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As easy as flipping a switch?

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Proteins that behave as switches help to establish the complex molecular logic that is central to biological systems. Aspiring to be nature's equal, researchers have successfully created protein switches of their own design; in particular, numerous and varied zinc-triggered switches have been made. Recent studies in which such switches have been readily identified from combinatorial protein libraries support the notion that proteins are primed to show allosteric behavior and that newly created ligand-binding sites will often be functionally coupled to the original activity of the protein. If true, this notion suggests that switch engineering might be more tractable than previously thought, boding well for the basic science, sensing and biomedical applications for which protein switches hold much promise.

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Introduction

Proteins with a function that can be modulated by external signals (e.g. ions, small molecules and other proteins) are often described as 'switches'. Many switches function through allosteric effects, whereby signal recognition occurs at a site distal to the functional site of the protein and is transmitted by a conformational change. Allosteric effects are termed 'heterotropic' when the signaling molecule is distinct from the substrate or ligand of the protein. Such allosteric effects are central to how complex functions are manifested in biological systems.

Accordingly, protein engineers have been motivated to build switches of their own design to test their understanding of natural switches, to test hypotheses concerning the molecular basis of protein form and function, to develop tools for elucidating cellular function and behavior, and to create switches for sensing and biomedical applications. The challenges in switch design are that of any protein-engineering endeavor — that is, our understanding of proteins is incomplete and the potential design space is astronomically large — but have the added complication that the design must incorporate two states that are interconvertible in a signal-dependent manner.

In this review, we take the position that protein switch design — although obviously not as easy as 'flipping a switch' — might be inherently simpler than first imagined. We illustrate this idea by focusing on the breadth of approaches that have created protein switches for one particular ligand: namely, divalent zinc (Table 1). Zn^{2+} has been a popular ligand to base switches around, perhaps because the binding geometry of this metal ligand is relatively well understood. By focusing on Zn^{2+} switches, we cover only a small portion of the growing literature on protein switches. The reader is directed to other recent reviews covering protein switches derived from natural allosteric proteins [1], switches designed for analytical molecular sensing [2], peptides and proteins designed to adopt multiple, specific conformations [3], and switches created by domain insertion [4]. Zinc biosensors created by adapting Zn^{2+}-binding proteins and peptides with fluorescent groups are also not comprehensively covered, because this topic has been recently reviewed in this journal [5].

Zn^{2+}-triggered switching between distinct protein folds

Conformational changes that take place on effector binding are a hallmark of protein switches and allosteric effects. The ability to design substantial conformational changes in proteins would be invaluable to the construction of switches. The most marked conformational change is that between the unfolded and folded state of a protein. The presence of Zn^{2+} triggers this conformational change in proteins that require Zn^{2+} for stability, and this phenomenon can be exploited for sensing applications by the judicious incorporation of an environmentally sensitive fluorophore [6,7]. In general, however, natural allosteric proteins function by an effector-induced conformational change between distinct folded states, which — although more difficult to design — is more practical, particularly for *in vivo* applications. As a first step in this direction, peptides that can adopt two substantially different conformations depending on the presence of Zn^{2+} have been designed [8,9•,10].

One approach is to superimpose the consensus sequences for two different folds, one of which requires Zn^{2+}. Hori and Sugiura [8], for example, searched for sequences of proteins of known structure that could be converted to zinc-fingers with a minimal amount of mutations. They

Table 1

Engineered Zn^{2+}-dependent switches

Method of creating switch	Function modulated	K_d for Zn^{2+}	Modulation extent[a]	Refs
Merge sequences between homeodomain and zinc-finger	Binding to DNA	13 nM	10-fold (–)	[8]
Merge sequences between consensus sequences of coiled-coil and zinc-finger	Protein conformation	3–5 μM	Not quantified	[10]
Computationally design to switch from trimeric coiled-coil to zinc-finger	Protein conformation	0.44 nM	Not quantified	[9•]
Computationally redesign β_2-adregenic receptor to respond to Zn^{2+}	Cellular cAMP levels	21 μM (EC_{50})	4-fold (+)	[12]
Computationally redesign ligand-binding site in fluorophore-labeled periplasmic binding proteins	Fluorescence	1–5 μM	2-fold (+) (RBP)	[14,15]
Incorporate Zn^{2+}-binding site by randomizing surface loops of BLA, selecting for binders, and screening for allosteric effects	BLA activity	1–90 μM	1.2-fold (+) to 28-fold (–)	[17,18••]
Serendipitously discovered in an engineered maltose-activated BLA	BLA activity	2 μM	>100-fold (–)	[19•]

