Efficient Synthesis and In Vivo Incorporation of Acridon-2-ylalanine, a Fluorescent Amino Acid for Lifetime and FRET/LRET Studies

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General Information. Boc-L-thionophenylalanine-1-(6-nitro)benzotriazolide and Fmoc-β-(7-methoxycoumarin-4-yl)-alanine (Mcm) were purchased from Bachem (Torrance, CA, USA). Rink amide resin, Fmoc-Ala-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Leu-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Phe-OH, Fmoc-Ile-OH, and Fmoc-Trp(Boc)-OH

purchased from Novabiochem (currently EMD Millipore; Billerica, MA, USA). Piperidine and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from American Bioanalytical (Natick, MA, USA). Sigmacote, *N*,*N*-diisopropylethylamine (DIPEA), L-tyrosine, thionyl chloride, and di-tert-butyl dicarbonate (Boc anhydride) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ni-NTA resin was purchased from Qiagen (Hilden, Germany); BD Talon™ resin was purchased from Clontech (Mountain View, CA, USA). *E. coli* BL21(DE3) cells and a QuickChange® site-directed mutagenesis kit were purchased from Stratagene (La Jolla, CA, USA). *E. coli* DH10B cells were purchased from Invitrogen (Grand Island, NY, USA). DNA oligomers were purchased from Integrated DNA Technologies, Inc (Coralville, IA, USA). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Milli-Q filtered (18 MΩ) water was used for all solutions (Millipore; Billerica, MA, USA). All peptide synthesis reaction vessels (RVs) were treated with Sigmacote® prior to use.

DNA sequencing was performed at the University of Pennsylvania DNA sequencing facility. Matrix-assisted laser desorption ionization (MALDI) mass spectra were collected with a Bruker Ultraflex III MALDI-TOF-TOF mass spectrometer (Billerica, MA, USA). "Low resolution" electrospray ionization (ESI) mass spectra (LRMS) were obtained on a Waters Acquity Ultra Performance LC (Milford, MA, USA) connected to a single quadrupole detector (SQD) mass spectrometer. "High resolution" ESI mass spectra (HRMS) were obtained on a Waters LCT Premier XE LC/MS. UV absorbance spectra were obtained with a Hewlett-Packard 8452A diode array spectrophotometer (currently Agilent Technologies; Santa Clara, CA, USA). Fluorescence spectra were collected with a Varian Cary Eclipse fluorescence spectrophotometer (currently Agilent Technologies) fitted with a Peltier multicell holder, a Photon Technologies

International (PTI) QuantaMaster™ fluorometer (Birmingham, NJ, USA), a Tecan M1000 plate reader (Mannedorf, Switzerland), or a BioTek Synergy2 plate reader (Winooski, VT, USA) with a UV transparent 96 well plate. Steady-state Stern-Volmer experiments were conducted on a PerkinElmer Envision Xcite multilabel reader (PerkinElmer Health Sciences Inc.; Shelton, CT, USA) with a black bottomed 96 well plate. Circular dichroism experiments were conducted with an Aviv 410 CD spectrometer (Aviv Biomedical; Lakewood, NJ, USA). Automated peptide synthesis was performed on a Liberty 1 system (CEM Corp.; Matthews, NC, USA) in the UPenn Biological Chemistry Resource Center. Protein purification was conducted on an ÄKTA fast protein liquid chromatography (FPLC) system (GE Healthcare Life Sciences; Piscataway Township, NJ, USA). Peptides were purified with a Varian ProStar High-Performance Liquid Chromatography (HPLC) instrument outfitted with a diode array detector (currently Agilent Technologies) using aqueous (H_2O + 0.1% CF_3CO_2H) and organic (CH_3CN + 0.1% CF_3CO_2H) phases. Small molecule analyses were performed on an Agilent 1100 HPLC instrument outfitted with a diode array detector using the same aqueous and organic phases.

Screening of Cross-Coupling Conditions. The experimental procedures in this work were similar to those previously reported.[1] The reaction screens were carried out in a Vacuum Atmospheres glove-box (Topsfield, MA, USA) operating under a nitrogen atmosphere (oxygen and water content typically < 0.5 ppm). The experimental design was accomplished using Accelrys Library Studio (San Diego, CA, USA). Screening reactions were carried out in 1 mL flat bottom glass vials (30 mm x 8 mm) in a 96-well plate aluminum reactor block from Analytical Sales and Services (Pompton Plains, NJ, USA).

Liquid chemicals were dosed using multi-channel or single-channel pipettors. Solid chemicals were dosed as solutions or slurries in appropriate solvents. Undesired addition solvent was removed using a JKem blow-down system (St. Louis, MO, USA) located inside the glovebox. The reactions were heated and stirred on a heating block with a tumble-stirrer (V&P Scientific; San Diego, CA, USA) or IKA magnetic stirrer hot plate (Wilmington, NC, USA) using 1.32 mm diameter x 1.57 mm length parylene stir bars. The reactions were sealed in the 96-well plate during reaction. Below each reactor vial in the aluminum 96-well plate was a 0.062 mm thick silicon-rubber gasket. Directly above the glass vial reactor top was a Teflon perfluoroalkoxy copolymer resin sealing gasket and above that, two more 0.062 mm thick silicon-rubber gaskets. The entire assembly was compressed between an aluminum top and the reactor base with nine screws. The conditions for the coupling of **4** and **5** included variations of the following:

Bases	Solvents	Pd loading (mol %)
Cesium carbonate	*n*-Butanol	5.2
Potassium Carbonate	*N*-Methylpyrolidine	2.5
Sodium Carbonate	Cyclopentylmethylether	1
Lithium Carbonate	*N*,*N*-Dimethylacetamide	0.1
	Toluene	
	1,4-Dioxane	

The following procedure is representative of the high-throughput experimentation reaction described in this publication. Palladium diacetate (10, 25 and 50 μL of a 0.01 M solution in THF and 101 μL of a 0.001 M solution in THF) was used in all cases. A racemic BINAP (10, 25 and 50 μL of a 0.014 M solution in THF and 101 μL of a 0.0014 M solution in THF) was added to the reaction vials and this was evacuated to dryness on a JKem-blow-down block. Base (30 μmol, stock solutions gave an approximate 25 mg/mL slurry in THF) was then added to the ligand/catalyst mixture, and this was evacuated to dryness on a JKem-blow-down block. A parylene stir-bar was added to each reaction vial. The aryl triflate **4** (10 μmol/reaction) and methyl-2-aminobenzoate **5** (10 μmol/reaction) were then dosed together in the reaction solvents (100 μL of a 0.1 M solution). The vials were then sealed and heated at 120 ºC for 4 h. After cooling to ambient temperature, the reaction mixtures were diluted with a solution of internal standard in CH_3CN (2 μmol biphenyl, 500 μL of solution), and the contents were stirred. Into a separate 96-well plate LC block was added 700 μL of CH_3CN and then 25 μL of the diluted reaction mixtures. The 96-well plate LC block was then sealed with a polypropylene 1 mL cap mat. The reactions were analyzed using an Agilent Technologies 1200 series HPLC with a 96-well plate auto-sampler. The results in Figure S1 show product formation as measured against an internal standard (IS).

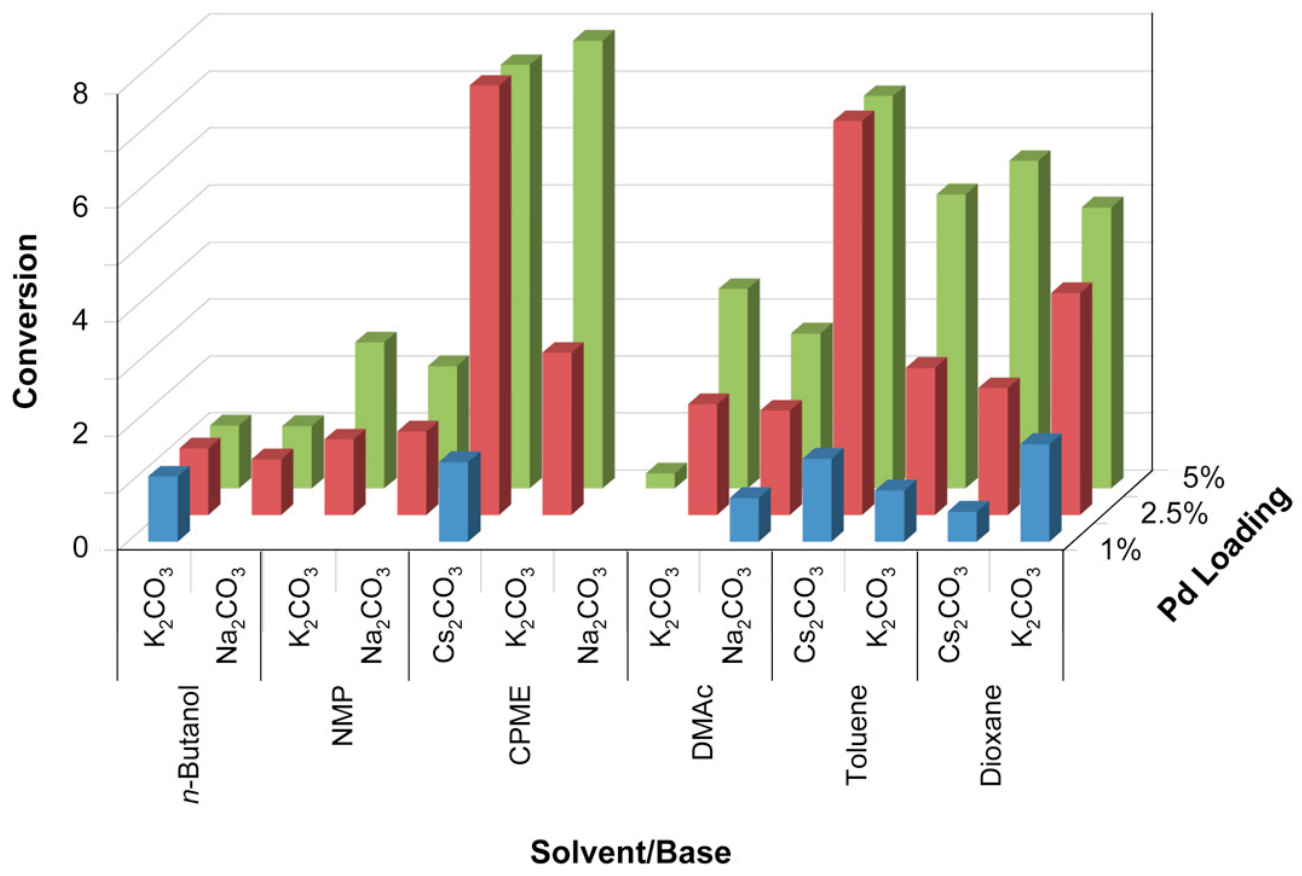
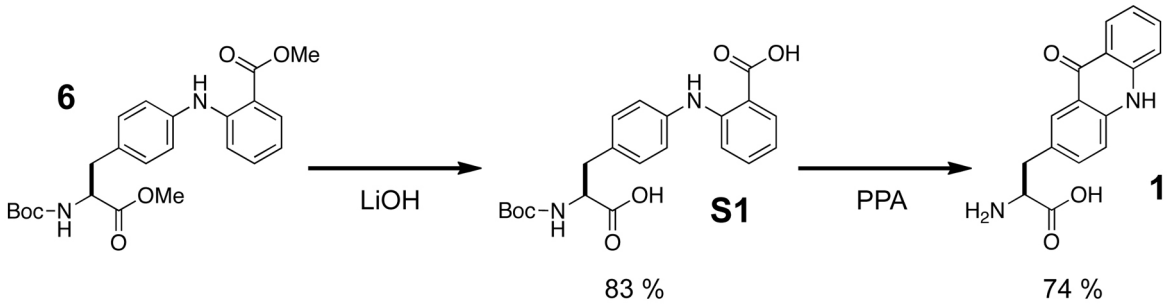


Figure S1. Screening Results. Coupling of compounds **4** and **5** carried out in 96 well plates with varying solvent, base, and Pd(II) acetate compositions. Conversion determined as the ratio of the absorbances at 215 nm of the product peak and a standard peak in HPLC chromatograms.

Alternate Route to Acd Using Polyphosphoric Acid (PPA)



(*S*)-2-((4-(2-((tert-butoxycarbonyl)amino)-2-carboxyethyl)phenyl)amino)benzoic acid (S1). Diester **6** (2.1600 g, 5.042 mmol) was added to a 250 mL round bottom flask with a stir bar and 50 mL of tetrahydrofuran (THF). This solution was then cooled to 4 °C. Lithium hydroxide (3.31 g, 138.15 mmol) was dissolved in a separate flask with 150 mL water and vigorous stirring. The LiOH solution was then slowly added to the solution containing **6**. The flask was fitted with a rubber septum and a balloon filled with argon and stirred magnetically at 4 °C. After 24 h, the solution was warmed to ambient temperature and the pH was adjusted to 3 with 42 mL of 3 M HCl. Crude product was extracted with CH_2Cl_2 (3 x 100 mL, 1 x 50 mL), dried with magnesium sulfate, and concentrated under reduced pressure. The resulting crude oil was re-dissolved in 20 mL DCM and cooled at 4 °C for 16 h. A first crop of recrystallized product (a fine white powder) was collected by vacuum and washed with 100 mL of cold CH_2Cl_2 (1.6326 g, 4.077 mmol, 80.8%). The filtrate was then concentrated under reduced pressure, and a second crop was collected by repeating the recrystallization procedure with 10 mL CH_2Cl_2 (0.0370 g, 0.092 mmol, 1.8%). The total yield for the two crops of fine white powder was 82.6%. ^{1}H NMR (500 MHz, CD_3OD) δ 7.96 (dd, *J* = 8.0, 1.4, 1H), 7.32 – 7.27 (m, 1H), 7.24 – 7.11 (m, 5H), 6.71 (t, *J* = 7.5 Hz, 1H), 4.36 (dd, *J* = 8.8, 5.0 Hz, 1H), 3.14 (dd, *J* = 8.9, 5 Hz, 1 H), 2.90 (dd, *J* = 9.0, 4.7 Hz, 1 H), 1.4 (s, 9 H); ^{13}C NMR (125 MHz, CD_3OD): δ 175.5 (np), 171.9 (np), 157.9 (np), 149.5

(np), 140.9 (np), 135.2 (+), 133.7 (np), 133.4 (+), 131.5 (+), 123.3 (+), 118.1 (+), 114.9 (+), 113.4 (np), 80.7 (np), 56.4 (+), 38.3 (-), 28.8 (+); HRMS (ESI) *m/z* calcd for $C_{21}H_{25}N_2O_6$ $[M+Na]^+$ 423.1532, found 423.1525.

(*S*)-2-amino-3-(9-oxo-9,10-dihydroacridin-2-yl) propanoic acid (Acd, 1). 51.71 g polyphosphoric acid (PPA) was weighed into a 250 mL flask. A stir bar was added and the material was heated to 135 °C for 15 min in an oil bath. **S1** (1.00 g, 2.5 mmol) was added directly to the flask of PPA and allowed to react for 2 h. 50 mL water was slowly added (2 mL portions over 10 min) and the reaction was allowed to cool to 60 °C. After stirring for 1 h at 60 °C, the flask was removed from the oil bath, the solution decanted into a 500 mL beaker, and cooled to ambient temperature. Insoluble impurities were removed by vacuum filtration and the clarified solution was returned to a 500 mL beaker with a stir bar. The pH of the solution was adjusted to 5.5 by addition of 100 mL of 10 M NaOH (aq) and then the solution was cooled to 4 °C for 16 h. Next, crude product was collected by vacuum. After drying on the filter cake, the crude material was suspended in 50 mL water and brought into solution by drop-wise addition of 5 M NaOH (bringing the pH to 9.0). Insoluble impurities were removed by vacuum filtration. The pH of the clarified solution was then adjusted to 5.5 with 3 M HCl, cooled to 4 °C, and let stand for 4 hours. Bright yellow precipitate was collected by vacuum filtration and dried overnight by vacuum yielding 0.5260 g (1.86 mmol, 74.4%). The purity was > 99% by analytical HPLC. Characterization of this compound matched previous reports.[2]

Peptide Synthesis and Purification. The CaM binding peptide pOCNC has the sequence FRRIARLVGVLREFAFR; two derivatives in which the N-terminal phenylalanine has been replaced with either Mcm or Trp are named Mcm-pOCNC and Trp-pOCNC respectively. The general sequence of these peptides is derived from the bOCNCp peptide fragment described by Contessa *et al*. (sequence: GGFRRIARLVGVLREWAYR).[3] The pOCNC and pOCNC-F′$_1$ peptides (where F′ indicates thiophenylalanine, see page S10) were constructed using a combination of automated and manual synthesis. Residues R_{17}-R_2 were added to 100 μmol of Rink amide resin (100 – 200 mesh; 0.6 mmol substitution/g; 100 μmol) using a Liberty 1 peptide synthesizer as follows.

