Original Article

Modular protein switches derived from antibody mimetic proteins

N. Nicholes, A. Date, P. Beaujean, P. Hauk, M. Kanwar, and M. Ostermeier*

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

*To whom correspondence should be addressed. E-mail: oster@jhu.edu

Edited by Shohei Koide

Received 21 July 2015; Revised 28 October 2015; Accepted 4 November 2015

Abstract

Protein switches have potential applications as biosensors and selective protein therapeutics. Protein switches built by fusion of proteins with the prerequisite input and output functions are currently developed using an *ad hoc* process. A modular switch platform in which existing switches could be readily adapted to respond to any ligand would be advantageous. We investigated the feasibility of a modular protein switch platform based on fusions of the enzyme TEM-1 β -lactamase (BLA) with two different antibody mimetic proteins: designed ankyrin repeat proteins (DARPs) and monobodies. We created libraries of random insertions of the gene encoding BLA into genes encoding a DARPin or a monobody designed to bind maltose-binding protein (MBP). From these libraries, we used a genetic selection system for β -lactamase activity to identify genes that conferred MBP-dependent ampicillin resistance to *Escherichia coli*. Some of these selected genes encoded switch proteins whose enzymatic activity increased up to 14-fold in the presence of MBP. We next introduced mutations into the antibody mimetic domain of these switches that were known to cause binding to different ligands. To different degrees, introduction of the mutations resulted in switches with the desired specificity, illustrating the potential modularity of these platforms.

Key words: antibody mimetic proteins, DARPs, monobodies, protein switches

Introduction

Protein switches can be built by the fusion of protein domains in a manner that couples the domains' activities. Ligand-activated protein switches are protein fusions in which the switch activity is controlled by the presence of a specific ligand. Ligand-activated protein switches typically consist of an input domain that binds a particular ligand and an output domain whose function is modulated in response to a binding event in the input domain. Such ligand-activated protein switches have many potential applications as biosensors and therapeutics (Stein and Alexandrov, 2015).

We have previously created protein switches by inserting β -lactamase (BLA) into maltose-binding protein (MBP) from *Escherichia coli*. Successful fusions resulted in BLA activity that was a function of the concentration of maltose (Guntas *et al.*, 2004). The degree to which a fusion behaved as a switch was a function

not only of the insertion site in MBP but also of the relative orientation of the two protein domains, which was altered through circular permutation of the BLA domain. BLA was circularly permuted by fusing its normal N- and C-termini with a DKS linker and opening up the protein at different points to create new N- and C-termini. BLA circularly permuted at residue 170 (BLA170) produced the largest maltose-dependent difference in BLA activity when properly fused with MBP (Guntas *et al.*, 2005). These switches served as a proof of principle of the possibility of coupling the enzymatic activity of one protein to the presence of a specific small molecule by directly combining two proteins through domain insertion. MBP presumably proved very useful for creating switches because it undergoes a large conformational change upon binding maltose. Since our initial studies with MBP and BLA, our lab has produced several other examples of ligand-activated protein switches using this strategy (Tullman *et al.*, 2011; Wright *et al.*,

2011; Choi *et al.*, 2015); however, all these switches were built from input domains that undergo significant conformational changes upon ligand-binding. Whether analogous switches can be created via domain insertion using input domains that bind ligands with insubstantial conformational change is an open question.

Switches with such domains have been successfully designed based on the ‘mutually exclusive folding’ approach (Radley *et al.*, 2003), a different strategy in which only one of the two domains can be folded simultaneously. Ligand binding drives the folding of the ligand-binding domain and the concomitant unfolding of the enzyme domain. However, there are two main drawbacks to this approach. First, it is a ‘signal-off’ system since the presence of ligand turns off the enzyme. Secondly, the switching mechanism requires one domain to remain unfolded, which might have negative consequences in a cellular environment (i.e. the fusions might be prone to degradation or aggregation). Here, we investigate whether ‘signal-on’ switches can be built from ligand-binding domains that possess little or no conformational change upon binding ligand.

We have demonstrated that the switches developed in our lab function by one of two non-mutually exclusive mechanisms. In the allosteric mechanism, effector-binding causes a change in the conformation of the input domain that impacts the conformation and activity of the output domain in a manner completely analogous to natural allosteric proteins (Guntas *et al.*, 2004; Kim and Ostermeier, 2006; Wright *et al.*, 2010). In the protein abundance mechanism, effector-binding increases the cellular stability of the switch (i.e. increases the thermodynamic or proteolytic stability) causing the switch to accumulate at higher levels in the presence of the effector compared with its absence (Heins *et al.*, 2011; Choi *et al.*, 2013). Our recent structural and thermodynamic studies of particular fusions of MBP and BLA indicate that switching by either mechanism can emerge from fusions in which effector-binding alters the conformational entropy of the output domain (Choi *et al.*, 2015). This result suggests that switches may be created using input domains that lack significant conformational changes upon ligand-binding—a hypothesis we test in the study presented here.

Protein switch development through library creation, selection and screening is time and labor intensive. Once the properties of a desired switch are defined, an appropriate input domain must be identified that can recognize the desired effector with high affinity and specificity. This input domain must then be fused with the desired output domain in a manner that results in ligand-dependent activation. Owing to the difficulty in predicting such fusion topologies, we create combinatorial libraries of gene fusions that consist of one domain inserted into the other, often with variable linker sequences and variable circular permutation of the insert protein. An efficient selection or screening methodology must be developed to successfully identify those rare fusions that behave as switches. Following the identification of promising switch candidates, these protein fusions must be characterized and subjected to additional rounds of mutation to optimize their activity and stability. The development of a second switch that is activated by a different effector requires identification of an appropriate input domain. The library creation and selection process is then repeated in full. This library creation and selection process must even be used to create new switches with the same output domain already present in developed switches unless the second effector is very similar to the first (Guntas *et al.*, 2005).

