Bioconjugate Chemistry

Article

pubs.acs.org/bc

Bioorthogonal Chemoenzymatic Functionalization of Calmodulin for Bioconjugation Applications

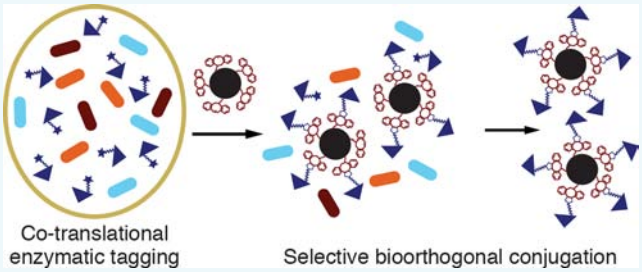
Chethana Kulkarni,[†,#] Megan Lo,[†,¶] Julia G. Fraseur,[‡] David A. Tirrell,[†] and Tamara L. Kinzer-Ursem*[,†,‡]

[†]Division of Chemistry and Chemical Engineering, California Institute of Technology, 1200 East California Blvd., Pasadena, California 91125, United States

[‡]Weldon School of Biomedical Engineering, Purdue University, 206 South Martin Jischke Drive, West Lafayette, Indiana 47907, United States

Supporting Information

ABSTRACT: Calmodulin (CaM) is a widely studied Ca^{2+}-binding protein that is highly conserved across species and involved in many biological processes, including vesicle release, cell proliferation, and apoptosis. To facilitate biophysical studies of CaM, researchers have tagged and mutated CaM at various sites, enabling its conjugation to fluorophores, microarrays, and other reactive partners. However, previous attempts to add a reactive label to CaM for downstream studies have generally employed nonselective labeling methods or resulted in diminished CaM function. Here we report the first engineered CaM protein that undergoes site-specific and bioorthogonal labeling while retaining wild-type activity levels. By employing a chemoenzymatic labeling approach, we achieved selective and quantitative labeling of the engineered CaM protein with an N-terminal 12-azidododecanoic acid tag; notably, addition of the tag did not interfere with the ability of CaM to bind Ca^{2+} or a partner protein. The specificity of our chemoenzymatic labeling approach also allowed for selective conjugation of CaM to reactive partners in bacterial cell lysates, without intermediate purification of the engineered protein. Additionally, we prepared CaM-affinity resins that were highly effective in purifying a representative CaM-binding protein, demonstrating that the engineered CaM remains active even after surface capture. Beyond studies of CaM and CaM-binding proteins, the protein engineering and surface capture methods described here should be translatable to other proteins and other bioconjugation applications.



INTRODUCTION

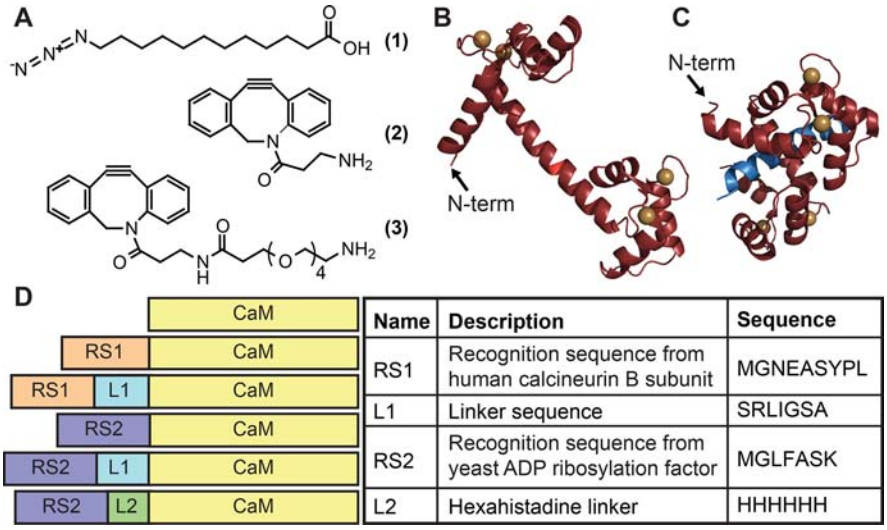
Chemoenzymatic protein labeling exploits the inherent selectivity of enzymes to achieve site-specific incorporation of functional tags into proteins of interest. Proteins can be labeled co-translationally or post-translationally *in vivo*, or in complex *in vitro* mixtures, with bioorthogonal functional groups that are useful for downstream bioconjugation applications (see Rashidian et al.[3] for a recent review). Previously, Tate and co-workers showed that the N-myristoyl transferase (NMT) enzyme, when recombinantly co-expressed with a natural substrate protein, appends functionalized fatty acid analogs to the substrate protein.[4,5] In a recent report, we advanced the utility of NMT for protein labeling purposes by demonstrating that NMT also transfers a bioorthogonal tag, 12-azidododecanoic acid (ADA; Figure 1A, compound **1**), to the N-terminus of non-natural substrate proteins engineered to display an NMT recognition sequence.[6] In the current work, we investigated whether we could engineer a model calcium (Ca^{2+})-binding protein, calmodulin (CaM), for NMT-mediated labeling without loss of activity.

Ca^{2+}-binding proteins play important roles in mediating intracellular and intercellular communication, as well as in activating various protein−protein interactions and corresponding biological processes. Perhaps the most widely studied Ca^{2+}-binding protein is CaM, a small (16 kDa) and ubiquitous Ca^{2+} sensor that binds and activates more than 100 other proteins.[7−10] CaM is highly conserved across evolution: its amino acid sequence is identical in all vertebrates.[11,12] CaM is also found in nearly every tissue of the human body.[11,13] Indeed, many cellular and physiological processes—ranging from cell proliferation, vesicle release, and apoptosis to neuronal signaling and muscle contraction—depend on Ca^{2+}/CaM-activated proteins.[9,14−18] While there are many CaM-binding proteins with well-characterized functions, especially in neuronal and muscle tissues,[9,10,17] there are also a number of Ca^{2+}/CaM-activated proteins whose biochemical functions and physiological roles are poorly understood.[18−21] Thus, there is a need for streamlined methods that enable investigations of CaM, CaM-binding proteins, and their respective interactions.