The peptide RRIARLVGVLREWAYR was synthesized (100 micromole scale) by solid phase peptide synthesis, using Fmoc chemistry, on a Liberty1 Automated Microwave Peptide Synthesizer. Piperidine (20%) in DMF was used as the deprotection agent, 0.5 M HBTU in DMF was used as the activator, and 2 M DIPEA in NMP was used as the activator base. Five molar equivalents of the amino acid were used for each coupling on Rink Amide MBHA resin (0.59 mmol/g substitution, Novabiochem). Residues Arg_1, Arg_2, Arg_5, Arg_{11}, and Arg_{16} were coupled using Method 1. Residues Ile_3, Leu_6, Val_7, Gly_8, Val_9, and Phe_{15} were coupled using Method 2. Residues Ala_4, Leu_{10}, Glu_{12}, Phe_{13}, Ala_{14} were coupled using Method 3. Method 1: Initial 30 s microwave deprotection (40 W, 75 ° C), followed by 3 min microwave deprotection (40 W, 75 ºC). Two consecutive coupling cycles were used. The first coupling was at room temperature for 25 min followed by an additional 5 min under microwave power (25 W, 75 ° C). The second coupling was performed under microwave power for 5 min (25 W, 75 ° C). Method 2: Initial 30 s microwave deprotection (40 W, 75 ºC), followed by 3 min microwave deprotection (40 W, 75 ºC). A single microwave coupling was performed for 5 min (25 W, 75 ºC). Method

3: Initial 30 s microwave deprotection (40 W, 75 ºC), followed by 3 min microwave deprotection (40 W, 75 ºC). Two consecutive microwave couplings were performed for 5 min (25 W, 75 ºC).

Before removal of the Fmoc-group from R_2, the resin beads were divided into two equal portions and transferred to clean RVs for manual coupling of either Fmoc-L-Phe-OH or Boc-L-thionophenylalanine-1-(6-nitro)benzotriazolide. The coupling of Fmoc-L-Phe-OH to R_2-R_{17} to form pOCNC was performed for 45 min with 5 equiv HBTU and 10 equiv DIPEA. The addition of Boc-L-thionophenylalanine-1-(6-nitro)benzotriazolide to form pOCNC-F'_1 was performed for 45 min with 10 equiv DIPEA. After removal of the *N*-terminal Fmoc-group from pOCNC with 20% piperidine, the resin beads were washed with CH_2Cl_2, dried under vacuum, and incubated on a rotisserie for 120 min with 10 mL TFA/TIPSH (95:5 v/v). The thioamide-containing pOCNC-F'_1 was cleaved from the resin (without piperidine treatment) and deprotected by washing the resin with CH_2Cl_2, drying under vacuum, and incubating for 90 min with 10 mL TFA/TIPSH (95:5 v/v). After each incubation period, the cleavage cocktail for the peptide was expelled from the RV with nitrogen and dried by rotary evaporation. Crude peptide was redissolved in 10 mL CH_3CN/H_2O (3:2 v/v) and purified by reverse phase HPLC with gradients 1 and 2 (Table S2). Using this method, the peptide eluted at approximately 19.5 min. Peptide identity was confirmed by MALDI-MS (Table S1).

Similarly, the peptides Mcm-pOCNC and Trp-pOCNC (see page S13) were made on the 50 μmol scale by manual solid phase peptide synthesis. Rink amide resin (100 – 200 mesh; 0.6 mmol substitution/g; 50 μmol) was added to a dry clean RV. Two successive 90 min incubations of 10 mL dimethylformamide (DMF) and magnetic stirring were used to swell the resin. Following swelling, DMF was removed by vacuum. 20% piperidine in DMF (5 mL) was used to deprotect the resin in two successive 15 min incubations with magnetic stirring. Then the resin

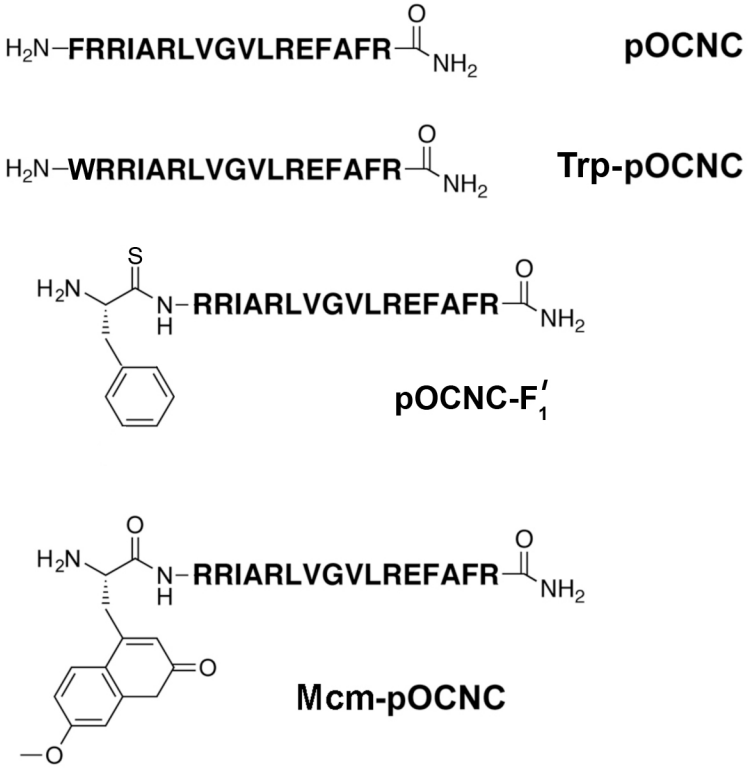
was washed extensively with DMF. The first amino acid (5 equiv) and HBTU (5 equiv) were dissolved in DMF (5 mL) and added to the RV along with DIPEA (10 equiv; 44 μL). The mixture was allowed to react for 45 min with magnetic stirring. Spent solution was removed with vacuum suction and the resin beads were washed thoroughly with DMF. The coupling was repeated with an additional 5 equiv of amino acid and HBTU and 10 equiv of DIPEA (in 5 mL DMF). The second spent solution was removed with vacuum suction and the resin beads were again washed thoroughly with DMF. The *N*-terminal Fmoc protecting group was removed by two successive 15 min treatments of 20% piperidine in DMF (5 mL). Subsequent amino acid couplings and deprotections were conducted as described above until R_2. Prior to the the *N*-terminal Fmoc deprotection of R_2, the beads were split into two 25 μM portions in separate vessels. In one vessel, the beads were treated twice with 5 equiv of Fmoc-Mcm-OH, 5 equiv HBTU, and 10 equiv DIPEA for 45 min while the beads in the other vessel were given the same treatment with the replacement of 5 equiv Fmoc-Trp(Boc)-OH for Fmoc-Mcm-OH. The peptides were then cleaved from the resin separately by incubating the beads with 10 mL TFA/TIPSH (95:5 v/v) for 120 min on a rotisserie. After this treatment, the resulting solutions were expelled from the RV with nitrogen and concentrated by rotary evaporation. The resulting residues were resuspended in 2 mL acetonitrile and split into two 1 mL portions. Then 14 mL diethyl ether was added to each portion to precipitate the peptide after 10 min of incubation at -20 ºC. The precipitates were collected by centrifuging for 5 min at 3000 rpm and decanting the supernatant. This precipitate was air dried and resuspended in 10 mL H_2O/CH_3CN/TFA (10:10:1) for HPLC purification. The crude peptide was purified by reverse phase HPLC with gradient 3 (Table S2). Using this method, the peptides eluted at approximately 17 min. Peptide identities were confirmed by MALDI MS (Table S1).

Table S1. Calculated and Observed Peptide and Protein Masses.

Peptide	Calculated m/z $[M+H]^+$	Observed m/z $[M+H]^+$	Calculated m/z $[M+Na]^+$	Observed m/z $[M+Na]^+$
pOCNC	2106.3	2106.3	2128.6	2128.8
pOCNC-F'$_1$	2122.3	2122.6	2144.2	2144.7
Mcm-pOCNC	2204.6	2204.3	2226.6	2226.2
Trp-pOCNC	2145.6	2145.2	2167.6	2167.2
GFP-WT	27828	27828		
GFP-N$_{150}\delta$	27978	27978		
αS-WT	14461	14461		
αS-Y$_{39}\delta$	14562	14563		
αS-F$_{94}\delta$	14578	14578		
CaM-WT	16707	16707		
CaM-F$_{13}\delta$	16824	16825		
CaM-G$_{41}\delta$	16915	16914		
CaM-Y$_{100}\delta$	16808	16808		
CaM-L$_{113}\delta$	16858	16859		
TIM-WT	31112	31113		
TIM-F$_{74}\delta$	31229	31230		
TIM-Y$_{101}\delta$	31213	31214		
TIM-F$_{74}$Y$_{101}\delta$	31330	31331		

Table S2. Solvent Gradients Used for Peptide Purification and Analysis.

Gradient	Time (min)	Buffer A (%)	Gradient	Time (min)	Buffer A (%)
1	0:00	98	2	0:00	98
	5:00	98		5:00	98
	8:00	71		8:00	71
	29:00	59		19:00	64
	33:00	0		23:00	0
	38:00	0		28:00	0
	43:00	98		33:00	98
3	0:00	98			
	5:00	98			
	12:00	50			
	22:00	38			
	25:00	0			
	30:00	0			
	34:00	98			



Absorption and Fluorescence Spectroscopy of Fluorophore Pairs. UV/Vis absorbance and fluorescence spectra were acquired for Trp, 7-methoxycoumarin-4-yl alanine (Mcm), and Acd peptides, and BODIPY Fl, 5-carboxyfluorescein (Fam), rhodamine 6G (R6G), and 2-((7-nitrobenzo[1,2,5]oxadiazol-4-yl)amino)ethanol (NBD). Absorption spectra were collected using quartz cells with 1 cm path lengths. Fluorescence spectra were collected as the average of three scans at 25 °C of three samples of each solution using quartz cells with 1 cm path lengths. Excitation and emission slit widths were 5 nm, scan rate 120 nm/min, averaging time 0.5 s, and data interval 1.0 nm.

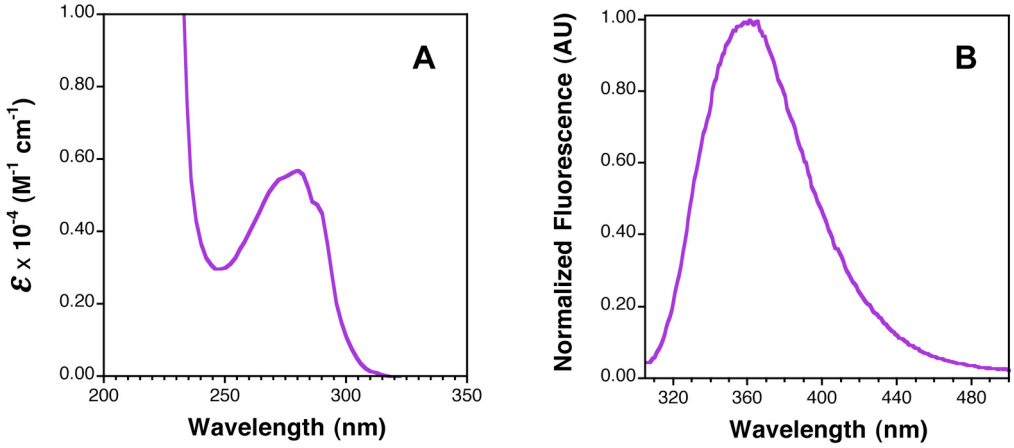


Figure S2. Absorption and Fluorescence Spectra of Trp. Absorbance (A) and fluorescence (B) spectra of 7.5 μM LeuProProTrp in pH 7.0 phosphate buffered saline (PBS; 10 mM Na_2HPO_4, 150 mM NaCl, pH adjusted with HCl). Concentration determined using published extinction coefficient of Trp (ε_{278} = 5, 700 M^{-1}•cm^{-1}).[4] Peptide synthesized as described.[5]

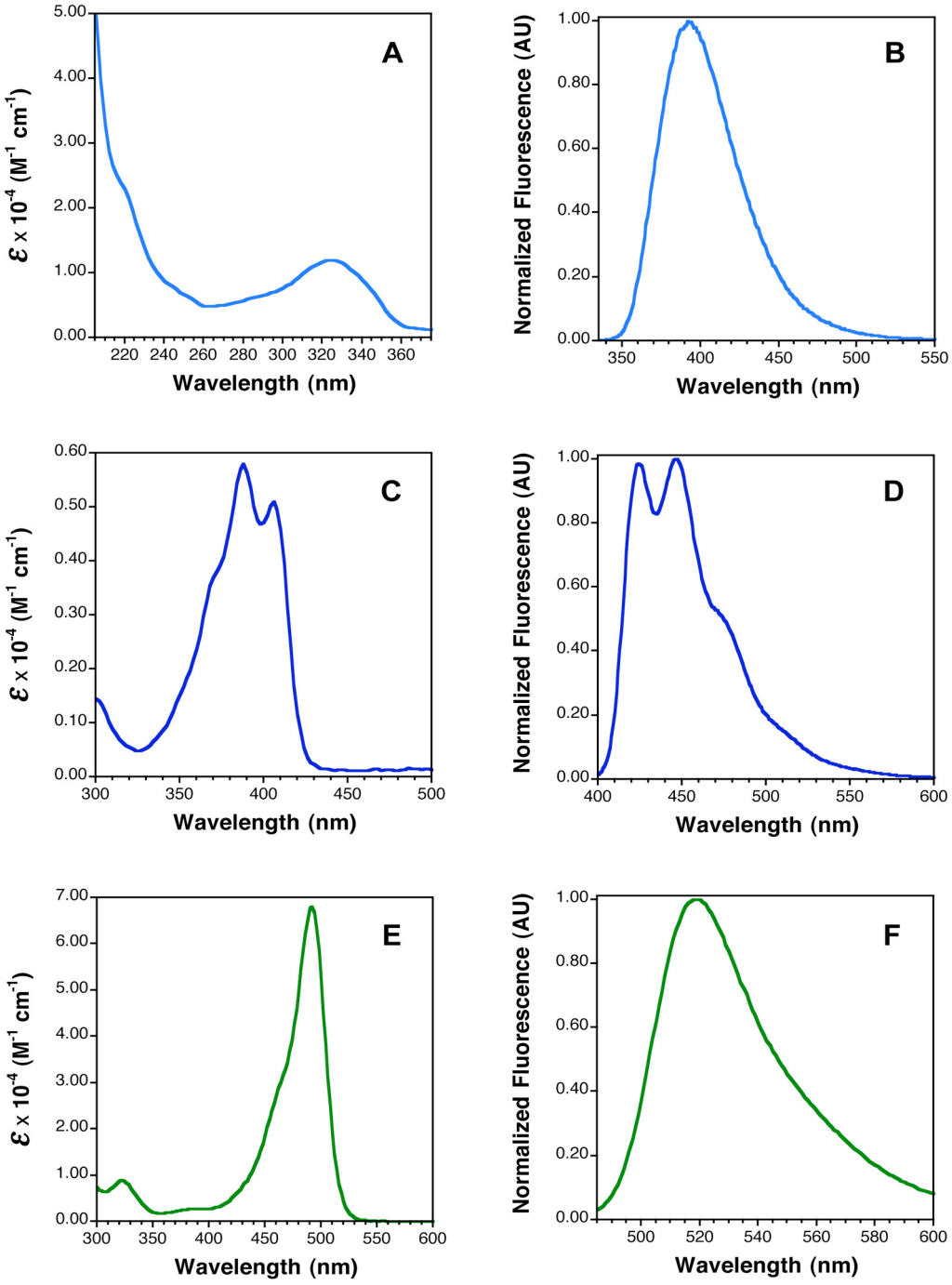


Figure S3. Absorption and Fluorescence Spectra of Mcm, Acd, and Fam. Absorbance (A) and fluorescence (B) spectra of 8.8 μM LeuProProMcm in pH 7.0 PBS . Peptide synthesized as described.[2] Absorbance (C) and fluorescence (D) spectra of 0.26 mM Acd and 2 μM Acd, respectively, in PBS, pH 7.0. Absorbance (E) and fluorescence (F) spectra of 9.5 μM Fam and 1.5 μM Fam, respectively, in 100 mM phosphate buffer, pH 7.0. Concentrations determined using published extinction coefficients of Mcm (ε_{325} = 12, 000 M^{-1}•cm^{-1}), Acd (ε_{386} = 5, 700 M^{-1}•cm^{-1}), and Fam (ε_{492} = 68, 000 M^{-1}•cm^{-1}).[6-8]

