *bla* was inserted into various sites of *mbp*. Screening of the resulting library on plates containing variable concentrations of ampicillin and maltose identified a MBP−BLA chimera with ∼600-fold higher β-lactamase activity in the presence of maltose as compared to the background.[28] This maltose-dependent β-lactamase was redesigned into a sucrose-responsive catalyst by randomizing five residues proximal to the maltose-binding site in MBP and growing the resulting mutants on plates supplemented with ampicillin and sucrose. Colonies from sucrose-containing plates were then screened for nitrocefin hydrolysis. The best mutant identified in the screen had high affinity (K_d = 0.7 μM) and relatively high specificity for sucrose (33-fold greater than that of maltose). The observed allosteric interaction is thought to originate from conformational change that occurs upon carbohydrate binding to MBP, which in turn favors the more active state of BLA.[28] These sugar-activated switches may find application in delivering drugs to tumor cells.[13,14]

Jones et al. developed a combinatorial approach to construction of allosterically regulated β-lactamases. Instead of cutting a target DNA with DNase I, their method relies on random insertion of engineered transposon MuDel.[29,30] After a library of transposition of MuDel into β-lactamase gene has been created, a restriction endonuclease was used to remove MuDel, creating a single cut in the gene sequence. A domain insertion library was then generated by the ligation of the cytochrome gene into the opening within the β-lactamase gene. The ability of the resulting mutants to hydrolyze ampicillin in the presence and absence of hemin was then characterized. Several of the designed fusion proteins demonstrated an ≤128-fold increase in β-lactamase activity when the cytochrome domain of the allosterically regulated β-lactamases bound the exogenous heme ligand.

Firefly luciferase provides a high detection sensitivity and rapid signal readout, making this enzyme suitable for high-throughput screening of large libraries. This enzyme's activity depends on the relative position of its two domains located on the opposite ends of the hinge region (Figure 3B). To eliminate the native activity that can interfere with the readout, the enzyme was subjected to circular permutation. As a result, the movement of the two domains around the hinge region was restricted, which led to inactivation of the enzyme. A random transposon insertion method identified a site within the luciferase sequence that tolerated structural modifications, and new termini were relocated to this site.[31] In the next step, the cAMP-binding (RIIβB) domain was inserted close to the hinge region using peptide linkers of variable length. A resulting library of constructs with different linkers and circular permutation sites was screened for luminescence enhancement in the presence of cAMP. The best construct displayed a 70-fold increase in luciferase activity in the presence of 100 μM cAMP relative to the background. Such allosterically regulated luciferases can be used to design bioluminescent sensors for a range of physiological mediators and may find applications in visualizing cellular processes.

A similar approach was used to create focal adhesion kinase (FAK) that is regulated by a small molecule rapamycin (Figure 3C). Protein kinases are involved in a large variety of cellular signaling processes that are sometimes hard to identify using conventional biochemical techniques. The ability to selectively turn on a kinase in response to a small molecule can be an indispensible chemical biology tool. To engineer a protein kinase that can be specifically activated by rapamycin, a short rapamycin-responsive protein (iFKBP) was inserted near the catalytic site of FAK.[32] Molecular dynamics studies showed that iFKBP's terminal domains are flexible in the absence of rapamycin, but addition of the ligand leads to the increased rigidity of its structure. Therefore, insertion of iFKBP into the catalytic domain of FAK was predicted to dramatically destabilize FAK's activity. The binding of rapamycin to the allosteric domain was expected to restore the structural integrity of the active site and rescue the activity. On the basis of molecular dynamic simulations, several constructs with variable insertion sites and linkers connecting the allosteric

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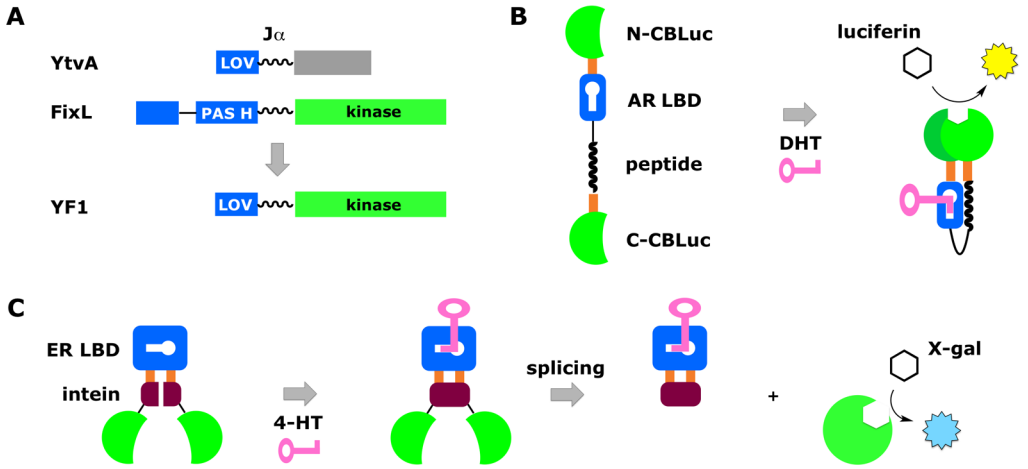

Figure 4. Strategies used to allosterically regulate assembly of the active enzyme. (A) Design of a fusion protein by exchanging PAS domains. Light-sensitive YtvA protein contains LOV domain and regulates gene transcription. Oxygen sensor FixL has two PAS domains, one of which contains a heme (PAS H), and a histidine kinase. YF1 is a fusion of the YtvA LOV domain and FixL kinase. Sensor and effector domains are connected through the Jα linker. (B) Agonist-dependent bioluminescent enzyme designed by a complementation strategy. Two parts of a split click beetle luciferase (C-CBLuc and N-CBLuc) flanking the ligand recognition domain (peptide and AR LBD) reassemble when an agonist [5α-dihydroxytestosterone (DHT)] binds, restoring the enzyme's activity. (C) Binding of the ligand at the estrogen receptor ligand-binding domain (ER LBD) triggers splicing and reassembly of the dissected protein into a functional enzyme.