Previous studies of CaM biochemical function have generally employed established protein labeling/protein fusion strategies to measure the binding of Ca^{2+} to CaM, and subsequent

Received: August 10, 2015
Revised: September 11, 2015
Published: October 2, 2015

DOI: 10.1021/acs.bioconjchem.5b00449
Bioconjugate Chem. 2015, 26, 2153−2160



Name	Description	Sequence
RS1	Recognition sequence from human calcineurin B subunit	MGNEASYPL
L1	Linker sequence	SRLIGSA
RS2	Recognition sequence from yeast ADP ribosylation factor	MGLFASK
L2	Hexahistadine linker	HHHHHH

Figure 1. Schematic representation of reagents and proteins. (A) Reagents used in this study: 12-azidododecanoic acid (ADA, **1**), azadibenzocyclooctyne-amine (ADIBO-NH_2, **2**), and azadibenzocyclooctyne-$(PEG)_4$-amine (ADIBO-PEG-NH_2, **3**). (B,C) CaM crystal structures, with CaM shown in red and Ca^{2+} ions shown as gold spheres; arrows point to the N-terminus of CaM. (B) Ca^{2+}-bound CaM, PDB ID: 1CLL.[1] (C) CaM bound to a peptide (blue helix) derived from the regulatory domain of calcineurin, PDB ID: 4Q5U.[2] (D) CaM was engineered to display NMT recognition sequences (RS1 and RS2) and linker regions (L1 and L2) to produce a small library of enzymatically labeled, ADA-tagged proteins.

binding of CaM to downstream proteins.[22−28] Many of these studies have taken advantage of the fact that CaM is a small protein with relatively few reactive amino acids; vertebrate CaM, in particular, possesses no Cys residues. Thus, selective labeling can be achieved at Cys sites that are introduced in mutated vertebrate CaM or that are present in nonvertebrate CaM.[26,28] Through the precise introduction into CaM of small fluorescent probes that are sensitive to their immediate microenvironment, researchers have elegantly elucidated the biochemical mechanisms underpinning the binding steps in which CaM participates. However, a survey of these extrinsic labeling techniques reveals three main drawbacks: they generally require purification of CaM both before and after labeling; they are nonselective in either amino acid site or side-chain chemistry; and most significantly, they can diminish the ability of CaM to bind and activate its target proteins.[25,27] Therefore, a method enabling bioorthogonal and site-specific functionalization of CaM, without disruption of CaM activity, would greatly benefit the Ca^{2+} and CaM research communities. For example, controlled attachment of CaM to biosensor surfaces in a single orientation would permit downstream functional assays with a uniform CaM array. Furthermore, the use of a bioorthogonal tag would allow for selective conjugation in lysate samples or cells, thus circumventing an extra purification step and providing a platform for live-cell conjugation experiments with CaM.

We sought to achieve site-specific, bioorthogonal labeling of CaM while maintaining wild-type levels of binding and activity by taking advantage of recent advances in chemical biology. We selected an NMT-based chemoenzymatic labeling strategy in part because the N-terminus of CaM appears solvent-accessible in both the Ca^{2+}-bound and protein-bound states (Figure 1B and C). Our previous work also demonstrated that quantitative labeling of engineered, non-natural substrate proteins may be attained with NMT.[6] In addition, NMT-mediated protein labeling is especially well-suited for a key objective of the current studies: surface capture of CaM for downstream applications. Because NMT is orthogonal toward bacterial proteins,[29] surface capture of an ADA-tagged protein can be achieved directly from lysate,[6] thus simplifying experimental protocols and potentially improving protein yields.

Here, we report the preparation of an engineered CaM protein that is labeled site-specifically, selectively, and quantitatively by NMT with ADA with no loss of wild-type binding and activity levels. We also present a surface capture strategy enabling rapid generation of CaM-affinity resins that are highly effective for purifying a representative CaM-binding protein. The methods and advances described here provide a promising platform for future studies of CaM. Additionally, they constitute a general set of translatable techniques that should aid in the labeling and surface capture of other proteins.

RESULTS AND DISCUSSION

Preparation and LC-MS Characterization of Engineered CaM Proteins. We designed a family of CaM proteins that were engineered to display different N-terminal amino acid sequences (Figure 1D). An NMT recognition sequence was incorporated into each construct in order to achieve NMT-mediated protein labeling.[30] The selected recognition sequences were derived from two well-known NMT substrate proteins: human calcineurin B (CaN) and yeast ADP ribosylation factor.[31,32] In addition, two linker sequences were chosen for inclusion between the NMT recognition sequence and CaM itself: a linker employed in a previously reported CaM fusion protein,[33] and a hexahistadine tag that could aid in protein purification and identification.

In total, five engineered CaM constructs were cloned and co-expressed with human NMT1 in *Escherichia coli* using LB medium supplemented with ADA. Wild-type (WT) CaM and the engineered CaM proteins were purified on phenyl sepharose resin (SI Figure S1). Of the five engineered CaM proteins, the highest yield of purified protein was consistently achieved for ADA-RS1-CaM (SI Table S1). Notably, the ADA label did not interfere with CaM purification, which depends on hydrophobic interactions between the protein and resin.[34] In addition, the ADA label does not appear to affect protein solubility; both in the current work and in our previous

DOI: 10.1021/acs.bioconjchem.5b00449
Bioconjugate Chem. 2015, 26, 2153−2160

research, we have not observed ADA-labeled proteins precipitating from solution,[6] a result that is in agreement with related reports.[4,5,35]

We have previously shown that NMT appends ADA specifically to the N-terminus of non-natural substrate proteins engineered to display an NMT recognition sequence.[6] To determine the extent of NMT-mediated labeling of the engineered CaM proteins, purified CaM proteins were analyzed by whole-protein liquid chromatography−mass spectrometry (LC-MS). The results confirmed that all five engineered constructs were labeled with ADA by NMT in a near-quantitative manner (Table 1).

Table 1. Engineered CaM Proteins Are Labeled with ADA *In Situ* with High Efficiency

protein	expected mass (Da)	observed mass (Da)	% labeled[a]
ADA-RS1-CaM	17,735.02	17,731.59	>98%
ADA-RS1-L1-CaM	18,419.83	18,416.77	93%
ADA-RS2-CaM	17,506.84	17,504.05	88%
ADA-RS2-L1-CaM	18,191.65	18,188.88	>98%
ADA-RS2-L2-CaM	18,329.68	18,327.36	>98%

[a]Calculated from the relative abundance of peaks corresponding to labeled and unlabeled forms of each protein.

