Available online at www.sciencedirect.com
ScienceDirect
Current Opinion in Structural Biology

Designing switchable enzymes

Marc Ostermeier

The modulation of enzyme function is a key regulatory feature of biological systems. The ability to engineer synthetic enzymes that can be controlled by any arbitrary signal would enable a wide array of sensing applications and therapeutics and provide us with powerful tools for the basic study of biology. Here several recent advances in the engineering of switchable enzymes through domain fusion are discussed.

Address
Department of Chemical and Biomolecular Engineering, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218, United States

Corresponding author: Ostermeier, Marc (oster@jhu.edu)

Current Opinion in Structural Biology 2009, **19**:442–448

This review comes from a themed issue on
Engineering and Design
Edited by Bill DeGrado and Brian Kuhlman

Available online 25th May 2009

0959-440X/$ – see front matter

DOI 10.1016/j.sbi.2009.04.007

Introduction

The modulation of enzyme activity is a common mechanism through which regulatory functions have been achieved in biology. Although the regulation of enzyme activity can occur at the production (i.e. transcription/translation) or degradation steps (i.e. proteolytic degradation) during the lifetime of a protein, direct regulation of enzyme activity at the protein level through allosteric effects offers two important advantages: regulation does not depend on other cellular components and the regulation can be executed on much shorter time scales — essentially instantaneously. These advantages have motivated the engineering of synthetic switchable enzymes for biotechnological, biomedical, and basic science applications.

A common approach to engineer switchable enzymes has been to create fusion proteins between enzyme domains (the output domain) and domains that recognize/respond to input signal (the input domain). In general, the modulation of enzyme activity is sought through signal-induced conformational changes in the input domain affecting the enzyme domain. Conceptually, the design of a switchable enzyme has three constraints, which will be described here for a ligand-activated enzyme. The first two seem tractable in isolation: (1), create a fusion protein in which the enzyme domain is highly active when the input domain is bound to its ligand and second, create a fusion protein in which the enzyme domain is inactive (or less active) when the input domain is in its ligand-unbound state. The difficulty lies in the third constraint: (3), the primary sequence of the designed protein must be the same for constraints (1) and (2) Furthermore, not only must a single sequence achieve the prerequisite structure to satisfy constraints (1) and (2), but there also must be an energetically favorable pathway by which one structure changes to the other upon ligand binding. Ideally, this conversion should be reversible once the ligand is removed. Intimately connected to this pathway is the mechanism by which the states are converted.