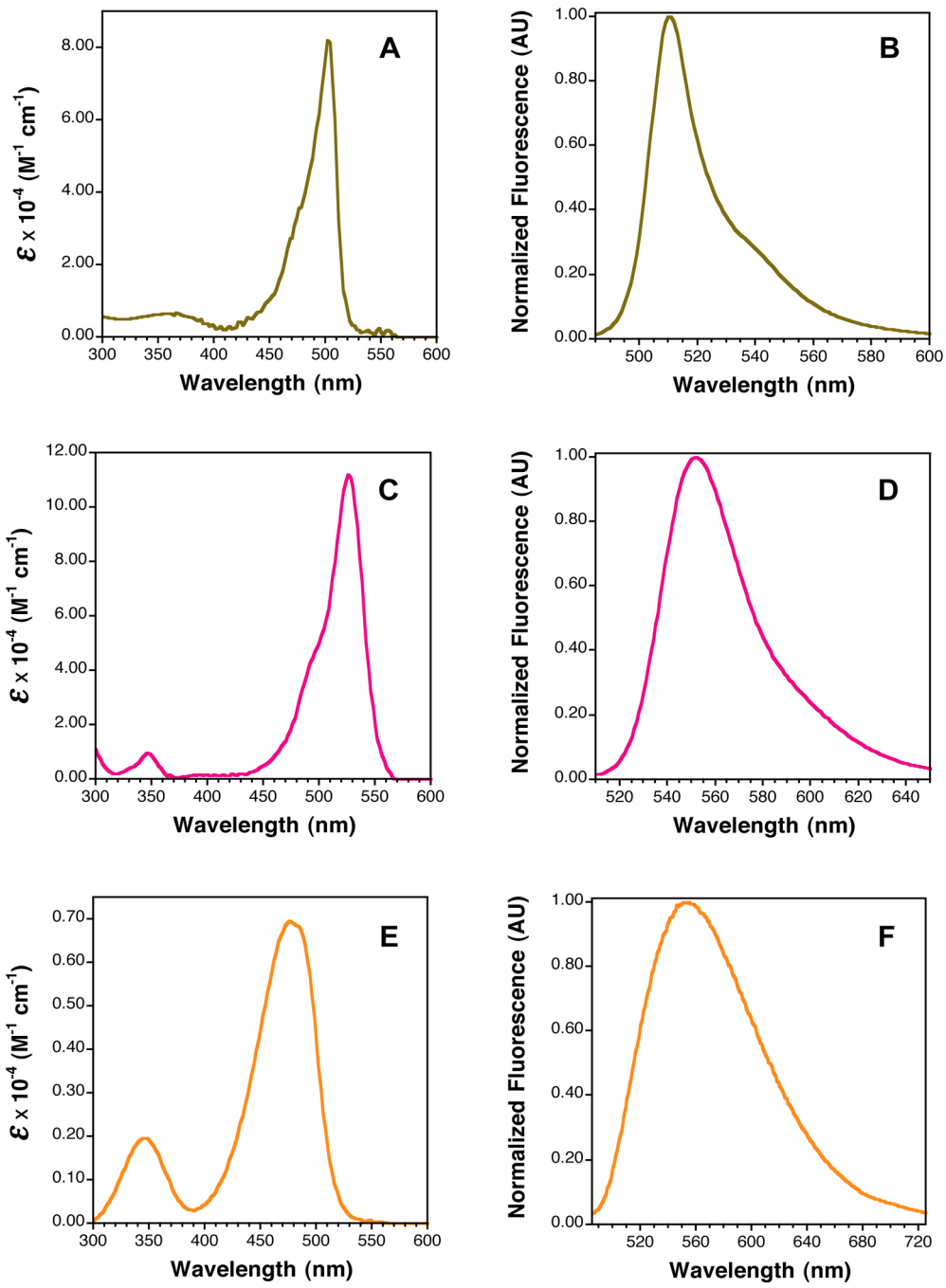


Figure S4. Absorption and Fluorescence Spectra of BODIPY Fl, R6G, and NBD in 100 mM phosphate buffer, pH 7.0. Absorbance (A) and fluorescence (B) spectra of 4.3 and 1.2 μM BODIPY Fl, respectively. Absorbance (C) and fluorescence (D) spectra of 4.5 and 0.25 μM R6G, respectively. Absorbance (E) and fluorescence (F) spectra of 86 and 15 μM NBD, respectively. Concentrations determined using published extinction coefficients of BODIPY Fl (ε_{502} = 82, 000 M^{-1}•cm^{-1}), R6G (ε_{530} = 116 000 M^{-1}•cm^{-1}), and NBD (ε_{484} = 6835 M^{-1}•cm^{-1}).[9-12]

Acd Steady-State Stern-Volmer Experiments. Stocks of 50 μM Acd and 100 mM tyrosine methyl ester (Tyr-OMe), cysteine, histidine, glycine, and tryptophan (83.6 mM stock solution) in 100 mM sodium phosphate buffer, pH 7.00, were used to prepare samples that were uniform in Acd concentration (5 μM) and variable in amino acid concentration (0, 2.5, 5, 15, 25, 35, 50, and 65 mM). The fluorescence of each sample was measured by exciting the solution at 385 nm and recording the emission at 450 nm. Measurements were made in Corning 3650 black flat-bottom microplates on a Perkin Elmer Envision Xcite instrument at 25 and 40 °C. Excitation and emission bandwidths were 8 nm and the gain was calculated automatically from a plate containing 5 μM Acd in four wells. The samples were allowed to equilibrate for several min at each temperature before scans were taken. The fluorescence intensity at 450 nm was averaged from three separate trials for Stern-Volmer calculations. The data for a given temperature were fit to Equation S1a or S1b with KaleidaGraph (Synergy Software; Reading, PA, USA).

$$\frac{F_0}{F} = 1 + K_{\mathrm{St}}[Q] \qquad \text{(S1a)}$$

$$\frac{F_0}{F} = \left(1 + K_{\mathrm{St}}[Q]\right)\left(1 + K_{\mathrm{Dy}}[Q]\right) \qquad \text{(S1b)}$$

F_0 is the average fluorescence intensity at 450 nm in the absence of quencher; F is the fluorescence intensity at 450 nm at each amino acid concentration step; K_{St} is the static Stern-Volmer constant in M^{-1}; K_{Dy} the dynamic Stern-Volmer constant in M^{-1}, and $[Q]$ is the quencher concentration in M. Only Trp and Tyr data were fit to equation S1b since all other quenching was negligible. K_{St} for Trp is 21.25 ± 0.28 M^{-1} at 25 °C. K_{St} for Tyr-OMe is 8.81 ± 0.24 M^{-1} at 25 °C. K_{Dy} was fixed at the value obtained from lifetime experiments (see below) for both Trp and Tyr-OMe.

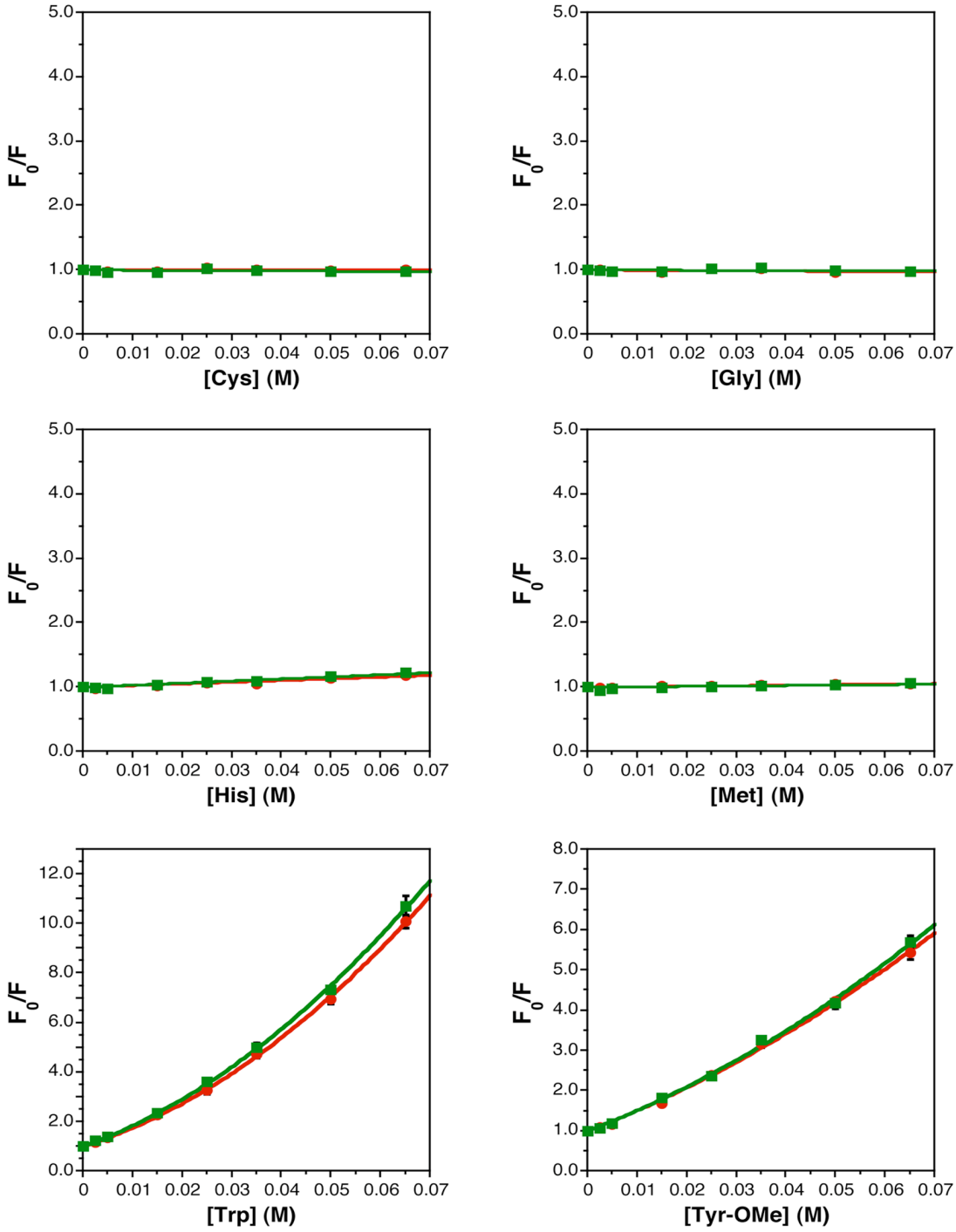


Figure S5. Steady-State Stern-Volmer Titrations of Acd with Amino Acids. Acd fluorescence (λ_{ex} = 385 nm, λ_{em} = 450 nm) recorded with varying concentrations of Cys, Gly, His, Met, Trp, or Tyr-OMe in 100 mM sodium phosphate buffer, pH 7.00 at 25 °C (red) or 40 °C (green). Average of three trials, error bars are calculated from standard error. Fits to equation S1a shown for Cys, Gly, His, and Met. Fits to equation S1b shown for Trp (R^2 = 0.99961 at 25 °C) and Tyr-OMe (R^2 = 0.99814 at 25 °C).

Acd Fluorescence Lifetime Stern-Volmer Experiments. Fresh solutions of each sample were prepared at identical concentrations for Stern-Volmer lifetime experiments. Time-resolved fluorescence measurements were performed using the Time-Correlated Single Photon Counting (TCSPC) method. The TCSPC system consisted of a blue diode laser (PicoQuant GmbH; Berlin, Germany) generating 10 MHz output pulses at 405 nm, a subtractive double monochromator with an MCP-PMT (Hamamatsu Photonics R2809U; Bridgewater, NJ, USA), and a TCSPC computer board (Becker and Hickl SPC-630; Berlin, Germany). Emission at 450 nm was monitored. All samples were thermostatted at 25 °C. Data analysis was performed with FluoFit software (PicoQuant) using a single-exponential decay model (Eq. S2).

$$I(t) = \sum_{i=1}^{n} A_i e^{-\frac{t}{\tau_i}} \tag{S2}$$

Here, $n = 1$, for a single-exponential fit, and the parameters A_i and τ_i are the amplitude and lifetime of the i^{th} component, respectively. Reduced χ^2 values were calculated for each fit according to Equation S3.

$$\chi^2 = \frac{1}{N-p}\sum_{j=1}^{N} W(j)^2\left[decay(j) - fit(j)\right]^2 \qquad W(j) = \frac{1}{\sqrt{decay(j)}} \tag{S3}$$

In this equation, N is the number of fitted data points j (i.e. measured photon counts at a given delay time, a bin); p is the number of adjustable, fitted parameters; $W(j)$ is a Poisson weighting factor; $decay(j)$ is the experimentally determined decay curve; and $fit(j)$ is the fitted model. The residuals were calculated according to Equation S4 using these values.

$$R = W(j)\left[decay(j) - fit(j)\right] \tag{S4}$$

Fluorescence lifetime data were used to construct a Stern-Volmer plot according to Equation S5

$$\frac{\tau_0}{\tau} = 1 + K_{\text{Dy}}[Q] \tag{S5}$$

where τ_0 is the fluorescence lifetime in the absence of quencher (15.73 ± 0.05 ns); τ is the lifetime at each quencher concentration; K_{Dy} is the Stern-Volmer constant in units of M^{-1}; and [*Q*] is the concentration of the quencher (Tyr-OMe or Trp) in M (Fig. S6). Using this equation, we found K_{Dy} = 38.20 ± 0.28 M^{-1} for Tyr-OMe and K_{Dy} = 48.98 ± 0.22 M^{-1} for Trp.

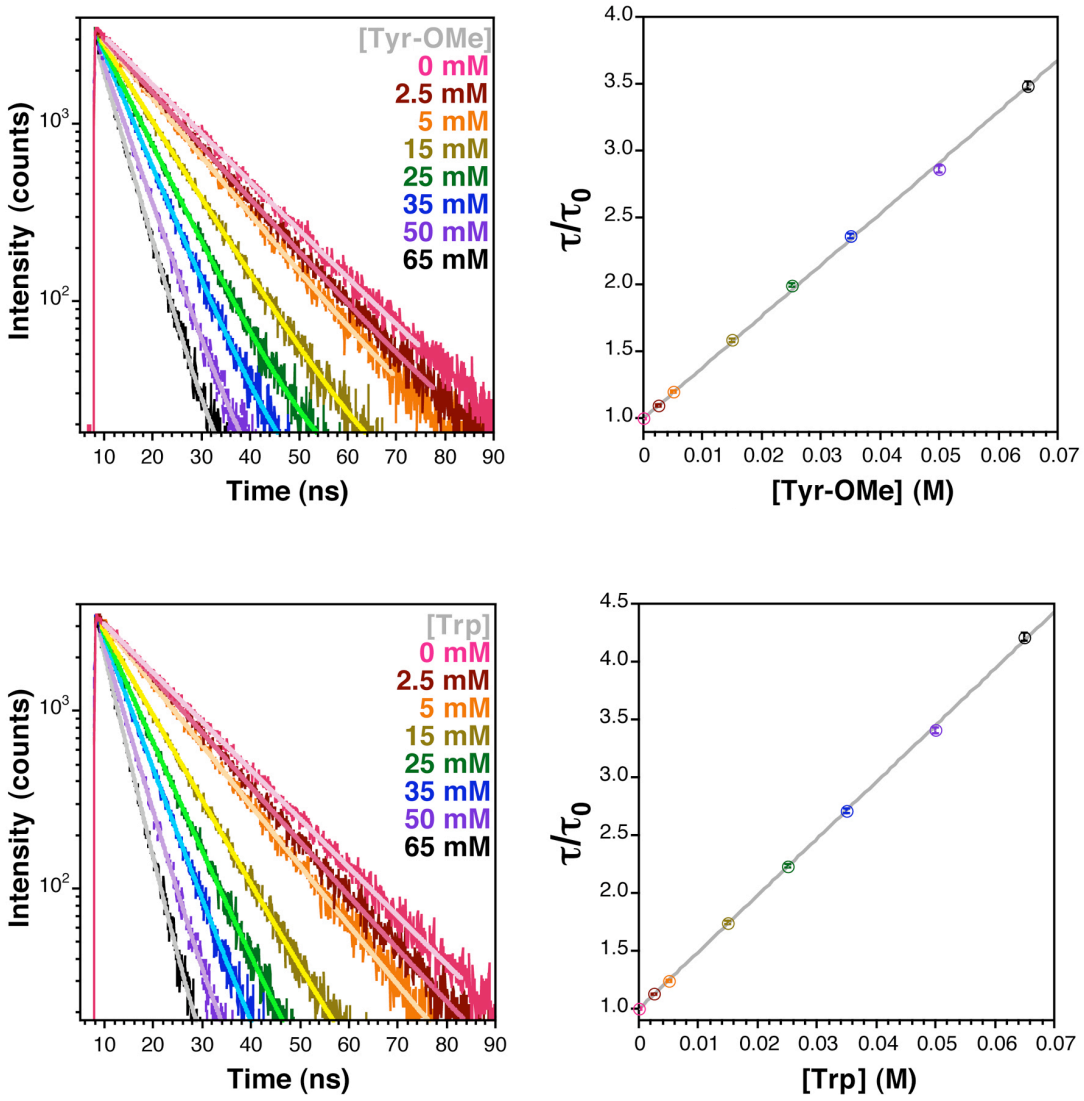


Figure S6. Lifetime Stern-Volmer experiments. Left: Fits to normalized Acd fluorescence lifetime data with varying concentrations of tyrosine methyl ester (Tyr-OMe) or tryptophan in 100 mM sodium phosphate buffer, pH 7.00 (λ_{ex} = 405 nm; λ_{em} = 450 nm). Right: Stern-Volmer plots of lifetime data at 25 °C. Error bars are calculated from fits of the raw data; R^2 = 0.99997 for Tyr-OMe, R^2 = 0.99997 for Trp.