domain and kinase were created and tested for rapamycin-induced activation. The best construct (RapR-FAK) has indeed shown nearly complete recovery of FAK's activity upon addition of 4 μM rapamycin. To lower the activation threshold for this allosterically activated kinase, rapamycin was added in combination with FRB, a small protein that associates with iFKBP in the presence of rapamycin (Figure 3C). Additional stabilization of the iFKBP domain by FRB binding lowered the concentration of rapamycin required to induce activation of kinase to 50 nM. RapR-FAK was successfully activated by rapamycin inside living cells to demonstrate that FAK is involved in the regulation of membrane dynamics. Src and p38 kinases fused to the iFKBP cassette were also activated by treatment with rapamycin in the presence of FRB, suggesting that this strategy will allow observation of transient signals induced by a wide variety of kinases.[33−35]

Tucker et al. demonstrated how a domain insertion sensor could be applied to screening small molecules for their ability to bind estrogen receptor α (ERα).[36] Human ERα was inserted into mouse DHFR at residue 107 using a flexible linker. This construct was then introduced into DHFR-deficient *Saccharomyces cerevisiae* strain TH5. The strain showed an increased level of growth in the presence of estrogen and estrogen analogues, which also correlated with relative binding affinities of estrogens for ERα. In addition, this growth assay was used to identify mutations in ERα important for analyte binding. This proof-of-principle study showed the utility of domain insertion for high-throughput screening to identify small molecules that bind receptors of interest.

Domain Swapping. An alternative approach to functionally linking allosteric and catalytic domains relies on domain exchange between homologous proteins. Signaling proteins containing the PAS sensor domain detect various signals, including light, redox potential, and the presence of chemicals.[37] These signals are propagated to response domains such as kinases, transcription factors, and phosphodiesterases. Despite their functional diversity, these proteins share similar structures and signaling mechanisms. To show that PAS domains are interchangeable, Möglich et al. replaced the oxygen-sensing PAS domain of the histidine kinase FixL with a blue light PAS sensor domain (LOV) from YtvA and optimized the linker between the domains (Figure 4A).[38] The most successful fusion protein YF1 phosphorylated a response regulator (FixJ) with almost the same rate as the parent enzyme FixL and showed a 1000-fold decrease in kinase activity upon illumination with light. Further studies suggested that switching is mediated by the helical Jα linker that connects LOV and kinase domains (Figure 4A). When the flavin mononucleotide (FMN) cofactor of the LOV domain absorbs light, the Jα linker partially unfolds, resulting in rotation of the histidine kinase domain relative to the sensor domain. Because a family of PAS signaling proteins comprises numerous members with various combinations of sensor and effector domains, swapping domains can potentially be used to reprogram allosteric regulation of enzymes or even to design receptors that respond to multiple signals.[39]

Control of Enzyme Assembly. The approach outlined in this section is based on enzyme splitting and reassembly. The reporter enzyme is split into two parts that cannot assemble into an active protein on their own. These two fragments are then fused to an allosteric domain, which by interacting with the analyte brings the fragments together to facilitate proper folding. As a result, a catalyst whose activity is controlled by a ligand in a concentration-dependent manner is created. Using this method, Kim et al. designed a bioluminescent probe for detection of androgen.[40] First, they identified the dissection site of click beetle luciferase (CBLuc) to generate inactive N- and C-terminal fragments of the enzyme that can recover bioluminescence activity through intramolecular complementation. Suitable dissection points were selected near the hinge region between two subdomains, a place essential for catalytic function. The ligand-binding domain of the androgen receptor (AR LBD) was fused to a coactivator peptide, and the resulting fusion construct was placed between the C- and N-terminal parts of CBLuc (Figure 4B). A library of constructs with varying peptide linkers and CBLuc dissection points was screened for luminescence intensities in the presence and absence of 5α-dihydroxytestosterone (DHT), an androgen