Engineered CaM Proteins Bind Ca^{2+} and Activate Calcineurin. We used an electrophoretic gel-shift assay[33,36] to examine the Ca^{2+}-binding ability of the ADA-labeled CaM constructs. CaM undergoes a shift in electrophoretic mobility in the presence of either excess Ca^{2+} or excess EDTA, a Ca^{2+} chelator. The shift is caused by the conformational change of CaM upon binding Ca^{2+}, which results in the protein running to a relatively lower mass during SDS-PAGE analysis. Conversely, in excess EDTA, CaM gives up its Ca^{2+} ions and runs to a relatively higher mass. Notably, the Ca^{2+}-induced conformational change is also required for binding by CaM of most target proteins. We observed the same shift for the five labeled, engineered CaM proteins as for WT CaM, suggesting that the engineered constructs bind Ca^{2+} in a manner similar to WT CaM (Figure 2A and B). The ADA label does not interfere with the conformational change.

Upon binding calcineurin (CaN), CaM activates CaN phosphatase activity by regulating the enzyme's response to Ca^{2+} transients and increasing its turnover rate (V_{max}).[37,38] To further characterize the behavior of the engineered CaM proteins, we tested their ability to activate CaN. Specifically, we performed CaN phosphatase activity assays, which measure the amount of free phosphate (PO_4^{3-}) generated upon incubation of CaN with a phosphopeptide substrate in the presence of CaM and Ca^{2+}; appreciable dephosphorylation of the phosphopeptide occurs only when CaN is bound and activated by CaM. For our assays, we varied either the Ca^{2+} concentration or the CaM concentration, while holding the other components constant (Figure 2C and D). Both the Ca^{2+}- and CaM-dependent assays demonstrated that ADA-RS1-CaM maintains the ability to activate CaN in a manner similar to WT CaM. However, CaN phosphatase activity does plateau at a lower level in the presence of some of the other engineered CaM constructs as compared to WT CaM. The EC_{50} and maximal percent activity values relative to WT CaM, summarized in Table S2, were estimated based on best fit to the Hill equation (eq S1, SI Materials and Methods) for each labeled, engineered CaM protein. Notably, previous studies have demonstrated that increasing Ca^{2+} and CaM concentrations increase the enzymatic activity of CaN but have little effect on the K_{act} or EC_{50} for CaN activity.[37] Similarly, our studies show that WT CaM and the engineered CaM proteins increase CaN enzymatic activity in a concentration-dependent manner (Figure 2). Together, the results of the activity assays show ADA-RS1-CaM to be equivalent to WT CaM; considering the small size of CaM, as well as its documented sensitivity to structural modifications,[25,27,39] it is quite significant that the labeled, engineered ADA-RS1-CaM protein

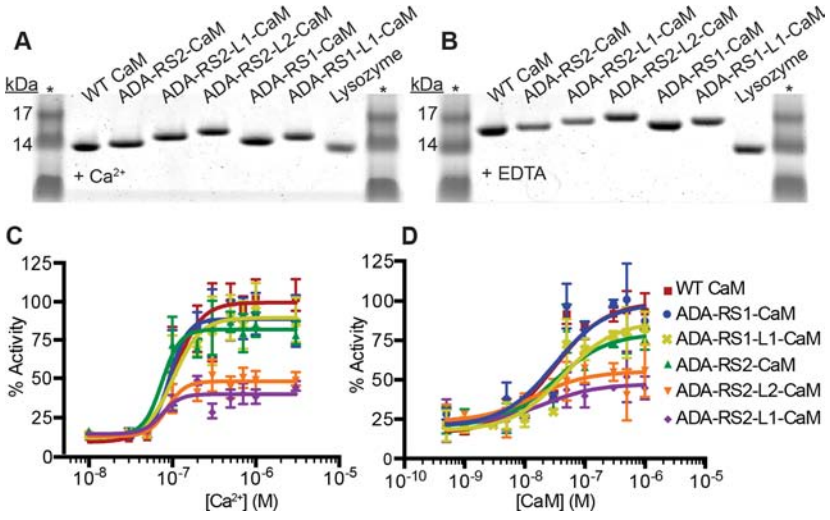


Figure 2. Ca^{2+}-binding and CaN activation by ADA-labeled CaM proteins. (A,B) Ability of ADA-labeled CaM proteins to bind Ca^{2+} was examined in an electrophoretic mobility assay. SDS-PAGE buffers contained (A) excess Ca^{2+} or (B) excess EDTA. Coomassie staining revealed a Ca^{2+}-dependent shift in apparent mass for all CaM proteins. Lysozyme served as a negative control for Ca^{2+}-dependent mobility. * = protein marker. (C,D) Ability of ADA-labeled CaM proteins to activate 10 nM CaN phosphatase activity at (C) varying Ca^{2+} ([CaM] = 1 uM) or (D) varying CaM ([Ca^{2+}] = 0.5 mM) concentrations is presented as % activity of WT CaM; $n \geq 4$. Error bars correspond to SD. The lines represent the best fits of the experimental data to the Hill equation (eq S1, SI Materials and Methods). Best-fit parameters are summarized in Table S2. No difference in activity is observed between ADA-RS1-CaM and WT CaM.

DOI: 10.1021/acs.bioconjchem.5b00449
Bioconjugate Chem. 2015, 26, 2153−2160

retains wild-type properties. Gratifyingly, the highest protein yields were also achieved with ADA-RS1-CaM, as noted earlier.

ADA-RS1-CaM Reacts Selectively in Lysate with an Alkyne Probe. After establishing that ADA-RS1-CaM is the most active of the labeled, engineered CaM constructs, we sought to assess the bioorthogonal reactivity of the ADA-RS1-CaM azide group. RS1-CaM and hNMT1 were coexpressed in the presence of ADA, and the cells were harvested and lysed. Then alkyne-TAMRA, a fluorescent probe (SI Figure S2), was reacted with the lysate in the standard copper-catalyzed azide–alkyne "click" reaction.[40−42] SDS-PAGE analysis of the probe-treated lysate revealed a single fluorescent band at the expected mass of ADA-RS1-CaM (Figure 3A, lane 2), confirming that

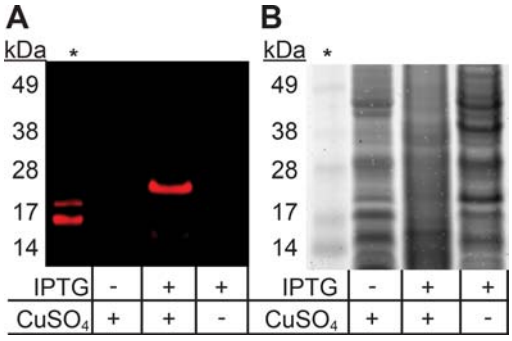


Figure 3. NMT selectively labels RS1-CaM in *E. coli*. RS1-CaM and NMT were co-expressed in the presence of ADA; expression of both RS1-CaM and NMT was controlled by IPTG-inducible promoters. Resultant lysates were reacted with alkyne-TAMRA in a copper-catalyzed "click" reaction, then analyzed via SDS-PAGE. * = protein marker (17 kDa band is myoglobin red). (A) Fluorescent band appears only at the MW of ADA-RS1-CaM (~18 kDa), only when protein expression is induced and the copper catalyst is included. (B) Coomassie staining shows protein loading across all lanes. The structure of alkyne-TAMRA is presented in Figure S2.

the azide moiety of ADA-RS1-CaM is reactive, and that the orthogonality of NMT in bacteria enables selective labeling of only ADA-RS1-CaM. Negative controls, in which protein expression was not induced or the copper catalyst was omitted from the reaction mixture, are dark (Figure 3A, lanes 1 and 3). These results suggest that it should be possible to achieve selective, covalent surface capture of a single protein, in this case ADA-RS1-CaM, directly from lysate, with no need for intermediate purification.