Förster Distance Calculations. The Förster distance, R_0, is given in Å by equation (S6)

$$R_0^6 = \frac{9000(\ln 10)\kappa^2 \Phi_D J}{128\pi^5 n^4 N_A} \quad \text{(S6)}$$

where κ^2 is a geometrical factor that relates the orientation of the donor and acceptor transition moments, Φ_D is the quantum yield of the donor, n is the index of refraction of the solvent, N_A is Avogadro's number, and J is the spectral overlap integral defined in units of $M^{-1}\bullet cm^{-1}\bullet nm^4$. Combining constants and rearranging gives R_0 as

$$R_0 = 0.211\{\Phi_D \kappa^2 n^{-4} J\}^{1/6} \quad \text{(S7)}$$

J is formally defined as

$$J = \int_0^\infty f_D(\lambda)\varepsilon_A(\lambda)\lambda^4 \, d\lambda \quad \text{(S8)}$$

where $\varepsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor at each wavelength λ and $f_D(\lambda)$ is the normalized donor emission spectrum given by

$$f_D(\lambda) = \frac{F_{D\lambda}(\lambda)}{\int_0^\infty F_{D\lambda}(\lambda) \, d\lambda} \quad \text{(S9)}$$

where $F_{D\lambda}(\lambda)$ is the fluorescence of the donor at each wavelength λ. Fluorescence spectra of Trp (LeuProProTrp), Mcm (LeuProProMcm), and Acd (LeuProProAcd) were integrated with KaleidaGraph from 305 to 500 nm for Trp, 336 to 600 nm for Mcm, and 400 to 600 nm for Acd to calculate $f_D(\lambda)$. UV/Vis spectra of Acd, BODIPY Fl, Fam, R6G, and NBD in water were used to determine $\varepsilon_A(\lambda)$. J values were obtained using Equation (S8) for various donor/acceptor pairs are given in Table S3. Substituting these results into Equation (S7), as well as the donor quantum yields in Table S3, 1.33 for the index of refraction of water, and 2/3 for κ^2 gives the Förster distances listed in Table S3. These R_0 values were used to generate plots of FRET efficiency as a function of distance using Equation (S10).

$$E_{FRET} = \frac{1}{1+\left(\frac{R}{R_0}\right)^6} \quad \text{(S10)}$$

Here, E_{FRET} is the FRET efficiency and R is the separation between the chromophores. Plots of FRET efficiency for each chromophores are shown in Figure S7.

Table S3. Photophysical Characteristics of Acd FRET Pairs.

FRET Pair (Donor, Acceptor)	Φ_D	J (M^{-1}•cm^{-1}•nm^4)	R_0 (Å)	ε_λ (M^{-1}•cm^{-1}) @ λ_{max} (nm)
Trp, Acd	0.13[13]	5.47 x 10^{13}	22.6	5, 700 @ 386
Mcm, Acd	0.18[6]	7.96 x 10^{13}	25.4	5, 700 @ 386
Acd, Fam	0.95	9.64 x 10^{14}	50.8	68, 000 @ 492
Acd, BODIPY	0.95	8.18 x 10^{14}	49.4	82, 000 @ 504
Acd, R6G	0.95	7.86 x 10^{14}	49.1	116, 000 @ 530
Acd, NBD	0.95	5.47 x 10^{14}	37.0	6, 835 @ 484

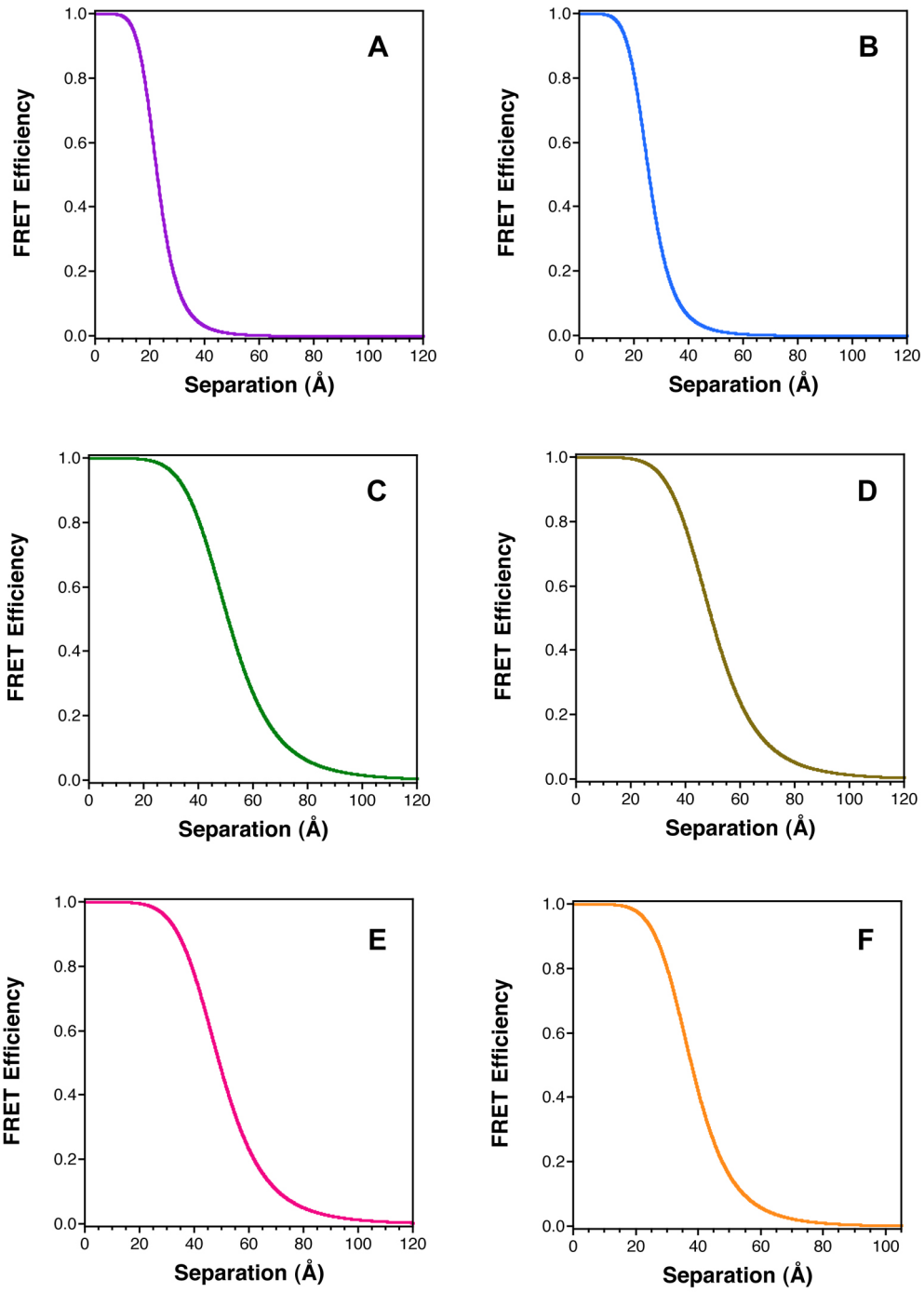


Figure S7. Distance Dependence of Acd FRET Interactions. FRET efficiencies of donor/acceptor pairs Trp/Acd (A), Mcm/Acd (B), Acd/Fam (C), Acd/BODIPY (D), Acd/R6G (E) and Acd/NBD (F) calculated according to Equations (S7), (S8), and (S10) using the values in Table S3.

Synthetase Sequences

M. jannaschii TyrRS

```
   1                                                          60
  MDEFEMIKRNTSEIISEEELREVLKKDEKSAYIGFEPSGKIHLGHYLQIKKMIDLQNAGF

  61                                                         120
  DIIILLADLHAYLNQKGELDEIRKIGDYNKKVFEAMGLKAKYVYGSEFQLDKDYTLNVYR

 121                                                         180
  LALKTTLKRARRSMELIAREDENPKVAEVIYPIMQVNDIHYLGVDVAVGGMEQRKIHMLA

 181                                                         240
  RELLPKKVVCIHNPVLTGLDGEGKMSSSKGNFIAVDDSPEEIRAKIKKAYCPAGVVEGNP

 241                                                         300
  IMEIAKYFLEYPLTIKRPEKFGGDLTVNSYEELESLFKNKELHPMDLKNAVAEELIKILE

 301
  PIRKRL
```

Synthetase Mutants

	31	32	65	107	108	109	158	159	161	162	164	167
TyrRS	Ala	Tyr	Leu	Glu	Phe	Gln	Asp	Ile	Tyr	Leu	Val	Ala
BrbRS1		Gly	Glu				Gly	Cys				
BrbRS2		Gly	Val		Trp	Met	Gly	Pro				
BrbRS3		Gly	Glu		Trp	Met	Ser					
BzfRS1		Gly		Pro			Thr	Ser	Arg			
BzfRS2		Gly		Pro			Thr	Ser	Arg	Ala		
BzfRS3		Gly		Pro			Ser	Ser	Arg			
BzfRS4	Val	Gly		Pro			Ser	Ser	Arg			
BzfRS4		Gly		Pro			Ser	Ser	Arg		Ala	
NapRS1		Ala		Pro			Pro	Ala				Val

Figure S8. Sequences of Candidate Synthetases. Top: Amino acid sequence of parent *M. jannaschii* TyrRS. Residues where mutations were examined are highlighted in red. Bottom: Sequences of mutant synthetases screened for selective Acd incorporation using GFP assay. Amino acids listed by position number. Blank cells indicate no mutation. BrbRS1 = AcdRS.

Cloning of Calmodulin Expression Constructs. pCaM, A plasmid containing the wild-type chicken calmodulin (CaM) gene was provided by Joshua Wand (Perelman School of Medicine at the University of Pennsylvania). An insert containing the CaM gene (458 base pairs from start to stop codon) was cloned into a pET15b vector (Novagen; Gibbstown, NJ, USA) between the NcoI and XhoI cut sites. Quikchange® site-directed mutagenesis was used to generate CaM mutants by inserting a TAG codon at the site of interest: $F_{13}\delta$, $G_{41}\delta$, $Y_{100}\delta$, and $L_{113}\delta$. The mutant plasmids were verified by DNA sequencing analysis with a T7 promoter primer.

A. CaM Gene

```
5'-atggctgatcaactgacagaagagcagattgcagaattcaaagaagctttttcactattt
gacaaggatggtgatggtactataactacaaaggagttggggactgtgatgagatcacttggt
cagaaccccacagaagcagaattacaggacatgatcaatgaagtagacgctgatggcaatggc
acaattgacttcccagagtttctgacaatgatggcaagaaaaatgaaagatacagatagcgaa
gaagaaattagagaagcgttccgtgtgtttgacaaggatggtaatggttacattagtgctgca
gaacttcgtcatgtgatgacaaatcttggggagaagctaacagatgaagaagttgatgaaatg
attagggaagcagacattgatggtgatggtcaagtaaactatgaagagtttgtacagatgatg
acagcgaagtga-3'
```

B. Chicken CaM Amino Acid Sequence

MADQLTEEQIAE**F**KEAFSLFDKDGDGTITTKELGTVMRSL**G**QNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKM
KDTDSEEEIREAFRVFDKDGNG**Y**ISAAELRHVMTN**L**GEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAK

C. DNA Oligomers used for Quikchange® Mutagenesis

F13δ_For	5'-ACTGACAGAAGAGCAGATTGCAGAATAGAAAGAAGCTTTTTCACTATTTGAC-3'
F13δ_Rev	5'-GTCAAATAGTGAAAAAGCTTCTTTCTATTCTGCAATCTGCTCTTCTGTCAGT-3'
G41δ_For	5'-TTCTGTGGGGTTCTGCTAAAGTGATCTCATCACAGTCCCCAACTCC-3'
G41δ_Rev	5'-GGAGTTGGGGACTGTGATGAGATCACTTTAGCAGAACCCCACAGAA-3'
Y100δ_For	5'-GACAAGGATGGTAATGGTTAGATTAGTGCTGCAGAACTTCG-3'
Y100δ_Rev	5'-CGAAGTTCTGCAGCACTAATCTAACCATTACCATCCTTGTC-3'
L113δ_For	5'-CTGCAGAACTTCGTCATGTGATGACAAATTAGGGGGAGAAGCTAACA-3'
L113δ_Rev	5'-TGTTAGCTTCTCCCCCTAATTTGTCATCACATGACGAAGTTCTGCAG-3'

Figure S9. A) DNA sequence of chicken CaM gene from start to stop codon. B) Amino acid sequence of chicken CaM. F_{13}, G_{41}, Y_{100}, L_{113} highlighted in bold red. C) Forward and reverse DNA oligomers used for site-directed mutagenesis.

Wild-Type Calmodulin Protein Expression. The plasmid containing the chicken CaM gene, pCaM, was used to transform *E. coli* BL21(DE3) cells. Transformed cells were selected on the basis of ampicillin resistance. Single colonies were used to inoculate 5 mL of LB media supplemented with ampicillin (100 μg/mL). To an autoclaved solution containing 42 mM Na_2HPO_4, 22 mM KH_2PO_4, 19 mM NH_4Cl, and 86 mM NaCl (M9 salts), the following autoclaved solutions were added per liter of M9 salts: 1 mL of 2 M $MgSO_4$, 1 mL of 15 mg/mL $FeCl_2$ (in 1.0 M HCl), 1 mL of 15 mg/mL $ZnCl_2$ (in acidified H_2O), 6.25 mL of 40% glucose, 100 μL of 1M $CaCl_2$ and 2 mL of 10% Bacto™ Yeast Extract. The primary 5 mL culture was incubated at 37 °C with shaking at 250 rpm for 4 h. Upon the primary culture reaching saturation, it was added to 1 L of M9 minimal media supplemented with ampicillin (see concentrations). The 1 L culture was incubated at 37 °C with shaking at 250 rpm until the OD_{600} reached 0.7 AU. Protein expression was induced with isopropyl D-galactoside (IPTG), and the culture was incubated at 37 °C with shaking at 250 rpm for an additional 16 h. The cells were again harvested by centrifuging at 5000 x g for 15 min and the resulting pellet was suspended in 15 mL of MOPS resuspension buffer (50 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS), 100 mM KCl, 1 mM ethylenediamine tetraacetic acid (EDTA), pH 7.5). Following sonication, the cell lysate was allowed to cool on ice for 5 min. $CaCl_2$ was added to the sonicated lysate to a final concentration of 5 mM prior to centrifugation for 20 min at 30,000 x g, 4 °C.

Acd Mutant Calmodulin Protein Expression. The pCaM-$F_{13}\delta$, -$G_{41}\delta$, -$Y_{100}\delta$, or -$L_{113}\delta$. plasmid (with Amp resistance) was used to transform *E. coli* BL21(DE3) cells. These cells were previously transformed with the pDule2-Acd plasmid and made competent following the Hanahan method.[9] The pDule2-Acd plasmid encodes the AcdRS and cognate tRNA (streptomycin resistant). Transformed cells were selected on the basis of Amp and Strep

resistance. Single colonies were used to inoculate 5 mL of LB media supplemented with Amp and Strep (100 μg/mL each). To an autoclaved solution containing 42 mM Na_2HPO_4, 22 mM KH_2PO_4, 19 mM NH_4Cl, and 86 mM NaCl (M9 salts), the following autoclaved solutions were added per liter of M9 salts: 1 mL of 2 M $MgSO_4$, 1 mL of 15 mg/mL $FeCl_2$ (in 1.0 M HCl), 1 mL of 15 mg/mL $ZnCl_2$ (in acidified H_2O), 6.25 mL of 40% glucose, 100 μL of 1M $CaCl_2$ and 2 mL of 10% Bacto™ Yeast Extract. The primary 5 mL culture was incubated at 37 °C with shaking at 250 rpm for 4 h. Upon reaching saturation, the primary culture was added to 1 L of M9 minimal media supplemented with Amp and Strep. The 1 L culture was incubated at 37 °C with shaking at 250 rpm until the OD_{600} reached 0.7 AU. At this point, a solution of 282 mg Acd in 4 mL sterile water (with 4 drops 10 M NaOH to solubilize Acd) was added and protein expression was induced with IPTG. The culture was incubated at 37 °C for an additional 16 h. The cells were harvested at 5000 x g for 15 minutes and the resulting pellet was suspended in 15 mL of MOPS resuspension buffer (50 mM MOPS, 100 mM KCl, 1 mM EDTA, pH 7.5). Following sonication, the cell lysate was allowed to cool on ice for 5 min. $CaCl_2$ was added to the sonicated lysate to a final concentration of 5 mM prior to centrifugation for 20 minutes at 30,000 x g, 4 °C.

