SUPPORTING INFORMATION FOR:

Bioorthogonal Chemoenzymatic Functionalization of Calmodulin for Bioconjugation Applications

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SUPPORTING FIGURES AND TABLES

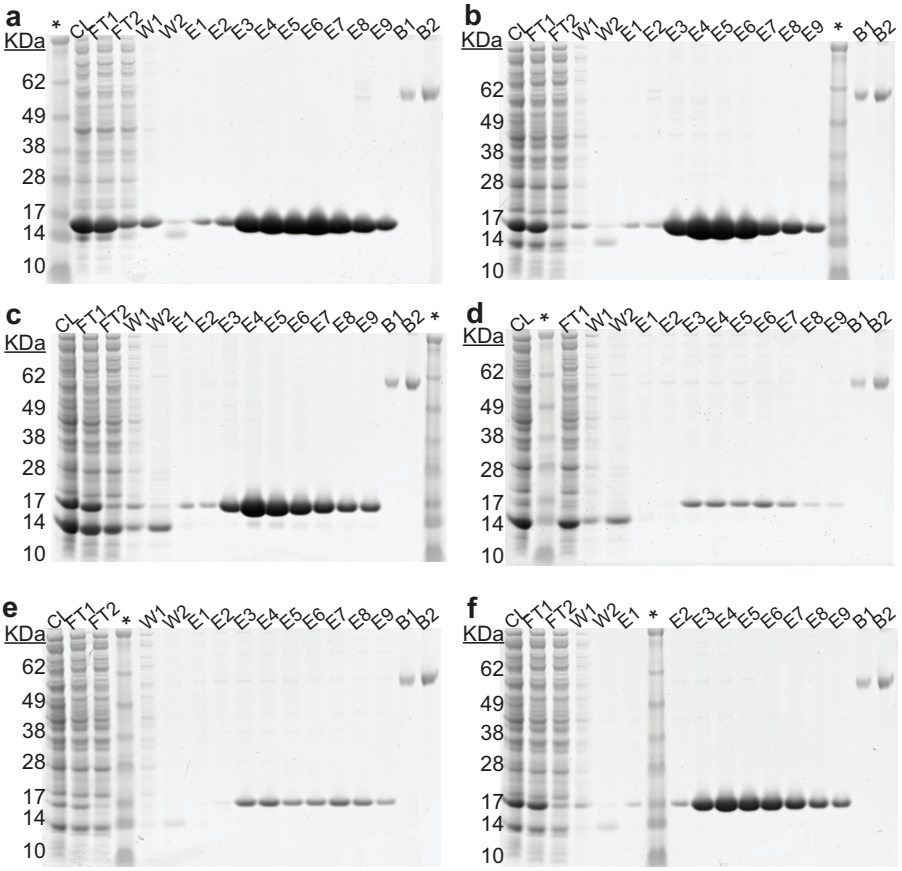


Fig. S1. SDS-PAGE analysis was performed for phenyl sepharose purification fractions collected during CaM purification from expression cultures of (a) WT CaM, (b) ADA-RS1-CaM, (c) ADA- RS1-L1-CaM, (d) ADA-RS2-CaM, (e) ADA-RS2-L1-CaM, and (f) ADA-RS2-L2-CaM. All CaM proteins except WT CaM were expressed in the presence of ADA for N-terminal labeling by NMT. Bands for the pure CaM proteins appear in the expected MW range of 16–19 kDa. *=protein marker, CL=clarified lysate, FT=flow-through, W=wash, E=elution, B1=0.05 mg mL^{-1} BSA, B2=0.1 mg mL^{-1} BSA.

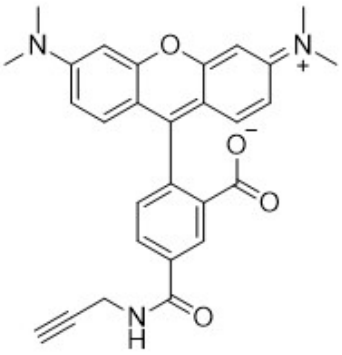
Table S1. Purified yields of CaM proteins.

