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A Fluorogenic Peptide Probe Developed by *In Vitro* Selection Using tRNA Carrying a Fluorogenic Amino Acid

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Materials and Methods

Preparation of NBD-C6-AF-tRNA (NBDaa-tRNA)

NBD-C6-AF-tRNA was synthesized as follows (Scheme S1). DMSO solution of NBD-X-succinimide ester (25 mM, 40 μL, Invitrogen, USA) was mixed with DMSO solution aminophenylalanyl-pdCpA (AF-pdCpA) (5 mM, 40 μL)[1] in aqueous pyridine-HCl (5M, pH 5.0, 80 μL). After incubation at 37°C for 3 h, NBD-C6-AF-pdCpA was purified by reverse-phase HPLC (Waters XTerra C18; 2.5 μm, 4.6 mm × 20 mm) at a flow rate of 1.5 mL/min, with a linear gradient of 0%-100% acetonitrile in 0.1% trifluoroacetic acid for 10 min. The product was confirmed by MALDI-TOFMS (Voyager, Applied Biosystems: for the calculated value of $[M-H]^-$ 1073.27, we obtained a value of 1073.83.

The resulting NBD-C6-AF-pdCpA was ligated to an amber suppressor tRNA derived from *Mycoplasma capricolum* Trp_1 tRNA without the 3' dinucleotide by chemical ligation method as described[2]. The NBDaa-tRNA can be obtained as commercially available reagent (CoverDirect tRNA reagents for site-directed protein labeling, ProteinExpress, Chiba, Japan).

Construction of DNA library for ribosome display

A DNA pool was constructed to incorporate the signaling probe, NBD, into the translated peptide. We constructed a plasmid named 13Trx, which possessed the promoter sequence for T7 RNA polymerase, *E. coli* ribosome-binding sequence (RBS), *Sfi*I restriction sequence, a protein linker sequence and a ribosome arrest sequence (SecM). In parallel, we prepared a random double-stranded DNA (dsDNA). Two custom synthesized single-stranded DNA (ssDNA) were obtained from Operon (Japan);

5’-ATATGGCCATGCAGGCC$(NNB)_m$TAG$(NNB)_n$GGCCAGCTAGGCCAGTT-3’

where the repetitive number sets are (m; 3, n; 7) or (m; 7, n; 3), and N and B represent G, C, T, or A, and T, G or C, respectively. We prepared dsDNA from an equimolar mixture of the ssDNAs using Ex Taq (Takara, Japan) in the presence of a reverse primer rp-2 (5’-AACTGGCCTAGCTGGCC-3’) primer. The resulting dsDNA and 13Trx plasmid were digested with a restriction enzyme (*Sfi*I, New England Biolabs, USA) and fused (DNA Ligation Kit, Takara, Japan). Finally, a double-stranded DNA library was prepared by PCR using primer T7-fp-rec-1 (5’-TTAATACGACTCACTATAGAACATGAGGATCACCCATGTAAAAGTCGAC AATAATTTTGTTTAACTT-3’) and primer rp-fp-M13-NS (5’-AAACAGCTATGACCATGATTA-3’).

Part of 13 Trx Plasmid for DNA library insert and amplification:

GTCGACAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCCATGC
AGGCCAGCTAGGCCAGTTCGAAGGTAGCGATAAAATTATTCACCTGACTGA
CGACAGTTTTGACACGGATGTACTCAAAGCGGACGGGGCGATCCTCGTCGA

TTTCTGGGCAGAGTGGTGCGGTCCGTGCAAAATGATCGCCCCGATTCTGGA
TGAAATCGCTGACGAATATCAGGGCAAACTGACCGTTGCAAAACTGAACAT
CGATCAAAACCCTGGCACTGCGCCGAAATATGGCATCCGTGGTATCCCGAC
TCTGCTGCTGTTCAAAAACGGTGAAGTGGCGGCAACCAAAGTGGGTGCAC
TGTCTAAAGGTCAGTTGAAAGAGTTCGAAGGCGGCGGCTCTGGTGGTGGT
TCTGGTGGCGGCTCTGAGGGTGGCGGCTCTGAGGGTGGCGGTTCTGAGGG
TGGCGGCTCTGAGGGTGGCGGTTCCGGTGGCGGCTCCGGTTCCGGTGATTT
TGATTATGAAAGAATGGCAAACGCTAATAGAGGGGCTATGACCGAAAATGC
CGATGAAAACGCGCTACAGTCTGACGCTAGAGGCAGACTTGATTCTGTCGC
TACTGATTACGGTGCTGCTATCGATGGGATCCAGTTTTCTACTCCTGTTTGGA
TTTCTCAAGCTCAAGGTATTCGTGCTGGTCCTCAACGTCTCACTCAGCTTG
GCGTAATCATGGTCATAGCTGTTT (*Sfi*I enzyme digest sites are colored with green background)