CaM was purified from the cleared cell lysate using a phenyl-sepharose (PhS) CL-4B column with EDTA as eluent. Using a total resin bed volume of 10 mL, the column was first equilibrated with 4 column volumes of PhS Buffer A (50 mM Tris base, 1 mM $CaCl_2$, pH 7.5). After the cleared cell lysate was loaded and allowed to pass through the resin, the column was washed with 4 column volumes of PhS Buffer A, 4 column volumes of high-salt PhS Buffer B (50 mM Tris base, 0.5 M NaCl, 0.1 mM $CaCl_2$, pH 7.5), and an additional 2 column volume washes of PhS Buffer A to restore low-salt conditions. CaM was eluted with PhS Buffer C (10

mM Tris base, 10 mM EDTA, pH 7.5) and collected in 1 mL fractions and the presence of protein was detected by SDS-PAGE. Fractions containing protein were combined and dialyzed against water for 16 h at 4 ºC and dialyzed into 15 mM HEPES, 140 mM KCl, 6 mM $CaCl_2$, pH 6.7.

For CaM-Y_{100}δ, additional purification was performed. Following PhS purification, fractions containing protein were directly loaded onto a HiTrap Q-Column (GE Healthcare) on an ÄKTA FPLC system. The running buffer was 50 mM Tris pH 7.5 and the eluent was 50 mM Tris, 500 mM NaCl, pH 7.5. Fractions containing CaM-Y_{100}d eluted between 44 and 47 minutes (corresponding to 31 to 34 % elution buffer). These fractions were combined and dialyzed against water and then against 15 mM HEPES, 140 mM KCl, pH 6.70.

Cloning of Triose Phosphate Isomerase Expression Constructs. The tpiA gene from *E. coli* genome was cloned into pBad vector (Invitrogen) between the NcoI and KpnI cut sites. Quikchange® site-directed mutagenesis was used to generate the TAG mutants $F_{74}\delta$ and $Y_{101}\delta$. The mutant plasmids were verified by DNA sequencing analysis with a pBad primer below.

A. TIM Gene

```
5'atgggggttctcatcatcatcatcatggtatggctagcatgactggtggacagcaaatg
ggtcgggatctgtacgacgatgacgataaggatcgatggggatccgagctcgagcgacatccttt
agtgatgggtaactggaaactgaacggcagccgccacatggttcacgagctggtttctaacctgc
gtaaagagctggcaggtgttgctggctgtgcggttgcaatcgcaccaccggaaatgtatatcgat
atggcgaagcgcgaagctgaaggcagccacatcatgctgggtgcgcaaaacgtggacctgaacct
gtccggcgcattcaccggtgaaacctctgctgctatgctgaaagacatcggcgcacagtacatca
tcatcggtcactctgaacgtcgtacttaccacaaagaatctgacgaactgatcgcgaaaaaattc
gcggtgctgaaagagcagggcctgactccggttctgtgcatcggtgaaaccgaagctgaaaatga
agcgggcaaaactgaagaagtttgcgcacgtcagatcgacgcggtactgaaaactcagggtgctg
cggcattcgaaggtgcggttatcgcttacgaacctgtatgggcaatcggtactggcaaatctgca
actccggctcaggcacaggctgttcacaaattcatccgtgaccacatcgctaaagttgacgctaa
catcgctgaacaagtgatcattcagtacggcggctctgtaaacgcgtctaacgctgcagaactgt
ttgctcagccggatatcgacggcgcgctggttggtggtgcttctctgaaagctgacgccttcgca
gtaatcgttaaagctgcagaagcggctaaacaggcttaa-3'
```

B. *E. coli* TIM Amino Acid Sequence

```
MGGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGSELERHPLVMGNWKLNGSRHMVHELVSNLRKELAGVAGCAVA
IAPPEMYIDMAKREAEGSHIMLGAQNVDLNLSGAFTGETSAAMLKDIGAQYIIIGHSERRTYHKESDELIAKKFAVL
KEQGLTPVLCIGETEAENEAGKTEEVCARQIDAVLKTQGAAAFEGAVIAYEPVWAIGTGKSATPAQAQAVHKFIRDH
IAKVDANIAEQVIIQYGGSVNASNAAELFAQPDIDGALVGGASLKADAFAVIVKAAEAAKQA
```

C. DNA Oligomers used for Quikchange® Mutagenesis

F74δ_For	5′—CTGAACCTGTCCGGCGCATAGACCGGTGAAACCTCTGCTG-3′
F74δ_Rev	5′—CAGCAGAGGTTTCACCGGTCTATGCGCCGGACAGGTTCAG-3′
Y101δ_For	5′—CTGAACGTCGTACTTAGCACAAAGAATCTGAC-3′
Y101δ_Rev	5′—GTCAGATTCTTTGTGCTAAGTACGACGTTCAG-3′
pBad_Seq	5′—ATGCCATAGCATTTTTATCC—3′

Figure S10. A) DNA sequence of *E. coli* tpiA gene from start to stop codon. B) Amino acid sequence of *E. coli* TIM. F_{74},Y_{101} highlighted in bold red. C) Forward and reverse DNA oligomers used for site-directed mutagenesis and sequencing.

Wild-Type Triose Phosphate Isomerase Expression. The wt-TIM plasmid containing the *E. coli* TIM gene with (with Amp resistance) was used to transform *E. coli* DH10B cells via electroporation. Single colonies were then picked and grown overnight at 37°C with 250 rpm shaking in 5 mL of LB media (with Amp antibiotic). These starter cultures were then used to inoculate 500 mL of autoinduction media (created as previously described).[14] The cultures were grown for 24 h under these conditions. The cells were harvested at 5000 x g for 15 min and the resulting pellet was suspended in 15 mL 20 mM Tris Base, 300 mM KCl, and 15 mM imidazole. Following sonication, the cell lysate was allowed to cool on ice for 5 min. The cell lysate was cleared of debris by centrifugation for 20 min at 30,000 x g, 4 °C. The cleared lysate was then added to 3 mL of Ni-NTA slurry and incubated at 4 °C for 1 hour with moderate shaking to ensure thorough mixing. Following this incubation, the column flow-through was collected and the slurry was washed with the following buffers: 20 mL of 20 mM Tris Base, 300 mM KCl, and 50 mM imidazole, 20 mL of 20 mM Tris Base, 300 mM KCl, and 75 mM imidazole, and 10 mL of 20 mM Tris Base, 300 mM KCl, and 100 mM imidazole. Acd-TIM mutants were eluted from the Ni-NTA column by 4 x 1.5 mL and 1 x 5 mL portions of 20 mM Tris Base, 300 mM KCl, and 250 mM imidazole. Elution fractions containing the protein of interest were identified by SDS-PAGE analysis.

Acd Mutant TIM Expression. The TIM-$F_{74}\delta$ or -$Y_{101}\delta$ plasmid (with Amp resistance) along with a pDule1 plasmid containing the AcdRS and cognate tRNA (with Tet resistance) was used to transform *E. coli* DH10B cells via electroporation. Single colonies were then picked and grown overnight at 37 °C with 250 rpm shaking in 5 mL of LB media (with Amp and Tet antibiotics). These starter cultures were then used to inoculate 500 mL of autoinduction media (created as previously described).[14] After 30 minutes of growth at 37 °C with 250 rpm shaking, a

solution of 282 mg Acd in 4 mL sterile water (with 4 drops 10 M NaOH to solubilize Acd) was added. The cultures were grown for 24 h under these conditions. The cells were harvested at 5000 x g for 15 minutes and the resulting pellet was suspended in 15 mL 20 mM Tris Base, 300 mM KCl, and 15 mM imidazole. Following sonication, the cell lysate was allowed to cool on ice for 5 min. The cell lysate was cleared of debris by centrifugation for 20 minutes at 30,000 x g, 4 °C. The cleared lysate was then added to 3 mL of Ni-NTA slurry and incubated at 4 °C for 1 h with moderate shaking to ensure thorough mixing. Following this incubation, the column flow-through was collected and the slurry was washed with the following buffers: 20 mL of 20 mM Tris Base, 300 mM KCl, and 50 mM imidazole, 20 mL of 20 mM Tris Base, 300 mM KCl, and 75 mM imidazole, and 10 mL of 20 mM Tris Base, 300 mM KCl, and 100 mM imidazole. Acd-TIM mutants were eluted from the Ni-NTA column by 4 x 1.5 mL and 1 x 5 mL portions of 20 mM Tris Base, 300 mM KCl, and 250 mM imidazole. Elution fractions containing the protein of interest were identified by SDS-PAGE analysis.

Cloning of α-Synuclein Expression Constructs. A plasmid containing the human wild-type αS gene cloned between NdeI and HindIII in the expression vector pRK172 was provided by Dr. Virginia Lee (Perelman School of Medicine, University of Pennsylvania). Quikchange® site-directed mutagenesis was used to generate the TAG mutants $Y_{39}\delta$ and $F_{94}\delta$. Sequencing was confirmed using a T7 promoter.

A. αS Gene

```
5'atggatgtattcatgaaaggactttcaaaggccaaggagggagttgtggctgctgctgagaaa
accaaacagggtgtggcagaagcagcaggaaagacaaaagagggtgttctctatgtaggctccaa
aaccaaggagggagtggtgcatggtgtggcaacagtggctgagaagaccaaagagcaagtgacaa
atgttggaggagcagtggtgacgggtgtgacagcagtagcccagaagacagtggagggagcaggg
agcattgcagcagccactggctttgtcaaaaaggaccagttgggcaagaatgaagaaggagcccc
acaggaaggaattctggaagatatgcctgtggatcctgacaatgaggcttatgaaatgccttctg
aggaagggtatcaagactacgaacctgaagcctaa-3'
```

B. Human αS Amino Acid Sequence

```
MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKTKEGVVHGVATVAEKTKEQVTNVGGAVVTGVTAV
AQKTVEGAGSIAAATGFVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGYQDYEPEA
```

C. DNA Oligomers used for Quikchange® Mutagenesis

Y39δ_For	5'-AAAAGAGGGTGTTCTCTAGGTAGGCTCCAAAACCAAG-3'
Y39δ_Rev	5'-CTTGGTTTTGGAGCCTACCTAGAGAACACCCTCTTTT-3'
F94δ_For	5'-GCATTGCAGCAGCCACTGGCTAGGTCAAAAAGGACCAGTTGGG-3'
F94δ_Rev	5'-CCCAACTGGTCCTTTTTGACCTAGCCAGTGGCTGCTGCAATGC-3'

Figure S11. A) DNA sequence of human αS gene from start to stop codon. B) Amino acid sequence of human αS. Y_{39} and F_{94} are highlighted in bold red. C) Forward and reverse DNA oligomers used for site-directed mutagenesis.

Wild-Type α-Synuclein Expression. The plasmid containing the αS gene was transformed into *E. coli* BL21(DE3) cells. Single colonies were used to inoculate 4 mL of LB media supplemented with ampicillin (100 μg/mL). The primary culture was grown at 37 °C with shaking at 250 rpm for 4 h. A 500 mL secondary culture was inoculated using 1 mL of the primary culture and grown at 37 °C with shaking at 250 rpm for 20 h. The cells were harvested at 5000 x g for 15 min and the resulting pellet was resuspended in 20 mM Tris, pH 8.0 with 1 mM PMSF and sonicated. Following sonication, the cell lysate was boiled for 30 min prior to centrifugation for 20 min at 30,000 x g, 4 °C. The cleared lysate was dialyzed overnight against 20 mM Tris pH 8.0 at 4 °C prior to loading on a Superdex 200 column (25 cm) connected to a BioCad Sprint (FPLC) system. FPLC fractions were dialyzed against 20 mM Tris pH 8.0 at 4 °C and further purified using a HiTrap Q HP column (GE Healthcare) on the BioCad FPLC. Fractions were dialyzed against Milli-Q water at stored at 4 °C. Protein was concentrated using 3KDa Amicon Ultra-0.5 mL centrifugal filters (EMD Millipore) and adjusted to 500 μM by BCA assay and stored at -80 °C until further use. SDS-PAGE analysis was performed to analyze the purity of αS.

Acd Mutant α-Synuclein Expression. *E. coli* BL21-Gold (DE3) cells were transformed with the αS-Y_{39}δ or -F_{94}δ plasmid and a pDule2 plasmid containing the AcdRS and $tRNA_{CUA}$ pair. Cells were selected for resistance to both ampicillin (100 μg/mL) and streptomycin (100 μg/mL). Single colonies were used to inoculate 4 mL of LB media. The primary culture was grown at 37 °C with shaking at 250 rpm for 4 h. A 500 mL secondary culture was inoculated using 1 mL of the primary culture and grown at 37 °C with shaking at 250 rpm until OD_{600} 0.8. Protein expression was induced with 1 mM Acd (282 mg in 4 mL sterile water solubilized with 4 drops 10 M NaOH) and 1 mM IPTG, then cells were grown at 37 °C for 18 h. The cells were

harvested at 5000 x g for 15 min and the resulting pellet was resuspended in 20 mM Tris, pH 8.0 with 1 mM PMSF and sonicated. Following sonication, the cell lysate was boiled for 30 min prior to centrifugation for 20 min at 30,000 x g, 4 °C. The cleared lysate was dialyzed overnight against 20 mM Tris, pH 8.0 at 4 °C prior to loading on a Superdex 200 column (25 cm). FPLC fractions were dialyzed against 20 mM Tris, pH 8.0 at 4 °C and further purified using a HiTrap Q HP column (GE Healthcare). Fractions were dialyzed against Milli-Q water and stored at 4 °C. Protein was concentrated using 3 KDa Amicon Ultra-0.5 mL centrifugal filters (EMD Millipore) and adjusted to 500 μM by BCA assay. Protein was then stored at -80 °C until further use. SDS-PAGE analysis was performed to analyze the purity of αS.

Table S4a. CaM Protein Expression

protein	yield (mg/L)	% yield relative to WT
WT	30.0	-
13	16.8	56.0
41	18.1	60.3
100	6.1	20.3
113	20.7	69.0

Table S4b. TIM Protein Expression

protein	yield (mg/L)	% yield relative to WT
WT	26.4	-
74	4.0	15.2
101	0.8	3.0

Table S4c. αS Protein Expression

protein	yield (mg/L)	% yield relative to WT
WT	30.0	-
39	2.0	6.7
94	5.0	16.7

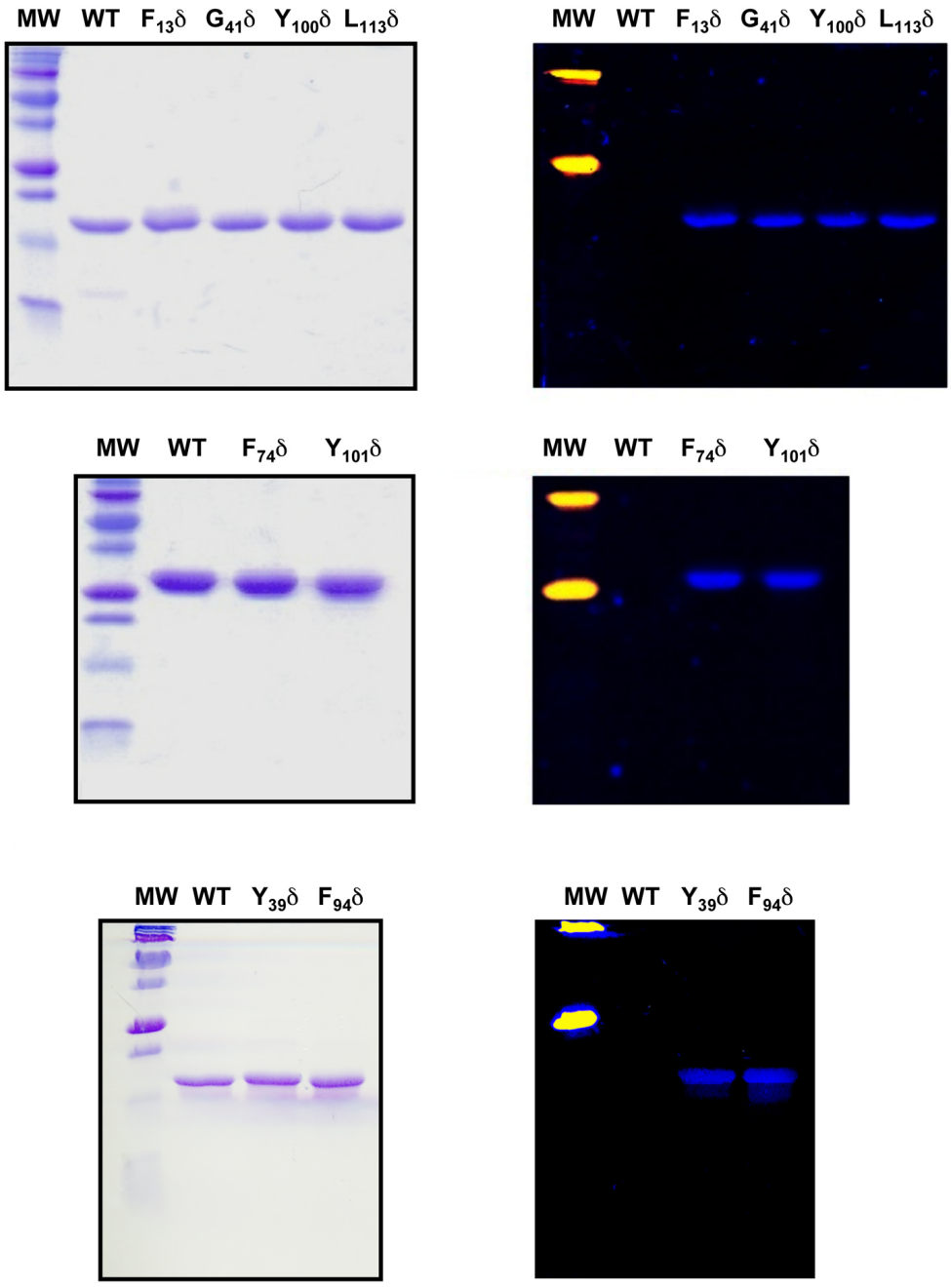
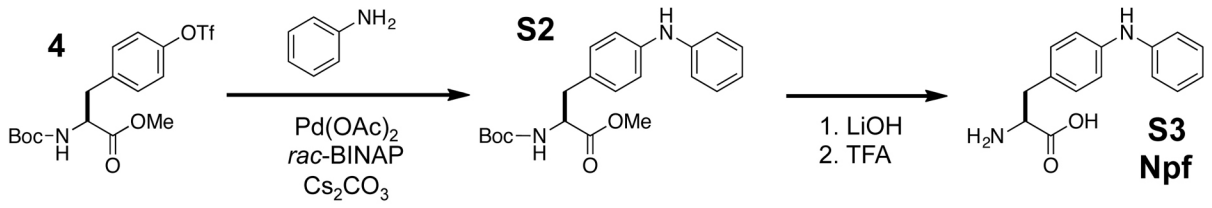


