An Engineered Calmodulin-Based Allosteric Switch for Peptide Biosensing

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An Engineered Calmodulin-Based Allosteric Switch for Peptide Biosensing

Glenna E. Meister and Neel S. Joshi*^[a,b]

This work describes the development of a new platform for allosteric protein engineering that takes advantage of the ability of calmodulin to change conformation upon binding to peptide and protein ligands. The switch we have developed consists of a fusion protein in which calmodulin is genetically inserted into the sequence of TEM1 β-lactamase. In this approach, calmodulin acts as the input domain, whose ligand-dependent conformational changes control the activity of the β-lactamase output domain. The new allosteric enzyme exhibits up to 120 times higher catalytic activity in the activated (peptide bound) state compared to the inactive (no peptide bound) state in vitro. Activation of the enzyme is ligand-dependent – peptides with higher affinities for wild-type calmodulin exhibit increased switch activity. Calmodulin’s ability to “turn on” the activity of β-lactamase makes this a potentially valuable scaffold for the directed evolution of highly specific biosensors for detecting toxins and other clinically relevant biomarkers.

Introduction

The development of new technologies for molecular sensing and signal transduction is critical to advancements in fields of synthetic biology,^[1] medical diagnostics,^[2] and environmental data collection^[3] among others. At the most basic level, a molecular sensor should be able to couple two distinct functions: specific molecular recognition (input) and generation of a readily detectable signal (output). Although there exist various methods to accomplish this using advanced analytical techniques such as immunoassays, biology relies heavily on allosteric systems for cellular sensing and signal transduction. Here we broadly define an allosteric system as any protein whose activity is modulated by a binding event at a location distinct from the active site. The study of biological signaling has inspired new methods to engineer allosteric systems,^[4] especially those that can sense biomedically relevant targets^[5] or interface with established signaling mechanisms in whole cells or organisms.^[6]

The complexities of protein design have made it difficult to construct allosteric systems de novo, but researchers have circumvented this problem by separating the input and output functions into separate but coupled domains. In this type of approach, an ideal input domain is one that undergoes large but predictable conformational changes upon target binding. The input domain is then fused to an output domain in such a way that the function (i.e. signal generation) of the output domain is conformationally dependent. Despite the abundance of engineered allosteric enzymes and sensors present in the literature,^[7] few are designed to respond to protein or peptide-based binding targets^[8] – rather, most are activated by ions and small molecules.^[9] Nevertheless, there is great interest in developing sensors for peptides and proteins because of their central role in cellular signaling. More widespread use of these techniques is hindered by other barriers, such as output signals that are limited to FRET or the need to painstakingly optimize fusion points for the desired output protein, which can require screening of large libraries of randomized fusion points.^[10]

Here we report the rational design and development of a new allosteric switch protein that responds to peptide-based binding targets. Based on the relatively straightforward optimization of the switch compared to related examples, our design could represent a promising platform for modular switch development. The switch we have developed (BLACaM) makes use of the domain insertion technique^[7] and consists of calmodulin (CaM) inserted into the sequence of TEM1 β-lactamase (BLA, EC 3.5.2.6). BLACaM is controlled by the conformational changes of the CaM input domain. CaM is a small (~17 kDa) globular protein that exists in three conformational states. In the absence of Ca^[2+]^{[7a]}, it adopts a “closed” conformation with its N- and C-termini spatially separated by only 9 Å. In the presence of Ca^[2+]^{[7b]}, CaM adopts an “open” conformation with the termini separated by ~31 Å. In the presence of Ca^[2+]^{[7a]} and a peptide binding target, CaM once again adopts a “closed” conformation (Figure 1a). The target-bound forms of CaM for different peptides are similar but not identical and structural data suggests that the distance between the termini may vary between 13-24 Å for different targets. Because of its well-characterized conformational dynamics, CaM has been used in molecular sensors such as genetically encodable calcium sensors for decades. However, its ability to bind with high affinity to proteins and peptides has yet to be exploited in allosteric biosensors. Here we show that the new switch, BLACaM, is mostly inactive when Ca^[2+]^{[7a]} is present, and

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Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.
becomes activated after binding to a range of target peptides. The switch demonstrates specificity in that it is only activated by peptides that are known to bind to wt-CaM.