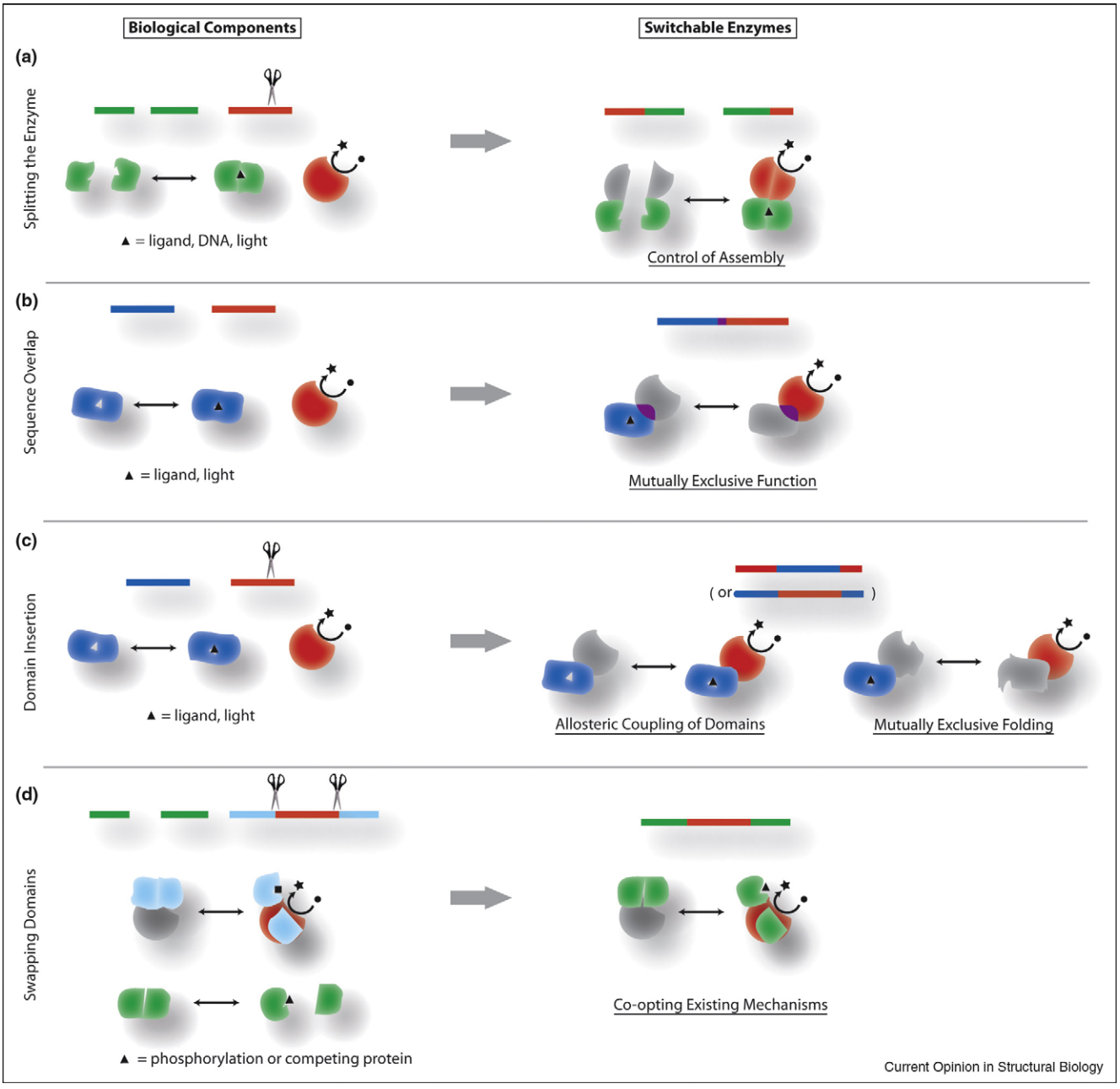
Allosteric effects must depend on the details of a protein's sequence, structure, and energetics. Despite this, most successes at creating switchable enzymes have resulted from a more abstract view of proteins — with designs based on conceptually simple mechanisms that often rely on the modularity of protein functions and the ability to readily form new connections between protein domains. Here, a review and discussion of recent successes in the creation of switchable enzymes is organized by the mechanisms through which switching was designed to occur.

Control of protein assembly

One way to control a protein's function is to control its assembly into the correct three-dimensional structure (Figure 1a). This approach derives from the development of protein-fragment complementation assays (PCAs) for interrogating protein–protein interactions [1]. In PCAs, the reporter protein (e.g. ubiquitin, β-galactosidase, dihydrofolate reductase (DHFR), β-lactamase, luciferase, or GFP) is split such that it can no longer assemble into a functional protein at the concentration the assay is performed. The fragments are fused to two proteins of interest. If the two proteins associate, this brings the two reporter fragments into proximity and allows them to adopt their active three-dimensional structure. The primary goal of PCAs is to detect protein–protein interactions and not to control the reporter's function, but the strategy can be adapted for creating switchable enzymes. If the two proteins require a third molecule for association, then the enzyme activity can be controlled by the concentration of the third molecule.