A modular switch platform in which a single switch fusion topology could be easily adapted to respond specifically to any ligand could greatly accelerate switch development. Such a modular platform would require (i) an input protein domain capable of being engineered

to recognize a variety of different ligands, and (ii) a fusion topology between this input domain and a desired output domain that robustly results in switching activity regardless of changes in the input domain implemented for recognition of new ligands. In such a platform, once a switch is created for a ligand, a second switch that is activated by a different ligand could be created by the introduction of mutations known to cause the input domain to bind to the new ligand (Fig. 1). Such mutations could be identified outside the context of a switch by traditional methods for creating ligand-binding proteins such as directed evolution (e.g. phage display and yeast surface display) and rational design of the input domain. Such a switching platform would decrease the time required to develop protein switches, provided that the switching property and ligand affinity remain when the binding interface is changed to recognize the new ligand.

In an attempt to develop a modular switch platform, we demonstrate here that switches can be created using monobodies (Koide *et al.*, 1998) and designed ankyrin repeat proteins (DARPin) (Binz *et al.*, 2003; Forrer *et al.*, 2003) as input domains. Monobodies and DARPins undergo relatively minimal conformation changes upon binding their respective ligands. We also demonstrate that these switches, particularly those built from DARPins, show promise as a modular switch platform.

Materials and methods

Monobody YS1 library creation and selections

The gene encoding the MBP-binding monobody YS1 was obtained from Dr Koide’s group. A library of insertions of BLA into the monobody was created by designing abutting primers to amplify and linearize the plasmid according to the inverse PCR method described in Kanwar *et al.* (2013). These primers were used in different combinations to either produce perfect insertions (i.e. no addition or deletion of amino acids) or additions or deletions of up to three amino acids. The linearized plasmid DNA from each of these 672 PCR reactions was combined into a single tube, concentrated and purified using the Zymo Clean and Concentrate 5 kit (Zymo Research, Irvine, CA, USA). BLA170 was PCR amplified using Phusion High Fidelity polymerase (New England Biolabs, Ipswich, MA, USA). The BLA170 PCR product was also purified using the Zymo Clean and Concentrate 5 kit. The DNA containing the linearized plasmid was recircularized by ligation with the BLA170 insert using T4 DNA ligase (New England Biolabs) and then used to transform *E.coli* SNO301D (Sohka *et al.*, 2009) cells along with the pTS2-pBAD-MBP plasmid. The naïve library was first plated on LB agar plates containing 0.5 µg/ml Amp, 20 µg/ml Tet, 50 µg/ml Cm, 50 µg/ml Kan. This plate was swept and dilutions from this negative selection were plated on LB agar plates containing 0.05% arabinose and an ampicillin gradient from 0 to 256 µg/ml. Colonies were observed on the plates up to 32 µg/ml amp. These colonies were picked and grown in a 96-well plate. The colonies identified showed a 4- to 16-fold difference in their MIC on ampicillin in the presence and absence of arabinose-induced MBP co-expression.

DARPin off7 library creation and selections

Since the DARPin consists of a consensus repeat sequence with a few variable positions, we anticipated that it would be difficult to use the inverse PCR library creation technique to reliably generate insertions in the highly repetitive DNA that encodes this protein. Therefore, the DNA encoding the MBP-binding DARPin off7 (Binz *et al.*, 2004) was designed by our group using synonymous mutations to minimize the

Fig. 1 A modular protein switch platform with an antibody mimetic protein serving as the input domain. Once an initial switch is developed using directed evolution (here illustrated as a switch activated by MBP), mutations known to cause the antibody mimetic protein to bind other ligands are introduced to change switch specificity. These mutations are identified by traditional directed evolution or protein design methods for creating specific binding proteins.

repetitiveness of the DNA sequence. This enabled the use of the inverse PCR technique for generation of the library of BLA insertions into off7 (Kanwar *et al.*, 2013). Primers were designed to amplify and linearize the pRH-DsbA-off7 plasmid between every codon in the gene and before the STOP codon, to create an end-to-end fusion of the two proteins. The BLA and BLA170 domains were PCR amplified with primers that were designed to add 0–3 NNK random codons to both ends of the BLA domain using Phusion High Fidelity Polymerase (New England Biolabs, Ipswich, MA, USA). The linearized plasmid containing off7 was then recircularized by ligation with the amplified BLA or BLA170 DNA with the attached linkers that vary in both their length and amino acid composition. DNA from this ligation was used to transform electrocompetent DH5 α cells that were plated on LB agar plates containing 50 μ g/ml chloramphenicol. The next day, the colonies from this plate were swept from the plate and the DNA was extracted using the Qiagen Miniprep Kit (Qiagen, Venlo, The Netherlands). This DNA was used to transform electrocompetent SNO301D cells along with the pTS2-pBAD-MBP band-pass plasmid.

The naïve library was first plated on tryptone agar plates with 20 μ g/ml tetracycline, 12.5 μ g/ml chloramphenicol, 12.5 μ g/ml kanamycin and 500 μ M IPTG on plates containing 0, 2, 4, 8 and 16 μ g/ml

ampicillin. Under these conditions, MBP is not expressed, since it is under the arabinose promoter. The highest number of colonies was observed on the 4 μ g/ml ampicillin plate for the off7/BLA170 library and 8 μ g/ml ampicillin for the off7/BLA library. These cells were again plated on larger tryptone agar plates with the same conditions (20 μ g/ml tetracycline, 12.5 μ g/ml chloramphenicol, 12.5 μ g/ml kanamycin and 500 μ M IPTG with 4 μ g/ml ampicillin for the off7/BLA170 library and 8 μ g/ml ampicillin for the off7/BLA library). A total of 713 colonies were observed for the off7/BLA library and only seven colonies were observed for the off7/BLA170 library from an estimated 30 000 CFU and 45 000 CFU's plated for each library, respectively. Based on the very low number of colonies that passed through the negative selection from the off7/BLA170 fusion, selections from this point continued only with the off7/BLA library. A total of 10 000 CFUs from this negative selection of off7/BLA were then plated on tryptone agar plates containing 12.5 μ g/ml chloramphenicol, 12.5 μ g/ml kanamycin, 500 μ M IPTG and 0.025% (w/v) arabinose with 0, 8, 16, 32, 64, 128, 256, 512, 1024 μ g/ml ampicillin. Colonies were observed on the plates up to 128 μ g/ml ampicillin. A total of 32 colonies were picked from the plates with 64 and 128 μ g/ml ampicillin and growth was tested again on plates with 12.5 μ g/ml chloramphenicol, 12.5 μ g/ml kanamycin, 500 μ M IPTG with and without 0.025% (w/v) arabinose.