![Diagram of Calmodulin Conformation Change](image)

**Figure 1.** Calmodulin (CaM) and TEM1 β-lactamase (BLA) structures. a) Structures showing the conformation changes of CaM in the apo, or closed state (pdb ID: 1CFD), calcium bound, or open state (pdb ID: 1CLL), and bound to calcium and the M13 peptide (pdb ID: 2BMM). b) Structure of the TEM1 β-lactamase (pdb ID: 1BTL) with the protein complementation fragments BLF1 and BLF2 shown in purple and green, respectively. The insertion points for all switch constructs are indicated with arrows and labelled orange.

## Results and Discussion

### Calmodulin – TEM1 β-lactamase fusions

Previous attempts at switch engineering using domain insertion required high throughput screening techniques in order to identify a suitable insertion point.\(^9\)\(^-\)\(^10\) We sought to avoid the time consuming process of library construction associated with most domain insertion efforts by making use of the known split points of TEM1 β-lactamase (BLA). Therefore, we synthesized eight BLACaM variants using the BLA split points reported by Galarneau et al.\(^{21}\) and Guntas et al.\(^9\)\(^,\)\(^12\) (Figure 1b). We initially constructed genes encoding for a small panel of BLACaM variants (P1-P8, Figure 2S1). P1 and P2 consisted of the N-terminal fragment of BLA (1-194) (a.k.a. BLF1) fused to the N-terminal of CaM, and the C-terminal fragment of BLA (196-286) (a.k.a. BLF2) fused to the C-terminal of CaM, while P3 and P4 had the order of the domains reversed. P5/P6 and P7/P8 were analogous to P1/P2 and P3/P4, respectively, except we used insertion points identified by Guntas et al. in the construction of a maltose binding protein-β-lactamase switch.\(^9\)\(^,\)\(^12\) Our panel included constructs that had no linkers (direct fusion), two very short linkers (S and SG), and constructs that used both a short stiff linker (DKS) and a short flexible linker (GSGGG). We rationalized that the Galarneau, et al. split points would be good initial insertion points because the fragments had been pre-optimized to: 1) resist spontaneous association in solution, and 2) be individually non-functional but reconstitute an active enzyme when brought in close proximity to one another by fused domains. Additionally, the Guntas, et al. split points, which were identified using a large library (10^6 members) screening process, have already been proven to be suitable insertion sites that allow for modulation of the BLA activity. We reasoned that the open conformation of CaM inserted at these points would be able to separate the two BLA fragments enough to inactivate them. Once CaM adopted a closed conformation in response to ligand binding, the two β-lactamase fragments would be brought together to activate the enzyme.

The P1-P8 domain insertion variants were initially screened for switching activity in crude cellular lysates. To do this, plasmids encoding for each variant were separately transformed into \textit{E. coli} and overexpression was induced. Following induction, the cells were lysed and the switching properties of the variants were assayed directly in the clarified lysate using a colorimetric indicator (nitrocefin) for BLA activity. The screening process for switching activity was performed by monitoring each variant’s BLA activity under sets of conditions known to induce the three different conformational states of wt-CaM (Figure 1a): no calcium or target (1 mM EGTA), calcium only (5 mM Ca\(^{2+}\)), and calcium plus peptide target (5 mM Ca\(^{2+}\), 1 μM target). The target in this case was the calmodulin binding peptide M13, a 26 amino acid peptide derived from myosin light chain kinase that is known to bind to and induce the “closed” conformation of CaM.\(^{13}\) Based on the activity assays, the variants were classified into three types: always on (P1 and P3), always off (P2, P5, P7 and P8), and switch (P4 and P6) (Figure 2). The P4 variant exhibited “on” states in both the non-calcium bound and M13 bound states with activity close to baseline (“off”) in the presence of calcium while the P6 variant only showed increased activity in the M13 bound state. Notably, both P1 and P3 did exhibit higher activity under the EGTA and M13-bound conditions, but also showed significant activity under the calcium only conditions. Furthermore, the range of responses observed for the variants even in this small panel highlights the importance of insertion point and linker flexibility for effective switch construction. We theorized that too much linker flexibility would allow the BLA fragments to associate regardless of the conformational state of the CaM domain, effectively decoupling input from output, while rigid linkers or no linkers may confine the BLA fragments in orientations that do not allow them to re-associate properly. Performing the screen in lysates also enabled us to probe the specificity of the switch. Variants identified as “hits” had to respond specifically to the target peptide in the presence of the complex lysate mixture, which contained proteins, DNA, RNA, lipids, and small molecules.