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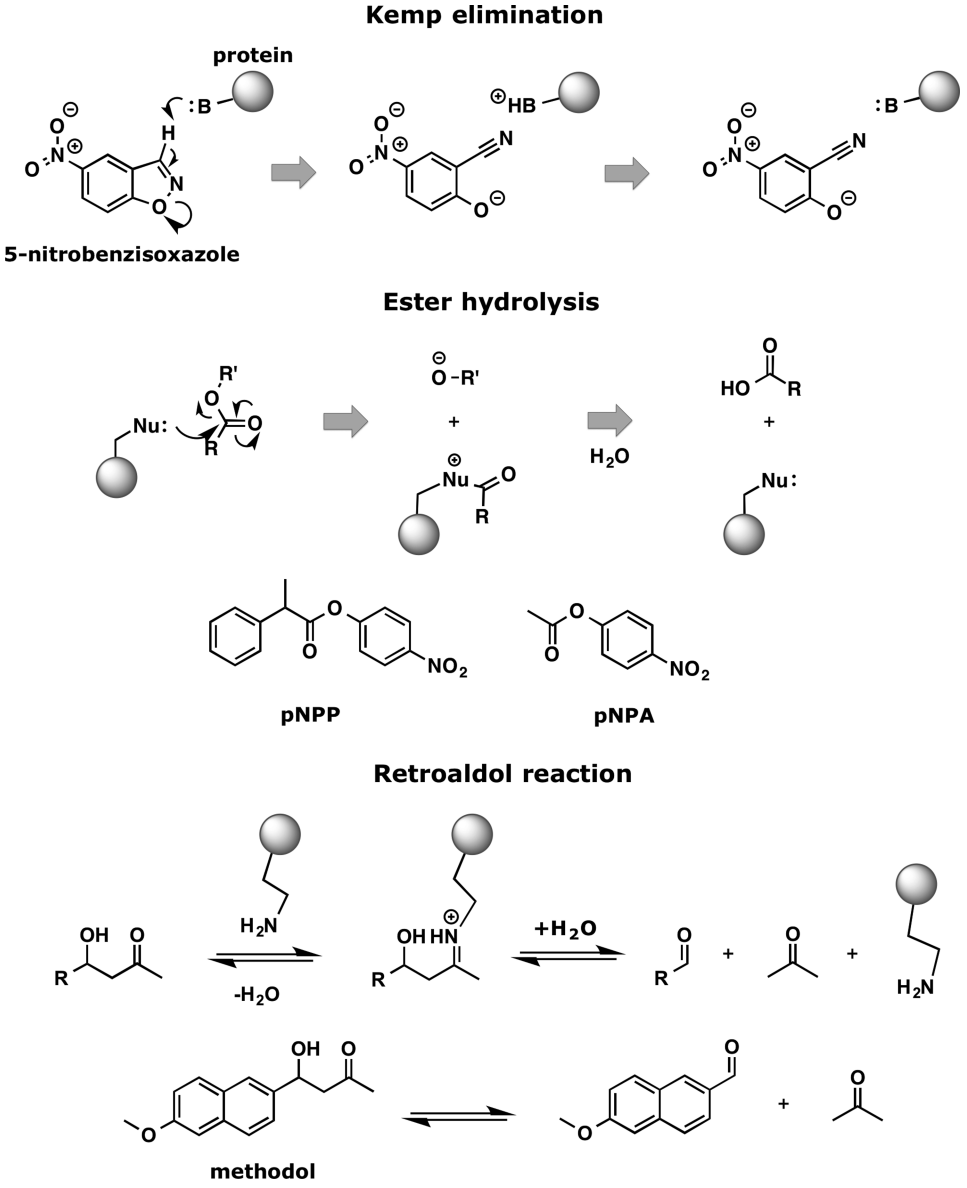
Scheme 1. Reaction Mechanisms and Model Substrates Used To Test Designed Protein-Based Catalysts



receptor agonist. DHT stimulates the interaction between AR LBD and the coactivator peptide and restores the activity of split CBLuc. One of the constructs exhibited a bioluminescense signal 30 times above the background after a 20 min stimulation with 10 μM DHT; the signal dropped to background levels upon withdrawal of DHT. Because the probe is agonist-selective and generates reversible bioluminescence light insensitive to pH and metal ions, it can be applied to monitoring the androgen receptor signaling *in vivo*[41] or screening for androgen receptor agonists.

Another bioluminescent sensor was rationally designed by splitting photoprotein aequorin, an enzyme that oxidizes the coelenterazine substrate with the emission of light.[42] Glucose-binding protein (GBP) was inserted into a flexible loop of aequorin separating two fragments of this protein. The resulting hybrid protein undergoes conformational changes when GBP binds glucose that allows reassembly of aequorin and recovery of bioluminescence. Because of the high sensitivity of bioluminescence methods, glucose was detected at concentrations as low as 100 nM.

Protein splicing is another widely used tool to control the assembly of individual protein units into a functional enzyme. An enzyme is rendered inactive by insertion of an intein, a protein that can excise itself from the host via a process called protein splicing. As a result of such transformation, two parts of the enzyme are joined together by a peptide bond restoring the enzyme's activity. Inteins are controlled by small molecules and flanked by protein−host fragments and allow an attractive strategy for designing allosterically controlled enzymes, where protein function depends on the presence of the small molecule. Buskirk et al. inserted the estrogen receptor ligand-binding domain (ER LBD) into intein RecA, and this fusion was introduced into several different enzymes.[43] Upon binding to 4-hydroxytamoxifen (4-HT), ER LBD undergoes a conformational shift moving N- and C-termini of the flanking enzyme in a position favorable for splicing (Figure 4C). To optimize the splicing efficiency and the regulation of enzyme function by 4-HT, a large library of constructs was created by introducing mutations into the intein sequence. The library was subjected to several rounds of positive and negative selection

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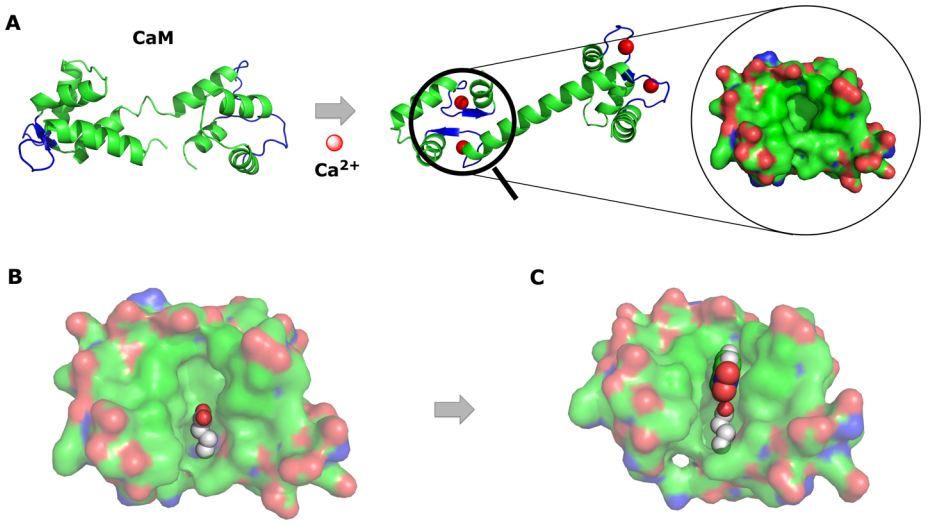