CaM-Sepharose Affinity Resins Prepared from ADA-RS1-CaM are Superior to Conventional Resins Prepared from Purified WT CaM. Since we had determined that ADA-RS1-CaM is highly expressed, quantitatively and selectively labeled in bacteria, similar to WT CaM in activity, and reactive toward alkyne probes, we proceeded to prepare CaM-affinity resins from ADA-RS1-CaM. Protein-affinity resins are critical components of many protein-purification protocols. For example, CaM-affinity resin is often employed in the purification of CaM-binding proteins—including CaN, CaM kinase, muscle light chain kinase (MLCK), and nitric oxide synthetases (eNOS and nNOS)—and has been utilized in the pull-down purification of proteins engineered to display CaM-binding peptides.[43−49] The preparation of protein-affinity resins is generally labor-, time-, and resource-intensive, partly because the protein used for resin preparation must itself be purified prior to reaction with the resin. Instead, we sought to prepare a protein-affinity resin directly from cell lysate (Figure 4A). We also anticipate that our methods could be translated to proteins other than CaM and to bioconjugation applications other than production of protein-affinity resins.

Six CaM-affinity resins were prepared and evaluated for their ability to bind and purify CaN, which served as a representative CaM-binding protein. Two resins were produced following the conventional approach of attaching purified WT CaM via its Lys side-chains to amine-reactive sepharose beads; specifically, we worked with amine-reactive resins that had been activated by treatment with cyanogen bromide (CNBr) or activated with *N*-hydroxy succinimidyl ester (NHS).[43,44] In addition, four resins were produced with lysate prepared from a recombinant expression of a CaM protein: NHS-activated resin was first functionalized with ADIBO-NH_2 or ADIBO-PEG-NH_2 (Figure 1A, compounds **2** and **3**) to take advantage of the strain-promoted azide–alkyne cycloaddition reaction,[50,51] then treated with lysate containing ADA-RS1-CaM or WT CaM. To ensure that all six resins were exposed to the same amount of CaM, whether purified or in lysate, the concentration of CaM in each lysate was determined (SI Figure S3). Finally, all six CaM-affinity resins were incubated with lysate resulting from a recombinant expression of CaN, washed, and CaN was eluted from the resin with EGTA buffer.

To evaluate the effectiveness of each resin for purifying CaN, we analyzed the CaN purification fractions by SDS-PAGE (Figure 4B–E; SI Figure S4). We were pleased to find that both resins produced from lysate containing ADA-RS1-CaM were highly effective in purifying CaN (Figure 4B; SI Figure S4A). Interestingly, a higher yield of CaN was obtained with ADA-RS1-CaM-resin prepared from ADIBO-PEG-resin versus ADIBO-resin (Table 2). The higher CaN yield associated with ADIBO-PEG-resin could be due to the increased distance between RS1-CaM and the resin beads, which could improve the accessibility of RS1-CaM for CaN binding. The hydrophilic PEG spacer may also reduce nonspecific hydrophobic interactions between the RS1-CaM protein and the resin, again improving the probability of successful CaM/CaN binding events. Both negative control resins, in which ADIBO-PEG-resin or ADIBO-resin was treated with WT CaM lysate, showed no evidence of eluted CaN (Figure 4C; SI Figure S4B). These data strongly suggest that the CaN purification results observed with ADA-RS1-CaM-resins are due to specific capture of ADA-RS1-CaM from lysate via the azide–alkyne cycloaddition. Finally, we found that both resins prepared from ADA-RS1-CaM lysate were much more effective for CaN purification than the two positive control resins prepared from purified WT CaM (Figure 4D and E). Measurement of purified CaN yields from each resin across independent replicates revealed that, on average, a 10-fold enhancement in CaN yield was achieved when using either ADA-RS1-CaM-resin versus either WT CaM resin (Table 2).

Taken together, our CaM-affinity resin experiments show that RS1-CaM remains active through protein engineering, labeling with ADA, and surface immobilization from lysate, thus establishing a streamlined approach to the preparation of CaM-affinity resins for purification of CaM-binding proteins. Our CaM-affinity resins would also be useful for pull-down of proteins engineered to display CaM-binding peptides; in fact, CaM-based tandem affinity purification strategies have been employed extensively to purify and identify protein complexes from bacterial, plant, and mammalian systems.[47,48] Furthermore, two key advantages of our methods are applicable to any protein engineered to display an NMT recognition sequence:

DOI: 10.1021/acs.bioconjchem.5b00449
Bioconjugate Chem. 2015, 26, 2153−2160

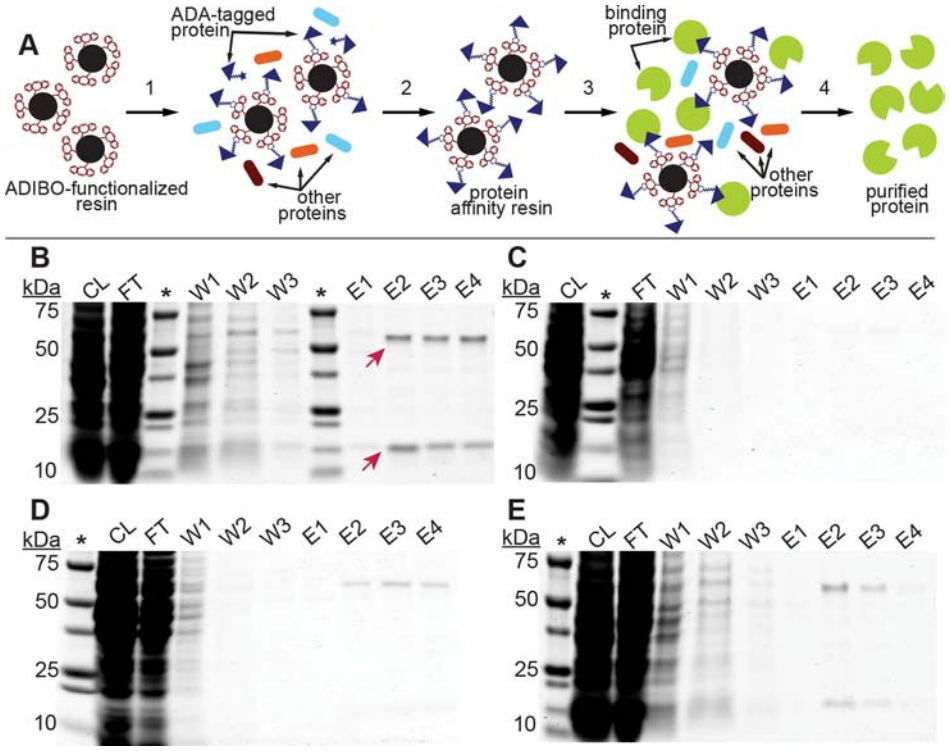


Figure 4. Preparation and use of protein-affinity resins. (A) Schematic representation showing preparation of a protein-affinity resin directly from bacterial lysate, followed by purification of a binding protein from lysate. (1) ADIBO-functionalized resin is incubated with clarified bacterial lysate containing an ADA-tagged protein, which reacts covalently with the ADIBO-resin via the strain-promoted azide−alkyne reaction. (2) Protein-affinity resin is washed and equilibrated with binding buffer. (3) Protein-affinity resin is incubated with clarified lysate containing the protein to be purified (binding protein). (4) Protein-affinity resin is washed, and the binding protein is eluted. (B−E) Representative results of CaN purification from lysate with the following CaM-affinity resins: (B) ADIBO-PEG-resin prepared by reacting NHS-activated resin with ADIBO-PEG-NH_2 and incubated with lysate containing ADA-RS1-CaM, (C) ADIBO-PEG-resin incubated with lysate containing WT CaM, (D) NHS-activated resin reacted with purified WT CaM, (E) CNBr-treated resin reacted with purified WT CaM. A larger quantity of CaN is recovered with resin prepared from ADA-RS1-CaM lysate than with either control resin prepared from purified WT CaM. CaN bands appear at the expected MW values for the CaN-A subunit (60 kDa) and CaN-B subunit (19 kDa), highlighted by red arrows. * = protein marker, CL = clarified lysate, FT = flow-through, W = wash, and E = elution.

Table 2. Purified CaN Yields from CaM Resins

resin	functionalization	CaM source	quantity of CaN[a] (μg)
NHS	ADIBO-PEG-NH_2	ADA-RS1-CaM lysate	190.0 ± 70.8
NHS	ADIBO-PEG-NH_2	WT CaM lysate	N.D.
NHS	ADIBO-NH_2	ADA-RS1-CaM lysate	127.1 ± 82.1
NHS	ADIBO-NH_2	WT CaM lysate	N.D.
NHS	-	Purified WT CaM	14.7 ± 12.8
CNBr	-	Purified WT CaM	16.6 ± 10.8

[a]Quantity of CaN purified from each resin in μg (mean ± SD); n = 4. Representative SDS-PAGE gels are shown in Figures 4 and S4. N.D. = not detected.

(1) NMT-mediated labeling enables surface capture directly from lysate, owing to the orthogonality of NMT toward endogenous bacterial proteins, and (2) site-specific conjugation of an ADA-labeled protein to a reactive partner, particularly a functionalized surface, may confer significant advantages over random attachment via primary amine chemistry or other nonspecific methods. Indeed, the latter point may be responsible for the difference in purified CaN yields between the ADA-RS1-CaM resins and the conventionally prepared WT CaM resins, since primary amine chemistry gives rise to a mixture of attachment points and, as a result, a variety of protein orientations on the resin. Thus, our work enables the purification of other CaM-binding proteins with ADA-RS1-CaM resins and the production of other protein resins in a similar fashion.

In summary, we have successfully engineered a CaM protein to accept a bioorthogonal chemical label (ADA) in an *E. coli* expression system, and we have shown that neither the RS1 peptide sequence nor the ADA tag interferes with the ability of CaM to bind Ca^{2+} and activate a downstream binding partner, calcineurin. Additionally, we have demonstrated that ADA-RS1-CaM retains its wild-type function even after surface immobilization. In ongoing work, we are implementing this strategy to incorporate CaM proteins into other surface-based assay formats; in particular, we are interested in probing the mechanisms underlying the ability of CaM to bind and activate other downstream partners in varying Ca^{2+} conditions. Based on the current work as well as previous research,[4−6,35] we anticipate that natural NMT substrate proteins and proteins that are engineered for NMT labeling may be tagged with chemically functional myristic acid analogs without reduction in their inherent function. Furthermore, labeling a protein with a bioorthogonal tag allows for conjugation of the protein directly from lysate without harsh purification conditions that may detrimentally affect protein function or result in decreased protein recovery. Such methods facilitate streamlined assay development and protein bioconjugation to fluorophores, enrichment tags, or biosensors to investigate protein structure,

DOI: 10.1021/acs.bioconjchem.5b00449
Bioconjugate Chem. 2015, 26, 2153−2160

measure protein function, and investigate protein−protein interactions.

MATERIALS AND METHODS

Full details regarding reagents and experimental procedures used in this study can be found in the Supporting Information file.

Cloning, Protein Expression, and Protein Labeling. The template plasmid for all engineered CaM constructs was pDrosCaM, pET-15b encoding *Drosophila melanogaster* WT CaM.[39,52] Primers encoding the NMT recognition sequences and linker sequences were used with the QuikChange site-directed mutagenesis kit (Agilent) to prepare plasmids encoding the engineered CaM proteins, following the Two-Step PCR method.[53] Plasmids were transformed into chemically competent BL21(DE3) *E. coli* cells already containing the pHV738 plasmid encoding human NMT1.[54] Expression cultures were grown in an incubator/shaker (37 °C, 250 rpm) in LB medium supplemented with 50 μg mL^{-1} kanamycin and 100 μg mL^{-1} ampicillin. Once cultures grew to an OD_{600} of 0.8−1.0, protein expression was induced with 1 mM IPTG, with simultaneous addition of 500 μM ADA (compound **1**, from a 500 mM stock in DMSO). After 3−4 h of protein expression, cells were harvested via centrifugation. Cell pellets were washed with cold PBS and stored at −80 °C until use. (See SI Table S3 for a summary of plasmids and SI Table S4 for a summary of strains.)

