Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information For:

Selenomethionine, *p*-Cyanophenylalanine Pairs Provide a Convenient, Sensitive, Non-Perturbing Fluorescent Probe of Local Helical Structure

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1. Supplementary Materials & Methods

Peptide Synthesis and Purification

Peptides were synthesized on a 0.1 mmol scale using 9-fluoronylmethoxycarbonyl (Fmoc) chemistry on a CEM Liberty microwave peptide synthesizer. An amidated C-terminus was incorporated using a 5-(4'-Fmoc-aminomethyl-3',5-dimethoxyphenol)valeric acid (Fmoc-PAL-PEG-PS) resin. An acetylated N-terminus was incorporated into the designed helical peptide using acetic anhydride. For the 21-residue helical peptide, the C-terminal alanine and the proline were double coupled. In HP36, all β-branched amino acids along with the C-terminal Phe and the Pro were double coupled. A standard trifluoroacetic acid (TFA) protocol with thio-scavengers was used to cleave the peptides from the resin. Crude peptides were dissolved in 20% acetic acid (v/v) and lyophilized. Crude peptides were purified by reverse-phase high performance liquid chromatography (HPLC) using a Higgins Analytical Proto 300 C18 preparative column (10 mm X 250 mm). A two buffer gradient system was used where buffer A consisted of 0.1% TFA (v/v) in H_2O, and buffer B consisted of 9:1 acetonitrile:H_2O with 0.1% TFA (v/v). Purified dry peptides were dissolved in hexafluoro-2-propanaol (HFIP) at a concentration of 10 mg/mL and allowed to sit overnight at room temperature. This step is included to remove residual scavengers. Peptides in HFIP were diluted into 18 MΩ water and purified by HPLC using the same protocols used for the first purification step. Analytical HPLC was used to check the purity of the peptides and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry confirmed the correct molecular weight. For the 21 residue helical peptide, the expected monoisotopic molecular weight is 2002.0 Da and the observed value is 2001.0 Da. For HP36-W24F_{CN}-N28M_{Se}, the expected monoisotopic mass is 4237.1 Da while the observed value is 4237.2 Da.

NMR Spectroscopy

The M_{Se}, F_{CN} HP36 variant was dissolved in a 100% D_2O solution containing 20 mM sodium acetate (pre-exchanged with D_2O) at pD 4.6 (uncorrected pH reading). The protein concentration was 750 µM. 1D ^1H NMR spectra were recorded on a Bruker 700 MHz spectrometer at 25 °C. 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) (0.5 mM) was used as an internal reference. The data was analyzed using the commercially available software package Mnova 7.

Circular Dichroism Spectroscopy

CD wavelength spectra were recorded on an Applied Photophysics Chirascan instrument. For wavelength scans, the dry 21 residue helical peptide was dissolved in a 10 mM sodium acetate buffer at pH 5.5 with or without 8 M urea for a final peptide concentration of 25 µM. Measurements were performed at 25 °C. Dry HP36 was dissolved in 20 mM sodium acetate buffer with or without 10 M urea at pH 5.0 for a final peptide concentration of 25 µM. Data was collected at 25 °C. The concentration of urea was determined by measuring the refractive index. The concentration of the peptides and proteins was estimated using the F_{CN} absorbance measured at 280 nm based on an extinction coefficient (ε) of 850 $M^{-1}\,cm^{-1}$. For the helical peptide, the fraction of helical structure was determined using the measured mean residue ellipticity at 222 nm, $[\theta]_{obs}$, from the following expression:[1]

$$f_h = \frac{[\theta]_{\mathrm{obs}} - [\theta]_{\mathrm{C}}}{[\theta]_{\mathrm{H}} - [\theta]_{\mathrm{C}}} \quad (1)$$

Where $[\theta]_H$ corresponds to the mean residue ellipticity at 222 nm for 100% helical peptide and $[\theta]_C$ corresponds to the value for a random coil:

$$[\theta]_{\mathrm{H}} = -40{,}000 \times \left(1 - \frac{2.5}{n}\right) + 100 \times T \quad (2)$$

$$[\theta]_{\mathrm{C}} = 640 - 45 \times T \quad (3)$$

Where *n* is the number of residues in the peptide and *T* is the temperature in °C.