### BLACaM switch purification and characterization

Based on the switching activity observed in the lysate assays, the P4 variant (hereafter referred to as BLACaM) was selected for further characterization (Figure 3b). While the P6 fusion also exhibited higher activity when bound to M13 (but not in the presence of EGTA), this switch was found to be highly toxic to \textit{E. coli} cells even in low amounts and was not characterized further.
The BLACaM switch also seemed to exhibit a low level of toxicity in *E. coli* cells, however we were able to obtain sufficient amounts of BLACaM using a T7 promoter by expressing at a lower temperature (18°C) and subsequently purifying using a C-terminal 6xHis tag. SDS-PAGE characterization of the affinity-purified samples revealed that there was extensive truncation of the protein, possibly resulting from the translation of short fragments from internal start codons. The c-terminal gene encoding for the CaM domain originated from humans, and consequently contained several non-optimal codons for *E. coli* that we believe enabled the use of internal start codons and hindered the full translation of BLACaM. Therefore, we altered the gene to include only those codons common in *E. coli*, allowing for isolation of a BLACaM sample that was ≥95% pure after His-tag affinity purification (Figure S2).

The purified BLACaM protein was then characterized *in vitro* to determine its switching activity with different analytes. For these experiments, activity was measured by tracking the hydrolysis of a commercially available BLA substrate (CENTA<sup>TM</sup>). In order to confirm our hypothesis about the mechanism of switch activation, we ran several control experiments to monitor the activity of BLACaM sub-domains individually. The wt-BLA alone established a baseline for enzyme activity, while wt-CaM alone was not active under any conditions, as expected (Figure S3a). Notably, the activity of pure BLA was decreased slightly in the presence of EGTA. Neither wt-BLA nor wt-CaM was affected by the presence of the target peptide, M13 (Figure S3b). Finally, we conducted activity assays with wt-BLA and wt-CaM together in one pot in the presence of a variety of peptides. In total, the control experiments confirmed three important points: 1) on its own, BLA is largely unaffected by the various conditions expected to induce switching in BLACaM; 2) the CaM domain does not exhibit any ability to hydrolyze the CENTA substrate; 3) points 1 and 2 are not affected by non-covalent interactions between BLA and CaM under any of the tested conditions.

With a clear understanding of the behavior of the wild-type proteins, we proceeded to characterize the purified BLACaM switch. As expected from the lysate assays, BLACaM exhibited ligand-dependent activity. In a purely qualitative sense, it was immediately clear that BLACaM was only moderately active in the absence of calcium (EGTA), mostly inactive in the presence of Ca<sup>2+</sup> only, and highly active in the presence of Ca<sup>2+</sup> plus M13 (Figure 3a-c). Notably, BLACaM exhibits much higher switching activity when pure compared to when in crude lysate. Also, while the EGTA and Ca<sup>2+</sup> plus M13 states appear to be equally active in lysates, they are quite different when pure, suggesting that complex mixtures could have unexpected effects on the switch behavior.

**BLACaM activity is due to intramolecular interactions of BLF1 and BLF2**

In a final set of control experiments we wanted to confirm that switch activity was not a result of multimerization of BLACaM during the activity assays. Although the split BLA fragments (BLF1 and BLF2) are designed to have a sufficiently high K<sub>i</sub> so as not to associate at low concentrations, they can spontaneously refold through intermolecular interactions at sufficiently high concentrations. Therefore, we constructed two truncation mutants of BLACaM (Figure 3b), one with the BLF1 fragment removed (BLF2-CaM) and one with the BLF2 fragment removed (CaM-BLF1). In order to probe the potential for intermolecular complementation to contribute to the observed activity of BLACaM, the two truncated mutants were combined at various equimolar concentrations and subjected to activity assays. Some complementation did occur at concentrations above 1 µM (Figure S4c), but no significant substrate turnover was observed at concentrations that were used for the BLACaM activity assays (125 nM) (Figure 3d).