The idea is best exemplified by the rapamycin-induced interaction between FRB and FKBP [2] — a prototypical interaction often used to demonstrate PCAs. The heterodimeric proteins in such PCAs are rapamycin-activated enzymes. Recently, other signals for dimerization have

Figure 1



Strategies for designing switchable enzymes in which switch properties are predictable based on the properties of the biological components from which they were assembled. Strategies are organized by the manipulations performed (left-hand side vertical text) and the switching mechanism (right-hand side underlined text). DNA sequences are depicted as lines and their corresponding proteins as geometric shapes. The enzyme domain is depicted in red. Gray shapes indicate that the domain is inactive or less active. The signal that modulates the switch is depicted as a black triangle. For each mechanism, signals that have been demonstrated are listed. Note that sequence overlap has not yet been demonstrated for enzymes but has been demonstrated in switches with ligand-affinity as the output [8,12•].

been exploited to create switchable enzymes. By fusing the enzymes fragments to proteins that bind to specific DNA or RNA sequences, these nucleotide sequences can serve as a template for the enzymes to assemble (Figure 1). Thus, a specific DNA sequence can control the activity of the enzyme and conversely the enzyme can report on the presence of the DNA sequence. In particular, split firefly luciferase (FLuc) has been shown to be very useful for the detection of DNA and RNA sequences and can be used to detect methylated DNA [3]. An interesting twist comes from the plant phytochrome family of chromo-proteins in which light controls dimerization. Light at 660 nm causes

the association of PhyB and PIF3 and 750 nm light causing their disassociation [4]. With PhyB and PIF3 each fused to one-half of a split intein, Tyszkiewicz and Muir [5] demonstrated how intein-mediated protein splicing could be activated by light, offering a convenient method for spatiotemporal control over the production of a protein product.

Light is also a convenient output for the detection of cellular processes; thus, switchable luciferases are desired for sensing applications. Split click beetle luciferase (CBLuc) has been exploited in an intramolecular complementation strategy using nuclear hormone receptors (NRs). The binding of the ligand to the ligand-binding domain (LBD) of the NR induces the association between the LBD and a specific peptide motif of the N-terminal domain (NTD). In the case of the androgen receptor (AR), the peptide sequence is FQNLF. Tao and colleagues have built AR-agonist-dependent CBLuc by inserting a fusion of the AR LBD and the FQNLF peptide between two halves of FLuc [6]. In the presence of antagonist, or in the absence of agonist, the FQNLF peptide and the AR LBD do not associate, leaving the two halves of CBLuc far apart. Agonist binding brings the two halves of CBLuc, reconstituting enzyme activity. Most recently, Tao and colleagues have developed a multicolor format for detecting agonists and antagonists of the estrogen receptor (ER) using separate but similar probes for each type of ligand [7]. The construct for detecting ER agonists is analogous to the AR sensor design, with the ER LBD fused to the ER NTD inserted into CBLuc. Agonist binding results in green bioluminescence through the assembly of the two halves of CB. In order to detect antagonists, the researchers made use of a red-shifted variant of CBLuc (CBLuc Red) into which the ER LBD fused to SH2 domain of Src was inserted. Antagonist binding to the ER LBD results in its phosphorylation, enabling the association of ER LBD and Src SH2 to bring the two halves of CBLuc Red together. The two probes were used in COS-7 cells to simultaneously evaluate agonistic and antagonistic activities of a ligand. The sensors required different insertion sites into CB for the different NR in order to exhibit the best signal-to-background ratios. Some constructs were found to be reversible, whereas others were irreversible.

One or the other

Long linkers are placed between domains when the domains are designed to function independently. Thus, it makes intuitive sense that one should bring the domains closer together to engineer coupling between two domains; they need to be close but not too close. Going a step further one can fuse the domains such that they share a feature — providing an intimate contact between the two domains and potentially a mechanism for allosteric signal propagation through the fusion site (Figure 1b). For example, Sallee *et al.* [8] designed ligand-binding switches by overlapping the functionally important sequences of a pair of proteins such that the fusion protein can bind to either of the two respective ligands but not to both simultaneously. The design of fusions of protein domains such that both domains cannot be folded simultaneously (mutually exclusive folding) is a related approach [9] (Figure 1c). For example, the GCN4 DNA-binding domain was inserted into barnase in a manner that was compatible with activity in the absence of DNA, but disruptive of enzyme activity when the GCN4 bound its target sequence [10]. Studies on another switch designed to function by mutually exclusive folding (ubiquitin inserted into barnase) indicate that linker length is critical to switch function [11]. Too long a linker causes uncoupling of the structure, whereas too short linkers prevent intramolecular folding of the barnase domain under any condition and favor dimers and higher order multimers — probably through three-dimensional domain swapping — and thus prevent any regulation of enzyme activity [11].