For CD monitored thermal unfolding, the HP36 variant was dissolved in 20 mM sodium acetate and 150 mM NaCl at pH 5.0 for a final peptide concentration of 25 µM. Thermal denaturation data was fit to the following expression:

$$\Delta G^{\circ}(T) = \Delta H^{\circ}_{T_m}\left(1 - \frac{T}{T_{\mathrm{m}}}\right) - \Delta C_p^{\circ}\left[(T_{\mathrm{m}} - T) + T\ln\left(\frac{T}{T_{\mathrm{m}}}\right)\right] \quad (4)$$

Where T_m is the midpoint temperature, *T* is the temperature, $\Delta H^{\circ}_{T_m}$ is the change in enthalpy for unfolding at T_m, and ΔC_p^o is the change in heat capacity, which was set to 0.38 kcal mol^{-1} deg^{-1}, as determined by previous experiments.[2]

Steady-State Fluorescence

Fluorescence emission spectra were measured using a Photon Technologies fluorimeter. The same samples used to collect the CD data were used. Measurements were made at 25 °C. For pH dependent measurements, HP36 was dissolved in 20 mM phosphate buffer for a final peptide concentration of 17 µM. F_{CN} fluorescence was excited at 240 nm and spectra were recorded from 265 to 400 nm. Experiments were performed at 20 °C.

Time-resolved Fluorescence

Time-resolved experiments were performed at the University of Massachusetts Medical School in Worcester, MA. Helical peptide was dissolved in 10 mM sodium acetate buffer at pH 5.5 with or without 8 M urea. HP36 was dissolved in 20 mM sodium acetate buffer at pH 5.0 with or without 10 M urea. Final peptide and protein concentrations were 25 µM. Decays were measured on a home-built time correlated single photon counting (TCSPC) apparatus. The tripled output of a 10 W Verdi (Coherent) pumped Ti:sapphire laser (Coherent Mira) was used to preferentially excite F_{CN} at 240 nm. The repetition rate was reduced to 3.8 MHz. The detection utilized a bandpass filter (FF01-292/27, Semrock, Rochester, NY) and Glan-Taylor polarizer at magic angle. A PMH-100-6 photomultiplier tube connected to an SPC150 photon counting card (Becker-Hickl, Berlin, Germany) was used for TCSPC. All measurements were collected at 20 ±1 °C. The decay curves were analyzed by fitting to a two-exponential model

reconvoluted with the measured instrument response function.[3] Decays were also fit using a maximum entropy model (MEM Laplace Inversion version 2.0c developed by Bilsel).[3]