Selection of fluorogenic peptides specific for the Ca^{2+}-calmodulin complex

The DNA library was transcribed into mRNA using RiboMAX (Promega, USA) and purified with RNeasy® Mini kit (Qiagen, Germany). *In vitro* translation was performed using Puresystem ΔRF1 (Wako, Japan) in the presence of 320 pmol of NBDaa-tRNA (Figure 1), which was obtained as a custom synthesis from ProteinExpress (Chiba, Japan). After the reaction was stopped by placing 50 µl of the reaction mixture on ice for 10 min, 250 µL of the selection buffer with Tween 20 (60 mM Tris-acetate, pH 7.5; 180 mM KCl; 60 mM magnesium acetate; 6 mM $CaCl_2$; 0.06 % Tween 20; BSA 1.2 mg/ml) was added. The diluted translate solution was incubated with calmodulin-agarose (Sigma-Aldrich, USA) at 4°C for 1 h. The agarose

beads were collected by centrifugation and washed four times with washing buffer (50 mM Tris-acetate pH 7.5, 150 mM KCl, 50 mM magnesium acetate; 0.05 % Tween 20; 1.0 mg/ml BSA) at 4°C. Then the agarose beads were incubated with elution buffer 1 (50 mM Tris-acetate pH7.5, 150 mM KCl, 50 mM EGTA) at 4°C for 1 h. The supernatant was collected after centrifugation. Finally, mRNA was eluted using elution buffer 2 (50 mM Tris-acetate, pH 7.5; 150 mM KCl; 600 mM EDTA) for 10 min at room temperature. The eluted mRNA was purified using the RNeasy® Mini kit (Qiagen, Germany) and reverse transcribed by PrimeScript (Takara, Japan). Preparative PCR was performed to amplify the reverse transcription products using primers T7-fp-rec-1 and rp-fp-M13-NS. The DNA product was purified by PCR Purification kit (Qiagen, Germany) and the quality and concentration were verified by 6% PAGE and UV absorbance. The purified dsDNA was used as a template for the next round of selection.

ELISA

The binding activity of the selected peptides to Ca^{2+}-bound CaM was measured by an enzyme-linked immunosorbent assay (ELISA). Specifically, we amplified the respective peptide-encoding sequence by PCR using primer fp-2 (5'-ATATGGCCATGCAGGCC-3') and rp-2 (5'-AACTGGCCTAGCTGGCC-3') and inserted each sequence into the 13Trx plasmid. Subsequently, we obtained PCR amplified double-stranded DNAs using the fp-3 (5′-TAATACGACTCACTATAGGGGTCGACAATAATTTTGTTTAAC TT-3′) and primer rp-flag (5′-CTACTTGTCGTCATCGTCCTTGTAGTCCGCAATCAGACTG ATCATACC-3′), which introduced a FLAG tag and an UGA stop-codon (opal)

sequences. The amplified DNAs were purified, transcribed and then translated. We confirmed the *in vitro*-translated FLAG-tagged peptides from the western blotting result using an anti-FLAG M2 monoclonal antibody-peroxidase conjugate (Sigma-Aldrich, USA). Briefly, the solutions of translated peptides (25 μL) were combined with washing buffer (25 μL) and selection buffer (250 μL) and incubated with calmodulin-agarose with gently rotating for 1 h at 4°C. Unbound peptides were removed by washing with washing buffer, and an anti-FLAG M2 monoclonal antibody-peroxidase conjugate (Sigma-Aldrich, USA) was added. After the incubation for 0.5 h at 4°C and several washings with washing buffer, chemiluminescence reactions were performed using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Sweden). Chemiluminescent imaging for the respective FLAG-tagged peptides was observed by Light-Capture (ATTO, Japan). All the experiments were performed in 1.5 mL tubes.