Protein	Yield from 100 mL Culture (mg)
WT CaM	7.3
ADA-RS1-CaM	6.2
ADA-RS1-L1-CaM	4.5
ADA-RS2-CaM	0.2
ADA-RS2-L1-CaM	0.4
ADA-RS2-L2-CaM	3.5

Table S2. Summary of best-fit parameters for CaM proteins in Ca^{2+}-dependent and CaM-dependent CaN activity assays. [a]

Protein	[Ca^{2+}]-Dependent Assay		[CaM]-Dependent Assay	
	EC_{50} (nM)	Max Activity (%)	EC_{50} (nM)	Max Activity (%)
WT CaM	107 ± 12	100 ± 14	31 ± 14	100 ± 11
ADA-RS1-CaM	93 ± 12	89 ± 12	31 ± 14	99 ± 11
ADA-RS1-L1-CaM	106 ± 12	90 ± 14	43 ± 16	87 ± 13
ADA-RS2-CaM	75 ± 12	82 ± 8	33 ± 17	80 ± 11
ADA-RS2-L2-CaM	87 ± 12	48 ± 5	15 ± 34	55 ± 12
ADA-RS2-L1-CaM	79 ± 13	40 ± 4	22 ± 34	47 ± 10

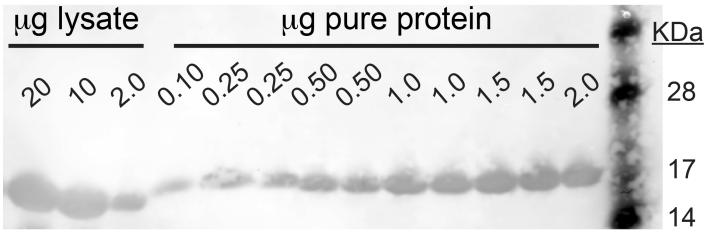
[a] Data from the CaN activity assays were fit to a Hill equation (Eqn. S1, SI Materials and Methods), yielding the EC_{50} (the Ca^{2+} or CaM concentration giving 50% maximal activity), maximal activity, and N (the Hill coefficient). For the Ca^{2+}-dependent assay, N was found to be 2.5-4 for all CaM proteins, similar to previously published values [1, 2]. For the CaM-dependent assay, N was set to 1. All data are presented as mean ± SD. The corresponding graphs of activity data are presented in Fig. 2.



Alkyne-TAMRA

Molecular Weight: 467.52

Fig. S2. The alkyne-TAMRA probe was reacted with lysates containing ADA-RS1-CaM for selective visualization of the ADA-tagged protein, via the copper-catalyzed azide-alkyne "click" reaction. The corresponding SDS-PAGE results are presented in Fig. 3.



Total protein loaded (μg)	ADA-RS1-CaM calculated (μg)	ADA-RS1-CaM in cell lysate (%)
2	0.5	25
10	1.6	16
20	3.0	15
		Average 18.6

Fig. S3. Quantitative Western blot analysis of cell lysates containing ADA-RS1-CaM.

Western blot: The indicated amounts of clarified cell lysate from a bacterial expression culture of ADA-RS1-CaM were analyzed alongside known quantities of purified CaM.

Table: The amount of ADA-RS1-CaM in each lysate sample was determined based on a standard curve prepared from the pure CaM band intensities.