Figure 5. Summary of the AlleyCat design strategy. (A) Binding of calcium to CaM triggers conformational changes that result in formation of the hydrophobic pocket (EF-hands that bind Ca^{2+} are colored blue). (B) The F92E mutation introduced a catalytic base into an otherwise hydrophobic pocket (E92 is shown as spheres). (C) Transition state docking based on a superrotamer library for the transition state. Images were prepared using structures of Protein Data Bank entries 1CFD (no Ca^{2+}) and 1CLL (with Ca^{2+}).

based on the response of the extein protein, such as kanamycin resistance enzyme and GFP. The screen identified an intein with a strong ligand dependence and high splicing activity that was further characterized in combination with β-galactosidase and Ade2p enzymes. In both cases, the function of the enzyme was restored upon treatment with 4-HT, suggesting that an intein, once developed, can be used to regulate the activity of any protein of interest. In a different study, Skretas et al. used random mutagenesis combined with selection through growth phenotype to design an intein whose splicing activity was controlled by a thyroid hormone.[44] The ability to control protein function by ligand-dependent protein splicing can serve as a tool to activate enzyme in living cells with small cell-permeable molecules. Recently, ligand-dependent inteins have been improved to splice within mammalian cells with high yields and fast kinetics extending applications of this method.[45] Among advantages of the protein splicing approach is its generality, because the same intein–ligand couple can be adapted to regulate many different enzymes. However, intein splicing is irreversible as it can only activate enzymes and does not provide control over spliced proteins.

To summarize this part, the key challenge in a design of an allosterically regulated enzyme by domain insertion is to identify a site of domain insertion that would both tolerate structural manipulations and maximize the connection between the allosteric site and the catalytic site. The following conclusions can be drawn from the existing work. (1) The allosteric and catalytic domains should be located close to each other in the resulting construct to be efficiently coupled. (2) The structure and location of the linker regions are critically important for the design. Switching between the active form and the inactive form is more likely to happen when inserts are placed in short loops. When peptide linkers are used to assist the insertion of one domain into another, their length becomes a critical variable: the linkers that are too short may result in protein unfolding, but the linkers that are too long could uncouple the two separate functions. (3) Rational prediction of efficient linkers, circular permutation sites, and insertion points is still in its infancy. Essentially every single successful example of allosterically regulated proteins developed by this approach relied to some degree on combinatorial optimization. (4) While catalytic efficiencies of the resulting allosterically regulated enzymes are excellent, the degree of allosteric regulation, i.e., the difference in activity between the on state and the off state, is still relatively low compared to those of some natural proteins. Future optimization of this method is likely to focus on the development of more sophisticated linker domains that can improve communication between ligand binding and catalysis.

■ ALLOSTERICALLY REGULATED CATALYSTS DESIGNED BY CREATION OF A NEW CATALYTIC SITE

This section is focused on the design of novel catalytic activity that is linked to ligand-promoted conformational change. The idea behind this approach is *de novo* design of a catalytic site into an existing allosterically regulated protein. This path offers a major advantage for the creation of regulated catalysts for unnatural reactions but is still limited by the current capabilities of protein design.[5,6] Nonetheless, several notable successes in the *de novo* design of allosterically regulated catalysts have been reported. Efforts in this area are represented by the *de novo* design of a family of calcium-dependent catalysts for Kemp elimination, ester hydrolysis, and retroaldol reaction (Scheme 1).

Kemp elimination is a benchmark reaction catalyzed by abstraction of a proton from benzisoxazole followed by cleavage of the isoxazole ring and formation of the yellow 2-cyano-phenolate product. Carboxylic acids in organic solvents are good catalysts for this reaction[46] because the hydrophobic environment increases the basicity of carboxylate. Therefore, introduction of a carboxylate residue into a protein's hydrophobic pocket was expected to result in Kemp eliminase activity. The small allosterically regulated protein calmodulin (CaM) was chosen as a design scaffold because of its ability to form a hydrophobic pocket when Ca^{2+} binds to its EF-hands (Figure 5A). CaM itself does not have enzymatic activity, providing an excellent reference point for catalysis. The design strategy used a combination of minimalist rational and

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computational approaches. The entire cavity of CaM was computationally screened to identify positions that can form a Michaelis complex with a substrate without disrupting the protein's packing. The best mutant [F92E (Figure 5B,C)] predicted by computation, named AlleyCat, was recombinantly expressed and characterized. AlleyCat's catalytic efficiency (k_{cat}/K_M) of 6 M^{-1} s^{-1} in catalyzing Kemp elimination is within an order of magnitude of those of the most successful Kemp eliminases.[5,47] AlleyCat shows excellent allosteric regulation; no activity above the background is observed in the absence of calcium. AlleyCat was further improved using directed evolution.[48] First, the residues around the active site were subjected to saturation mutagenesis. Mutants with increased activity were subjected to gene shuffling and an additional two rounds of error-prone polymerase chain reaction (PCR) followed by saturation mutagenesis. In just seven rounds of mutagenesis, the enzymatic efficiency of AlleyCat was improved by more than 200-fold to 1280 M^{-1} s^{-1} without any loss of allosteric regulation. Importantly, the increase in the k_{cat}/K_M value originated essentially exclusively from the increase in k_{cat}. The turnover number of the resulting allosterically regulated catalyst of Kemp elimination is higher than that of catalytic antibodies,[49−51] is on par with the best examples of sophisticated computational studies coupled with directed evolution,[52] and is only 2 orders of magnitude lower than that of the state-of-the-art Kemp eliminase designed using the iterative approach developed by Hilvert and Mayo.[53,54] Examples outlined above demonstrate successful use of the combination of computational and directed evolution methods to introduce new catalytic function into proteins. Computational design generally creates a good starting model of the active site that can be further fine-tuned by rational mutations and directed evolution.[55] The advantage of CaM-based Kemp eliminase is the simplicity of its design and allosteric regulation. The ability of this calcium-modulated protein to convert colorless substrate into yellow product can be used for calcium sensing *in vivo*.