2. Supporting Figures

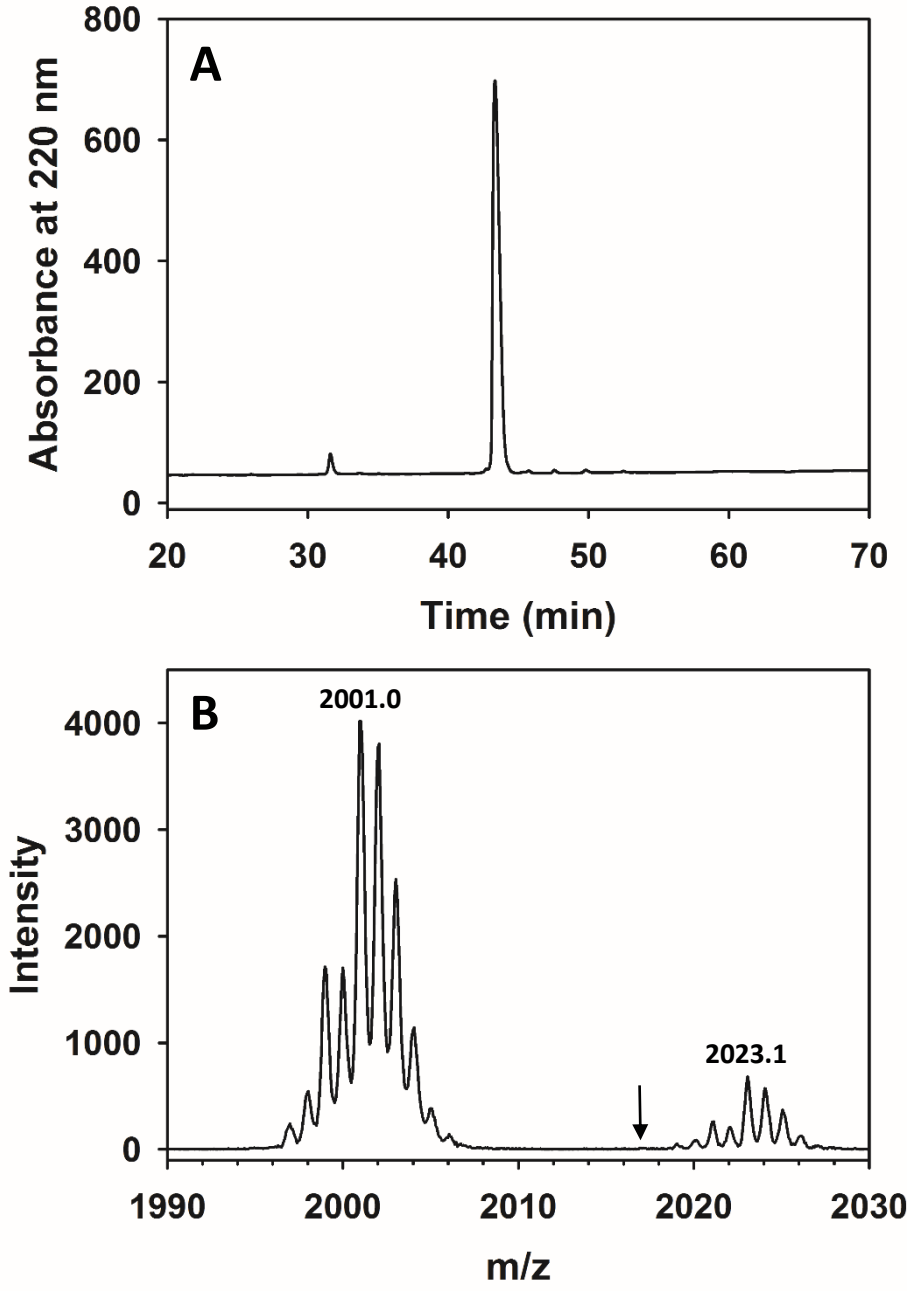

Figure S1. (A) Analytical HPLC trace of the helical peptide after 72 hours in buffer. A single peak is obsevered with a retention time of 43.3 minutes which corresponds to unmodified material. No additional peptide peak is observed. **(B)** MALDI-TOF mass spectrum of the helical peptide after 72 hours in buffer. The arrow indicates where a peak would appear if the selenomethionine was oxidized. The set of peaks with lower intensity centered near 2023.1 Da are the sodium adduct. The peptide was incubated in 10 mM sodium acetate at pH 5.5 and room temperature.