Those cells that still showed a difference in their minimum inhibitory concentration (MIC) on ampicillin were picked and grown in 1 ml tryptone media with 25 µg/ml chloramphenicol and 25 µg/ml kanamycin in 96-well plates at 37°C overnight. The next day, these cultures were spotted on tryptone agar plates with 16, 32, 64, 128, 256, 512 µg/ml ampicillin. The library members that showed a >2-fold difference in their MIC in the presence and absence of arabinose were sent for sequencing.

MIC tests

To determine the MIC values conferred by the protein switches with the different ligands, SNO301D cells were cotransformed with plasmids designed to express every combination of protein switch and ligand. After confirming the correct sequence of both plasmids in a single colony, the colony harboring the pairing of plasmids for co-expression of a switch and ligand was used to inoculate an overnight of 10 ml of tryptone-only media with 25 µg/ml chloramphenicol and 25 µg/ml kanamycin. The following morning, this overnight was pelleted via centrifugation (4500g, 5 min, 4°C), the supernatant was discarded and the pellet was resuspended in 1 ml of fresh tryptone only media. The cell suspension was diluted to allow for the plating of 100–200 CFUs on tryptone agar plates with 25 µg/ml chloramphenicol and 25 µg/ml kanamycin, a range of ampicillin concentrations (0 and 1–512 µg/ml in 2-fold increments) with and without arabinose. After ~40 h of growth at 37°C, the MIC values were observed and recorded. The median MIC measurements represent the median MIC value of at least three replicates.

Expression and purification of the DARPin switch candidates

The three DARPin switch candidates (off7BLAC2, AR_3b-BLAC2 and 3G86.32-BLAC2) were cloned into the pHFT2 expression plasmid (Huang *et al.*, 2006; Koide *et al.*, 2007) under the control of the T7 promoter. This DNA was used to transform BL21(DE3) cells. An overnight culture of a single colony was started in LB media with 50 µg/ml kanamycin. The next day, this overnight was used to inoculate 1 l M9 + glycerol media (12.8 g/l Na₂HPO₄·7H₂O, 3.0 g/l KH₂PO₄, 0.5 g/l NaCl, 1.0 g/l NH₄Cl, 2.0 mM MgSO₄, 100 µM CaCl₂, 30 µM thiamine-HCl, 0.20% v/v glycerol). Once the culture reached an OD₆₀₀ value of 0.6, protein expression was induced by the addition of 500 µl of 1 M IPTG. The culture flask was transferred to an incubator shaker (250 RPM, 18°C) for overnight protein expression.

The next day, the cells were pelleted (10 min, 4°C, 5353g) and the supernatant was discarded. The cell pellet was resuspended in 20 ml 1 × PBS pH = 7.40 with 20 mM imidazole and 200 µl protease inhibitor (P8849 Sigma Aldrich, St Louis, MO, USA). The cells were lysed by passing them through a ThermoScientific French Press three times at >20 000 psi. The lysate was then clarified by centrifugation (75 600g, 4°C, 30 min). The protein was purified using a 1 ml HisTrap HP column in a GE Äkta FPLC (GE Healthcare, Piscataway, NJ, USA). After loading the clarified lysate, the column was washed with 30 ml of 1 × PBS with 20 mM Imidazole (pH = 7.40). The protein switch was eluted using a 20–500 mM imidazole gradient in 1 × PBS (pH = 7.40) executed over a 10 min period. One milliliter fractions were collected during purification and run on 4–12% Bis–Tris NuPAGE gels (Life Technologies, Carlsbad, CA, USA). These gels were stained with Coomassie brilliant blue to determine which fractions contained the protein of interest.

Expression and purification of the monobody switch candidates

The fibronectin switches were expressed in the same manner as the DARPin switches. However, an alternative osmotic shock cell lysis

protocol was used (adapted from Neu and Heppel, 1965; Nossal and Heppel, 1966). The cells were pelleted (10 min, 4°C, 5353g). The cell pellet was resuspended in 20 ml 33 mM Tris–HCl pH = 8.0 at 4°C. The cells were again pelleted (10 min, 4°C, 5353g). The cell pellet was resuspended in 8 ml 33 mM Tris–HCl pH = 8.0 at 4°C. Twenty milliliters of 33 mM Tris–HCl with 1 mM EDTA and 40% w/v sucrose pH = 8.0 were added 1 ml at a time to the cell suspension over 15 min. The cells were pelleted again (15 min, 4°C, 6000g). The supernatant was disposed of and any remaining liquid was pipetted out of the centrifuge bottle. The cell pellet was then resuspended in 20 ml 0.1 mM MgCl₂ at 4°C with 200 µl protease inhibitor (P8849 Sigma Aldrich). The cells were incubated with gentle rotation (150 RPM) on ice for 10 min. The cells were then pelleted one final time (75 600g, 4°C, 30 min). The supernatant was transferred to a 10 000 MWCO Amicon Centrifugal Filter Unit to exchange the buffer with 50 mM Tris with 150 mM NaCl at pH = 8.0. The supernatant was centrifuged multiple times with addition of fresh 50 mM Tris 150 mM NaCl pH = 8.0 buffer between centrifuge steps until the original buffer had been diluted by a factor of at least 20-fold (<5% original buffer). This sample was then purified using a 1 ml HisTrap HP column in a GE Äkta FPLC (GE Healthcare). After loading the clarified lysate, the column was washed with 30 ml of 50 mM Tris 150 mM NaCl pH = 8.0 and then eluted using a 0–1 M imidazole gradient in 50 mM Tris 150 mM NaCl pH = 8.0 executed over a 10 min period. One milliliter fractions were collected during purification and run on a 4–12% Bis–Tris NUPAGE gels (Life Technologies). The fractions containing the protein were then concentrated and buffer exchanged in a 10 000 MWCO Amicon Centrifugal Filter Unit with the assay buffer: PBS pH = 7.40. The centrifuge steps were carried out for 20 min at 4°C (4500g).