A similar computational approach was applied to the design of an allosterically regulated esterase. Ester hydrolysis reactions are widespread in nature and find many practical applications ranging from chemical synthesis to biofuel production. A benchmark reaction for ester hydrolysis is formation of the yellow product from *p*-nitrophenyl esters (Scheme 1). This reaction was employed to test if allosterically regulated CaM could assume esterase activity. Esterases catalyze hydrolysis using multiple residues arranged in catalytic dyads or triads and are aided by an oxyanion hole that stabilizes developing charges of the transition state. However, the histidine residue itself can catalyze ester hydrolysis as was demonstrated with engineered thioredoxin, although with a modest catalytic efficiency (k_{cat}/K_M) of 3 M^{-1} s^{-1}.[56] Using a minimalist approach, a single histidine was introduced into the hydrophobic pocket of CaM. The design was accomplished in several steps. First, the model substrate, *p*-nitrophenyl (2-phenyl)propionate (pNPP), was computationally docked into CaM's hydrophobic pocket to ensure the overall feasibility of catalysis, and then a search was performed to identify positions where a histidine residue could form a transition state without disrupting the overall protein fold. A single position was identified (M144H) to satisfy the conditions given above. Experimental characterization confirmed the prediction that a single mutation, M144H, converted CaM into an allosterically regulated esterase capable of hydrolyzing pNPP with a k_2/K_S of at least 1500 M^{-1} s^{-1}.[57] This value compares well to those previously reported for rational, computational,[58] and combinatorial designs[59] (between 10 and 270 M^{-1} s^{-1}).

Finally, calmodulin was redesigned to serve as an allosterically regulated retroaldolase using the approach outlined above. Introduction of a strategically placed single reactive lysine into calmodulin's hydrophobic pocket conferred retroaldolase activity on this protein.[60] The resulting allosterically regulated catalyst, dubbed AlleyCatR, is capable of converting methodol (Scheme 1) into a fluorescent product. Importantly, AlleyCatR is active only in the presence of calcium. AlleyCatR is active enough to produce a fluorescence signal above the background in the *E. coli* crude cell lysate, making it suitable for high-throughput screening for further optimization of retroaldol activity by directed evolution.

As described in the next chapter, the EF-hands in the calmodulin-based allostrerically regulated AlleyCat can be reprogrammed to respond to a variety of different metals, paving the way to creating biocompatible metal sensors.[61]

In a different study, another Ca-responsive protein, recoverin, was used as a scaffold to design an allosterically regulated selenoenzyme.[62] Docking of glutathione (GSH) into the N-terminal domain of recoverin identified positions that could accommodate the catalytic selenocysteine residue and stabilize substrate binding. Mutation of A128, normally buried in the absence of calcium, to selenocysteine converted recoverin into Ca-regulated glutathione peroxidase. Subsequent mutagenesis improved the enzyme's activity by 5-fold. The resulting allosterically regulated peroxidase was shown to protect mitochondria from oxidative damage in the presence of Ca^{2+}.

Examples of allosterically regulated enzymes described in this chapter rely on the introduction of novel functionalities into the existing allosteric scaffolds. This approach yields outstanding regulation, and the limiting factor is our ability to efficiently create catalytic functionality. Each enzyme is a complex system, and designing such molecules from scratch can be daunting. Despite much effort, artificial enzymes are still inferior to their natural counterparts, but rapidly improving protein design and engineering methods will likely advance the field.[5,8] Computational[63] and rational[64−66] design approaches can already efficiently provide an adequate starting point for subsequent optimization using directed evolution.[67,68]

■ ALLOSTERICALLY REGULATED CATALYSTS DESIGNED BY THE CREATION OF NEW OR MODIFICATION OF EXISTING ALLOSTERIC SITES

Many naturally occurring sensors undergo significant conformational changes when they undergo the transition from the ligand-free state to the ligand-bound state. However, this type of switching cannot be easily designed into the enzyme scaffold *de novo*. One of the solutions to this challenge, outlined in the previous section, is to insert a ligand-binding domain into the enzyme. Another strategy is to adapt an existing allosterically controlled enzyme to bind any arbitrary ligand of choice. Several examples of this approach are described below.

Chemical Rescue. This method is based on the ability of a small molecule to partially replace the functionality of an amino acid side chain.[69] Normally, a residue with a side chain important for catalysis is mutated (often to alanine), and the molecule complementing the lost functionality, e.g., imidazole for histidine, is added. The chemical rescue approach was used to create a catalytically impaired tyrosine-protein kinase that

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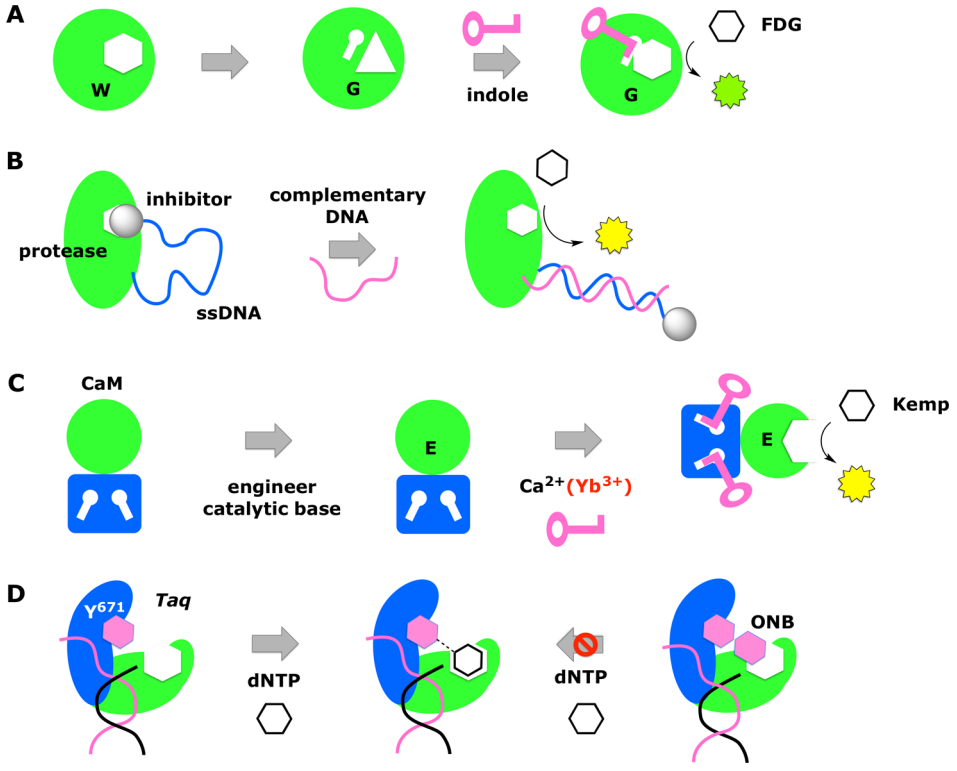