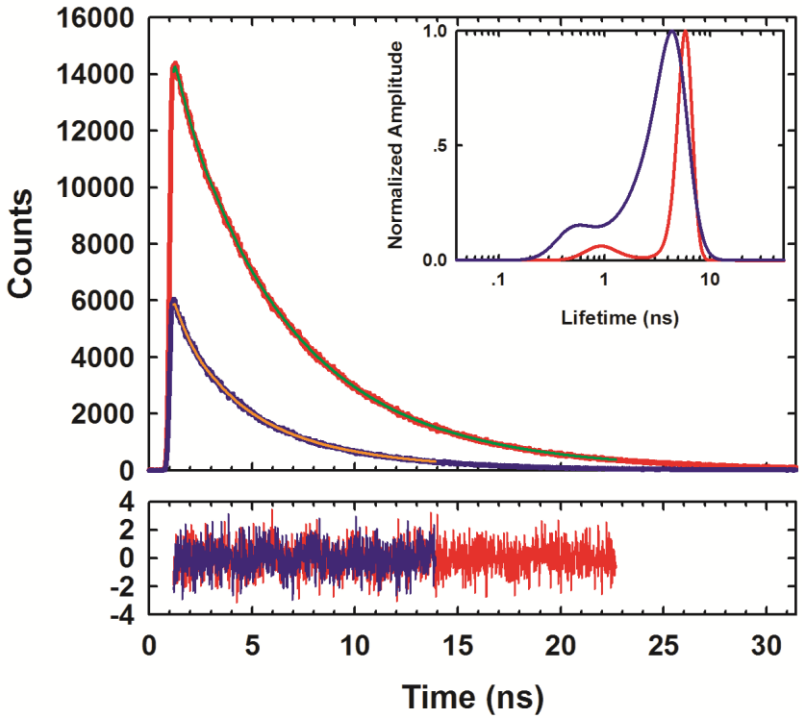

Figure S2. Time-resolved fluorescence decays for peptide in buffer (blue) and in 8 M urea (red) fit using a maximum entropy model. For the helical state in buffer the fits yield time constants of 4.37 ns and 0.59 ns for the slow and fast components, respectively, with relative amplitudes of 0.87 and 0.13, respectively. In 8 M urea, the fit yields time constants of 5.84 and 0.93 ns for the slow and fast components, respectively, and relative amplitudes are 0.94 and 0.06, respectively. Shown in the inset is the lifetime distribution. Residuals are plotted below.