Peptide synthesis

We synthesized NBD labeled aminophenylalanine for the chemical synthesis of the peptides. We added 5 M aqueous pyridine-HCl pH 5.0 (20 mL) to a solution of 6-[(7-Nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoic acid N-succinimidyl ester (650 mg, 1.66 mmol) and Fmoc-4-amino-L-phenylal-anine (555 mg, 1.38 mmol) in DMSO (20 mL) and stirred the solution at 37°C overnight. The solvent was evaporated under reduced pressure and the crude residue was purified by flash chromatography on silica gel (CH_2Cl_2:CH_3OH, 100:1, v/v) to obtain NBD labeled aminophenylalanine (895.0 mg, 1.32 mmol, yield 95.6%) as an orange solid. We confirmed the NBD labeled aminophenylalanine using NMR and mass spectrum analysis. ^{1}H NMR (400 MHz,

DMSO) δ(ppm): 12.7168 (br, 1H), 9.792 (s, 1H), 9.535 (s, 1H), 8.478 (d, J = 8.8 Hz, 1H), 8.864 (d, J = 7.6 Hz, 2H), 7.687 (d, J = 8.4 Hz, 1H), 7.631 (t, J = 8.4 Hz, 2H), 7.480 (d, J = 8.8 Hz, 2H), 7.396 (q, J = 6.4 Hz, 2H), 7.328 ~ 7.259 (m, 2H), 7.163 (d, J = 8.0 Hz, 2H), 6.396 (d, J = 9.2 Hz, 1H), 4.214 ~ 4.065 (m, 5H), 3.460 (br, 2H), 3.017 (dd, J_1 = 4.4 Hz, J_2 = 14.0 Hz, 1H), 2.802 (dd, J_1 = 9.8 Hz, J_2 = 13.6 Hz, 2H,), 2.298 (t, J = 7.6 Hz, 2H), 1.174 ~ 1.603 (m, 4H), 1.444 ~ 1.370 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ(ppm): 173.313, 170.918, 155.874, 145.125, 144.409, 144.138, 143.718, 143.685, 140.615, 137.916, 137.677, 132.385, 129.225, 127.562, 127.019, 125.249, 125.167, 120.501, 120.032, 118.797, 99.078, 65.573, 55.582, 46.520, 43.204, 36.224, 35.903, 27.410, 26.027, 24.793. ESI-Q-TOF: for calculated $[M+Na]^+$ 701.2330, obtained 701.2342.

The peptides incorporating NBD aminophenylalanine were synthesized using the Fmoc chemical method in RIKEN Brain Science Institute. The synthesized peptide were confirmed by MALDI-TOF mass analysis (Table S1)

Fluorescence Measurements

All fluorescence measurements were performed on a FP-6500 Spectrofluorometer (JASCO, Japan) at 20°C. For measurement of fluorescence intensity change, various concentrations of CaM were added to 5 μM (B4, B8, C5 and D9) or 2 μM (A3) peptide in a buffer (50 mM Tris-acetate, 150 mM KCl, 50 mM magnesium acetate, 2% DMSO, pH 7.5). The calcium concentration was adjusted by addition of $CaCl_2$. The fluorescence spectra emission between 500 and 600 nm were measured by excitation at 488 nm with the slit with set at 3 nm and 3 nm. Relative fluorescence intensity changes (RFIC) at 535 nm were calculated by RFIC = $(F_x\text{-}F_0)/F_0$, where F_x

and F_0 are the fluorescence intensity of the peptide with and without CaM (or BSA), respectively. The estimated RFIC were plotted using GraphPad Prism 5 (GraphPad Software, Inc., USA).