Figure S12. SDS PAGE Gel Analysis of Mutant Protein Expression. Top: CaM mutants, Middle, TIM mutants, Bottom: αS mutants. Coomassie stained gels are shown at left, fluorescence images at right. Molecular weight (MW) markers: 7, 10, 17, 25, 40, 50, 75, 100, and 150 kDa. The 25 and 75 kDa markers are fluorescently stained.

Synthesis of (*S*)-2-amino-3-(4-(phenylamino)phenyl)propanoic acid (Npf, S3).



HPLC Analysis of Acd Batches. Amino acid batches for protein expression were dissolved in a solution of 9:1 water/CH_3CN with 0.1% TFA and centrifuged at 13,200 rpm for 15 min. The supernatant was collected and diluted to a concentration of approximately 10 μM as determined by UV/Vis spectroscopy. Acd batches were produced either by the sulfuric acid or PPA route. *N*-phenyl-*p*-aminophenylalanine (Npf) was synthesized by coupling of aniline to **4** using the Pd-catalyzed conditions described for Acd synthesis. LRMS (calcd m/z $(M+H)^+$ 257.13, found 275.15) and crude (90 % purity) ^{1}H NMR confirmed product identity. Full characterization will be reported elsewhere. These samples were analyzed using an Agilent 1100 HPLC with a Luna $C_{18}(2)$, 150 x 4.60 mm, 3 μm column (Phenomenex; Torrance, CA, USA). The solvent gradient is shown in Table S5.

No evidence of Npf was seen in the HPLC chromatogram of the "H_2SO_4" batch, but the mass of Npf (m/z 257.1) was found during LRMS analysis of this batch, and protein containing Npf was obtained from expression of the CaM-$L_{113}\delta$ construct with this amino acid batch. (Fig. S13) Expression of the construct with the Npf batch confirmed that Npf could be incorporated by AcdRS. HPLC analysis and expression of the CaM-$L_{113}\delta$ construct with a pure batch of Acd produced by the PPA route is shown for comparison.

Table S5. Acd HPLC Analysis Gradient

Time (min)	% Solvent A
0:00	99
6:00	98
12:00	95
18:00	90
30:00	50
35:00	1
40:00	1
50:00	99

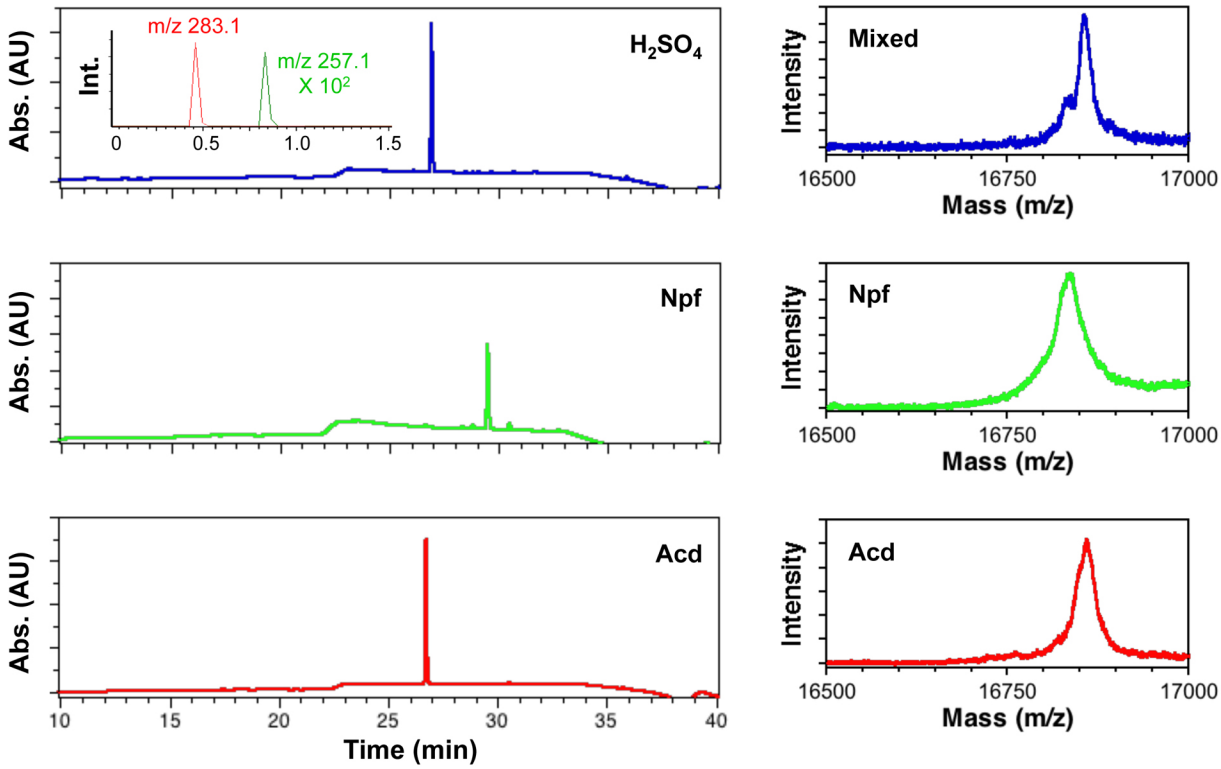


Figure S13. HPLC, LRMS, and MALDI MS Analysis of Amino Acids and Protein Expression. Left: HPLC chromatograms of amino acid monomer batches. H_2SO_4: Acd batch produced by global deprotection and cyclization in sulfuric acid. Inset shows LRMS selected ion chromatograms for Acd, calcd $(M+H)^+$ m/z 283.1, and Npf calcd $(M+H)^+$ m/z 257.1. Weaker Npf ion chromatogram signal scale multiplied by 10^2. Npf: Authentic Npf sample produced by coupling of aniline to **4**. Acd: Pure Acd batch produced by PPA route. No mass corresponding to Npf was observed in LRMS analysis of this batch. Right: MALDI MS analysis of full-length CaM-$L_{113}\delta$ construct expressed using either a "Mixed" Npf/Acd batch from H_2SO_4 cyclization; authentic Npf, calcd m/z 16833, found 16835; or pure Acd, calcd m/z 16858, found 16859.

Trypsin Digest Analysis of Acd Mutants. Protein (CaM-$L_{113}\delta$, αS-$Y_{39}\delta$, or αS-$F_{94}\delta$) was precipitated using 1:4 8.75 M trichloroacetic acid/protein sample and incubated at 4 °C for 15 minutes. The precipitate was centrifuged for 15 min at 13,200 rpm to pellet protein. The protein pellet was then washed three times with cold acetone to remove trace trichloroacetic acid. Trace acetone was removed by incubating protein pellets in a 95 °C water bath for 5 min open to the atmosphere. Protein pellets were then re-suspended in 6 M guanidinium hydrochloride with 50 mM Tris pH 8.0, and denatured by boiling at 95 °C for 10 minutes. Protein samples were then diluted to 0.75 M guanidinium hydrochloride with 50 mM Tris pH 7.6 and 1 mM calcium chloride. Sequencing grade modified trypsin (0.6 µg, Promega) was used to digest samples for 24 hours at 37 °C. Trypsin digest aliquots (1 µL) were combined with α-cyano-4-hydroxycinnamic acid (1 µL of a saturated solution in 1:1 H_2O/CH_3CN with 1 % TFA) and analyzed by MALDI MS.

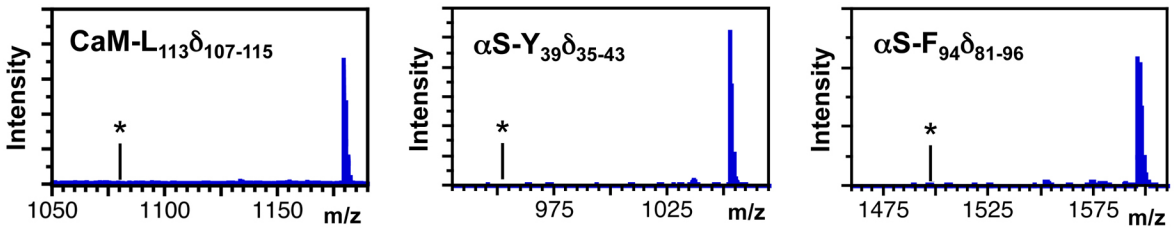


Figure S14. MALDI MS of Trypsinized Proteins. Asterisks indicate the absence of peaks corresponding to Tyr incorporation at the site of interest. CaM-$L_{113}\delta_{107-115}$: Calcd m/z $(M+H)^+$ 1179.3, found 1179.5; αS-$Y_{39}\delta_{35-43}$: Calcd m/z $(M+H)^+$ 1052.5, found 1052.5; αS-$Y_{94}\delta_{81-96}$: Calcd m/z $(M+H)^+$ 1595.8, found 1595.8. No evidence of Npf incorporation is observed.

Native PAGE Gel Analysis of CaM Mutants. CaM peptide binding can be detected by native (non-denaturing) PAGE gel analysis.[15] In order to determine whether mutant CaM was capable of binding peptide, 25 μL samples were prepared containing 10 μM of each CaM mutant, with or without a stoichiometric equivalent of pOCNC-F′$_1$. Prepared samples were then incubated for one hour at 4 °C and loaded into a non-denaturing gel with 2.5 μL of 60% glycerol and 2.5 μL of .01% bromophenol blue to assist loading. The gel shown in Figure S15 was stained with Coomassie blue and scanned.

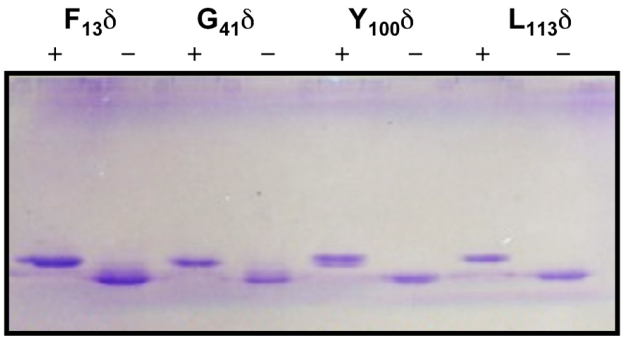


Figure S15. Native PAGE Gel Analysis of CaM Peptide Binding. PAGE analysis demonstrates upward shift of the CaM protein band upon addition of pOCNC-F′$_1$.

Calmodulin Circular Dichroism Measurements. Mutant stability was evaluated using temperature-dependent circular dichroism (CD) spectroscopy. Since calcium-bound CaM (holo-CaM) is thermostable (T_m > 90 °C), we specifically examined the thermal unfolding of the apo-enzyme. To compare the apo- and holo-forms, purified protein was dialyzed against 2 mM EDTA in 50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 6.70, or 10 mM $CaCl_2$ in 50 mM HEPES pH 6.70. CD data were obtained from 20 μM protein samples diluted in the appropriate buffer monitoring at 222 nm, between 5 and 95 °C, using the variable temperature module provided with the Aviv 410 CD spectrometer. Protein concentrations were determined by using the BCA assay. Data were collected with a 1 °C/min temperature slope, 30 s averaging time, 2 min temperature equilibration time, 5 s response time, and 1 nm band width. The resulting raw ellipticity (θ_D) measurements were transformed to molar residue ellipticity values (θ) using

$$\theta = \theta_D/(c\ell n_R) \qquad \text{(S11)}$$

where c is concentration (M), ℓ is the path length (cm), and n_R is the number of residues. To determine fraction folded (f_f) for the apo-enzyme, linear baselines were fit to the low temperature ($\theta_F = m_F T + b_F$) or high temperature ($\theta_U = m_U T + b_U$) data. The full data range was then fit to equation (S12) where $K = e^{-(\Delta H - T\Delta S)/RT}$, ΔH and ΔS are adjustable parameters and R = 8.3145 $J \bullet mol^{-1} \bullet K^{-1}$. Plots are shown for each mutant in Figure S16.

$$\theta = \theta_F(T)(1 - f_f(T)) + \theta_u(T)f_f(T) \qquad f_f = K/(1+K) \qquad \text{(S12)}$$

Since the holo-enzyme did not melt, only the molar residue ellipticity as a function of temperature is shown for each mutant (Fig. S16).

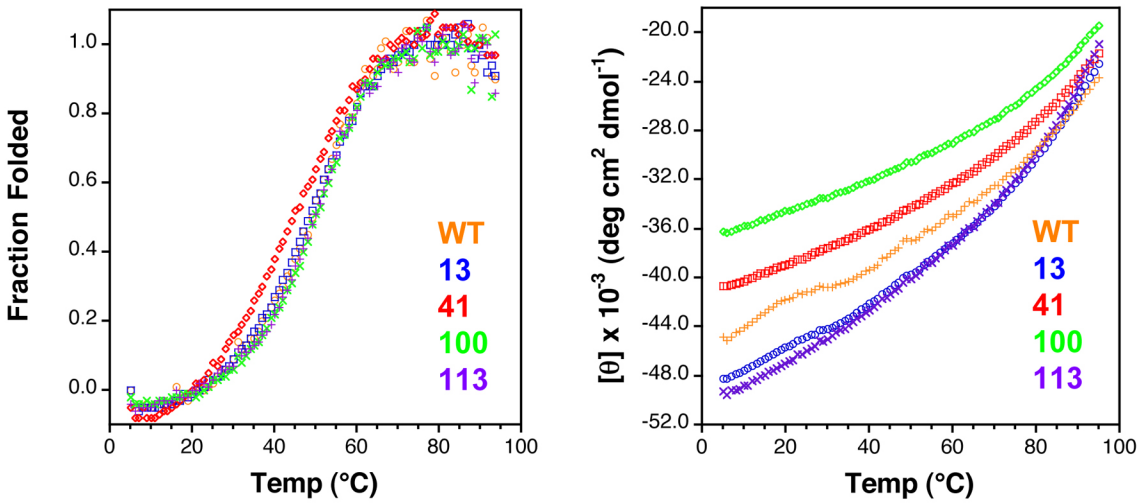


Figure S16. Temperature-Dependent Circular Dichroism Spectroscopy of Wildtype CaM and Mutants. Left: Fraction folded as a function of temperature as determined by molar residue ellipticity (θ) at 222 nm measured for 20 μM solutions of each protein in the absence of Ca^{2+}. Right: Molar residue ellipticity (θ) at 222 nm measured for 20 μM solutions of each protein in the presence of Ca^{2+}.