Based on these experiments, it is clear that multimerization was not a factor in the switching activity experiments. However, it should be noted that the switch needed to be “primed” by calcium-induced inactivation before rigorous switching activity experiments could take place. BLACaM required 50+ minutes of incubation with Ca<sup>2+</sup> at room temperature to reach its lowest activity levels (Figure S5a), possibly due to residual intramolecular fragment complementation. In contrast, after being in the “off” state, the switch could be reactivated again in less than 5 minutes upon addition of the target peptide M13 (Figure S5b), suggesting that fragment association from an unfolded state occurs much more rapidly than dissociation of the folded fragments. It is known that certain assisted protein complementation fragments are stabilized in their fully folded state, which could explain the longer times required for inactivation.[15] Notably, the time required for switch inactivation was dramatically reduced by increases in temperature. BLACaM that was assayed following a 10 minute incubation with only Ca<sup>2+</sup> at 37°C followed by re-equilibration at 25°C was inactive, but remained active when incubated with M13 (Figure S6a). Activity assays performed at various temperatures confirmed that BLACaM retains most of its activity up to 30°C in the EGTA or calcium plus peptide states. However, at 37°C, the switch loses
Activity of the BLACaM switch is dose dependent

In order to determine the sensitivity of the switch towards a target ligand, we monitored substrate hydrolysis rates for BLACaM at various concentrations of M13. A plot of initial rates versus M13 concentration reveals that the peptide can be detected at tens of nanomolar concentrations and is dose dependent up to 150 nM (Figure 4). There was ~10-20% decrease in initial rates for M13 concentrations above 175 nM, hindering the effectiveness of traditional Michaelis-Menten-type kinetic models to fit the data (data not shown). We attribute this rate decrease to aggregation of M13, which is a known problem in CaM binding assays because many canonical CaM binders are hydrophobic peptides prone to aggregation in aqueous solutions.

Table 1. Switching Activity of BLACaM in Response to Peptide Targets

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<tr>
<th>Peptide Sequence</th>
<th>Switching Activity (SA)</th>
<th># of Binding Sites</th>
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<tr>
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<td>1.0 ± 0.1</td>
<td>4</td>
<td>N/A</td>
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<tr>
<td>EGTA</td>
<td>2.9 ± 0.2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>M13 (sm-MLCK fragment)</td>
<td>110 ± 12</td>
<td>1</td>
<td>0.15 nM</td>
</tr>
<tr>
<td>Mastoparan</td>
<td>120 ± 11</td>
<td>1</td>
<td>0.3 nM</td>
</tr>
<tr>
<td>Melittin</td>
<td>120 ± 11</td>
<td>1</td>
<td>3 nM</td>
</tr>
<tr>
<td>δ-toxin</td>
<td>16 ± 2</td>
<td>1</td>
<td>c</td>
</tr>
<tr>
<td>Neuropeptide Y (NPY)</td>
<td>100 ± 11</td>
<td>1</td>
<td>c</td>
</tr>
<tr>
<td>Luteinizing Hormone- Releasing Hormone (LH-RH)</td>
<td>0.97 ± 0.13</td>
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<td>---d</td>
</tr>
<tr>
<td>Angiotensin</td>
<td>1.1 ± 0.1</td>
<td>1</td>
<td>---d</td>
</tr>
<tr>
<td>Trifluoperazine (TFP)</td>
<td>2.2 ± 0.3</td>
<td>4+</td>
<td>---&quot;</td>
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</table>

[a] A description of the statistical analysis is available in the Supporting Information. [b] Published dissociation constants for CaM binding to M13, mastoparan, melittin. [c] While binding was confirmed for NPY and δ-toxin, accurate Kd determinations were not done. [d] There is no known interaction between CaM and these peptides. [e] Multiple TFP molecules bind cooperatively to CaM.