[a] Ratio of the magnitude of the modulated property: '+' or '–' indicates with or without Zn^{2+}, respectively. Abbreviations: BLA, TEM-1 β-lactamase; EC_{50}, half-maximal excitatory concentration; K_d, dissociation constant; RBP, ribose-binding protein.

found that making four mutations to the Antennapedia homeodomain created a protein that could switch from the normal α-helical fold to a zinc-finger (mixed α/β) fold on the addition of Zn^{2+} ions.

Likewise, ZiCo is a peptide designed by a similar approach that reversibly changes its folded conformation from a trimeric coiled-coil to a monomeric helix–loop–helix in the presence of micromolar concentrations of Zn^{2+} [10]. The peptide was designed by inspecting the sequences of coiled-coil and zinc-finger motifs and manually merging the residues in the proper patterning necessary to produce the desired folds. Analysis by circular dichroism and Fourier transform infrared spectroscopy indicated that ZiCo changes conformation on the addition of Zn^{2+}, increasing in α-helix and β-sheet content.

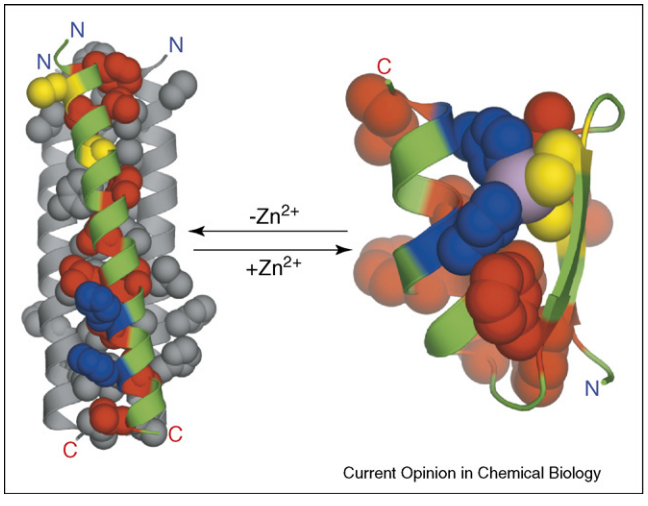
The approach of Ambroggio and Kuhlman [9•] differed in the use of a computational algorithm to optimize a sequence of amino acids to fold into two distinct structures depending on the presence of Zn^{2+} ions. Their target structures were a coiled-coil and a zinc-finger (Figure 1). This algorithm, which makes use of the RosettaDesign program (http://www.doe-mbi.ucla.edu/People/Software/rosetta_design.html), differs from traditional energy minimization algorithms in that it optimizes the amino acid sequence so that two target structures are favorable. The resulting protein, Sw2, was characterized in the absence and presence of Zn^{2+} and was found to switch from a tri- or tetrameric coiled-coil to a structure similar to a natural zinc-finger in the presence of Zn^{2+} ions, as designed.

Interestingly, other than two conserved residues in the zinc-finger motifs, there is no significant sequence similarity between Sw2 and ZiCo. In comparison to the hybrid sequence approach, the computational method of Ambroggio and Kuhlman [9•] holds more promise for the engineering of switches of arbitrary function, because it neither relies on existing proteins nor uses methods dependent on the design goal. The authors conclude by suggesting that improvements in the algorithm could lead to better stability of one target structure over the other.

Re-engineering natural switches to respond to Zn^{2+}

Zn^{2+}-dependent switches can be created by re-engineering natural switches to respond to Zn^{2+}. This approach is

Figure 1



Structural model of the Sw2 conformational switch. Sw2 was computationally designed to convert from a coiled-coil (left) to a zinc-finger (right) fold in the presence of Zn^{2+}. Hydrophobic residues are shown in red, cysteine ligands in yellow and histidine ligands in blue; the Zn^{2+} ion is represented as an enlarged sphere in violet. For clarity, only one of the three helices is colored in the coiled-coil model. Reproduced, with permission, from [9•].