CaM Peptide Binding Measurements from Steady-State Fluorescence. All peptide binding experiments shown on the following pages were conducted in 15 mM HEPES buffer, 140 mM KCl, and 6 mM $CaCl_2$, pH 6.70. Dry peptides were brought up in a minimal amount of buffer to make fresh concentrated stock solutions for each experiment. Protein concentrations were determined by use of Thermo Scientific's Pierce BCA protein assay kit using known concentrations of bovine serium albumin as the standard. Peptide concentrations were determined on the following basis: for pOCNC, Phe absorbance was used (ε_{257} = 600 M^{-1}•cm^{-1}); for Trp-pOCNC, Trp absorbance at was used (ε_{270} = 5237 M^{-1}•cm^{-1}), and for Mcm-pOCNC, methyl coumarin absorbance was used (ε_{325} = 12,000 M^{-1}•cm^{-1}).[6,13] For each mutant, solutions were prepared that contained approximately 10 μM protein and a variable amount of peptide, ranging from 0 to 15 μM and representing at least seven distinct concentrations. Each sample was prepared in triplicate.

Corrected fluorescence measurements of each sample were taken on a Cary Eclipse fluorometer at 25 °C using quartz fluorometer cells with 1 cm path lengths for experiments with pOCNC binding to CaM-G_{41}δ. Fluorescence spectra were obtained using a Tecan M1000 plate reader and a UV transparent 96 well plate for experiments with CaM- F_{13}δ, CaM-Y_{100}δ, CaM-L_{113}δ, Trp-pOCNC, and Mcm-pOCNC. The excitation wavelength was 385 nm and emission spectra were collected from 400 to 550 nm for pOCNC and Trp-pOCNC or from 350 nm to 550 nm for Mcm-pOCNC binding. For pOCNC binding to CaM-G_{41}δ experiments on the Cary instrument, the excitation and emission slit widths were 5 nm, the scan rate was 120 nm/min, the averaging time 0.5 s, and the data interval 1.0 nm. For the Tecan M1000 plate reader: excitation and emission bandwidths were 5 nm, 50 flashes at 400 Hz, 20 ms integration time, a manual gain setting of 75, and a 20 mm Z-position. For CaM/peptide pairs for which no significant change in

fluorescence was observed in the presence of 15 μM peptide, representative spectra are shown in Figure S17.

CaM Binding Data Fitting. All peptide binding affinities were calculated using Equation S13 as previously described.[5]

$$y = 1 - R\frac{\left(\frac{1}{K_a} + [P]_0 + [P]_0[L]_0\right) - \sqrt{\left(\frac{1}{K_a} + [P]_0 + [P]_0[L]_0\right)^2 - 4[P]_0^2[L]_0}}{2[P]_0} \qquad \text{(S13)}$$

$[L]_0$ and $[P]_0$ are the total concentration of peptide and protein, respectively. K_a is the equilibrium constant for peptide binding to the protein to form a 1:1 complex and R is an instrumental response parameter. K_a and R are adjustable parameters in the fit.

Eu^{3+} titrations with CaM-$Y_{100}\delta$ were fit to a two-site cooperative binding model according to Equation S14, where F_x, K_x, and n_x are the relative emissions, dissociation constants, and Hill parameters, respectively, of the two sites.[16]

$$F = F_1\left\{\frac{[Eu^{3+}]^{n_1}}{K_1^{n_1} + [Eu^{3+}]^{n_1}}\right\} + F_2\left\{\frac{[Eu^{3+}]^{n_2}}{K_2^{n2} + [Eu^{3+}]^{n2}}\right\} \qquad \text{(S14)}$$

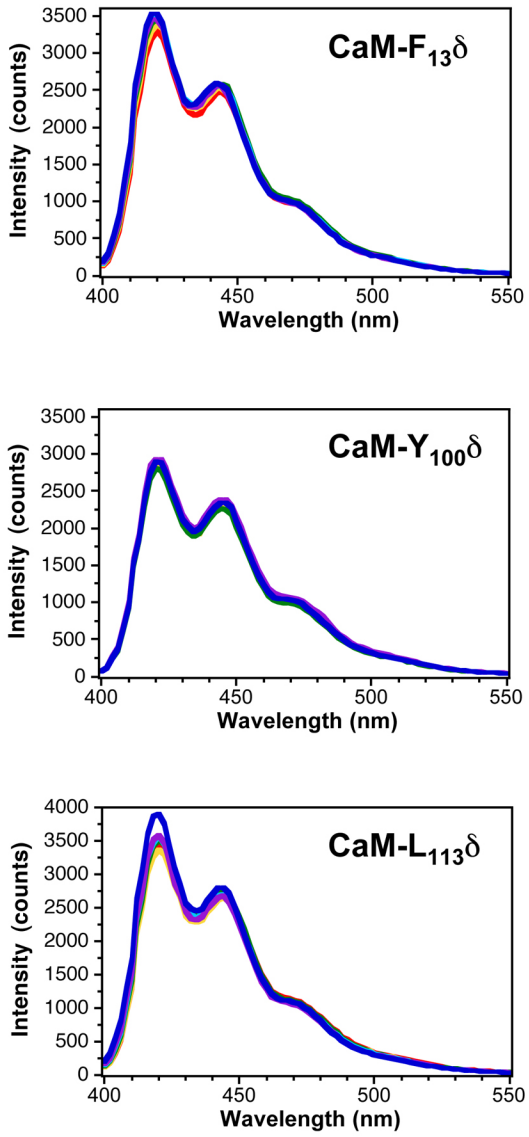


Figure S17. Fluorescence Spectra of CaM Mutants with Bound Peptides. Top: CaM-F_{13}δ/pOCNC, Middle: CaM-Y_{100}δ/pOCNC, Bottom: CaM-L_{113}δ/pOCNC. All spectra recorded for solutions of 0 (red), 2.5 (orange), 5 (yellow), 7.5 (green), 10 (blue), and 15 μM (purple) pOCNC solutions.

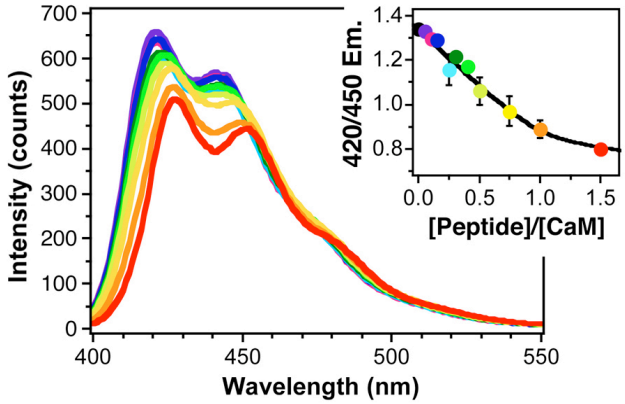


Figure S18. Monitoring CaM Binding By Changes in Acd Environment. Fluorescence spectra of 10 μM CaM-$G_{41}\delta$ in the presence of 0, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 0.75, 1.0, or 1.5 equivalents of pOCNC, colored black, purple, magenta, blue, aqua, green, lime, chartreuse, yellow, orange, and red, respectively. Inset: Peptide binding monitored by changes in the ratio of emission at 420 nm and 450 nm.

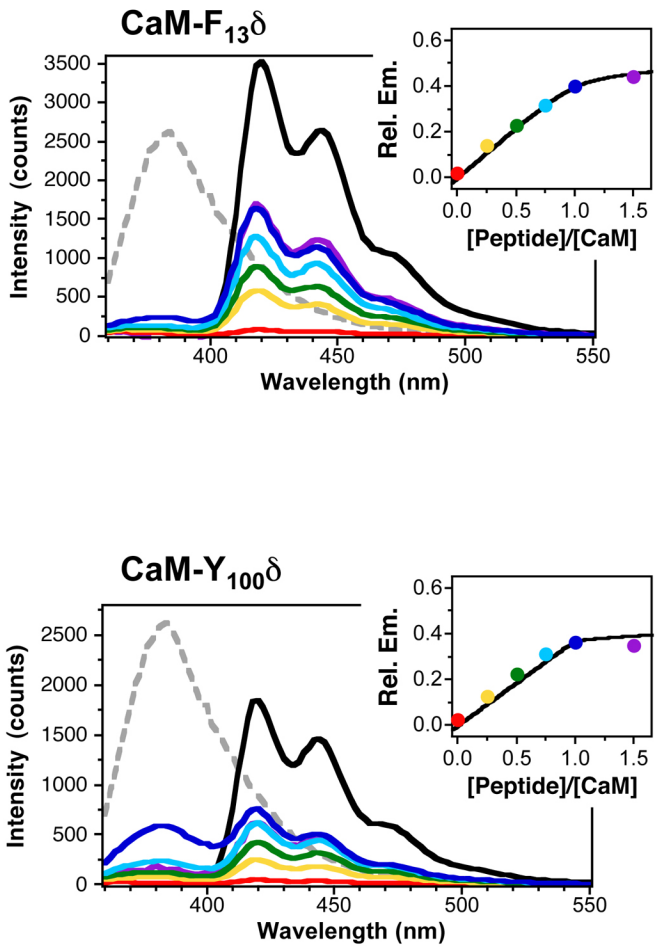


Figure S19. Fluorescence Spectra of CaM Mutants with Bound Peptides. Fluorescence spectra of 10 μM CaM-F_{13}δ or CaM-Y_{100}δ in the presence of 0, 0.25, 0.5, 0.75, 1.0, or 1.5 equiv Mcm-pOCNC, colored red, orange, green, aqua, blue, and purple, respectively. The fluorescence spectrum of 10 μM Mcm-pOCNC alone is shown in dashed grey. All of these spectra were recorded with excitation at 325 nm. The spectrum with 1.5 equiv Mcm-pOCNC is a difference spectrum in which the emission from 5 μM Mcm-pOCNC was subtracted from the spectrum of the 15 μM Mcm-pOCNC/10 μM CaM mutant mixture. The fluorescence spectrum of 10 μM CaM mutant alone with excitation at 385 nm is shown in black. Inset: Peptide binding monitored by changes in the emission at 450 nm due to excitation at 325 nm, normalized to the emission at 450 nm due to excitation at 385 nm.

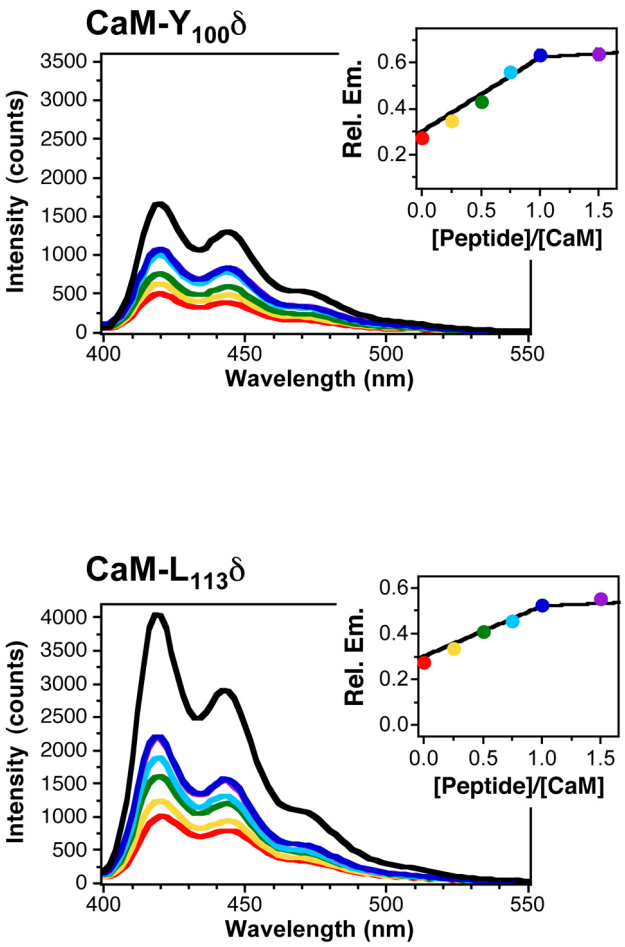


Figure S20. Fluorescence Spectra of CaM Mutants with Bound Peptides. Fluorescence spectra of 10 μM CaM-$Y_{100}\delta$ or CaM-$L_{113}\delta$ in the presence of 0, 0.25, 0.5, 0.75, 1.0, or 1.5 equiv Trp-pOCNC, colored red, orange, green, aqua, blue, and purple, respectively. The fluorescence spectra of 10 μM CaM mutant alone or in the 1:1 Trp-pOCNC mixture with excitation at 385 nm is shown in black and grey, respectively. Inset: Peptide binding monitored by changes in the emission at 450 nm due to excitation at 295 nm, normalized to the emission at 450 nm due to excitation at 385 nm.

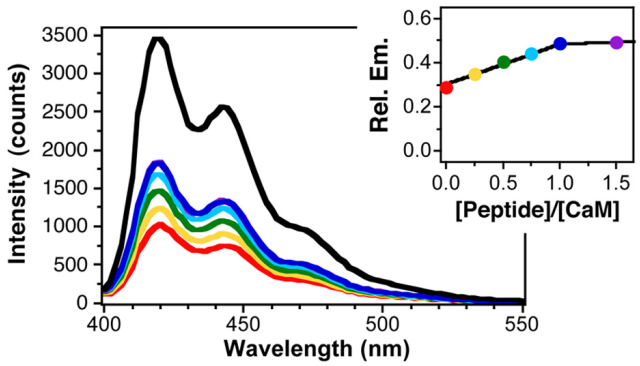


Figure S21. Monitoring CaM Binding By FRET. Fluorescence spectra of 10 μM CaM-$F_{13}\delta$ in the presence of 0, 0.25, 0.5, 0.75, 1.0, or 1.5 equiv Trp-pOCNC, colored red, orange, green, aqua, blue, and purple, respectively. The fluorescence spectra of 10 μM CaM-$F_{13}\delta$ alone or in the 1:1 Trp-pOCNC mixture with excitation at 385 nm is shown in black and grey, respectively. Inset: Peptide binding monitored by changes in the emission at 450 nm due to excitation at 295 nm, normalized to the emission at 450 nm due to excitation at 385 nm.

TIM Unfolding Measurements from Steady-State Fluorescence. Fluorescence spectra were obtained using a Tecan M1000 plate reader and a UV transparent 96 well plate with settings as described above. Acd was excited at 386 nm and emission monitored between 400 - 550 nm. The fluorescence intensity at 450 nm was used to monitor the dynamics of Acd fluorescence relative to the addition of urea.

TIM Unfolding from Fluorescence Lifetimes. Fluorescence lifetimes for TIM or Acd solutions in urea were recorded and fit to equations S2 – S4 as described for the Stern-Volmer experiments. Amplitude-weighted exponential fit data are shown in Figures S23 – S25 and Table S6.

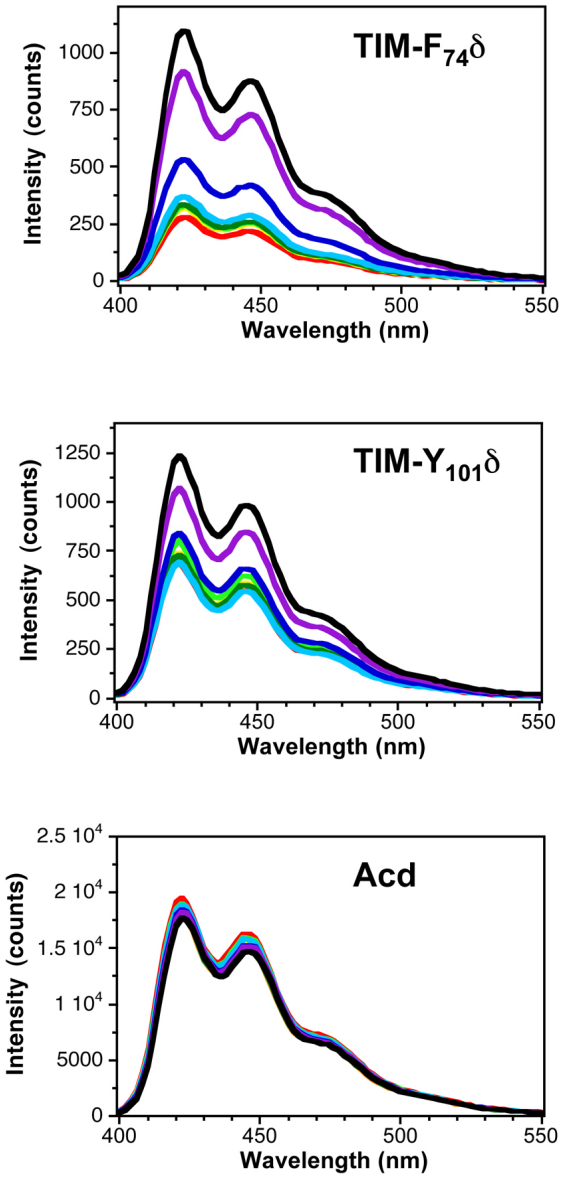


Figure S22. Fluorescence Spectra of TIM Denaturation in Urea. Top: TIM-$F_{74}\delta$, Middle: TIM-$Y_{101}\delta$, Bottom: free Acd. All spectra recorded for solutions TIM or Acd in 0 (red), 0.05 (pink), 0.1 (orange), 0.25 (yellow), 0.5 (light green), 0.75 (dark green), 1.0 (light blue), 2.0 (dark blue), 3.0 (purple), and 4.0 M (black) urea solutions.