Conclusion

We have developed a new allosteric enzyme by inserting CaM into a previously established split point for BLA. In contrast to previous attempts to create allosteric enzymes by domain insertion, the initial identification of this switch structure was accomplished without the need to synthesize and analyze large libraries of mutants. Furthermore, the switch is activated to various degrees (up to 120-fold) by several peptides and one small molecule target with reported affinity for wt-CaM. This magnitude of ligand-dependent activation is on par with the some of the highest values reported for both natural and engineered allosteric enzymes. This represents the first example of a calmodulin-based allosteric biosensor designed to respond to peptide targets. We anticipate that the BLACaM architecture will be a fruitful starting point for the rational design or directed evolution of new highly specific and sensitive biosensors for use inside cells and in vitro.

Experimental Section

General: All reagents were purchased from Sigma and all enzymes from New England Biolabs unless otherwise noted. Molecular cloning techniques and protein analysis such as PCR and electrophoresis were performed as described in Sambrook and Russell.
Gene Construction of Calmodulin and β-lactamase Fusions: Fusions of calmodulin and β-lactamase were created by overlap extension PCR using the Phusion® DNA Polymerase (NEB) and primers from either Eurofins MWG Operon or Bioneer Corp (see Table 1). All constructs were then transformed into E. coli (New England Biolabs). Cloning was performed in the Turbo electrocompetent cell line (New England Biolabs). The calmodulin gene (human variant calm1) was obtained from Origene Technologies Inc. and TEM1 β-lactamase gene (bla) was cloned from the pDIM2 vector.[7]

We created constructs fusing calmodulin (CaM) with TEM1 β-lactamase at four different fusion points: 1) the protein complementation assay (PCA) split points at BLA 194 and 196, 2) the BLA N- and C-termini using the (PCA) fragments,[8,9] 3) fusion points of the RG13,[10,11] and 4) MBP317-347[12] maltose binding protein(MBP)-BLA allosteric switches discovered by Gunatas. PCR fragments of all BLA fragments were made using the TEM1 β-lactamase gene from the pDIM2 vector as a template and using primers to either fuse fragments directly to CaM or with short linkers DKS and GSAGG. The BLA fragments utilized for fusions P1-P4 were the PCA fragments BLF1 (BLA 24-194) and BLF2 (BLA196-286), for fusions P5-P6 were fragments from RG13 (BLA 24-226 and 227-286), and for P7 and P8 from MBP317-347 (BLA 24-170 and BLA 170-286). All fragments were created by PCR using the following primers in Table S1. The human calmodulin gene was amplified for 0.5-0.6 and induced with 1 mM IPTG and protein was expressed for an additional 4 hours. Cells were then spun down and frozen overnight. Cells were then resuspended in 2.5 ml lysis buffer (50 mM Tris, pH 7.5 and cOmplete EDTA-free protease inhibitor cocktail (Roche)) and lysates were digested by freeze/thaw method and clarified by centrifugation. After clarification, the lysates’ total protein concentrations were determined using the Bio-Rad Quick Start Bradford Protein Assay kit. Samples were diluted to a total protein concentration of ~275 µg/ml and 25 µl was mixed with 100 µl of 50 mM Tris-CI buffer with mixtures containing final concentrations of either 1 mM EGTA, 5 mM CaCl₂, or 5 mM CaCl₂ plus 1 µM M13 peptide (N=3 for each condition). Mixtures were then incubated for 1 hr at room temperature. After incubation 100 µl of the mixture was removed to be used to determine Δε,

Construction of BLACaM gene constructs and controls: For purification of the BLACaM fusion, an optimized version of the gene for expression in *E. coli* was designed using Gene Designer software from DNA 2.0 and synthesized by Biotek. This gene version was cloned into the pET30a vector (EMD Millipore) appending a 6x His tag. All controls for expression were also cloned into the pET30a vector appending the 6x His tag and under control of a T7 promoter. CaM, BLA2-CaM, and CaM-BLA1 controls were created by cloning sections of the optimized BLACaM gene using primer pairs BLA2opt-for/CalmI(SpeI)-rev and CalmI(SpeI)-for/CalmI(SpeI)-rev primers, digesting and inserting into the pDIMC8 vector.