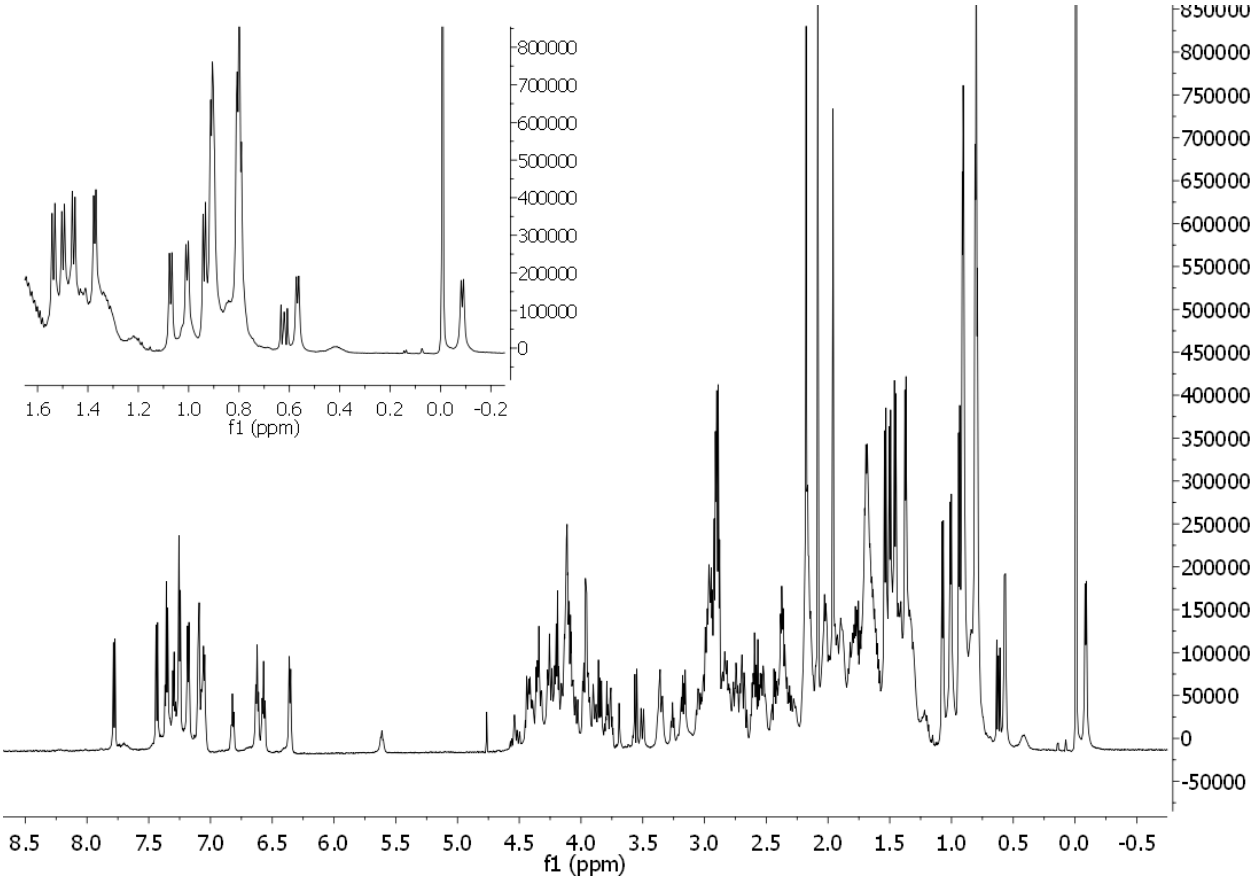

Figure S3. 1D ^{1}H-NMR spectrum of the M_{Se}, F_{CN} variant of HP36. The spectrum was recorded in D_2O at pD 4.6 (uncorrected pH reading) in 20 mM sodium acetate (pre-exchanged with D_2O) at 25 °C. The sharp peak at 0.00 ppm is the chemical shift standard. Note the characteristic ring current shifted methyl resonances and the distinctive up-field aromatic resonance. These peaks are indicative of the HP36 fold. The inset shows the upfield methyl resonances. We did not detect any resolved ^{77}Se-^{1}H J-couplings in the one-dimensional proton spectrum, but this is not surprising given the resolution of the one dimensional experiment.