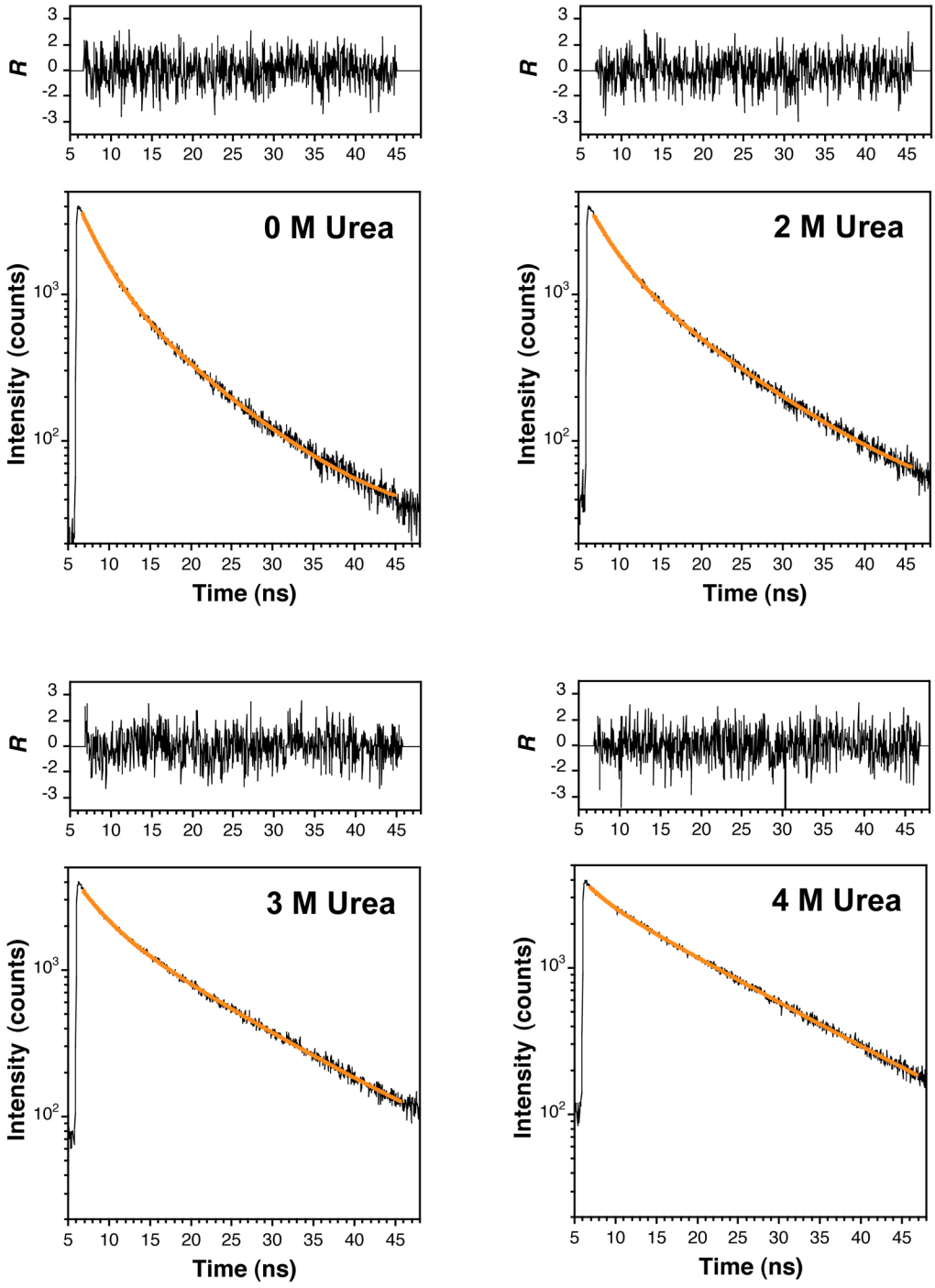


Figure S23. Lifetime TIM Urea Denaturation Experiments. Fits to TIM-$F_{74}\delta$ fluorescence lifetime data with varying concentrations of urea in 100 mM sodium phosphate buffer, pH 7.00 (λ_{ex} = 405 nm; λ_{em} = 450 nm) at 25 °C. Biexponential fit parameters are given in Table S5.

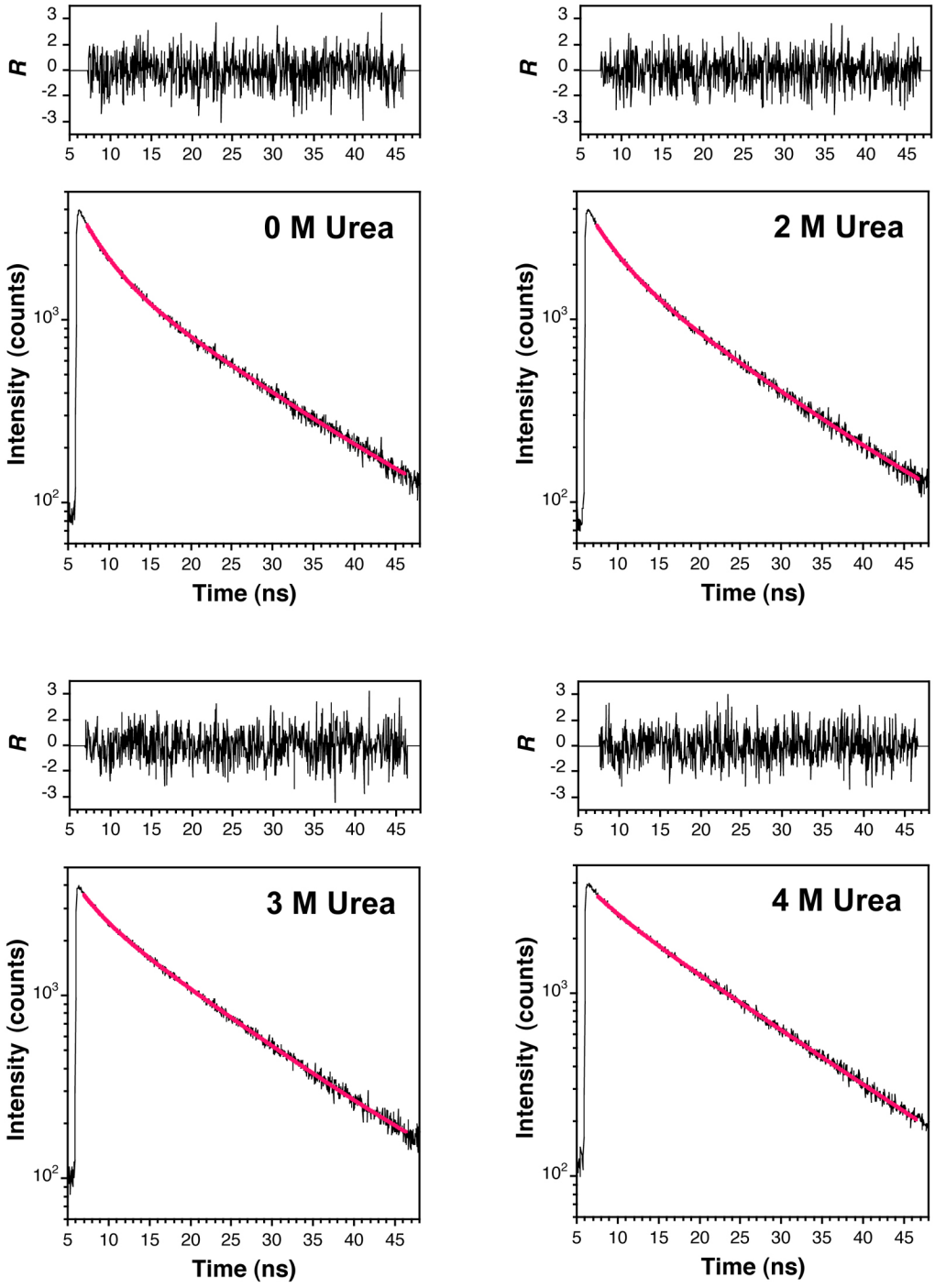


Figure S24. Lifetime TIM Urea Denaturation Experiments. Fits to TIM-$Y_{101}\delta$ fluorescence lifetime data with varying concentrations of urea in 100 mM sodium phosphate buffer, pH 7.00 (λ_{ex} = 405 nm; λ_{em} = 450 nm) at 25 °C. Biexponential fit parameters are given in Table S5.

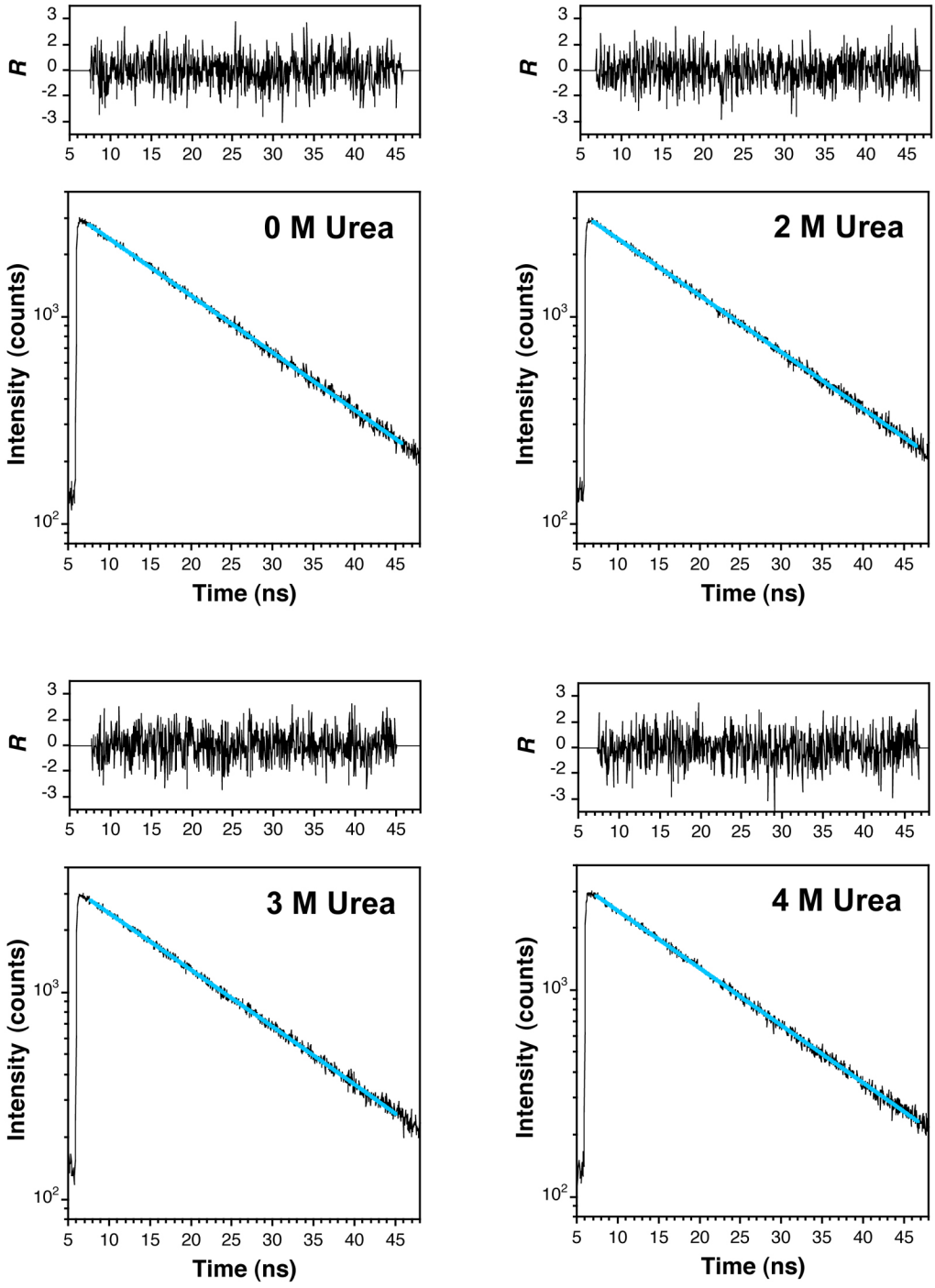


Figure S25. Lifetime TIM Urea Denaturation Experiments. Fits to Acd fluorescence lifetime data with varying concentrations of urea in 100 mM sodium phosphate buffer, pH 7.00 (λ_{ex} = 405 nm; λ_{em} = 450 nm) at 25 °C. Exponential fit parameters are given in Table S5.

Table S6. Exponential Fits to Lifetime TIM Urea Denaturation Data.

Sample	[Urea] (M)	τ_1 (ns)	% τ_1	τ_2 (ns)	% τ_2	τ_{avg} (ns)
TIM-$F_{74}\delta$	0	8.85 ± 0.07	37.6 ± 0.7	2.68 ± 0.05	62.4 ± 1.5	5.0 ± 0.1
	2	10.51 ± 0.07	45.5 ± 0.8	3.04 ± 0.07	54.5 ± 1.4	6.4 ± 0.1
	3	12.92 ± 0.07	61.0 ± 1.0	3.22 ± 0.11	39.0 ± 1.2	9.2 ± 0.2
	4	13.99 ± 0.07	83.6 ± 1.6	2.65 ± 0.28	16.4 ± 1.4	12.1 ± 0.3
TIM-$Y_{101}\delta$	0	14.32 ± 0.09	56.4 ± 1.0	3.23 ± 0.10	43.6 ± 1.4	9.5 ± 0.2
	2	13.54 ± 0.08	61.6 ± 1.0	3.44 ± 0.12	38.4 ± 1.3	9.7 ± 0.2
	3	13.37 ± 0.07	78.8 ± 1.4	2.83 ± 0.21	21.2 ± 1.3	11.1 ± 0.3
	4	14.61 ± 0.07	85.7 ± 1.4	3.89 ± 0.36	14.3 ± 1.1	13.1 ± 0.3

Sample	[Urea] (M)	τ_1 (ns)	% τ_1	τ_2 (ns)	% τ_2	τ_{avg} (ns)
Acd	0	15.82 ± 0.08				
	2	15.87 ± 0.08				
	3	15.99 ± 0.08				
	4	15.70 ± 0.08				

References

(1) Yonetani, M.; Nonaka, T.; Masuda, M.; Inukai, Y.; Oikawa, T.; Hisanaga, S.; Hasegawa, M. *J. Biol. Chem.* **2009**, *284*, 7940-7950.
(2) Goldberg, J. M.; Speight, L. C.; Fegley, M. W.; Petersson, E. J. *J. Am. Chem. Soc.* **2012**, *134*, 6088-6091.
(3) Contessa, G. M.; Orsale, M.; Melino, S.; Torre, V.; Paci, M.; Desideri, A.; Cicero, D. O. *J. Biomol. NMR* **2005**, *31*, 185-199.
(4) Edelhoch, H. *Biochemistry* **1967**, *6*, 1948-1954.
(5) Goldberg, J. M.; Wissner, R. F.; Klein, A. M.; Petersson, E. J. *Chem. Commun.* **2012**, *48*, 1550-1552.
(6) Brun, M. P.; Bischoff, L.; Garbay, C. *Angew. Chem. Int. Ed.* **2004**, *43*, 3432-3436.
(7) van Rooijen, B. D.; van Leijenhorst-Groener, K. A.; Claessens, M.; Subramaniam, V. *J. Mol. Biol.* **2009**, *394*, 826-833.
(8) Szymanska, A.; Wegner, K.; Lankiewicz, L. *Helv. Chim. Acta* **2003**, *86*, 3326-3331.
(9) Hanahan, D.; Jessee, J.; Bloom, F. R. *Methods Enzymol.* **1991**, *204*, 63-113.
(10) Giehm, L.; Lorenzen, N.; Otzen, D. E. *Methods* **2010**, *53*, 295-305.
(11) Fink, A. L. *Acc. Chem. Res.* **2006**, *39*, 628-634.
(12) Ferreon, A. C. M.; Moran, C. R.; Ferreon, J. C.; Deniz, A. A. *Angew. Chem. Int. Ed.* **2010**, *49*, 3469-3472.
(13) Du, H.; Fuh, R. C. A.; Li, J. Z.; Corkan, L. A.; Lindsey, J. S. *Photochem. Photobiol.* **1998**, *68*, 141-142.
(14) Miyake-Stoner, S. J.; Miller, A. M.; Hammill, J. T.; Peeler, J. C.; Hess, K. R.; Mehl, R. A.; Brewer, S. H. *Biochemistry* **2009**, *48*, 5953-5962.
(15) Stevens-Truss, R.; Marletta, M. A. *Biochemistry* **1995**, *34*, 15638-15645.
(16) Wu, X. C.; Reid, R. E. *Biochemistry* **1997**, *36*, 8649-8656.