Preparation of CaM-Affinity Resins. CNBr-Sepharose and NHS-Sepharose (GE Healthcare Biosciences) were prepared for primary amine reaction according to the manufacturer's instructions. Resins made with purified CaM were prepared according to previously published methods.[43,44] Briefly, 1 column volume of 0.5 mg mL^{-1} purified porcine brain CaM in 50 mM HEPES (pH 7.5) was incubated with either CNBr-treated resin or NHS-activated resin for 2−3 h at 4 °C on a rotator. To prepare ADIBO- and ADIBO-PEG-functionalized resins, one column volume of 12.5 mM ADIBO-NH_2 or ADIBO-PEG-NH_2 (compound **2** or **3**) in 50% DMSO and 10 mM HEPES (pH 7.5) was added to the NHS-activated resin. All resins were quenched with 4 column volumes of 1 M Tris (pH 8.0) for 1 h. ADIBO- and ADIBO-PEG-resins were equilibrated for CaM coupling with 4 column volumes of 50 mM Tris (pH 7.5). Frozen cell pellets from 100 mL *E. coli* cultures expressing ADA-RS1-CaM or WT CaM were lysed in CaM Lysis Buffer (50 mM Tris [pH 7.5], 10 mM KCl, 0.5 mM EGTA, 0.5 mM EDTA, 1 mg mL^{-1} lysozyme, 10 units DNase) via sonication, then clarified via centrifugation. The total protein concentration of each clarified lysate was quantified with a Pierce 660 nm Protein Assay. One column volume of clarified lysate at 3.5 mg mL^{-1} total protein concentration (0.5 mg mL^{-1} estimated CaM concentration) was incubated with ADIBO- or ADIBO-PEG-resin overnight at 4 °C on a rotator. CaM-conjugated resins were washed 3 times with 10 column volumes of the following wash buffers: Wash 1 (100 mM ammonium carbonate [pH 8.6], 2 mM EGTA), Wash 2 (10 mM Tris [pH 7.5], 1 M NaCl, 2 mM $CaCl_2$), and Wash 3 (100 mM sodium acetate [pH 4.6], 2 mM $CaCl_2$). The resins were either equilibrated for CaN purification with Buffer A (25 mM Tris [pH 7.5], 2 mM $CaCl_2$, 0.1 mM EDTA, 0.5 mM DTT) or stored in 20% ethanol.

Purification and Quantification of CaN. CaN purification was performed according to previously published methods.[55] Briefly, a frozen cell pellet from a 500 mL *E. coli* culture expressing CaN was lysed in CaN Lysis Buffer (25 mM Tris [pH 7.5], 3 mM $MgCl_2$, 100 mM NaCl, 2 mM $CaCl_2$, 0.5 mM PMSF, Roche Complete Protease Inhibitor Cocktail, and 1 mg mL^{-1} lysozyme). Clarified lysate (2 mL) and 1 mL of resin equilibrated with Buffer A were incubated for 30 min at 4 °C on a rotator. Resins were washed sequentially with Buffer A, Buffer A plus 1 M NaCl, and Buffer A. During the final wash, resins were transferred to BioRad MicroSpin columns that were prewashed with CaN Lysis Buffer. Elution was accomplished with sequential additions of 0.5 mL EGTA Elution Buffer (25 mM Tris [pH 7.5], 3 mM $MgSO_4$, 1 mM EGTA, 0.5 mM DTT). Elution fractions were analyzed via SDS-PAGE and pooled. *Quantification:* The concentration of CaN in the pooled elution sample for each resin was determined via SDS-PAGE. Resin elution samples, as well as BSA (66 kDa) and lysozyme (14 kDa) standards, were run in duplicate on protein gels. A Li-Cor Odyssey system was employed to image the gels and quantify the integrated band intensities. Concentrations (in μg mL^{-1}) of the CaN-A subunit (60 kDa) and the CaN-B subunit (19 kDa) were interpolated from the BSA and lysozyme standard curves, respectively, then added together to yield the total concentration of purified CaN for each resin. Finally, the quantity of purified CaN (μg) for each resin was calculated as the concentration (μg mL^{-1}) multiplied by the pooled elution volume (mL).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00449.

Supporting figures and tables; detailed Materials and Methods, including purification of CaM proteins, summary of best-fit parameters for CaM proteins in CaN activity assays, quantitative Western blot analysis of ADA-RS1-CaM lysates, purification of CaN with other CaM-affinity resins, and summary of plasmids and bacterial strains developed for this study (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: tursem@purdue.edu. Phone: (765) 494-7330. Fax: (765) 496-1459.

Present Addresses

[#]Chethana Kulkarni, New England Biolabs, 240 County Rd, Ipswich, MA 01938

[¶]Megan Lo, University of California, San Francisco (UCSF), 600 16th Street, Box 2240, San Francisco, CA 94158

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank members of D.A.T.'s and T.K.U.'s research groups for helpful discussions. We are grateful to Prof. Stephen Mayo (Caltech) and Prof. Richard Kahn (Emory University) for their gifts of plasmids. The staff of the Proteome Exploration Laboratory of the Caltech Beckman Institute kindly assisted with mass spectrometry. Support for this work was provided by the Caltech Innovation Initiative (CI^2), the Jacobs Institute for Molecular Engineering for Medicine, a Caltech Summer Undergraduate Research Fellowship (to M.L.), the Purdue

DOI: 10.1021/acs.bioconjchem.5b00449
Bioconjugate Chem. 2015, 26, 2153−2160

Research Foundation, and a Purdue HHMI Summer Research Fellowship (to J.G.F.).

■ ABBREVIATIONS

12-azidododecanioic acid, ADA; Azadibenzocyclooctyne, ADIBO; Calcium ion, Ca^{2+}; Calmodulin, CaM; Calcineurin, CaN; Cyanogen bromide, CNBr; Dalton, Da; *Escherichia coli*, *E. coli*; Liquid Chromatography Mass Spectrometry, LC-MS; *N*-hydroxy succinimidyl ester, NHS; N-myristoyl transferase, NMT; Polyethelyne glycol, PEG; Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE; Standard deviation, SD

■ REFERENCES

(1) Chattopadhyaya, R., Meador, W. E., Means, A. R., and Quiocho, F. A. (1992) Calmodulin structure refined at 1.7 A resolution. *J. Mol. Biol. 228*, 1177−1192.

(2) Dunlap, T. B., Guo, H. F., Cook, E. C., Holbrook, E., Rumi-Masante, J., Lester, T. E., Colbert, C. L., Vander Kooi, C. W., and Creamer, T. P. (2014) Stoichiometry of the calcineurin regulatory domain-calmodulin complex. *Biochemistry 53*, 5779−5790.