Expression and purification of the protein ligands

The pHFT2 construct containing the gene encoding MBP, ySUMO, eGFP or APH(3')IIIa under the control of the T7 promoter was used to transform BL21(DE3) cells. A single colony from this transformation was used to inoculate a 10 ml overnight culture of LB media with 50 µg/ml kanamycin. This tube was placed in an incubator/shaker overnight (37°C/250 RPM). The next morning, this culture was used to inoculate 1 l of M9 + Tryptone media (10 g/l tryptone, 12.8 g/l Na₂HPO₄·7H₂O, 3.0 g/l KH₂PO₄, 0.5 g/l NaCl, 1.0 g/l NH₄Cl, 2.0 mM MgSO₄, 100 µM CaCl₂, 30 µM thiamine-HCl, 25.0 mM glucose) with 50 µg/ml kanamycin in a 2 l baffled shake flask. This culture was returned to the incubator/shaker (37°C/250 RPM) until the culture reached an OD₆₀₀ ~0.6. Protein expression was then induced by adding 1 ml of 1 M IPTG to the culture.

For the expression of the ySUMO and APH(3')IIIa ligands, the shake flask was then transferred back to the incubator shaker at 37°C/250 RPM for 3 h. After this protein expression period, the cells were pelleted via centrifugation (5353g, 10 min, 4°C). The supernatant was discarded and the pellet was stored at –80°C.

For the MBP and eGFP expression cultures, following induction of protein expression, the shake flask was transferred to another incubator shaker (18°C/250 RPM) for overnight incubation. The next day, the cells were pelleted (5353g, 10 min, 4°C). The supernatant was discarded and the pellet was stored at –80°C.

The cell pellets were thawed on ice and resuspended in 20 ml of 1 × PBS (pH = 7.40) with 200 µl protease inhibitor (P8849 Sigma Aldrich) and 20 mM imidazole for ySUMO, eGFP and APH(3')IIIa, no imidazole was added to the resuspension buffer in the MBP purification. The cells were lysed by passing them through the French Press

Table I Antibody mimetic proteins used in this study

Scaffold	Protein name	Ligand	K_d (nM)	References
Monobody	YS1	MBP	81 ± 13	Gilbreth et al. (2008)
Monobody	ySUMO 53 (5)	ySUMO	7 ± 2	Koide et al. (2007)
Monobody	GFP GL4	eGFP	478 ± 72	Koide et al. (2012)
DARPin	off7	MBP	4.4	Binz et al. (2004)
DARPin	AR_3b	APH(3')IIIa	0.5 ± 0.4	Amstutz et al. (2005)
DARPin	3G86.32	eGFP	0.16	Brauchle et al. (2014)

three times at >20 000 psi. The crude lysate was collected on ice. Following the lysis step, the lysate was clarified by centrifugation (75 600g, 4°C, 30 min).

For the ySUMO, eGFP and APH(3')IIIa proteins, the supernatant from this step was purified using a 1 ml HisTrap HP column in a GE Äkta FPLC (GE Healthcare). After the protein was loaded onto the column, the column was washed with 30 ml of 1 × PBS (pH = 7.40) with 20 mM imidazole. The protein was then eluted from the column using a 20–500 mM imidazole gradient in 1 × PBS (pH = 7.40) over 10 min. One milliliter elution fractions were collected and run on a 4–12% Bis–Tris NuPAGE gel. The gel was stained with Coomassie brilliant blue (BioRad, Hercules, CA, USA) for 1 h and then destained in a solution of 50% methanol, 40% DI water, 10% glacial acetic acid for 2 h to overnight.

The MBP protein was purified using a 5 ml MBPTrap column and the GE Äkta FPLC (GE Healthcare). After the protein was loaded onto the column, the column was washed with 100 ml of 1 × PBS (pH = 7.40) until the UV absorbance dropped below 100 mAU. The protein was then eluted from the column using 10 mM maltose in 1 × PBS (pH = 7.40). One milliliter fractions were collected from this elution and run on a 4–12% Bis–Tris NuPAGE gel. The gel was stained with Coomassie Brilliant Blue (BioRad) for 1 h and then destained in a solution of 50% methanol, 40% DI water, 10% glacial acetic acid for at least 2 h. The gels showed bands at the appropriate molecular weight at a purity >95%.

The maltose and imidazole were removed from the purified proteins by overnight dialysis of 3 ml of purified protein in 3 l of 1 × PBS (pH = 7.40). Glycerol was then added to a final concentration of 10% prior to storing the proteins at –80°C.