Another simple mechanism for establishing an intimate connection between the two domains is to have them share a continuous structural element [12•]. The C-terminal alpha helix of the naturally photoactive LOV2 domain from *Avena sativa* phototropin 1 and the N-terminal alpha helix of the *E. coli* Trp repressor are both integral to the structure of these domains. Thus, by constructing end-to-end-fusions (LOV2-Trp) with the goal of creating a continuous alpha helix that functions as an 'allosteric lever arm,' Strickland *et al.* [12•] were able to create a fusion (named LovTAP) that functioned as light-controlled DNA-binding protein. Evidence suggests that the photoactivation of LovTAP decreases the affinity of the LOV domain for the shared helix, shifting the conformational ensemble toward structures in which the helix is associated with the Trp domain, increasing its apparent DNA-binding affinity sixfold.

Not going in circles

Insertion of one domain into another serves to make two connections between the fusion proteins and to take sequences near each other in the primary sequence and place them far apart (Figure 1c). Another method that relocates blocks of primary sequence relative to each other is circular permutation [13] — the removal of a C-terminal segment of the protein and joining it to the N-terminus through a peptide linker designed to span the original N-terminus and C-terminus. Circular permutation can be used to identify sites that are tolerant to insertion of other domains and thus is useful for switch construction [14]. More importantly, circular permutation is a powerful tool for switch creation because it can be used to achieve any number of relative orientations of the two domains in order to optimize the connectivity and allostery between the two domains [15,16]. Such rearrangements can serve to bring the active site of the enzyme closer to the input domain. The simplistic expec-

tation that connections made to the enzyme near the active site may be more suitable for switch construction proved correct in a combinatorial search for fusion topologies between maltose-binding protein (MBP) and TEM1 β-lactamase (BLA). The protein with the largest change in enzyme activity upon ligand binding (600-fold) occurred in a fusion in which the circular permutated BLA domain was inserted via its Ω-loop into MBP — a loop containing a key active site amino acid [15]. Switchable β-lactamase activity has also been achieved by the insertion of cytochrome b_{562} into BLA to create *E. coli* cells whose ampicillin resistance increased up to 128-fold in the presence of heme [17].

Circular permutation was used to change the connectivity of FLuc to create sensors for proteases and cAMP [18•]. Structural studies of luciferase show the monomeric protein consists of two domains connected through a hinge-like region about which the domains rotate and close upon binding substrate [19,20]. Connecting the N-termini and C-termini through a peptide linker (and locating the new termini at original amino acids 233 and 234) constrained motion about the hinge, resulting in a compromised enzyme. Cleavage by a protease at its target site — purposely located within the peptide linker — restored enzyme activity resulting in luminescence activity increase up to 2600-fold [18•]. Such a switch is not reversible because the sensor gets permanently switched to a new state by the activity it is designed to detect. Replacing the peptide linker with the cAMP-binding domain B from protein kinase regulatory subunit type IIβ (RIIβB) — a domain whose conformation change upon cAMP-binding has been previously used to develop FRET-based sensors for cAMP — resulted in a protein whose luminescence activity increased in the presence of cAMP. The difficulty in predicting the relationships between conformational changes in RIIβB and the resulting regulation of luciferase activity was overcome by testing different circular permutations of luciferase with a variety of flexible linkers joining RIIβB to luciferase. The optimal construct was circularly permuted at residue 359 and contained a GSSGGSGGSGGG linker on the N-terminal side of RIIβB and no linker on the C-terminal side. This construct displayed a 70-fold increase in enzyme activity upon the addition of saturating amounts of cAMP [18•]. The amplification of signal afforded by an enzyme results in high signal relative to fluorescent proteins making luciferase an attractive biosensor for intracellular and live animal applications.