(3) Rashidian, M., Dozier, J. K., and Distefano, M. D. (2013) Enzymatic labeling of proteins: techniques and approaches. *Bioconjugate Chem. 24*, 1277−1294.

(4) Heal, W. P., Wickramasinghe, S. R., Leatherbarrow, R. J., and Tate, E. W. (2008) N-Myristoyl transferase-mediated protein labelling in vivo. *Org. Biomol. Chem. 6*, 2308−2315.

(5) Heal, W. P., Wright, M. H., Thinon, E., and Tate, E. W. (2011) Multifunctional protein labeling via enzymatic N-terminal tagging and elaboration by click chemistry. *Nat. Protoc. 7*, 105−117.

(6) Kulkarni, C., Kinzer-Ursem, T. L., and Tirrell, D. A. (2013) Selective functionalization of the protein N terminus with N-myristoyl transferase for bioconjugation in cell lysate. *ChemBioChem 14*, 1958−1962.

(7) Villarroel, A., Taglialatela, M., Bernardo-Seisdedos, G., Alaimo, A., Agirre, J., Alberdi, A., Gomis-Perez, C., Soldovieri, M. V., Ambrosino, P., Malo, C., and Areso, P. (2014) The ever changing moods of calmodulin: how structural plasticity entails transductional adaptability. *J. Mol. Biol. 426*, 2717−2735.

(8) Tidow, H., and Nissen, P. (2013) Structural diversity of calmodulin binding to its target sites. *FEBS J. 280*, 5551−5565.

(9) Xia, Z., and Storm, D. R. (2005) The role of calmodulin as a signal integrator for synaptic plasticity. *Nat. Rev. Neurosci. 6*, 267−276.

(10) Chin, D., and Means, A. R. (2000) Calmodulin: a prototypical calcium sensor. *Trends Cell Biol. 10*, 322−328.

(11) Friedberg, F., and Rhoads, A. R. (2001) Evolutionary aspects of calmodulin. *IUBMB Life 51*, 215−221.

(12) Swan, D. G., Hale, R. S., Dhillon, N., and Leadlay, P. F. (1987) A bacterial calcium-binding protein homologous to calmodulin. *Nature 329*, 84−85.

(13) Toutenhoofd, S. L., and Strehler, E. E. (2000) The calmodulin multigene family as a unique case of genetic redundancy: multiple levels of regulation to provide spatial and temporal control of calmodulin pools? *Cell Calcium 28*, 83−96.

(14) Means, A. R., and Rasmussen, C. D. (1988) Calcium, calmodulin and cell proliferation. *Cell Calcium 9*, 313−319.

(15) Ui-Tei, K., Nagano, M., Sato, S., and Miyata, Y. (2000) Calmodulin-dependent and -independent apoptosis in cell of a Drosophila neuronal cell line. *Apoptosis 5*, 133−140.

(16) Maier, L. S., and Bers, D. M. (2002) Calcium, calmodulin, and calcium-calmodulin kinase II: heartbeat to heartbeat and beyond. *J. Mol. Cell. Cardiol. 34*, 919−939.

(17) Saucerman, J. J., and Bers, D. M. (2012) Calmodulin binding proteins provide domains of local Ca2+ signaling in cardiac myocytes. *J. Mol. Cell. Cardiol. 52*, 312−316.

(18) Pang, Z. P., Cao, P., Xu, W., and Sudhof, T. C. (2010) Calmodulin controls synaptic strength via presynaptic activation of calmodulin kinase II. *J. Neurosci. 30*, 4132−4142.

(19) Dadi, P. K., Vierra, N. C., Ustione, A., Piston, D. W., Colbran, R. J., and Jacobson, D. A. (2014) Inhibition of pancreatic beta-cell Ca2+/calmodulin-dependent protein kinase II reduces glucose-stimulated calcium influx and insulin secretion, impairing glucose tolerance. *J. Biol. Chem. 289*, 12435−12445.

(20) Zou, J., Salarian, M., Chen, Y., Veenstra, R., Louis, C. F., and Yang, J. J. (2014) Gap junction regulation by calmodulin. *FEBS Lett. 588*, 1430−1438.

(21) Berchtold, M. W., and Villalobo, A. (2014) The many faces of calmodulin in cell proliferation, programmed cell death, autophagy, and cancer. *Biochim. Biophys. Acta, Mol. Cell Res. 1843*, 398−435.

(22) Olwin, B. B., and Storm, D. R. (1983) Preparation of fluorescent labeled calmodulins. *Methods Enzymol. 102*, 148−157.

(23) Torok, K., Cowley, D. J., Brandmeier, B. D., Howell, S., Aitken, A., and Trentham, D. R. (1998) Inhibition of calmodulin-activated smooth-muscle myosin light-chain kinase by calmodulin-binding peptides and fluorescent (phosphodiesterase-activating) calmodulin derivatives. *Biochemistry 37*, 6188−6198.

(24) Torok, K., and Trentham, D. R. (1994) Mechanism of 2-chloro-(epsilon-amino-Lys75)-[6-[4-(N,N- diethylamino)phenyl]-1,3,5-triazin-4-yl]calmodulin interactions with smooth muscle myosin light chain kinase and derived peptides. *Biochemistry 33*, 12807−12820.

(25) Waxham, M. N., Tsai, A. L., and Putkey, J. A. (1998) A mechanism for calmodulin (CaM) trapping by CaM-kinase II defined by a family of CaM-binding peptides. *J. Biol. Chem. 273*, 17579−17584.

(26) Quintana, A. R., Wang, D., Forbes, J. E., and Waxham, M. N. (2005) Kinetics of calmodulin binding to calcineurin. *Biochem. Biophys. Res. Commun. 334*, 674−680.

(27) Meyer, T., Hanson, P. I., Stryer, L., and Schulman, H. (1992) Calmodulin trapping by calcium-calmodulin-dependent protein kinase. *Science 256*, 1199−1202.

(28) Mills, J. S., Walsh, M. P., Nemcek, K., and Johnson, J. D. (1988) Biologically active fluorescent derivatives of spinach calmodulin that report calmodulin target protein binding. *Biochemistry 27*, 991−996.

(29) Duronio, R. J., Jackson-Machelski, E., Heuckeroth, R. O., Olins, P. O., Devine, C. S., Yonemoto, W., Slice, L. W., Taylor, S. S., and Gordon, J. I. (1990) Protein N-myristoylation in Escherichia coli: reconstitution of a eukaryotic protein modification in bacteria. *Proc. Natl. Acad. Sci. U. S. A. 87*, 1506−1510.

