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Supplementary Information

for

An intrinsic FRET sensor of protein-ligand interactions

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MATERIALS AND METHODS:

Reagents

7-HCAA was obtained from Bachem (Torrance, CA) and 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) was obtained from Cayman Chemical Company (Ann Arbor, MI). Commercially available reagents were used without further purification.

Molecular Cloning, Protein Expression, & Purification:

The wild-type hexokinase gene from *Sulfolobus tokodaii*[1,2] was obtained from Integrated DNA Technologies (Coralville, IA) cloned into a pET29b(+) vector (Novagen) using Gibson assembly (New England Biolabs). First, the W76Y mutation was made with overlap extension PCR and Gibson Assembly using the wild-type gene as a template. The presence of the mutation was confirmed using Sanger sequencing (Genewiz; Plainfield, NJ). The E50X and K257X mutations were each made separately using overlap extension PCR followed by Gibson assembly. Sequence confirmed pET29b(+) plasmids containing the E50X and K257X mutations were transformed into BL21* (DE3) cells (Invitrogen) along with the pEVOL-CouRS[3,4] plasmid. The pEVOL-CouRS plasmid contains an orthogonal aminoacyl tRNA synthetase / tRNA pair that includes a tRNA with an anti-codon loop specific to the amber codon (termed $tRNA_{CUA}$) and a CouRS aminoacyl tRNA synthetase that can acylate the $tRNA_{CUA}$ with 7-HCAA. Because pET29b(+) contains a kanamycin resistance marker and pEVOL-CouRS contains a chloramphenicol resistance marker, BL21 Star(DE3) (Invitrogen; Carlsbad CA) cells containing either the E50X or K257X hexokinase mutant were plated on dual-antibiotic plates (kanamycin, 50 µg / mL and chloramphenicol, 34 µg / mL) and were allowed to incubate overnight at 37 ºC. Alternatively, BL21* (DE3) cells containing HexY were cultured on plates containing kanamycin alone.

A single colony was used to inoculate 5 mL of 2xYT media and cultures were incubated overnight at 37 ºC with 250 rpm shaking to an OD_{600} of ~6.0. The cultures were then used to inoculate in 50 mL of TB media supplemented with 100 mM Tris-HCl, pH 7.0. Arabinose (0.2% w/v) and the non-canonical amino acid, 7-HCAA (1 mM final concentration), were added to each culture and incubated for 1 hour at 37 ºC with 250 rpm shaking. This incubation period prior to HexY overexpression serves to induce production of CouRS. Expression of the HexY mutants was induced by addition of Isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. The cultures were then incubated between 8 and 16 hours at 25 ºC, with shaking at 180 rpm. Cells were harvested via centrifugation (4,200 xg for 10 minutes), resuspended in lysis buffer (25 mM Tris-HCl, pH 8.0, 10 mM NaCl) and frozen. Cells were thawed and lysozyme (1 mg / mL final concentration; Sigma Aldrich) and NaCl (500 mM final concentration) were added. Cells were then lysed via sonication for 5 minutes and centrifuged at 20,000 xg for 20 minutes to remove cell debris.

All proteins contained C-terminal 6x histidine tags and were purified on immobilized metal ion chromatography using a nickel-nitrilotriacetic acid resin (Ni-NTA, HisTrap FF, GE Healthcare). After loading on Ni-NTA, contaminant proteins were removed by washing the column with 5 column volumes (CV) of Ni-NTA buffer A (25 mM Tris-HCl pH 8.0, 20 mM Imidazole and 500 mM NaCl) followed by 5 CV of 90% Ni-NTA buffer A with 10% Ni-NTA buffer B (25 mM Tris-HCl pH 8.0, 500 mM Imidazole and 150 mM NaCl). Proteins were then eluted with five column volumes of 100% Ni-NTA buffer B. Eluted fractions that were fluorescent under UV illumination were consolidated, diluted 10x with anion exchange (IEC) wash buffer A