Surface Plasmon Resonance (SPR) Analysis

SPR measurements were performed on a Biacore T100 instrument with a CM5 sensor chip (Biacore, Sweden). Optimal immobilization of CaM on the chip was confirmed by the resonance values at 444.6 RU. All the experiments were performed at 25°C with a constant flow rate of 30 µl/min. To determine the binding kinetics of the C5 peptide to immobilized CaM, we injected 9.76, 19.53, 39.06, 78.125, 156.25, 312.5, 625, 1250, 2500, 5000, 7500, 625 nM solutions of the C5 peptide in a running buffer (50 mM Tris-HOAc pH 7.5, 150 mM KCl, 5 mM $CaCl_2$, 5% DMSO, 0.05% surfactant P20) over the sensor chip surface for 3 min; 450 µl of running buffer was then injected over the surface of sensor chip to associate C5 from the surface-bound CaM. The sensor chip was regenerated by injected 15 µl of 50 mM EGTA (pH 8.0) for 30 sec, followed by flushing with the running buffer for 5 min to stabilize the baseline. The sensor grams were analyzed using Biacore T100 Evaluation Software (version 2.0.3, Biacore). All of the binding curves were collected by subtraction of the curve for a reference flow cell and the data were fitted by heterogeneous ligand model.

NMR measurements

STD measurements

The NMR experiments for STD measurements and ^{1}H-^{1}H COSY were performed using a 700 MHz spectrometer equipped with cryogenic probe (BrukerBiospin) at 298K. Resonance assignments of C5 peptide were performed using a conventional set of 2D spectra: 2D ^{1}H-^{1}H COSY, TOCSY with 80 ms mixing time, NOESY with 400 ms mixing time, and ^{1}H,^{13}C HSQC. The sample condition was 0.5 mM C5 peptide in 5 mM Tris-HCl (pH7.5), 5 mM $Mg(OAc)_2$, 15 mM KCl, 5 mM $CaCl_2$, and 10% d_6-DMSO. For the STD experiments, selective saturation of the CaM protein was achieved by a train of Gauss shaped pulses of 50 ms, yielding a total saturation train of 2 s. The on-resonance irradiation of CaM was performed at a chemical shift of 0.14ppm. On the other hand, the off-resonance irradiation was set at 40 ppm, where no protein signals were present. The STD spectra were achieved by subtraction of the on- and off-resonance spectrum. The total scan number in the STD experiments was 1024 with 16384 time domain data points. The sample condition was 0.5 mM C5 peptide and 0.01 mM CaM in the same buffer as for the above 2D NMR measurements. For observation of the C5 peptide signal changes upon binding to CaM, 2D ^{1}H-^{1}H COSY spectra were employed. The sample conditions were 0.2 mM C5 peptide, 0.2 mM CaM, or 0.2 mM C5 peptide and 0.2 mM CaM in the same buffer. The spectra were processed using the programs Topspin (BrukerBiospin) or NMRPipe[3] and were analyzed using the programs Topspin or NMRView.[4]

Heteronuclear multidimensional NMR measurements

The NMR experiments for investigating the interactions of human CaM with C5 peptide were performed in a triple-resonance Cryoprobe fitted with a Z-axis pulsed

field gradient coil, using a 600 MHz Bruker Avance spectrometer. The NMR spectra obtained from these measurements were processed on Linux-PCs using the Azara 2.8 suite of software (Boucher, http://www.bio.cam.ac.uk/azara/), and visualized and analyzed on Linux-PCs using the CcpNmr Analysis 2.2.1 software.[5]

MD simulation

We used a complex structure of the *Drosophila melanogaster* CaM and a naturally existing CaM-binding peptide (PDB ID: 2BBM).[6] All MD simulations were performed using a constant number of molecules, a pressure of 1 atom, and a temperature of 310 K, according to Berendsen's algorithm with a coupling time of 0.2 ps after each system had been heated to 310 K over the first 100 ps. The size of the initial system was ~90 × 90 × 90 $Å^3$, and it contained 50,930 atoms. The time step was set at 1 fs. The bond lengths involving the hydrogen atoms were constrained to equilibrium lengths using the SHAKE method. Parm99 and gaff parameters were used for the amino acid residues and NBD modified aminophenylalanine (NBDaa). The electrostatic potential (ESP) of NBDaa residue was calculated by MP2/6-31G** levels using Gaussian03[7] and the restrained electrostatic potential (RESP) charges were assigned using the ANTECHAMBER module from amber 12.[8] The particle mesh Ewald method was used, and the direct space cutoff distance was set to 12 Å. The program package used for MD simulations was amber 12. The averaged root mean-square deviation (RMSD) of main chain of the complex from the solution structure during the last 10 ns was converged to 5.10 ± 0.32 Å. The RMSD value

indicated that the structure of CaM was flexible and that its conformation was changed by species of peptides.