(30) Boutin, J. A. (1997) Myristoylation. *Cell. Signalling 9*, 15−35.

(31) Kennedy, M. T., Brockman, H., and Rusnak, F. (1996) Contributions of myristoylation to calcineurin structure/function. *J. Biol. Chem. 271*, 26517−26521.

(32) Van Valkenburgh, H. A., and Kahn, R. A. (2002) Coexpression of proteins with methionine aminopeptidase and/or N-myristoyltransferase in Escherichia coli to increase acylation and homogeneity of protein preparations. *Methods Enzymol. 344*, 186−193.

(33) Li, C. J., Heim, R., Lu, P., Pu, Y., Tsien, R. Y., and Chang, D. C. (1999) Dynamic redistribution of calmodulin in HeLa cells during cell division as revealed by a GFP-calmodulin fusion protein technique. *J. Cell Sci. 112*, 1567−1577.

(34) Vogel, H. J., Lindahl, L., and Thulin, E. (1983) Calcium-dependent hydrophobic interaction chromatography of calmodulin, troponin C and their proteolytic fragments. *FEBS Lett. 157*, 241−246.

(35) Heal, W. P., Wickramasinghe, S. R., Bowyer, P. W., Holder, A. A., Smith, D. F., Leatherbarrow, R. J., and Tate, E. W. (2008) Site-specific N-terminal labelling of proteins in vitro and in vivo using N-myristoyl transferase and bioorthogonal ligation chemistry. *Chem. Commun. (Cambridge, U. K.)*, 480−482.

(36) Rhyner, J. A., Koller, M., Durussel-Gerber, I., Cox, J. A., and Strehler, E. E. (1992) Characterization of the human calmodulin-like protein expressed in Escherichia coli. *Biochemistry 31*, 12826−12832.

(37) Stemmer, P. M., and Klee, C. B. (1994) Dual calcium ion regulation of calcineurin by calmodulin and calcineurin B. *Biochemistry 33*, 6859−6866.

DOI: 10.1021/acs.bioconjchem.5b00449
Bioconjugate Chem. 2015, 26, 2153−2160

(38) Rumi-Masante, J., Rusinga, F. I., Lester, T. E., Dunlap, T. B., Williams, T. D., Dunker, A. K., Weis, D. D., and Creamer, T. P. (2012) Structural Basis for Activation of Calcineurin by Calmodulin. *J. Mol. Biol. 415*, 307−317.
(39) Shifman, J. M., and Mayo, S. L. (2002) Modulating calmodulin binding specificity through computational protein design. *J. Mol. Biol. 323*, 417−423.
(40) Tornoe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(i)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem. 67*, 3057−3064.
(41) Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem., Int. Ed. 41*, 2596−2599.
(42) Wang, Q., Chan, T. R., Hilgraf, R., Fokin, V. V., Sharpless, K. B., and Finn, M. G. (2003) Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J. Am. Chem. Soc. 125*, 3192−3193.
(43) Lukas, T. J., and Watterson, D. M. (1988) Purification of Calmodulin and Preparation of Immobilized Calmodulin. *Methods Enzymol. 157*, 328−339.
(44) Westcott, K. R., La Porte, D. C., and Storm, D. R. (1979) Resolution of adenylate cyclase sensitive and insensitive to Ca2+ and calcium-dependent regulatory protein (CDR) by CDR-sepharose affinity chromatography. *Proc. Natl. Acad. Sci. U. S. A. 76*, 204−208.
(45) Honey, S., Schneider, B. L., Schieltz, D. M., Yates, J. R., and Futcher, B. (2001) A novel multiple affinity purification tag and its use in identification of proteins associated with a cyclin-CDK complex. *Nucleic Acids Res. 29*, E24.
(46) Guerriero, V., Jr., Rowley, D. R., and Means, A. R. (1981) Production and characterization of an antibody to myosin light chain kinase and intracellular localization of the enzyme. *Cell 27*, 449−458.
(47) Gregan, J., Riedel, C. G., Petronczki, M., Cipak, L., Rumpf, C., Poser, I., Buchholz, F., Mechtler, K., and Nasmyth, K. (2007) Tandem affinity purification of functional TAP-tagged proteins from human cells. *Nat. Protoc. 2*, 1145−1151.
(48) Gavin, A.-C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A.-M., Cruciat, C.-M., Remor, M., Hofert, C., Schelder, M., Brajenovic, M., Ruffner, H., Merino, A., Klein, K., Hudak, M., Dickson, D., Rudi, T., Gnau, V., Bauch, A., Bastuck, S., Huhse, B., Leutwein, C., Heurtier, M.-A., Copley, R. R., Edelmann, A., Querfurth, E., Rybin, V., Drewes, G., Raida, M., Bouwmeester, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G., and Superti-Furga, G. (2002) Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature 415*, 141−147.
(49) Griffith, O. W., and Stuehr, D. J. (1995) Nitric oxide synthases: properties and catalytic mechanism. *Annu. Rev. Physiol. 57*, 707−736.
(50) Agard, N. J., Prescher, J. A., and Bertozzi, C. R. (2004) A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *J. Am. Chem. Soc. 126*, 15046−15047.
(51) Kuzmin, A., Poloukhtine, A., Wolfert, M. A., and Popik, V. V. (2010) Surface functionalization using catalyst-free azide-alkyne cycloaddition. *Bioconjugate Chem. 21*, 2076−2085.
(52) Shifman, J. M., and Mayo, S. L. (2003) Exploring the origins of binding specificity through the computational redesign of calmodulin. *Proc. Natl. Acad. Sci. U. S. A. 100*, 13274−13279.
(53) Wang, W., and Malcolm, B. A. (1999) Two-Stage PCR protocol allowing introduction of multiple mutations, deletions and insertions using QuikChange site-directed mutagenesis. *BioTechniques 26*, 680−682.
(54) Van Valkenburgh, H. A., and Kahn, R. A. (2002) Coexpression of proteins with methionine aminopeptidase and/or N-myristoyltransferase in Escherichia coli to increase acylation and homogeneity of protein preparations. *Methods Enzymol. 344*, 186−193.
(55) Mondragon, A., Griffith, E. C., Sun, L., Xiong, F., Armstrong, C., and Liu, J. O. (1997) Overexpression and purification of human calcineurin alpha from Escherichia coli and assessment of catalytic functions of residues surrounding the binuclear metal center. *Biochemistry 36*, 4934−4942.

DOI: 10.1021/acs.bioconjchem.5b00449
Bioconjugate Chem. 2015, 26, 2153−2160