Co-opting existing mechanisms: modular design

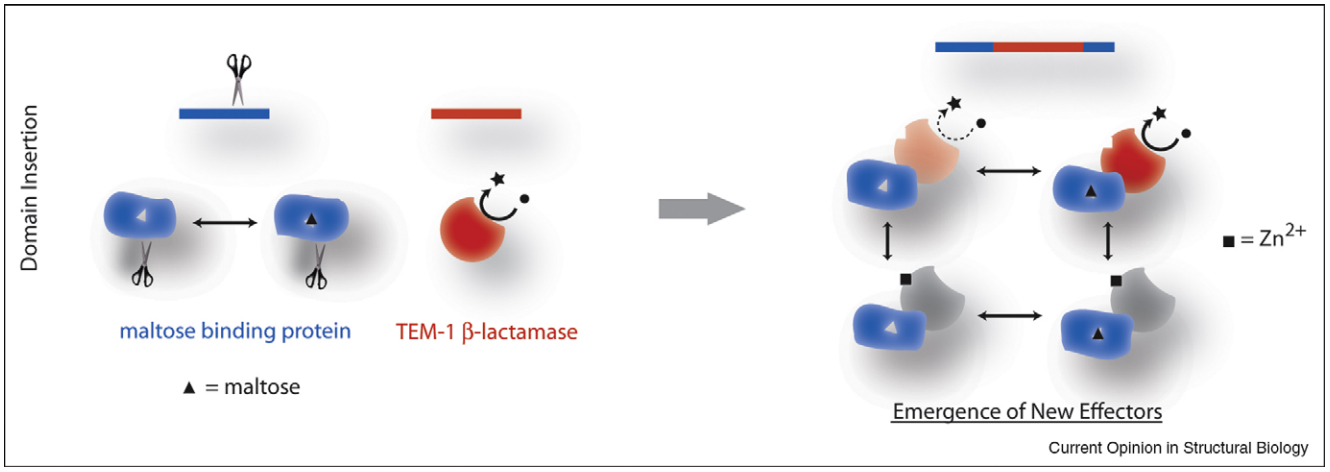
Most of the preceding examples make use of existing conformational changes in a protein but not necessarily the ones that allosterically regulate function in the natural protein. Alternatively, for enzymes that already have allosteric mechanisms in place for regulating function, a more straightforward approach to new regulatory functions is to adapt these natural mechanisms. Proteins with modular allosteric mechanisms have proven very receptive to this approach [21•,22,23••] (Figure 1d). The regulatory protein N-WASP activates the Arp2/3 complex, which stimulates its actin-nucleation activity [24]. This activation requires binding of Cdc42 and PIP2 to the regulatory domains of N-WASP, which serve to disrupt autoinhibitory interactions. By swapping the domains responsible for the autoinhibitory interaction with new interacting domain pairs that could be disrupted with new ligands, N-WASP activity could be switched on by new signals [22]. Most remarkably, by incorporating two pairs of interacting domains complex allosteric responses including AND-gating and OR-gating behavior could be established [22]. More recently the Lim lab has shown in this same system how multiple copies of the interacting pairs can be used to achieve cooperative behavior resulting in ultrasensitive switches that go from low to high activity over a very narrow range of activator concentration [21•]. This strategy of co-opting existing modular autoinhibitory allosteric mechanisms was successfully applied to the creation of new guanine nucleotide exchange factors (GEFs) that are activated by new ligands and thus can be used to reprogram cellular behavior that depends on signaling cascades involving these proteins [23••]. Remarkable modularity has also been shown using scaffold interactions to reshape MAP kinase pathway signaling dynamics using synthetic positive-feedback and negative-feedback loops [25].