BLACaM expression and purification: An optimized version of BLACaM for expression in *E. coli* in the pET30a vector (EMD Millipore) attached to a 6x His tag and transformed into Shuffle T7 LysY cells (New England Biolabs). Cells were grown in LB media supplemented with 50 µg/ml kanamycin until an OD of 0.6-0.8 at 30°C. Protein expression was then induced with 0.4 mM IPTG and expressed at 18°C for 16-18 hrs. Cells were resuspended in lysis buffer (50 mM Tris-CL, pH 7.4 with 300 mM NaCl, 20 mM imidazole, and cOmplete EDTA-free protease inhibitor cocktail (Roche),) lysed by sonication, and spun down to clarify lysate. Before application to the gravity column with NlNTa agarose (Qiagen), Tween-20 was added to the lysate to a 1% concentration. Lysate was applied to the column equilibrated with 5 column volumes of 50 mM Tris-CL, pH 7.4 with 300 mM NaCl, 20 mM imidazole and with 5 column volumes of the same buffer after lystate application. Column was then washed with an in addition 5 column volumes of buffer without teween but containing 50 mM imidazole, and then eluted with 3 column volumes of buffer containing 50 mM imidazole.

BLF2-CaM and CaM-BLF1 controls expression and purification: The pET30a BLF2-CaM vector was transformed into BL21 (DE3) cells (Invitrogen) while the pET30a BLA-CaM vector was transformed into SHuffle T7 lysiY cells (New England Biolabs) for expression. Controls were expressed and purified using the same protocol as for BLACaM except that the BL21 (DE3) cells were originally grown at 37°C instead of the 30°C at which SHuffle T7 lysiY cells are grown.

Calmodulin (CaM) expression and purification: The pET30a with optimized CaM was transformed into BL21 (DE3) cells and expressed using same procedure as BLACaM. Purification of CaM was performed essentially as described by Gopalakrishna and Anderson.[28]

TEM1 β-lactamase (BLA) expression and purification: The pET30a BLA was transformed into BL21 (DE3) cells for expression. Cells were grown to an OD of 0.6-0.8 and induced with 0.4 mM IPTG. BLA was expressed at 37°C for 4 hours. Protein was purified on a Ni-NTA agarose column in protocol similar to BLACaM however no TWEEN-20 was used in any buffers.

Lysate Screening Assays: DH5α cells were transformed with pDIMC8 plasmids with various CaM-BLA fusion constructs as well as a plasmid containing wt-bla and wt-calm1 were grown in 50 ml LB media supplemented with 50 µg/ml chloramphenicol. Cells were grown at 37°C to an OD at 600nm to approximately 0.5-0.6 and induced with 1 mM IPTG and protein was expressed for an additional 4 hours. Cells were then spun down and frozen overnight. Cells were then resuspended in 2.5 ml lysis buffer (50 mM Tris-CL, pH 7.5 and cOmplete EDTA-free protease inhibitor cocktail (Roche)) and lysates were lyzed by freeze/thaw method and clarified by centrifugation. After clarification, the lysates’ total protein concentrations were determined using the Bio-Rad Quick Start Bradford Protein Assay kit. Samples were diluted to a total protein concentration of ~725 µg/ml and 25 µl was mixed with 100 µl of 50 mM Tris-CL buffer with mixtures containing final concentrations of either 1 mM EGTA, 5 mM CaCl₂, or 5 mM CaCl₂ plus 1 µM M13 peptide (N=3 for each condition). Mixtures were then incubated for 1 hr at room temperature. After incubation 100 µl of the mixture was removed to be used to determine Δε, extinction coefficient:

BLACaM Activity Assays: Assays were done in the presence of 20 nM HEPES with either 5 mM CaCl₂, 1 mM EGTA, or 5 mM CaCl₂ and peptide at 500 nM or trifluoperazin (TFP) at 1µM unless otherwise noted. To determine switching activity, assays were performed for 3 different batches of purified BLACaM with multiple runs (n = 3 to 8) for each peptide target and buffer condition. Δε was calculated for each run using the Cary WinUV Software and batch switching activity (V<sub>0</sub>/V<sub>1</sub>) was calculated and standard deviation of the mean (SDM) determined. Reported SA values were then calculated using a weighted average of the three batch’s values. Dose dependent V<sub>0</sub> data using different concentrations of M13 peptide were averaged from multiple runs.
(n = 4). Alternatively, assays were also performed in 96 well microassay plates using the same concentrations and general procedures but with only 100 µl total volume and absorbances read using the Biotek Synergy H1 Hybrid plate reader.

Acknowledgements

The authors would like to thank the National Science Foundation (CBET - 1158784) for supporting this work.

Keywords: (allostery · biosensors · calmodulin · domain insertion · protein engineering)


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Glenna E. Meister and Neel S. Joshi*

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Engineered Calmodulin-Based Allosteric Switch for Peptide Biosensing

A calmodulin based switch detects peptides by switching on β-lactamase activity in the presence of both calcium and the peptide.
SI Table 1: Primers used in cloning. Primers were purchased from either Eurofins MWG Operon or Bioneer Corp.

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<td><strong>P5 and P6 Primers</strong></td>
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<td><strong>Control and Expression Primers</strong></td>
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<tr>
<td>BLA10p(XhoI)-rev</td>
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Figure S1. Schematic of β-lactamase-calmodulin fusions. Calmodulin (blue) fused to either PCA fragments BLF1 (purple) and BLF2 (green), or other BLA fragments (grey). P1 and P2 also contain the wt-BLA periplasmic export sequence (orange).
Figure S2. SDS-PAGE confirming the purity of BLACaM after codon optimization, expression in *E. coli*, and affinity purification.
Figure S3. Buffer condition effects on CENTA™ hydrolysis activity of wt-BLA and wt-CaM proteins. Substrate hydrolysis by BLA, CaM, and BLA and CaM combined is monitored as absorbance at 405 nm in conditions such as (a) EGTA or calcium only and (b) calcium and the peptides M13 (smMLCK), mastoparan, mellitin, δ-toxin, neuropeptide Y (NPY), luteinizing-hormone releasing-hormone (LH-RH), angiotensin, and small molecule trifluoperazine (TFP).
Figure S4. Results from control experiments to monitor the propensity for BLACaM fragments to spontaneously assemble in an intermolecular fashion and reconstitute an active enzyme. a) Schematic depicting the BLACaM fragments BLF1-CaM and CaM-BLF21. b) SDS page gel of the purified fragments after expression in *E. coli* and affinity purification. c) Total substrate hydrolysis was monitored in HEPES buffer for each of the fragments individually and combined at varying equimolar concentrations. Sample absorbances were normalized to blank samples of the assay substrate in the absence of any enzyme.
Figure S5. Optimization of activity assay conditions for BLACaM. a) Hydrolysis of CENTA™ substrate by BLACaM was monitored directly after the purification procedure after incubation for different lengths of time in the presence of 5 mM calcium. b) Comparison of BLACaM activity after preincubation for 60 min with calcium alone (red), or calcium plus M13 (purple). In order to monitor enzyme activation in real time, the assay was also performed with a preincubation for 60 min in calcium alone and addition of M13 to the assay solution 5 min after the start of the assay (green).
Figure S6. Effects of temperature on BLACaM activity. a) BLACaM with either 5 mM calcium (purple) or 5 mM calcium and M13 (green) was preincubated at 37°C for 10 min in 5 mM Ca²⁺, then equilibrated at 25°C before CENTA™ assays were performed at 25°C. Alternatively BLACaM was preincubated with calcium for 50 minutes at room temperature, followed by a 10 min equilibration at either 20, 25, 30 or 37°C before assays were performed at the same temperatures. Assays were performed in the presence of 1 mM EGTA (b), 5 mM calcium (c), or 5 mM calcium plus 500 nM M13 peptide (d).