In vitro characterization of protein switches

The enzymatic activity of the protein switches was characterized using the chromogenic β -lactam nitrocefin (Toku-E, Bellingham, WA, USA) in a Cary 50 UV–vis spectrophotometer. The assays were conducted by varying the concentrations of ligand to analyze its effect on the initial rate of nitrocefin hydrolysis catalyzed by the protein switch. Nitrocefin hydrolysis was quantified by measuring changes over time in the wavelength corresponding to the absorbance peak for hydrolyzed nitrocefin ($\lambda = 486$ nm). This change in absorbance was then converted to the number of micromoles of nitrocefin hydrolyzed per second by the enzyme using the molar extinction coefficient of hydrolyzed nitrocefin at $\lambda = 486$ nm ($20\,500\text{ M}^{-1}\text{ cm}^{-1}$) ([Jeon et al., 2011](#)). The protein switch (200 nM for DARPin switches and 25 nM for monobody switches), ligand and PBS buffer were added to a final volume of 1.8 ml in a quartz cuvette and mixed with a micro stir bar at 37°C for 5 min. Two hundred microliters of 1 mM nitrocefin in 1 × PBS (pH = 7.40) were then added and absorbance values at $\lambda = 486$ nm were collected at 0.1 s time intervals for 1 min. The initial rate of nitrocefin hydrolysis was approximated as the linear region of

the absorbance plots between 25 and 35 s after initiating the reaction by adding nitrocefin to the cuvette. We confirmed that all purified ligands [MBP, eGFP, APH(3')IIIa, ySUMO] lacked any ability to hydrolyze nitrocefin.

Results and discussion

Selection of input domains

We chose to explore the concept of a modular switch design for recognizing protein input signals. Two different binding protein scaffolds were selected to test as input domains for the modular protein switch: monobodies ([Koide et al., 1998](#)) and DARPins ([Binz et al., 2003](#); [Forrer et al., 2003](#)). We chose these two antibody mimetic binding scaffolds because they can be expressed in *E. coli*, they have already been engineered to bind a variety of different ligands (including those used in this study, see Table I), and they have a number of other advantages as binding scaffolds as enumerated by others ([Binz et al., 2004](#); [Zahnd et al., 2006](#); [Koide et al., 2007](#); [Schroeder et al., 2013](#)). Monobodies were developed from the 10th fibronectin type III domain, which consists of an immunoglobulin-like fold with a β sandwich core and three variable loops. DARPins were developed from the consensus sequence of ankyrin repeat proteins and consist of a series of repeats of two α -helices connected by structured loops. These individual repeats can be combined to create scaffolds of varying sizes ([Binz et al., 2003](#); [Forrer et al., 2003](#)). Since we are interested in identifying a single topology for a protein fusion that can recognize a wide variety of different protein targets, we elected to use a DARPin with three internal repeats in addition to the N- and C-terminal capping regions. Monobodies and DARPins that bind MBP from *E. coli* were previously identified from combinatorial libraries using phage display ([Koide et al., 2007](#)) and ribosomal display ([Binz et al., 2004](#)), and we chose these as the initial input domains from which to develop switches.

Library creation and selection

The genes encoding the MBP-binding monobody YS1 ([Koide et al., 2007](#); [Gilbreth et al., 2008](#)) and the MBP-binding DARPin off7 ([Binz et al., 2004](#)) were fused to the *E. coli* *dsbA* signal sequence for export into the *E. coli* periplasm. Multiplex inverse PCR ([Kanwar et al., 2013](#)) was performed on these plasmids in order to create a library of insertion sites throughout the genes encoding YS1 and off7 for insertion of the BLA or BLA170 gene. We designed the inverse PCR reactions such that direct insertions (both YS1 and off7 libraries) and insertions in which 1–3 amino acids of the antibody mimetic protein were deleted or tandemly duplicated (YS1 library only) were created. The BLA insert gene was amplified with (off7 library) or without (YS1 library) primers designed to add 1–3 random amino acids at either side of the insertion site. For the YS1 library, the insert gene was

BLA170. For the off7 library, both BLA and BLA 170 were used as insert genes.

We subjected these libraries to selections using our band-pass selection system for tunable selection of BLA activity and ampicillin resistance (Sohka *et al.*, 2009). The SNO301 strain was modified such that the strain no longer produced MBP from the chromosome. Instead, the pTS1 plasmid was modified to allow inducible expression of MBP using the pBAD promoter and named pTS2-pBAD-MBP. A two-tiered selection was performed to identify genes conferring both high levels of ampicillin resistance with MBP co-expression and low levels of ampicillin resistance in the absence of MBP co-expression. Library members surviving these selections were further screened to confirm that they exhibited increased ampicillin resistance with MBP co-expression. Library members passing this screen were sequenced (Supplementary Table SI) and plasmid DNA from select members was transformed into fresh SNO301D cells to verify that the switch genes conferred a switching phenotype.

In vivo characterization of the MBP-activated switches

For cells expressing the switch genes, coexpression of MBP increased ampicillin resistance from 2- to 8-fold (Supplementary Table SI). The lysates of cells expressing switches YS1-MBP5-BLA170, off7BLAC2 and off7BLAE2 contained MBP-dependent BLA activity, suggesting that these switch genes encoded switches that function by an allosteric

mechanism. For the monobody switch genes, we also tested whether coexpression of MBP would still increase ampicillin resistance when maltose was added to the growth media. Since YS1 binds MBP in the maltose-binding pocket (Fig. 2B), YS1 and maltodextrins are competitive binders of MBP (Koide *et al.*, 2007). We found that addition of 100 mM maltose to the growth media prevented or severely reduced the increase in MIC caused by MBP co-expression (Supplementary Table SII), presumably because maltose binding to MBP prevented switch binding to MBP. These data constitute good evidence that the MIC increase caused by MBP coexpression is a result of a specific interaction between monobody-derived switch proteins and MBP.

In vitro characterization of MBP-activated switches

We chose switches off7BLAC2 and YS1-MBP5-BLA170 for more extensive characterizations based on their superior MBP-dependent MIC ratios and potential allosteric mechanism. The BLA insertion sites in these two switches are shown in Fig. 2. Both switch genes conferred a switching phenotype to cells in which MBP co-expression (but not the co-expression of other control proteins or empty vector) increased ampicillin resistance 8-fold (Table II). We purified both switches and characterized the effect of MBP and control proteins on enzyme activity using nitrocefin as the substrate. Enzymatic activity of both switches increased over 10-fold in the presence of 10 μ M MBP (Fig. 3A and D). The relatively high concentration of MBP required for

Fig. 2 Structures of antibody mimetics bound to ligands indicating the site of BLA insertion that resulted in a switch. (A) The site of insertion of BLA into off7 in switch off7BLAC2 [structure: 1SVX (Binz *et al.*, 2004)]. (B) The site of insertion of BLA into YS1 in switch YS1-MBP5-BLA170 [structure: 2OBG (Koide *et al.*, 2007)]. Sites of insertion of BLA into the antibody mimetic are indicated with arrows.