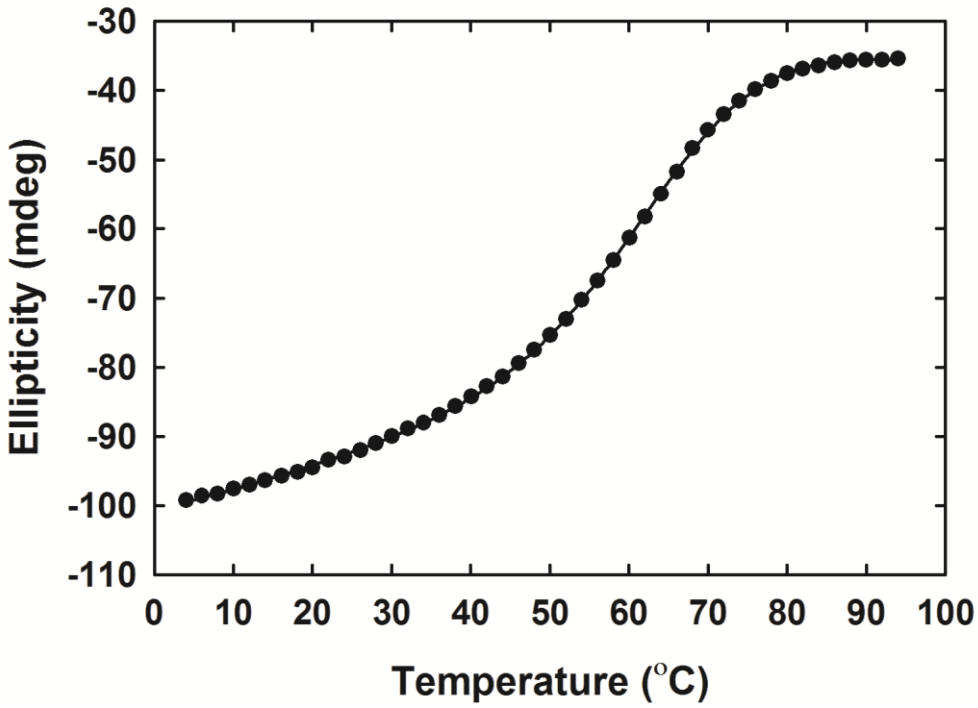

Figure S4. CD monitored thermal unfolding of the M_{Se}, F_{CN} variant of HP36 recorded in 20 mM sodium acetate and 150 mM NaCl at pH 5.0. The solid line is the best fit for a two-state unfolding transition. The T_m of the M_{Se} F_{CN} variant is 62 °C under these conditions. The reported T_m for the synthetic wild-type sequence with an amidated C-terminus is 70.5 °C in 10 mM phosphate.[4] Prior studies have shown that replacing Trp-24 with Leu or Ala destabilized the domain by 12 to 16 °C, so the change observed with the $W24F_{CN}$, $N24M_{Se}$ mutant is relatively modest.[5]

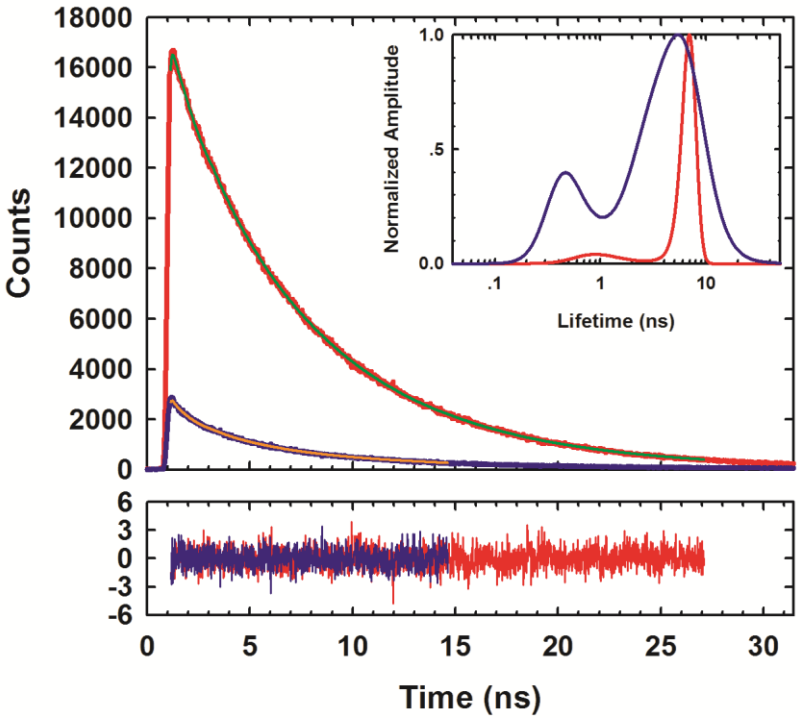

Figure S5. Time-resolved fluorescence decays for the villin protein in buffer (blue) and in 10 M urea (red) fit using a maximum entropy model. For the folded protein in buffer, the fit gave time constants of 5.38 ns for the slow component and 0.47 ns for the fast component with relative amplitudes of 0.72 and 0.28, respectively. For unfolded protein in 10 M urea, the time constants are 6.91 ns for the slow component and 0.89 for the fast component with relative amplitudes of 0.96 and 0.04, respectively. Shown in the inset is the lifetime distribution. Residuals are plotted below.