Reprograming can also be used in engineered switches with newly established allosteric mechanisms. A maltose-activated β-lactamase was re-engineered to respond to sucrose by introducing mutations to the maltose-binding site that convert MBP into a 'sucrose-binding protein' [15] — showing that switches created by domain fusion have their own degree of modularity.

Computational prediction of fusion sites

Do allosteric connections between fused protein domains result from connections between existing allosteric networks in the individual protein domains? The idea makes intuitive sense — it should be easier to make use of existing functional relationships than to create new ones. Statistical coupling analysis (SCA) quantitatively examines protein families and identifies the connected networks of coevolving amino acids that are proposed to exist because of conserved communication between the identified regions [26]. SCA might thus prove to be a useful design tool for identifying surface sites in a domain that are allosterically linked to the domain's function — sites that might serve as optimal fusion locations for establishing functional connections between two domains. Such an analysis was carried out on LOV2 and DHFR [27] — proteins that have been previously

Figure 2



Schematic illustrating that domain insertion can result in switch properties that are not predictable based on the properties of the biological components from which they are assembled. Insertion of a circular permuted TEM1 β-lactamase into maltose-binding protein resulted in a maltose-activated and Zn^{2+}-inactivated allosteric enzyme [30•].

shown to be amenable to switch construction by domain fusion with other proteins but not with each other [12•,28,29]. To test whether SCA could predict sites for successful switch construction between LOV2 and DHFR, two sets of fusions were created in which LOV2 was inserted into DHFR at two different surface loops — one predicted to establish coupling, the other predicted not to. Within in each set, the site of insertion was varied across the loop. All four insertions in the loop predicted to be unsuccessful showed light-independent DHFR activity comparable to wildtype DHFR. All three constructs inserted in the loop predicted to be successful had a large decrease in DHFR activity; but, one of the constructs showing a small increase in catalytic activity in the presence of light (1.6-fold increase in the hydride transfer rate and a 1.3-fold increase in the product release rate). The results are consistent with the idea that allosteric effects between domains can be achieved through connections via surface residues that are part of allosteric networks in the original domains. However, the idea needs to be examined with a larger data set such as that arising from combinatorial approaches to switch building [15,16], which offer a large number of successful and unsuccessful constructs found through an unbiased search for suitable fusion topologies.

Emergence of allostery

Domain insertion has been used to create new allosteric functions that are not predictable based on the properties of the individual proteins — a finding that has implications for how allosteric effects emerge and evolve [30•]. MBP does not bind Zn^{2+} and BLA neither binds Zn^{2+} nor is regulated by Zn^{2+}. Nevertheless, a maltose-activated β-lactamase (RG13) created by insertion of a circular permuted BLA into MBP has Zn^{2+} as a negative allosteric effector with K_d for Zn^{2+} of about 2 μM [30•] (Figure 2). Inhibition does not result from Zn^{2+}-induced precipitation of the protein, as RG13 remains folded and soluble at concentrations of Zn^{2+} that are well above the K_i and at concentrations of Zn^{2+} that precipitate BLA. The results suggest that effector-affinity and allostery may often emerge simultaneously rather than sequentially [30•] and provide an example of how seemingly improbable evolutionary events might instead be quite probable [31]. The origin of the Zn^{2+}-binding site in RG13 and the mechanism by which allostery was achieved should prove very interesting in this context.

Future directions

A common thread through many successful examples of switch construction is the necessity to try many fusions — making small variation in fusion sites and/or linker length/composition using combinatorial or trial-and-error methods. Simplistic mechanisms and an abstract view of proteins can serve us well, but only to point us to some of the interesting regions of sequence space to explore for switch construction. The fitness landscape in these regions is very rugged in terms of switch activity, and choosing exactly the right construct is still a combination of art and guesswork. This raises a number of questions. Are there other areas that we are not looking in that would be fruitful? How can we identify the best regions to explore? How can we correctly predict the exact fusions that will be switches (i.e. get the exact fusion site and linker right)? What proteins make good switch components? Here, rich data sets that can be provided by combinatorial experimental approaches

should be combined with computationally modeling to make significant progress.