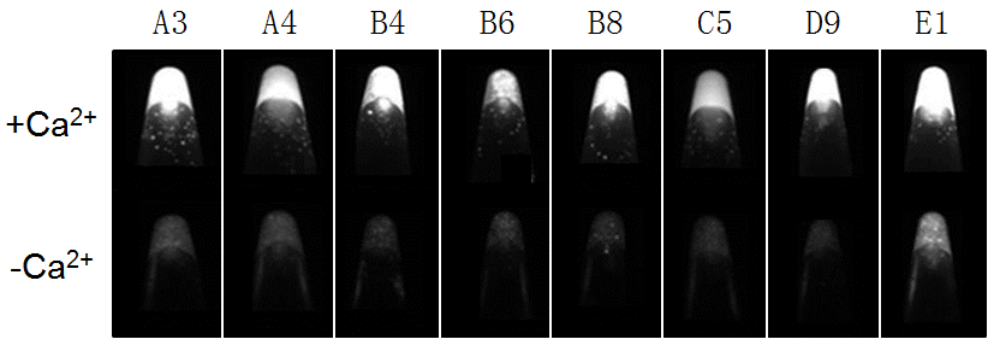


Fig. S1 ELISA shows that the selected eight peptides bind to CaM-immobilized beads in a Ca^{2+}-dependent manner.

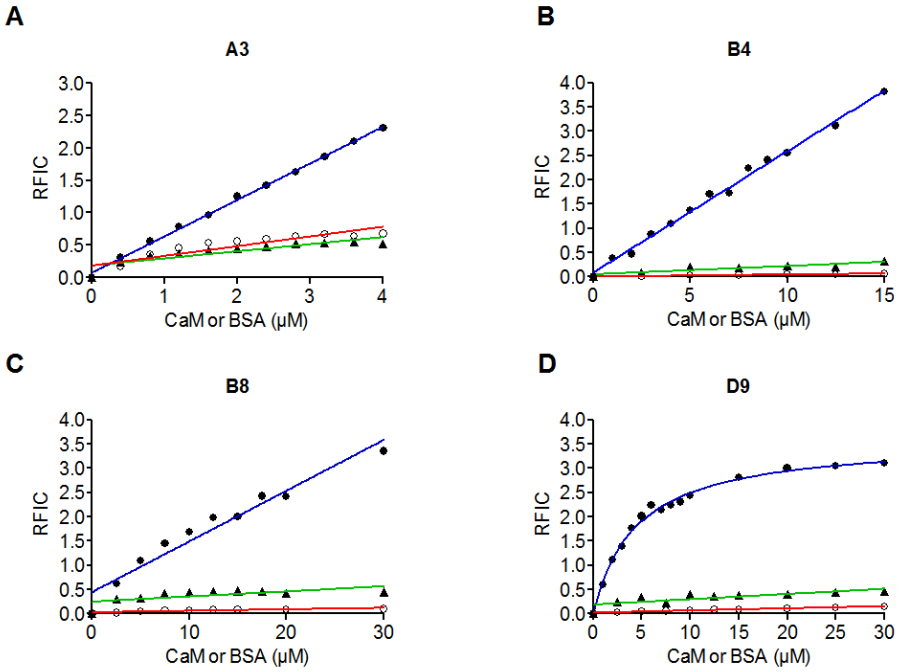


Fig. S2 The four water-soluble peptides (A3, B4, B8, D9) exhibit less signal than the C5 peptide (Figure 2b). We obtained the CaM concentration dependence of RFIC in the presence (black closed circle and blue line) and absence of Ca^{2+} (black closed triangle and green line), and the BSA concentration dependence of RFIC (open circle and red line).