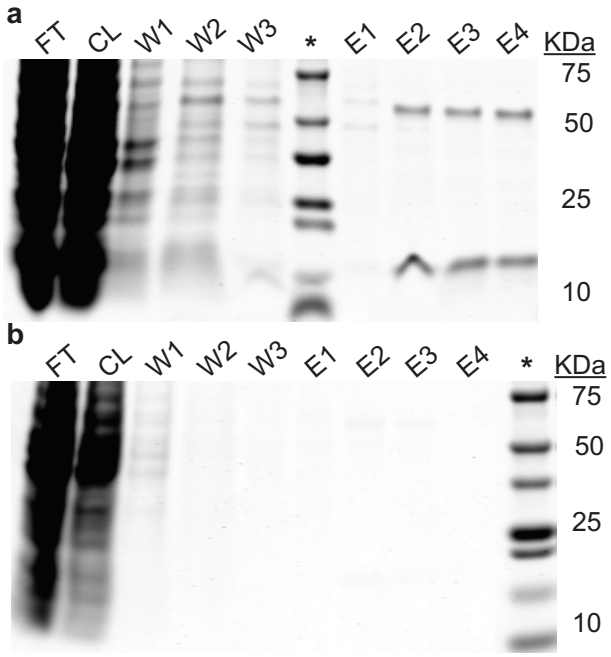


Fig. S4. Purification of CaN with CaM-affinity resins. Representative results of CaN purification from lysate with (a) ADIBO-resin prepared by reacting NHS-activated resin with ADIBO-NH_2 and incubated with lysate containing ADA-RS1-CaM, or (b) ADIBO-resin incubated with lysate containing WT CaM. Effective purification of CaN is achieved with ADIBO-resin that has been incubated with ADA-RS1-CaM, but not with WT CaM, suggesting that only ADA-RS1-CaM is covalently captured by ADIBO-resin. CaN bands appear at the expected MW values for the CaN-A subunit (60 kDa) and CaN-B subunit (19 kDa). *=protein marker, CL=clarified lysate, FT=flow-through, W=wash, and E=elution.

Table S3. Summary of plasmids developed for this study.

Construct[a]	NMT Recognition Sequence[b] Name (Sequence)	Linker Sequence[c] Name (Sequence)
pRS1CaM	RS1 (MGNEASYPL)	—
pRS1L1CaM	RS1 (MGNEASYPL)	L1 (SRLIGSA)
pRS2CaM	RS2 (MGLFASK)	—
pRS2L1CaM	RS2 (MGLFASK)	L1 (SRLIGSA)
pRS2L2CaM	RS2 (MGLFASK)	L2 (HHHHHH)

[a] The parent plasmid for the five engineered CaM constructs was pDrosCaM (pET-15b encoding *Drosophila melanogaster* WT CaM), with NMT recognition sequences and linker sequences inserted directly upstream of the CaM gene.

[b] RS1 (MGNEASYPL) was encoded by ATG GGT AAC GAA GCG TCT TAC CCG CTG in primers. RS2 (MGLFASK) was encoded by ATG GGT CTG TTC GCG TCT AAA in primers.

[c] L1 (SRLIGSA) was encoded by TCT CGT CTG ATC GGT TCT GCT in primers. L2 (HHHHHH) was encoded by CAT CAC CAT CAC CAT CAC in primers.

Table S4. Summary of bacterial strains developed for this study.

Strain	Proteins expressed upon induction with IPTG
BL21(DE3)/pRS1CaM–pHV738	RS1-CaM and hNMT1
BL21(DE3)/pRS1L1CaM–pHV738	RS1-L1-CaM and hNMT1
BL21(DE3)/pRS2CaM–pHV738	RS2-CaM and hNMT1
BL21(DE3)/pRS2L1CaM–pHV738	RS2-L1-CaM and hNMT1
BL21(DE3)/pRS2L2CaM–pHV738	RS2-L2-CaM and hNMT1