(25 mM Tris-HCl pH 8.0, 10 mM NaCl) and further purified on IEC resin (HiTrap Q FF, GE Healthcare). The column was washed with five CV of IEC wash buffer A, followed by five CV of 90% IEC buffer A and 10% IEC buffer B (25 mM Tris-HCl pH 8.0, 500 mM NaCl). Proteins were eluted with five column volumes of 100% IEC buffer B. Again, protein fractions that were found to be fluorescent were consolidated and concentrated in a 10kDa molecular weight cutoff spin column (Amicon) to 500 μl. The concentrated protein was then injected onto a size-exclusion column (Superdex 200 increase 10/300 gl, GE Healthcare) and eluted from the column with sizing buffer (50 mM Tris-HCl pH 9.0, 150 mM NaCl). Fractions that were fluorescent were collected, consolidated, and concentrated to a volume of ~1 mL on a 10 kDa molecular cutoff spin column (Amicon) and refrigerated at 4 ºC for use in experimental procedures.

Reducing Plus/Minus 7-HCAA SDS-PAGE: Single colonies of the E50X or K257X mutants of HexY were used to inoculate 5 mL cultures of 2xYT medium containing chloramphenicol and kanamycin. After growth overnight at 37 °C, 100 μl of confluent culture was used to inoculate two 5 mL cultures of each mutant. 7-HCAA (in 200 mM NaOH) was added to a final concentration of 1 mM in one of the two culture tubes containing cells harboring the E50X or K257X expression plasmids; an equivalent amount of 200 mM NaOH was added to the other tube. Protein expression was carried out as described above to the point of cell lysis. Rather than purifying the proteins, 100 μl of cell culture was removed from the 5 ml overnight cultures and centrifuged at 8,000 xg for 10 minutes. Pellets were lysed as previously described. Proteins were then purified using a gravity Ni-NTA column. 500 μl of Ni-NTA slurry (Qiagen; Hilden, Germany) was placed in a micro spin column (Bio-Rad; Hercules, CA). The resin was washed with 5 CV water and 5 CV Ni-NTA buffer A. The lysed cell supernatant was added to the column, washed with 5 CV of N-NTA buffer A and eluted with 5 CV of Ni-NTA buffer B. 15 μl of sample was mixed with 5 μl of 4x Laemmli buffer with β-mercaptoethanol (BME; Sigma Aldrich; St. Louis, MO) and brought to a final volume of 20 μl with water. The sample was heated to 95 ºC for 5 minutes and 15 μl of sample was loaded into each well of a 4% stacking/15% resolving polyacrylamide gel and run at 100 V for 90 minutes. Visualization was again carried out using the GelDoc XR followed by Coomassie staining.

Non-Reducing/Reducing SDS-PAGE: 15 μg of each of the purified HexY mutants was mixed with 5 μl of 4x Laemmli buffer with BME and brought to a final volume of 20 μl with water. The sample was heated to 95 ºC for 5 minutes and 15 μl of sample was loaded into each well of a 4% stacking/15% resolving polyacrylamide gel and run at 100 V for 90 minutes. Visualization was again carried out using the GelDoc XR followed by Coomassie staining.

Fluorescence Polarization Assay: 90 μl of protein sample (~10 μM) was mixed with 10 μl of 2-NBDG (1 mM) (Cayman Chemical Company; Ann Arbor, MI) in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM Bis-Tris pH 6.0, 150 mM NaCl. Polarization data were collected on a HORIBA Nanolog fluorimeter using 440 nm excitation, 520 nm emission, and 5 nm slit widths for both. Data was analyzed using the Fluoressence software package, version 3.5.1.991 (HORIBA Jobin Yvon).