Table II Switching phenotype of cells expressing switch proteins

Protein/switch expressed	Switch designed to be activated by	Fold-increase in MIC for ampicillin when the indicated protein is co-expressed ^a		
		MBP	eGFP	ySUMO
YS1	n/a ^b	1	1	1
YS1-MBP5-BLA170	MBP	8	1	1 ^c
GFP-GL4-BLA170	eGFP	1	2	1
ySUMO53(5)-BLA170	ySUMO	1 ^c	1	2
off7	n/a ^b	1	1	1 ^c
off7BLAC2	MBP	8	1	1

^aFold increase represents the median MIC ratio comparing the MIC with coexpression of the indicated protein to the MIC in the absence of coexpression of the protein. Supplementary Table SIII contains replicate MIC and MIC ratio values.

^bNot applicable since YS1 and off7 are the original MBP-binding monobody and DARPIn, respectively, and are not fused to BLA.

^cAlthough the median value was 1, at least one replicate indicated a 2-fold increase (see Supplementary Table SIII).

Fig. 3 Ligand-dependent enzyme activity of (A–C) monobody- and (D–F) DARPIn-derived switches. The nitrocefin hydrolysis activity of the switches was measured in the absence and presence of different concentrations of the indicated ligands. Switching is the ratio of the enzymatic rate in the presence of the ligand to that in the absence of any ligand. Error bars represent the standard deviation ($n=3$).

switch activation (relative to the antibody mimetics original K_d for MBP, Table I) indicate that MBP affinity had been significantly compromised in these fusion proteins. Activation of the off7BLAC2 switch derived from the DARPIn off7 by MBP (up to 11-fold) was substantially higher than the activation observed with the control proteins aminoglycoside phosphotransferase 3'IIIa [APH(3')IIIa] and eGFP, which showed at most a 2-fold difference in activation (Fig. 3D). Although activation of YS1-MBP5-BLA170 by MBP (14-fold) was higher than that observed for off7BLAC2, we also observed higher activation (4-fold) when either of the control proteins (eGFP and yeast small ubiquitin-like modifier protein (ySUMO)) were added (Fig. 3A).

The reasons for the background activation by control proteins are not clear. We confirmed that the preparations of purified MBP, APH(3')III, eGFP and ySUMO lacked BLA activity. The assays were not performed at a constant total protein level (e.g. we did not add a third unrelated protein to make up the difference when lower concentrations of the ligand protein were used). The ratio of protein ligand to switch protein in the assay ranged from 0 to 400 for the monobody switches and 0–50 for the DARPins. The control proteins may have a general, non-specific stabilizing effect on the switches.

Switch modularity

A modular switch platform would allow for conversion of a switch activated by ligand A into a switch activated by ligand B simply by introducing the mutations into the antibody mimetic domain known to cause the antibody mimetic to bind ligand B (Fig. 1). Such mutations could be identified through standard directed evolution or protein design methods on the antibody mimetic protein outside the context of a

switch. We wondered if YS1-MBP5-BLA170 and off7BLAC2 could function as modular switch platforms. Based on the sequences of the previously developed monobodies and DARPins (Table I), we modified YS1-MBP5-BLA170 to recognize ySUMO and eGFP, and we modified off7BLAC2 to recognize APH(3')IIIa and eGFP.

In order to test the *in vivo* performance of these constructs, the gene for MBP was replaced in the pTS2-pBAD-MBP plasmid with the genes encoding ySUMO and eGFP to be able to test the switching phenotype by periplasmic expression of these proteins. For unknown reasons, vectors for periplasmic expression of APH(3')IIIa and the DARPIn switches modified to recognize APH(3')IIIa and eGFP were toxic to *E. coli* and could not be constructed despite repeated attempts using a variety of different gene construction methodologies. All approaches resulted in very few transformants with plasmid DNA containing mutations or deletions in the desired gene—mutations that could not subsequently be repaired without the appearance of new mutations or deletions. Fortunately, vectors for cytoplasmic expression of these three proteins were not toxic. Thus, we could only test the switching phenotype conferred by the new monobody-derived fusions, as phenotype testing required periplasmic expression of both the switch and the ligand. As desired, modification of the YS1-MBP5-BLA170 switch to recognize eGFP and ySUMO eliminated the increase in Amp resistance with MBP co-expression and resulted in a small (2-fold) but reproducible increase in Amp resistance with co-expression of their intended effectors (Table II).

We purified the switches and their cognate ligands and performed BLA enzyme assays *in vitro*. For the monobody constructs, although the mutations designed to target the monobody domain to new ligands impaired MBP activation, we did not observe the desired

Table III Effect of the M182T stabilizing mutation on the switching phenotype

Protein	Median MIC for Amp (µg/ml) ^a		Fold increase in MIC when MBP is expressed
	–MBP	+MBP	
YS1-MBP5-BLA170	16	128	8
YS1-MBP5-BLA170 (M182T)	128	256	2
Off7BLAC2	32	256	8
Off7BLAC2 (M182T)	256	256	1

^aThe MIC values were determined with and without coexpression of MBP. Supplementary Table SIV contains replicate MIC values from which the median values were calculated.

change in switch specificity but instead observed some activation by all three ligands (Fig. 3B and C). In addition, the enzymatic activity was very low for the YS1-MBP5-BLA170 switch redesigned to be activated by ySUMO (Supplementary Fig. S1). We speculate that the nature of the monobody interface for this set of monobodies, whose variable loops are composed solely of serines and tyrosines, may result in non-specific binding and activation at the micromolar levels of ligand necessary for activation. Additionally, the YS1-MBP interaction is not mediated solely through the monobody's variable loops, as framework residues of YS1 significantly contribute to the interaction with MBP (Cheung *et al.*, 2015). The framework residues are the same in all three monobody domains.