SUPPORTING MATERIALS AND METHODS

Reagents

The pDrosCaM plasmid (pET-15b encoding *Drosophila melanogaster* wild-type CaM) was a gift from Prof. Stephen Mayo [3, 4]. The pHumCaN plasmid (pET-15b plasmid encoding human calcineurin (CaN)) was obtained from Addgene (reference number 11787) [5]. The pHV738 plasmid encoding human N-myristoyl transferase (hNMT1) and *E. coli* methionine aminopeptidase (MetAP) was a gift from Prof. Richard Kahn [6]. Primers were ordered from IDT. The QuikChange II Site-Directed Mutagenesis Kit was purchased from Agilent. Chemically competent DH5-alpha and BL21(DE3) *E. coli* cells were purchased from New England Biolabs. The Click-iT kit, which includes alkyne-TAMRA, was purchased from Invitrogen/Life Technologies. Sepharose resins were purchased from GE Healthcare Life Sciences. Porcine brain calmodulin was purchased from Enzo Life Sciences. Azadibenzocyclooctyne-amine (ADIBO-NH_2) and azadibenzocyclooctyne-$(PEG)_4$-amine (ADIBO-PEG-NH_2) were purchased from Click Chemistry Tools. All SDS-PAGE gels are NuPAGE Novex 4–12% Bis-Tris pre-cast gels purchased from Invitrogen/Life Technologies. The SeeBlue Plus2 (Invitrogen/Life Technologies) and Precision Plus Dual Color Protein Standard (BioRad) protein molecular weight markers were used for SDS-PAGE. For Western blotting, the Rabbit Anti-CaM monoclonal antibody (EP799Y, Abcam ab45689) and Goat Anti-Rabbit/Cy5 IgG antibody (Abcam ab6564) were purchased from Abcam. All other chemicals were reagent grade purchased from Sigma-Aldrich.

Synthesis of 12-Azidododecanoic Acid (ADA)

ADA was synthesized as previously described [7, 8] with minor modifications; in particular, flash chromatography was found to be unnecessary to obtain pure product. Briefly, 12-bromododecanoic acid (201.7 mg, 0.722 mmol) and sodium azide (140.8 mg, 2.166 mmol) were combined in a 1:3 molar ratio in 4 mL DMF. The resultant slurry was stirred at room temperature overnight. DMF was then removed via rotary evaporation. The residue was diluted with 10 mL diethyl ether, followed by addition of hydrochloric acid (1 M, 10 mL) to quench unreacted sodium azide. The organic layer was rinsed with brine, dried over sodium sulfate, filtered, and subjected to rotary evaporation, yielding the desired product (99 mg, 57% yield) at >95% purity as an off-white solid. Characterization data for the final product (ESI-MS, ^{1}H NMR, ^{13}C NMR, IR) matched published results.

Expression and Purification of CaN

CaN expression and purification were performed according to the method of Mondragon *et al* [5]. In summary, the pHumCaN plasmid encoding the human CaN-A and CaN-B subunits was transformed into chemically competent BL21(DE3) *E. coli* cells already containing the pHV738 plasmid encoding hNMT1 and MetAP. 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 myristic acid (from a 500 mM stock in DMSO). After 3–4 hr of protein expression, cells were harvested via centrifugation. Cell pellets were washed with cold PBS and stored at −80 °C until use.

Purification of CaM

Purification of CaM proteins was conducted with phenyl sepharose resin, taking advantage of Ca^{2+}-induced structural changes that increase the surface hydrophobicity of CaM. Protocols were adapted from Gaertner *et al* [9]. Cell pellets were resuspended in CaM Lysis Buffer (50 mM HEPES [pH 7.5], 100 mM KCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, Roche Complete Inhibitor Cocktail, 0.5–1 mg mL^{-1} lysozyme) according to the formula 50 µL lysis buffer / (mL culture * OD600). Cell pellets were lysed by sonication (3 min total sonication with 0.5 sec pulses), and resultant lysates were clarified by centrifugation at 10,000–12,000 x g for 10 min at 4 °C. The remaining purification steps were conducted at room temperature under gravity flow conditions. Clarified lysate was applied to phenyl sepharose resin equilibrated with CaM Lysis Buffer (omitting protease inhibitors and lysozyme). The flow-through was collected and applied to phenyl sepharose resin equilibrated with 50 mM HEPES (pH 7.5), 3 mM $CaCl_2$, 0.1 mM PMSF. The column was washed 3 times with 2 column volumes of Wash Buffer (50 mM HEPES [pH 7.5], 1 mM $CaCl_2$) and 3 times with 2 column volumes of Wash Buffer with 500 mM NaCl. CaM was eluted in CaM Elution Buffer (50 mM HEPES [pH 7.5], 1.5 mM EGTA). Elution fractions containing CaM were identified by SDS-PAGE, pooled, quantified, aliquotted, and stored at −80 °C.