A modular switch design would make the design of switches inherently easier. Toward this end, the adoption of a universal input domain would prove advantageous, should one exists. Such a universal input domain would have the ability to be readily engineered to bind whatever signal is desired for the switch, yet still retain the ability to regulate the output domain. Antibodies and antibody mimics come to mind as potential universal input domains, but switches built with these types of proteins have yet to be described. It remains to be seen whether the relative lack of conformational changes that these protein domains undergo preclude them from being useful switch components.

Acknowledgements

I thank Chapman W Wright and Richard A Heins for their valuable comments and discussions. I gratefully acknowledge the support from NIH #2RO1 GM066972 and NSF #CBET-0828724.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Michnick SW, Ear PH, Manderson EN, Remy I, Stefan E: **Universal strategies in research and drug discovery based on protein-fragment complementation assays**. *Nat Rev Drug Discov* 2007, **6**:569-582.

2. Chen J, Zheng XF, Brown EJ, Schreiber SL: **Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue**. *Proc Natl Acad Sci U S A* 1995, **92**:4947-4951.

3. Porter JR, Stains CI, Jester BW, Ghosh I: **A general and rapid cell-free approach for the interrogation of protein–protein, protein–DNA, and protein–RNA interactions and their antagonists utilizing split-protein reporters**. *J Am Chem Soc* 2008, **130**:6488-6497.

4. Quail PH: **Phytochrome photosensory signalling networks**. *Nat Rev Mol Cell Biol* 2002, **3**:85-93.

5. Tyszkiewicz AB, Muir TW: **Activation of protein splicing with light in yeast**. *Nat Methods* 2008, **5**:303-305.

6. Kim SB, Otani Y, Umezawa Y, Tao H: **Bioluminescent indicator for determining protein–protein interactions using intramolecular complementation of split click beetle luciferase**. *Anal Chem* 2007, **79**:4820-4826.

7. Kim SB, Umezawa Y, Kanno KA, Tao H: **An integrated-molecule-format multicolor probe for monitoring multiple activities of a bioactive small molecule**. *ACS Chem Biol* 2008, **3**:359-372.

8. Sallee NA, Yeh BJ, Lim WA: **Engineering modular protein interaction switches by sequence overlap**. *J Am Chem Soc* 2007, **129**:4606-4611.

9. Radley TL, Markowska AI, Bettinger BT, Ha JH, Loh SN: **Allosteric switching by mutually exclusive folding of protein domains**. *J Mol Biol* 2003, **332**:529-536.

10. Ha JH, Butler JS, Mitrea DM, Loh SN: **Modular enzyme design: regulation by mutually exclusive protein folding**. *J Mol Biol* 2006, **357**:1058-1062.

11. Cutler TA, Mills BM, Lubin DJ, Chong LT, Loh SN: **Effect of interdomain linker length on an antagonistic folding–unfolding equilibrium between two protein domains**. *J Mol Biol* 2009, **386**:854-868.

12. • Strickland D, Moffat K, Sosnick TR: **Light-activated DNA binding in a designed allosteric protein**. *Proc Natl Acad Sci U S A* 2008, **105**:10709-10714.
The authors uses end-to-end fusion to make a continuous a-helix that is designed to function like a 'lever arm' to couple light-activation in one domain to DNA affinity in the other.

13. Meister GE, Kanwar M, Ostermeier M: **Circular permutation of proteins**. In *Protein Engineering Handbook*. Edited by Lutz S, Bornscheuer U. Wiley; 2009.

14. Baird GS, Zacharias DA, Tsien RY: **Circular permutation and receptor insertion within green fluorescent proteins**. *Proc Natl Acad Sci U S A* 1999, **96**:11241-11246.