In contrast, the off7BLAC2 switch redesigned to be activated by APH(3')IIIa was activated solely by APH(3')IIIa (Fig. 3E). The level of activation was even stronger than the original switch, as the level of enzyme activity was 25-fold higher in the presence of APH(3')IIIa than in its absence. This improvement resulted from the mutations causing a significant decrease in the enzyme activity in the absence of ligand (Supplementary Fig. S2). However, the introduction of the mutations that recognize eGFP into off7BLAC2 resulted in a protein whose enzymatic activity was not significantly activated by any of the three ligands (Fig. 3F). Introduction of the mutations designed to allow off7BLAC2 to bind to eGFP increased enzyme activity in the absence of ligand (Supplementary Fig. S2). The lack of success with the eGFP-binding DARPins thus might result in part from the eGFP set of mutations relaxing the impairment of enzyme activity such that eGFP binding can no longer cause an increase in activity. Alternatively, since it is apparent that the DARPins fusions that do have switch properties have significantly reduced affinity for their ligands, perhaps the fusion cannot bind eGFP at all. The K_d of the GFP-binding monobody and GFP is weaker than the other monobody/ligand pairs (Table I).

Potential switch mechanisms

Switches YS1-MBP5-BLA170, off7BLAC2 and off7BLAC2-APH(3')IIIa (especially the two DARPins switches) exhibit clear activation of enzymatic activity *in vitro* with their intended ligand (Fig. 3), which suggests that they may function by an allosteric switching mechanism. We have often seen that switches that possess an allosteric mechanism also accumulate to higher levels *in vivo* in the presence of their respective ligands (for example, see Heins *et al.*, 2011). Thus, such switches also function by the protein abundance mechanism in conferring the switching phenotype to cells. We have shown that proteins that function by the protein abundance mechanism do so because the absence of bound ligand results in an increased unfolding rate, decreased conformational stability and increased susceptibility to proteases (Choi *et al.*, 2013). In the absence of ligand, decreased conformational stability and increased conformational entropy is a property that enables

the switching property (Choi *et al.*, 2015). Although we have not tested the ligand-dependence on switch cellular abundance for the monobody and DARPins switches described here, in the following experiment, we examined the role that protein stability plays in the switching phenotype.

The M182T mutation in BLA is a well-known mutation that increases thermodynamic stability and is an adaptive mutation that emerges evolutionarily to compensate for mutations that increase catalytic activity but decrease protein stability (Wang *et al.*, 2002). We introduced this mutation into the two MBP-activated switches YS1-MBP5-BLA170 and off7BLAC2 and quantified its effect on ampicillin resistance in the presence and absence of coexpressed MBP. The M182T mutation diminished or eliminated switching by increasing the MIC in the absence of coexpressed MBP (Table III). This result suggests that for fusions that confer a switching phenotype, insertion of BLA into the antibody mimetic destabilizes the BLA domain such that the domain's ability to provide resistance is compromised. Binding of MBP may stabilize the domain and reduce its conformational entropy, resulting in higher resistance. The M182T mutation might serve the same purpose, although the molecular details are presumably different.

Finally, although the antibody mimetic domains by themselves do not undergo appreciable ligand-induced conformational changes, this does not mean that the switches built from them do not undergo large conformational changes. For example, since the BLA domain splits the antibody mimetic domain into a discontinuous domain, the fusions might be prone to undergo 3D domain swapping (Liu and Eisenberg, 2002). In 3D domain swapping, protein monomers exchange identical domains resulting in oligomerization. Such a phenomenon could occur in both the ligand-bound and ligand-unbound state. Alternatively, ligand binding could alter the equilibrium between the monomer and the 3D domain swapped state. Switching would result if such states possessed different catalytic activities or other properties that contribute to the switching phenotype. In this case, a large conformational change upon ligand binding (monomer to oligomer) would be part of the switch mechanism. Such a mechanism might be more likely with the DARPins-derived switches since DARPins have a repeat structure and the insertion site of BLA in off7BLAC2 is at the border between the first and second repeat (Fig. 2A and B). The oligomeric state of these switches warrants future study.

Conclusions

There are two significant conclusions from our work. First, it is possible to create protein switches by domain insertion using input domains that do not undergo large conformational changes upon ligand binding. Second, DARPins and, to a lesser extent, monobodies show some promise as input domains for a modular platform for the rapid

development of switches. However, significant improvements need to be made to realize the potential of this platform. Switches with high affinity for their respective ligands need to be identified. Although the original antibody mimetic proteins had low nanomolar K_d 's, the switches described here required micromolar ligand concentrations for activation. The specificity of the monobody switches needs to be improved, perhaps by increasing the amino acid diversity of the loops. Although conversion of the MBP-activated switch to be activated by APH(3')IIIa succeeded, the conversion of the same switch to be activated by eGFP did not. Biochemical and biophysical studies on the set of DARPins switches described here may shed some light on the reasons for these differences. Finally, future studies could focus on testing new output domains such as fluorescent proteins for use as a diagnostic biosensing tool (Marvin *et al.*, 2011).

Supplementary data

Supplementary data are available at PEDS online.

Acknowledgements

We thank Shohei Koide for providing the DNA for YS1 and the expression plasmid for ySUMO. We thank Gerard Wright for providing the DNA for APH(3') IIIa.

Conflict of interest: M.O. declares a potential conflict of interest in the form of issued and pending patent applications on protein switches created by domain insertion and circular permutation.