Intact LC-MS Detection of CaN and CaM Proteins

Solutions of pure protein were concentrated using Microcon columns (MWCO = 30 kDa) (EMD-Millipore) and buffer-exchanged into 0.1% TFA (trifluoroacetic acid). For each protein, a final solution of 100 pmol protein in 100 µL was submitted for analysis on an Agilent 1100 MSD mass spectrometer at the Proteome Exploration Laboratory of the Caltech Beckman Institute. *Calculation of Masses.* The "Expected Mass (Da)" values listed in Table 1 were calculated from the corresponding masses for the unlabeled proteins by subtracting the mass of the initial Met residue (which is removed by methionine aminopeptidase prior to NMT labeling), adding the mass of ADA, and subtracting the mass of one water molecule to account for formation of an amide linkage between the protein N-terminus and ADA.

Data analysis. The "Percent Labeled" values reported in Table 1 are based on the area under the curve for the peak corresponding to each CaM protein species. For the three proteins listed as >98% labeled, only the ADA-labeled protein was detected, and no unlabeled protein was observed; the >98% descriptor is based on the detection limits of the mass spectrometer itself. For the two proteins listed as 93% or 88% labeled, both ADA-labeled and unlabeled forms of the protein were detected.

CaM-Dependent CaN Activity Assay

The Ca^{2+}/CaM-activated phosphatase activity of CaN was evaluated using the malachite green assay, which measures the amount of inorganic phosphate released upon dephosphorylation of a CaN-specific phosphopeptide substrate. Specifically, this colorimetric assay takes advantage of the green color of the complex formed by malachite green, molybdate, and free inorganic phosphate (PO_4^{-3}). The Calcineurin Phosphatase Assay Kit was purchased from Enzo Life Sciences. First, varying concentrations of WT CaM and the ADA-labeled engineered CaM proteins were incubated with saturating Ca^{2+} (10 µM $CaCl_2$) and 10 nM CaN in assay buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 6 mM $MgCl_2$, 0.5 mM DTT, 0.025% NP-40, 0.5 mM $CaCl_2$) for 10 min at 37 °C. To initiate the reaction, phosphorylated RII peptide substrate was added at a final concentration of 0.5 mM in 50 µL and allowed to react for 10 min. A standard curve of

phosphate (PO_4^{-3}) in Assay Buffer was made on each day of experiments. Biomol Green reagent (50 µL, containing malachite green and molybdate) was then added to the standard curve and experimental samples to terminate the reaction, and color was allowed to develop for 30 min. The absorbance of all samples was measured at 620 nm on a Tecan 96-well plate reader. The absorbance values of the standard curve were plotted against the (known) concentrations of the PO_4^{-3} standards and fit to a second-order polynomial, from which the quantity of CaN-mediated release of PO_4^{-3} in the samples was calculated. CaN activity was plotted as a function of CaM concentration using Prism (GraphPad Software). The dose-response of CaN activity at varying Ca^{2+} or CaM concentrations was calculated according to the Hill equation (Eqn. 1):

Eqn. 1: $Y = min + \frac{max - \min}{1+(\frac{EC50}{X})\text{^}N}$

where X is the concentration of Ca^{2+} (Fig. 2C) or CaM (Fig. 2D), Y is the response (% activity of CaN), min is the lower asymptote of the curve, max is the upper asymptote of the curve, N is the Hill coefficient, and EC_{50} is the x-coordinate of the inflection point (x, y). The EC_{50} value, which represents the concentration at which CaN is half-maximally activated, is directly related to the ability of CaM to bind and activate CaN. The Hill coefficient represents the cooperativity of binding.