15. Guntas G, Mansell TJ, Kim JR, Ostermeier M: **Directed evolution of protein switches and their application to the creation of ligand-binding proteins**. *Proc Natl Acad Sci U S A* 2005, **102**:11224-11229.

16. Guntas G, Mitchell SF, Ostermeier M: **A molecular switch created by in vitro recombination of nonhomologous genes**. *Chem Biol* 2004, **11**:1483-1487.

17. Edwards WR, Busse K, Allemann RK, Jones DD: **Linking the functions of unrelated proteins using a novel directed evolution domain insertion method**. *Nucleic Acids Res* 2008, **36**:e78.

18. • Fan F, Binkowski BF, Butler BL, Stecha PF, Lewis MK, Wood KV: **Novel genetically encoded biosensors using firefly luciferase**. *ACS Chem Biol* 2008, **3**:346-351.
Circular permutation, domain insertion, and linker-length optimization lead to a variety of luminescent biosensors for protease activity and cAMP.

19. Conti E, Franks NP, Brick P: **Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes**. *Structure* 1996, **4**:287-298.

20. Nakatsu T, Ichiyama S, Hiratake J, Saldanha A, Kobashi N, Sakata K, Kato H: **Structural basis for the spectral difference in luciferase bioluminescence**. *Nature* 2006, **440**:372-376.

21. • Dueber JE, Mirsky EA, Lim WA: **Engineering synthetic signaling proteins with ultrasensitive input/output control**. *Nat Biotechnol* 2007, **25**:660-662.
The authors demonstrate how multiple ligand-binding sites can lead to cooperativity in modular switch design.

22. Dueber JE, Yeh BJ, Chak K, Lim WA: **Reprogramming control of an allosteric signaling switch through modular recombination**. *Science* 2003, **301**:1904-1908.

23. •• Yeh BJ, Rutigliano RJ, Deb A, Bar-Sagi D, Lim WA: **Rewiring cellular morphology pathways with synthetic guanine nucleotide exchange factors**. *Nature* 2007, **447**:596-600.
Natural switches are reprogrammed using modular design principles to establish new cellular pathways: linking cytoskeletal responses to normally unrelated upstream signaling pathways and creating artificial cascades with improved signal processing behavior.

24. Mullins RD, Heuser JA, Pollard TD: **The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments**. *Proc Natl Acad Sci U S A* 1998, **95**:6181-6186.

25. Bashor CJ, Helman NC, Yan S, Lim WA: **Using engineered scaffold interactions to reshape MAP kinase pathway signaling dynamics**. *Science* 2008, **319**:1539-1543.

26. Suel GM, Lockless SW, Wall MA, Ranganathan R: **Evolutionarily conserved networks of residues mediate allosteric communication in proteins**. *Nat Struct Biol* 2003, **10**:59-69.

27. Lee J, Natarajan M, Nashine VC, Socolich M, Vo T, Russ WP, Benkovic SJ, Ranganathan R: **Surface sites for engineering allosteric control in proteins**. *Science* 2008, **322**:438-442.

28. Collinet B, Herve M, Pecorari F, Minard P, Eder O, Desmadril M: **Functionally accepted insertions of proteins within protein domains**. *J Biol Chem* 2000, **275**:17428-17433.

29. Tucker CL, Fields S: **A yeast sensor of ligand binding**. *Nat Biotechnol* 2001, **19**:1042-1046.

30. Liang J, Kim JR, Boock JT, Mansell TJ, Ostermeier M: **Ligand**
• **binding and allostery can emerge simultaneously**. *Protein Sci* 2007, **16**:929-937.
A hybrid protein between maltose-binding protein and TEM1 β-lactamase is shown to possess high affinity for zinc, which allosterically turns off enzyme activity. Since the parental proteins lack both zinc affinity and zinc-dependent allostery, this is an example of ligand-binding and allostery emerging simultaneously.

31. Kuriyan J, Eisenberg D: **The origin of protein interactions and allostery in colocalization**. *Nature* 2007, **450**:983-990.