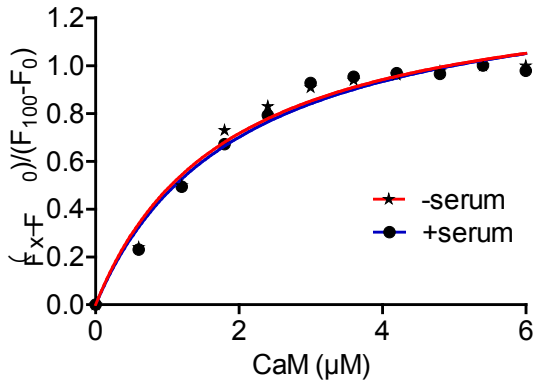


Fig. S3 The sensitivity of the C5 peptide for Ca^{2+}-bound CaM in a 50 % serum solution was indistinguishable from that in buffer.

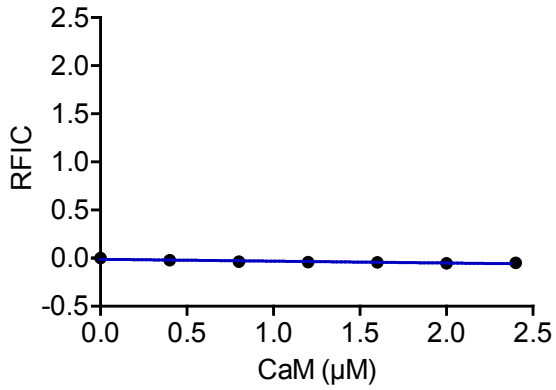


Fig. S4 NBD,6-[(7-Nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoic acid (NBD-C_5COOH), does not give a fluorescent signal even in the presence of Ca^{2+}-bound CaM.

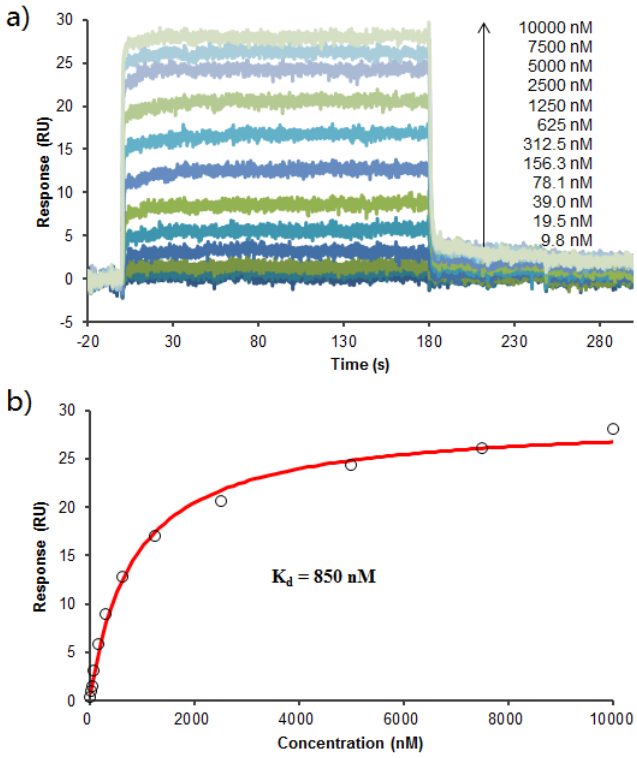


Fig. S5 Interaction of C5 with immobilized CaM. (a) SPR sensorgram using different concentrations of C5 peptide. (b) Plots of response values of RU against the concentration of C5 peptide. The dissociation constants K_d was determined using steady state affinity model.

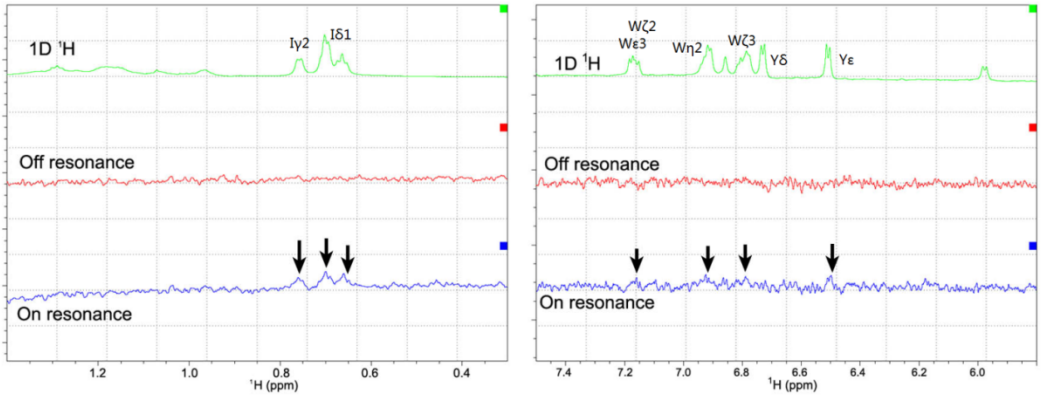


Fig. S6 The saturation transfer difference (STD) method shows that the N-terminal Tyr and Trp residues, and one of two Ile residues, are incorporated in the binding interface with CaM.