Figure 6. Sensors created by modifications of the existing or creation of a new allosteric site. (A) The chemical rescue approach is based on modulating the structure of the active site, which can be restored by small molecule binding. The W33G mutation inactivated β-glycosidase because of structural perturbations in the active site, which was restored by indole binding. (B) Sequence-specific sensor for low concentrations of DNA consisting of a covalently attached protease enzyme, a DNA oligomer (blue), and a protease inhibitor. The DNA analyte is shown as a purple line. (C) The Ca-regulated protein calmodulin (CaM) was redesigned into a Kemp eliminase by introducing a single F92E mutation in its hydrophobic pocket, and then EF-hands of this artificial enzyme were modified to respond to lanthanides. (D) Y671 (shown as a purple hexagon) of *Taq* DNA polymerase makes a hydrogen bond with the incoming dNTP (substrate) and helps to position it within the binding site. Photocaging of Y671 by the *o*-nitrobenzyl (ONB) group prevents substrate binding and leads to an inactive enzyme. Template DNA is colored purple and the growing DNA black.

could be rescued by a small molecule for signaling studies. Mutation of the arginine side chain that forms hydrogen bonds with a catalytic aspartate and with the hydroxyl group of the substrate (R318A) results in inactivated kinase (Csk), which could be rescued by imidazole *in vitro*.[70] Moreover, mutant R388A of Src protein kinase could be reversibly rescued (up to 50% of the wild-type activity) by imidazole in live cells, and this chemical rescue approach helped to identify new substrates of Src.[71] The methodology that allows activation of enzymes *in vivo* without sophisticated gene knockouts may find applications in identifying complex signal transduction mechanisms, given that the rescue molecule is nontoxic and cell-permeable.

A different approach to chemical rescue is based on destabilizing of the enzyme's active site by creating a cavity, which can be complemented by a small molecule, thus restoring the structure and function of the enzyme.[72] Using the available crystal structure of β-glycosidase, Karanicolas et al. predicted residue W33 to be critical for the integrity of the enzyme's active site. Mutating this single tryptophan residue to glycine strongly diminished β-glycosidase activity compared to that of the wild-type enzyme; however, addition of indole resulted in full recovery of activity (Figure 6A).[73] Comparison of the crystal structures of W33G and wild-type β-glycosidase provided an explanation for the observed chemical rescue. Replacing Trp with Gly created a cavity within the protein structure, and a key residue at the active site (W433) moved away from the substrate to fill the void. After the W33G crystal was soaked with indole, the crystal structure of W33G revealed that indole occupied the cavity and W433 reverted to its native position at the active site. Although in this example the mutation was introduced close to the active site and regulation is not purely allosteric, the strategy may be extended to remote sites as long as structural modifications are relayed to the active site.

Kohn and Plaxco used structure destabilization and target-activated folding to identify a phosphorylated peptide in blood serum.[74] The SH3 domain of Fyn tyrosine kinase was progressively truncated until the fourth deletion resulted in protein unfolding (ΔC4). Binding of the cognate 11-residue peptide at the binding pocket located relatively far from the mutation site induced protein folding. It should be noted that this study focused on protein folding monitored by fluorescence rather than the connectivity between allosteric interactions and catalysis; thus, no catalytic data are presented. It is, however, conceivable that restoring the fold gives the protein the ability to phosphorylate its substrate in an allosteric fashion.

An intrasterically regulated enzyme was developed by the Ghadiri group to detect low concentrations (10 pM to 10 μM) of specific DNA sequences.[75] The system consisted of a covalently attached enzyme [*Cereus* neutral protease (CNP)], a single-stranded DNA recognition sequence (24-mer oligonucleotide), and an enzyme inhibitor [phosphoramidite (Figure 6B)]. In the absence of a target analyte, a ssDNA chain is

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flexible and allows the attached inhibitor to block the active site of the enzyme. Annealing of the complementary DNA and ssDNA probe resulted in the removal of the inhibitor from the active site and enzyme activation. Protease activity was measured using a peptide substrate flanked by a fluorophore and a quencher as a function of the increase in fluorescence caused by endolytic cleavage of the peptide.