Funding

This work was supported by grants from the Defense Threat Reduction Agency (grant number HDTRA1-09-1-0016) and the National Institute of General Medicine at the National Institutes of Health (grant number R01 GM066972). P.H. was supported by the Brazilian National Council for Scientific and Technological Development and the Ministry of Science and Technology (Conselho Nacional de Desenvolvimento Científico e Tecnológico/Ministério de Ciencia e Tecnologia CNPq/MCT-Brazil) through (grant number 201979/2012-8).

References

Amstutz,P., Binz,H.K., Parizek,P., Stumpp,M.T., Kohl,A., Grütter,M.G., Forrer,P. and Plückthun,A. (2005) *J. Biol. Chem.*, **280**, 24715–24722.
 Binz,H.K., Stumpp,M.T., Forrer,P., Amstutz,P. and Plückthun,A. (2003) *J. Mol. Biol.*, **332**, 489–503.
 Binz,H.K., Amstutz,P., Kohl,A., Stumpp,M.T., Briand,C., Forrer,P., Grütter,M.G. and Plückthun,A. (2004) *Nat. Biotechnol.*, **22**, 575–582.
 Brauchle,M., Hansen,S., Caussinus,E., Lenard,A., Ochoa-Espinosa,A., Scholz,O., Sprecher,S.G., Plückthun,A. and Affolter,M. (2014) *Biol. Open*, **3**, 1252–1261.

Cheung,L.S., Shea,D.J., Nicholes,N., Date,A., Ostermeier,M. and Konstantopoulos,K. (2015) *Nat. Sci. Rep.*, **5**, 8247.
 Choi,J.H. and Ostermeier,M. (2015) *ACS Synth. Biol.*, **4**, 400–406.
 Choi,J.H., San,A. and Ostermeier,M. (2013) *Protein Sci.*, **22**, 475–485.
 Choi,J.H., Laurent,A.H., Hilser,V.J. and Ostermeier,M. (2015) *Nat. Commun.*, **6**, 6698.
 Firnberg,E., Labonte,J.W., Gray,J.J. and Ostermeier,M. (2014) *Mol. Biol. Evol.*, **31**, 1581–1592.
 Forrer,P., Stumpp,M.T., Binz,H.K. and Plückthun,A. (2003) *FEBS Lett.*, **539**, 2–6.
 Gilbreth,R.N., Esaki,K., Koide,A., Sidhu,S.S. and Koide,S. (2008) *J. Mol. Biol.*, **381**, 407–418.
 Guntas,G. and Ostermeier,M. (2004) *J. Mol. Biol.*, **336**, 263–273.
 Guntas,G., Mitchell,S.F. and Ostermeier,M. (2004) *Chem. Biol.*, **11**, 1483–1487.
 Guntas,G., Mansell,T.J., Kim,J.R. and Ostermeier,M. (2005) *Proc. Natl Acad. Sci. USA*, **102**, 11224–11229.
 Heins,R.A., Choi,J.H., Sohka,T. and Ostermeier,M. (2011) *PLoS One*, **6**, e27302.
 Huang,J., Koide,A., Nettle,K.W., Green,G.L. and Koide,S. (2006) *Prot. Expr. Purif.*, **47**, 348–354.
 Jeon,J.H., Kim,S.J., Lee,H.S., *et al.* (2011) *Appl. Environ. Microbiol.*, **77**, 7830–7836.
 Kanwar,M., Wright,R.C., Date,A., Tullman,J. and Ostermeier,M. (2013) *Met. Enzym.*, **523**, 369–388.
 Kim,J.R. and Ostermeier,M. (2006) *Arch. Biochem. Biophys.*, **446**, 44–51.
 Koide,A., Bailey,C.W., Huang,X. and Koide,S. (1998) *J. Mol. Biol.*, **284**, 1141–1151.
 Koide,A., Gilbreth,R.N., Esaki,K., Tereshko,B. and Koide,S. (2007) *Proc. Natl Acad. Sci. USA*, **104**, 6632–6637.
 Koide,A., Wojcik,J., Gilbreth,R.N., Hoey,R.J. and Koide,S. (2012) *J. Mol. Biol.*, **415**, 393–405.
 Liu,Y. and Eisenberg,D. (2002) *Protein Sci.*, **11**, 1285–1299.
 Marvin,J.S., Schreiter,E.R., Echevarria,I.M. and Looger,L.L. (2011) *Proteins*, **79**, 3025–3036.
 Neu,H.C. and Heppel,L.A. (1965) *J. Biol. Chem.*, **240**, 3685–3692.
 Nossal,N.G. and Heppel,L.A. (1966) *J. Biol. Chem.*, **241**, 3055–3062.
 Radley,T.L., Markowska,A.I., Bettinger,B.T., Ha,J.H. and Loh,S.N. (2003) *J. Mol. Biol.*, **332**, 529–536.
 Schroeder,T., Barandun,J., Flutsch,A., Briand,C., Mittl,P.R.E. and Grütter,M.G. (2013) *Structure*, **21**, 277–289.
 Sohka,T., Heins,R.A., Phelan,R.M., Greisler,J.M., Townsend,C.A. and Ostermeier,M. (2009) *Proc. Natl Acad. Sci. USA*, **106**, 10135–10140.
 Stein,V. and Alexandrov,K. (2015) *Trends Biotechnol.*, **33**, 101–110.
 Tullman,J., Guntas,G., Dumont,M. and Ostermeier,M. (2011) *Biotechnol. Bioeng.*, **108**, 2535–2543.
 Wang,X., Minasov,G. and Shoichet,B.K. (2002) *J. Mol. Biol.*, **320**, 85–95.
 Wright,C.M., Majumdar,A., Tolman,J.R. and Ostermeier,M. (2010) *Proteins*, **78**, 1423–1430.
 Wright,C.M., Wright,R.C., Eshleman,J.R. and Ostermeier,M. (2011) *Proc. Natl Acad. Sci. USA*, **108**, 16206–16211.
 Zahnd,C., Pecorari,F., Straumann,N., Wyler,E. and Plückthun,A. (2006) *J. Biol. Chem.*, **281**, 35167–35175.