accomplished by introducing mutations designed to bind Zn^{2+} into one of the two natural states of the allosteric protein. For example, the seven-transmembrane β_2-adrenergic receptor interacts with several different ligands and, as a result, its membrane-spanning helices move relative to each other, resulting in an increase in the production of intracellular cAMP. Elling et al. [11] have created mutant forms of the β_2-adrenergic receptor in which histidine and cysteine residues are substituted at selected sites on adjacent transmembrane (TM) helices — either TM-III and TM-VII, or TM-VI and TM-VII [12] — at the binding site for the partial agonist pindolol. These proteins transformed the cells into ones that no longer responded to pindolol but instead increase cAMP production in the presence of Cu^{2+} or Zn^{2+} ions. The introduction of allosteric Zn^{2+}-binding sites can also be used to investigate biological systems. For example, engineering Zn^{2+}-binding sites into TM-I and TM-III of the human seratonin transporter results in a decrease in seratonin transport in the presence of Zn^{2+} ions [13]. This finding supports a homology model of the structure of this protein in which these helices are proximal.

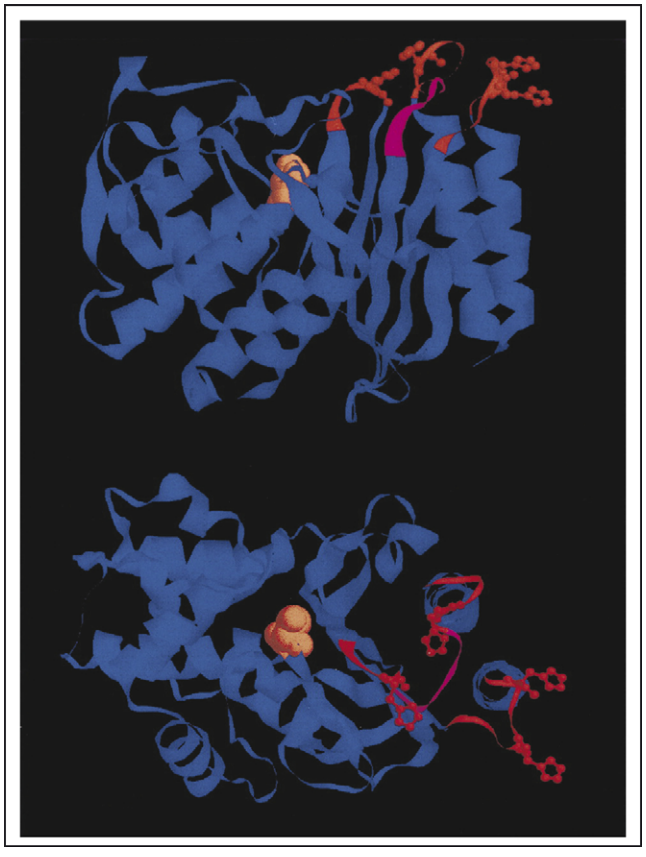
Periplasmic binding proteins such as maltose binding protein (MBP) and ribose-binding protein have an open conformation in the absence of ligand and a closed conformation in the presence of bound ligand. This conformational change is characterized by a bending motion about the hinge region between the two discontinuous domains of the protein. Hellinga and co-workers have computationally designed MBP [14] and ribose-binding protein [15] to bind Zn^{2+} in their closed state. The chemical attachment of fluorescent groups in the hinge region of these proteins results in fluorescent biosensors for Zn^{2+}. Unexpectedly, the MBP Zn^{2+} sensor (MBP-A*) binds Zn^{2+} in the unintended open conformation [16]. Despite lacking the large hinge-bending conformational change typical of MBP, MBP-A* still functions as a fluorescent sensor for Zn^{2+} [14]. This observation suggests (i) that simply creating a binding site for a new ligand can result in that ligand functioning as an effector, and (ii) that large conformational changes are not necessarily a requirement in the design of effective switches.