Redesign of an Existing Allosteric Site. Earlier in this review, we described how the binding site of maltose-regulated β-lactamase was redesigned to respond to sucrose.[28] In a different study, calmodulin-based allosterically regulated protein CuSeCat was reprogrammed to bind lanthanide ions (Figure 6C). First, noncatalytic protein calmodulin was modified to catalyze the Kemp elimination reaction as discussed in the previous section. Kemp elimination produces a colored product that can be easily measured spectrophotometrically. Binding of calcium to calmodulin triggers significant conformational change, which forms a solvent-exposed hydrophobic cavity that can accommodate substrate. Second, the EF-hands of CuSeCat were redesigned to bind trivalent metal ions. Previous work by Falke et al. established that both metal size and metal charge influence the selectivity of EF-hands and the higher negative charge of the metal-binding loop strengthens its preference for trivalent cations.[76,77] Mutation of neutral residues S101 and N137 to glutamates substantially lowered the affinity of CuSeCat for Ca^{2+} and simultaneously increased the affinity for Yb^{3+}.[61] This work demonstrates that the metal-binding domain and catalytic domain can be modified separately to improve selectivity and catalytic efficiency.

Rena et al. redesigned the serine protease thrombin to allosterically respond to K^+ instead of its native regulator Na^+.[78] The Na-binding site in thrombin is comprised of two loops starting at residues 186 and 220 as shown by the X-ray structure.[79] These cation-binding loops were replaced with complementary loops from the anticoagulant enzyme PC that can use both K^+ and Na^+ for the enhancement of its protease activity. The resulting loop chimera demonstrated a higher affinity for K^+ than for Na^+ and, more importantly, an improved enzymatic activity with K^+. More work is still required to clarify which specific residues within loops 186 and 220 control recognition of the monovalent ion.

Unlike rational design, high-throughput screening can sample numerous combinations and often leads to constructs with superb properties. Mathonet et al. used phage display to develop a β-lactamase with an allosteric site regulated by transition metal ions.[80] A library of phage clones was created by random mutations in three contiguous loops of β-lactamase. The library was subjected to immobilized metal affinity chromatography to isolate mutants with affinity for Ni^{2+}, Zn^{2+}, and Cu^{2+}. After five rounds of selection, phages with enhanced affinity for metal ions were screened for ampicillin hydrolysis and tested for activity modulation in the presence of metal. One of the mutants was 3-fold activated with Ni^{2+} and 10-fold inhibited by Cu^{2+} ion binding. Interestingly, one-third of tested mutants with ampicillin resistance were regulated by metal ions, suggesting that engineered binding sites have a high probability of affecting protein's function. In support of the hypothesis that ligand binding and allostery evolve simultaneously, Liang et al. showed that a chimeric protein created by fusion of MBP and BLA proteins has high affinity for Zn^{2+} and is negatively regulated by this metal ion.[81,82] The switch, named RG13, was designed by circular permutation of the *bla* gene, which was randomly inserted into *mbp*. Maltose binding increased β-lactamase activity of this protein 25-fold. Although neither MBP nor BLA has affinity for Zn^{2+}, RG13 bound Zn^{2+}, which turned off its β-lactamase activity. X-ray crystallography[83] and NMR[84] structural studies provided insight into the structure and allosteric mechanism of RG13.

Using a different approach, Reetz et al. were able to use the concept of allostery to reshape the binding pocket of a thermostable Baeyer−Villiger monooxygenase.[85] Knowledge-based saturation mutagenesis in two positions distal from the catalytic site identified a mutant that can accept 2-substituted cyclohexanone derivatives with high kinetic resolution.

Hellinga et al. computationally redesigned periplasmic binding proteins (PBP) to bind a number of small molecules such as trinitrotoluene, L-lactate, serotonin,[86] and pinacolyl methyl phosphonic acid (PMPA, the hydrolytic product of the nerve agent soman)[87] instead of their natural ligands. PBP consist of two domains connected by a hinge, and the structure adopts a closed conformation when the ligand binds in the interface between the two domains. Significant conformational changes between open and closed states make PBP an attractive scaffold for the design of allosteric regulation. Redesigned PBP receptors were introduced into a two-component system that regulates β-galactosidase expression, connecting ligand recognition and enzyme activity. However, these studies came under scrutiny because no binding between designed receptors and their ligands could be detected using direct experimental methods.[88]

Modular Creation of an Allosteric Site. Advances in structural and functional characterization of enzymes led to the realization that allosteric regulation is prevalent in natural systems.[4] In fact, whole families of enzymes are allosterically controlled by metabolites. Cross et al. applied the modular approach to engineering regulation into the first enzyme of the aromatic amino acid biosynthesis, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAH7PS).[89] The DAH7PS family includes enzymes that are regulated by various small molecules as well as unregulated orthologs. Cross et al. combined the tyrosine-regulated ACT domain from *Thermotoga maritima* (*Tma*DAH7PS) with the unregulated catalytic $(\beta/\alpha)_8$ barrel domain from *Pyrococcus furiosus* DAH7PS (*Pfu*DAH7PS) using simple gene fusion. The resulting chimera (*Tma*ACT−*Pfu*DAH7PS) preserves the original catalytic activity and undergoes a major conformational change in response to binding of tyrosine to the newly introduced regulatory domain.

Photocaged Systems. Finally, it should be mentioned that allosteric regulation of enzymatic activity could be achieved through caging. As opposed to the systems described above, caged enzymes are regulated in inherently nonreversible fashion, and thus, it can be turned on only once. There are quite a few reported examples of photocaged enzymes, where one of the residues at the active sites is protected;[90,91] however, publications in which a caged site can be considered to be allosteric are much more scarce. Recent developments in the incorporation of genetically encoded photocaged amino acids permitted the design of enzymes that can be turned on by light.[92] Enzyme function is abolished by replacing a residue involved in catalysis with an unnatural photocaged analogue of the same residue. A pulse of light is used to uncage this residue and restore its function. *Thermus aquaticus* DNA polymerase (*Taq*) was turned into a light-activatable enzyme by photocaging its Y671.[93] This residue helps to position incoming dNTPs in the binding pocket of the enzyme and prepare it to line up with the DNA template (Figure 6D).[94] Replacing Y671

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with photocaged *o*-nitrobenzyl (ONB) tyrosine inactivated *Taq*, because the ONB group occupied the place where incoming dNTP binds. Illumination of photocaged *Taq* at 365 nm followed by PCR showed that the enzyme's activity could be restored to 71% of that of the wild type by a 5 min exposure to UV light.