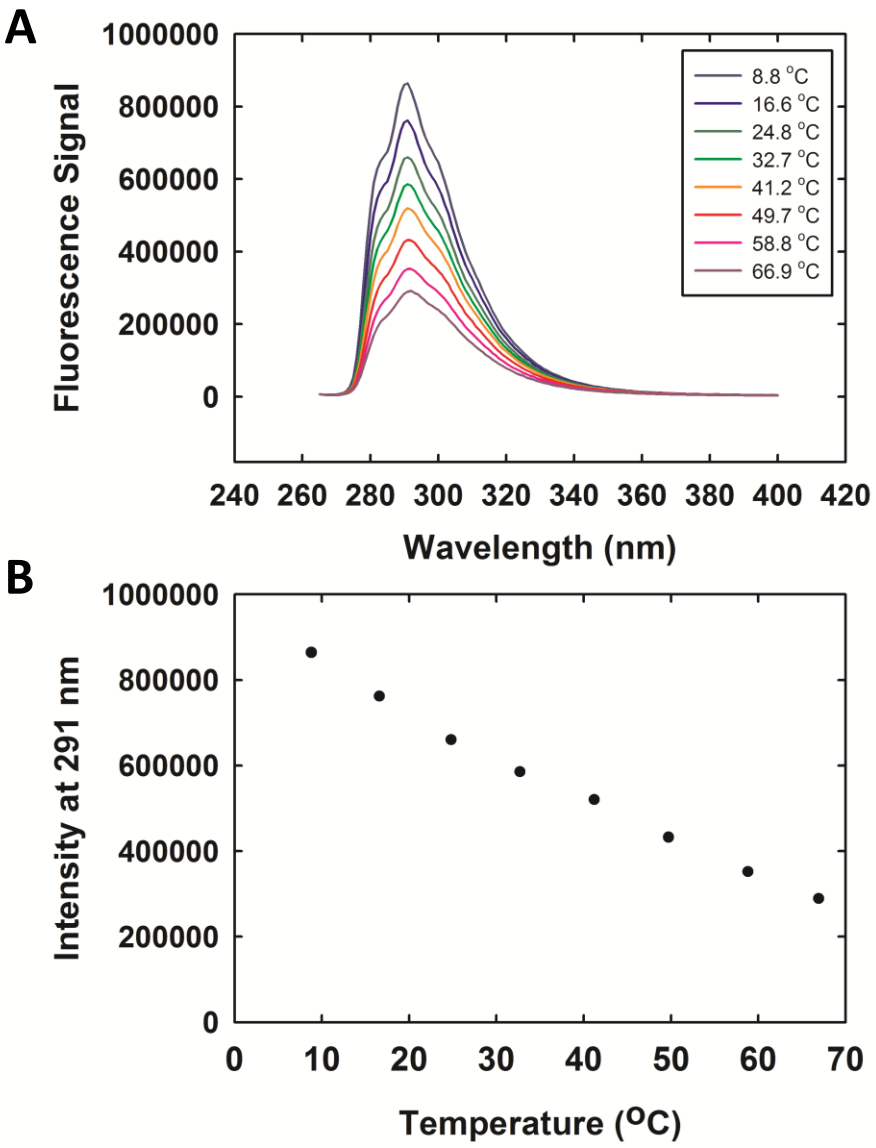

Figure S6. Temperature dependence of *p*-cyanophenylalanine fluorescence. **(A)** Fluorescence emission spectra of the free amino acid at different temperatures. **(B)** Plot of the intensity at the emission maximum versus temperature. Experiments were performed in 10 mM sodium acetate at pH 5.5 and 20 °C. The concentration of F_{CN} was 25 µM.

3. Supporting Information References

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[5] S. Xiao, D. P. Raleigh, *J. Mol. Biol.* **2010**, *401*, 274-285.