Engineering both allostery and effector binding

The recent work of Mathonet *et al.* [17,18••] directly supports the hypothesis that newly created binding sites have a high probability of allosterically affecting the original function of a protein. These researchers inserted random peptides into three contiguous surface loops of the TEM-1 β-lactamase (BLA) that were distal from the active site. From this phage-displayed library, they isolated transition metal ion-binding variants by panning on a metal ion affinity chromatography support. Of 30 selected library members that conferred resistance to ampicillin, one-third were found to show metal-ion-specific allosteric effects on β-lactamase catalytic activity [18••]. The activities of these β-lactamase mutants are both positively (β-lactam hydrolysis increases up to threefold) and negatively (hydrolysis activity decreases more than 20-fold) affected by metal binding. The startling allosteric complexity that can arise simply from creating ligand-binding sites is exemplified by one particular mutant, A5, that is activated by Ni^{2+}, nearly unaffected by Zn^{2+}, and inhibited by Cu^{2+} (Figure 2). This work suggests that the generation of allosteric proteins might be a straightforward process involving the creation of new ligand-binding sites in proteins with a function to be regulated (without necessarily explicitly designing for allosteric effects), followed by limited screening for ligand binders that also are allosterically regulated.

A Zn^{2+}-modulated BLA has also been created serendipitously during the engineering of a maltose-modulated BLA [19•]. RG13 is an engineered allosteric β-lactamase

Figure 2



Model of the A5 mutant of TEM-1 β-lactamase (BLA). A5 was engineered to bind metals by replacing three surface loops, resulting in metal-ion-dependent BLA enzyme activity. The engineered loops are shown in red with the histidine residue side chains (which presumably interact with the metal) in ball-and-stick notation. The nucleophilic serine of the active site is shown in space-filling notation. Two orthogonal views are shown. Reproduced, with permission, from [18••].

for which maltose is a positive effector [20]. It is a hybrid protein of BLA and MBP that was identified through the selection and screening of a combinatorial protein library in which random circular permutations of the gene encoding BLA were randomly inserted into the gene encoding MBP. The β-lactamase activity of RG13 is compromised in the absence of maltose and increases 25-fold in its presence. Unexpectedly, RG13 has an affinity of $\sim 10^6\ M^{-1}$ for Zn^{2+} — a property that neither MBP nor BLA possesses [19•]. Furthermore, Zn^{2+} is a negative effector that noncompetitively switches off β-lactam hydrolysis activity independent of maltose and without large conformational changes (as measured by circular dichroism). These results support the notion that Zn^{2+}-binding sites in particular might be relatively easy to evolve, as previously proposed [21] and observed in studies of random peptide libraries selected for ATP binders [22]. In addition, these results further support the hypothesis that the introduction of new ligand-binding sites will often result in allosteric effects involving that ligand. Changes that result in the ability to bind a new ligand might predispose the mutant protein to show allosteric behavior involving that site, making the co-emergence of ligand binding and allostery a viable path in the evolution of heterotropic allosteric effects [19•].

Conclusions

The number of successes and the variety of approaches used to create Zn^{2+}-modulated switches illustrate the inherent ability and untapped potential of proteins to show allosteric effects. This observation is in accord with the growing appreciation that all proteins are allosteric [23,24•], especially where heterotropic allostery is concerned. The incredible complexity of proteins (i.e. their large, intricate binding surface) and the marginal stability of the native state make it easy to imagine that establishing that a protein is allosteric is simply a matter of finding that protein's effector. It is partly for this reason that interest in heterotropic allosteric effectors as drug molecules has increased in recent years [25,26•].

The remarkable frequency at which Zn^{2+}-binding sites created in BLA have been allosterically linked with BLA catalytic activity [18••,19•] is also not so surprising when one considers the many studies describing proteins that have been evolved through random mutagenesis. A common occurrence is that mutations distal to the active site of the protein emerge as mutations that improve activity. What is rarely addressed specifically — but is widely appreciated — is that many more mutations distal from the active site result in a reduction of activity. One can view the effects of these positive and negative mutations as 'mutational allostery' — that is, they are chemical modifications at sites distal to the active site that are functionally coupled to the active site. The result of making this chemical modification is modulation of the activity, analogous to the modulation of an active site through the chemical modification of ligand–protein binding. An even more relevant analogy is the modulation of function through protein phosphorylation. Of course, mutational allostery is not practical because the signal does not operate on the protein level, it must be made through modification of the genes. But the preponderance of mutations that can be conceptually described as showing mutational allostery, together with the growing number of examples of engineered protein switches, brings hope that the regulation of protein activity by a ligand of our choosing might not be substantially more difficult than the creation of a binding site for that ligand.

Acknowledgements

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