An example of indirect two-step photoactivation was recently published by the Kragl group.[95] They have employed 2-nitrobenzaldehyde (2-NBA) that rearranges to 2-nitrosobenzoic acid with a concomitant release of a proton upon irradiation. The resulting pH jump activates hydrolytic activity of an acid phosphatase.

CONCLUSIONS

The design of an allosterically regulated protein arguably represents the most difficult task in the field of protein design and engineering. Indeed, introducing a new catalytic function into a protein is difficult enough; having it efficiently controlled by an external stimulus at the same time presents a formidable challenge. Not surprisingly, no examples of *de novo* designs of such systems from scratch have been reported so far. Nonetheless, there are plenty of reasons for optimism; many successful allosterically regulated enzymes have been reported over the past several decades. Existing strategies can be subdivided into three categories based on whether catalytic, allosteric, or linker sites are created, and all of them have their distinct advantages.

Linking naturally occurring or engineered allosteric and catalytic domains represents by far the most general approach to achieving catalysis regulated by an external stimulus as it taps into a vast resource of already very efficient catalysts and sensors. The challenge resides in the linker design; the signal from the allosteric domain is transmitted to the catalytic domain by means of conformational changes, and designing such communication is challenging. In fact, most rationally designed linkers have relatively small degrees of regulation despite the preservation of the natural efficiency of the catalyst to a great extent (Figure 7). Some degree of combinatorial

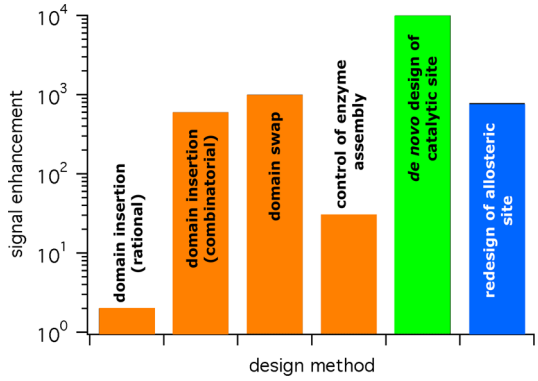

Figure 7. Degree of allosteric regulation provided by various design strategies discussed herein.

postoptimization is almost always required. This is perhaps not surprising as natural allosteric binding events result in sophisticated and concerted conformational changes that involve the whole protein. Such a degree of complexity is unlikely to be easily replicated by simple domain insertion or peptidic linkers; thus, future improvements in this strategy will likely focus on developing more efficient ways of domain coupling. Domain replication, commonly used in naturally occurring allosteric system, provides an enormous, completely untapped resource for future design efforts.

Introduction of a *de novo*-designed catalytic site into an existing allosterically regulated protein recently emerged as an exciting design possibility. This strategy provides the best degree of allosteric regulation (Figure 7) reported to date and a versatility of introduced functions. The ability to apply directed evolution to the allosteric domain of the *de novo*-designed enzyme to change the input ligand offers an additional advantage. Future work will be required to expand the repertoire of designable allosterically regulated scaffolds to test how general this approach can be. Recent advances in computational protein design and directed evolution methods will likely help improve this strategy.

Designing a new allosteric site into an existing catalyst remains a very complex problem. Successful examples of this approach are currently limited to chemical rescue, which requires prior structural information. Combinatorial methods of introducing new sites so far have not yielded very high levels of allosteric regulation because of the complexity of the requirement to have an efficient linker, which was discussed in detail above. Immediate improvement in the application of this strategy will probably come as a result of more efficient methods of reprogramming the existing sites to new substrates using the power of directed evolution. Coupling of selection with survival or efficient binding selection, e.g., phage display, will likely sample enough combinatorial space to sufficiently alter the existing selectivity of the allosteric site.

Concerted efforts to design allosterically regulated enzymes using already established and new approaches will advance our understanding of allostery[96] and will help in the development of more efficient tools for protein design and engineering. Studies of already developed *de novo*-designed and combinatorially engineered proteins[9,97,98] will undoubtedly lead to refinement of protein design principles.[6] A combination of rational design with sophisticated computational algorithms fine-tuned by focused directed evolution should create a powerful combination for development of new tools for chemical biology, sensing, and even drug delivery.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ikorendo@syr.edu. Telephone: (315) 443-1661.

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Notes

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ABBREVIATIONS

AR LBD, ligand-binding domain of the androgen receptor; BLA, β-lactamase; CaM, calmodulin; cAMP, cyclic adenosine monophosphate; CBLuc, click beetle luciferase; CNP, *Cereus* neutral protease; DHT, 5α-dihydroxytestosterone; DHFR, dihydrofolate reductase; ERα, estrogen receptor α; ER LBD, ligand-binding domain of estrogen receptor; FAK, focal adhesion kinase; FixJ, response regulator; FixL, oxygen-sensing domain of histidine kinase; FKBP, protein stabilized by rapamycin binding; FMN, flavin mononucleotide; FRB, rapamycin-binding domain; GBP, glucose-binding protein;

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GFP, green fluorescent protein; 4-HT, 4-hydroxytamoxifen; iFKBP, rapamycin-responsive protein; LOV, light-, oxygen-, or voltage-sensing; MBP, maltose-binding protein; 2-NBA, 2-nitrobenzaldehyde; ONB, *o*-nitrobenzyl; p38, kinase; PAS, Per-Arnt-Sim; PBP, periplasmic binding proteins; PC, anticoagulant enzyme; PMPA, pinacolyl methyl phosphonic acid; pNPP, *p*-nitrophenyl (2-phenyl)propionate; RIIβB, cAMP-binding; RecA, intein; SCA, statistical coupling analysis; Src, kinase.

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