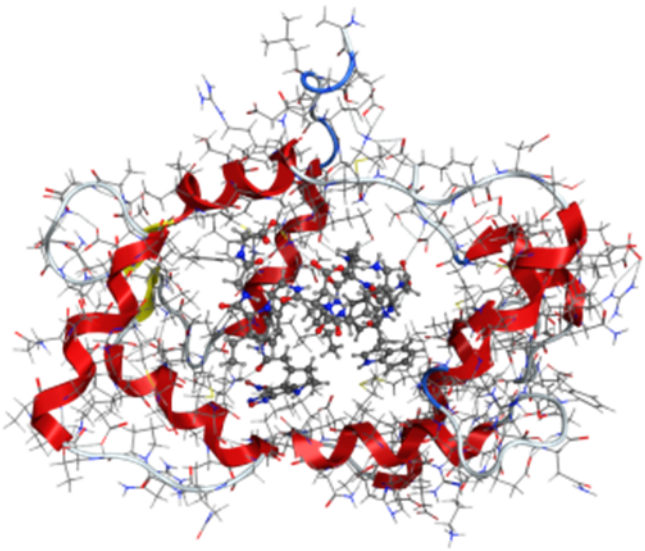
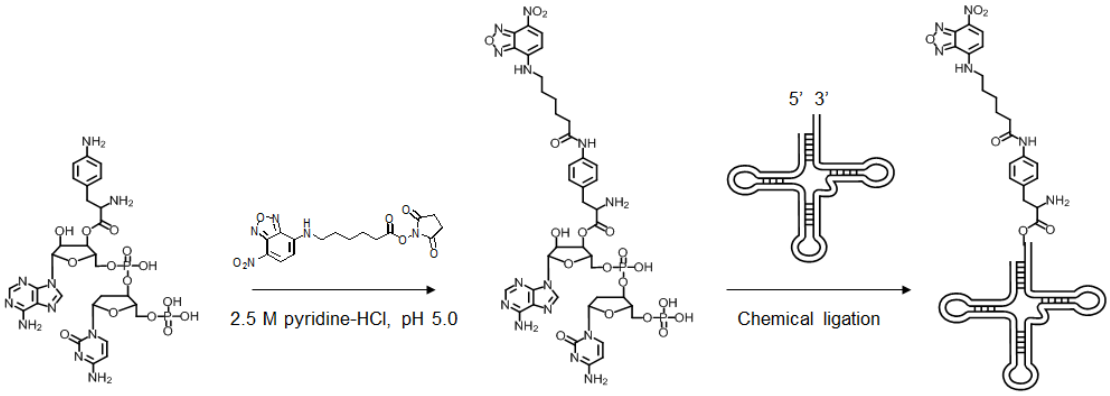


Fig. S7 Snapshot of CaM-C5 complex by MD simulation. C5 peptide is shown in ball and stick model.



Scheme S1 Synthetic scheme of NBD-C6-AF-tRNA (NBDaa-tRNA).

Table S1 Selected peptide sequences and their chemical synthesis mass values

Name	Sequence[a]	Clone	Calculated $[M+H]^+$	observed
A3	VVNXIMFLREV	4	1657.9	1657.9
A4	VVNXVMTQQAG	5	1484.7	1485.1
B4	SNIXYANKLRR	9	1672.9	1672.8
B6	VVNXVMVRNLE	3	1610.8	1611.3
B8	VVKXVMSRNVR	2	1625.9	1626.0
C5	YWDKIKDXIGG	4	1632.8	1632.8
D9	DMASLVAXVMD	2	1489.6	1489.1
E1	SNIXYANKLRR	2	1559.8	1559.7

[a] X is NBDaa.

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