Ca^{2+}-Dependent CaN Activity Assay

The ability of the ADA-labeled engineered CaM proteins to activate CaN in a Ca^{2+}-dependent manner was measured using the Calcineurin Phosphatase Assay Kit described above. Briefly, 10 nM CaN, 1 µM WT CaM or ADA-labeled engineered CaM protein, and varying concentrations of free Ca^{2+} were incubated for 10 min at 37 °C. The concentration of free Ca^{2+} was tightly controlled by titration of the calcium chelator EGTA according to the MaxChelator algorithm (maxchelator.stanford.edu, [10]). To initiate the reaction, phosphorylated RII peptide substrate was added at a final concentration of 0.5 mM in 50 µL and allowed to react for 10 min. A standard curve of phosphate (PO_4^{-3}) in Assay Buffer was made on each day of experiments. Biomol Green reagent (50 µL, containing malachite green and molybdate) was then added to the standard curve and experimental samples to terminate the reaction, and color was allowed to develop for 30 min. The absorbance of all samples was measured at 620 nm on a Tecan 96-well plate reader. The absorbance values of the standard curve were plotted against the (known) concentrations of the PO_4^{-3} standards and fit to a second-order polynomial, from which the quantity of CaN-mediated release of PO_4^{-3} in the samples was calculated. CaN activity was plotted as a function of Ca^{2+} concentration using Prism (GraphPad Software). The dose-response of CaN activity at varying Ca^{2+} concentrations was calculated according to the Hill equation (Eqn. 1).

Ca^{2+}-Dependent Electrophoretic Shift Assay

The SDS-PAGE electrophoretic mobility assay was carried out according to published protocols [11, 12]. Briefly, samples of pure WT CaM, ADA-labeled engineered CaM proteins, and lysozyme were prepared at equal concentrations, and 10 µg of each protein was loaded on 4–12% gradient gels. The gels were run in the presence of either 0.1 mM Ca^{2+} or 5 mM EGTA (*i.e.*, excess Ca^{2+} or excess EGTA, respectively). Gels were stained with Coomassie colloidal blue and imaged on a GE Typhoon fluorescence scanner.

Fluorescence Detection of ADA-Labeled Protein in Lysate

Cell pellets were resuspended in Lysis Buffer (50 mM Tris [pH 8.0], 1% SDS) according to the formula 50 µL lysis buffer / (mL culture * OD600). Cell pellets were lysed by sonication (3 min total sonication with 0.5 sec pulses), and resultant lysates were clarified by centrifugation at 10,000–12,000 x g for 10 min at 4 °C. Clarified cell lysates were reacted with Click-iT kit reagents, including alkyne-tetramethylrhodamine (alkyne-TAMRA, Fig. S2), according to the manufacturer's instructions; the only modification was the use of 15 µL of alkyne-TAMRA dye solution per reaction, rather than 100 µL. After reaction and methanol/chloroform precipitation, the resultant protein pellets were resuspended in a denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-Cl) and loaded on a 4–12% gradient gel for SDS-PAGE. The gel was imaged for TAMRA signal on a GE Typhoon fluorescence scanner, stained with Coomassie colloidal blue, then imaged for Coomassie signal.

Quantitative Western Blot Analysis of ADA-RS1-CaM in Lysate

Protein expression was performed as described in the "Cloning, Protein Expression, and Protein Labeling" section in the main text. Cell lysates were prepared as described in the "Purification of CaM" section above. Clarified cell lysates (2, 10, or 20 µg) prepared from ADA-RS1-CaM expression cultures and 0.1–2.0 µg of purified CaM were run on a 4–12% gradient gel and transferred to a nitrocellulose membrane. The Western blot was probed with primary and secondary antibodies, then analyzed on a GE Typhoon fluorescence scanner. The amount of ADA-RS1-CaM in each lysate sample was calculated based on a standard curve prepared from the pure CaM band intensities.

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