Spectroscopic Analysis: Spectroscopic experiments were performed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM Bis-Tris pH 6.0, 150 mM NaCl, or 50 mM Tris-HCl, pH 9.0, 150 mM NaCl using a 1 cm quartz cuvette (Starna Cells; Atascadero, CA) on a SpectraMax M5 spectrophotometer (Molecular Devices; San Jose, CA). Each glucose-free mutant was concentrated to a volume of 100 μl and then diluted to a final absorbance value of 0.05 at 325 nm for pH 7.5, 0.1 at 325 nm for pH 6.0, and 0.1 at 360 nm for pH 9.0. The samples were then divided into separate tubes into which glucose (10 mM final concentration) or an equivalent volume of buffer was added. Absorbance spectra were taken from 200 nm to 750 nm at 5 nm intervals, again using a SpectraMax M5 spectrophotometer. Fluorescence excitation spectra at (λ_{em} = 450 nm) were collected with excitation wavelengths ranging from 250 nm to 400 nm at 5 nm intervals. Fluorescence emission spectra were collected by exciting the proteins at either 280 nm, 325 nm or 360 nm and measuring the fluorescence intensity from 380 nm to 600 nm in 5 nm intervals. All spectra represent the average of three readings.

Calculation of Fluorescence Intensity Changes and FRET Efficiencies for E50X: The increase in fluorescence intensity from E50X apo to E50X holo was calculated using Equation 1. ΔI is the calculated change in fluorescence intensity and $E50X_{holo}$ and $E50X_{apo}$ represent the absolute fluorescent intensities of this mutant in the presence and absence of glucose, respectively. FRET efficiencies of E50X in the apo and holo forms were calculated using Equations 2 and 3, respectively. In these equations, $E50X_{apo}$, $E50X_{holo}$, $HexY_{apo}$, and $HexY_{holo}$ represent the fluorescent intensities of these proteins in the specified forms at 330 nm.

$$\Delta I = \frac{E50X_{holo} - E50X_{apo}}{E50X_{apo}} \quad \textbf{(Eq. 1)}$$

$$E = 1 - \left(\frac{E50X_{apo}}{HexY_{apo}}\right) \quad \textbf{(Eq. 2)}$$

$$E = 1 - \left(\frac{E50X_{holo}}{HexY_{holo}}\right) \quad \textbf{(Eq. 3)}$$

E50X Glucose Dependent Response: 10 μl of 1 M glucose was serial diluted in sterile water to concentrations of 100 mM, 10 mM, 1 mM, 100 μM, 10 μM and 1 μM. 10 μl of each glucose concentration was then mixed with 90 μl of E50X (10 μM) that had been dialyzed into the buffers listed above at pHs of 6.0, 7.5, and 9.0; final glucose concentrations were: 10 mM, 1 mM, 100 μM, 10 μM, 1 μM and 100 nM. Samples were excited at 280 nm and fluorescence was measured at 450 nm using a 1 cm quartz cuvette (Starna Cells; Atascadero, CA) on a SpectraMax M5 spectrophotometer (Molecular Devices; San Jose, CA). All data were normalized to the highest value at each pH and are the average of three readings. Curve fitting was performed with the Prism software package (Graphpad; San Diego, CA) using the 4-parameter logistic (4PL) non-linear regression function (Eq. 4). K_d values derived from these fits are listed in Table S2.

$$f(x) = d + \frac{a-d}{1+\left(\frac{x}{c}\right)^b} \quad \textbf{(Eq. 4)}$$

Circular Dichroism: 300 μl of protein sample (~10 μM) was placed into a 1 mm quartz cuvette (Starna Cells; Atascadero, CA) in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM Bis-Tris pH 6.0, 150 mM NaCl . Multi-wavelength CD measurements were taken every 1 nm from 300 to 200 nm. For melting curves, sample was incubated with 5 mM BME (Sigma Aldrich; St. Louis, MO) at room temperature for 1 hour prior to the reading. Measurements were taken from 80 to 100 ºC at 222 nm wavelength. All measurements were taken on a Jasco J-815 CD Spectrometer (Jasco Inc., Easton, MD) and processed with the Jasco Spectra Manager V1.54.03 software package. Curve fitting was performed with the Prism software package (Graphpad; San Diego, CA); the 4-parameter logistic (4PL) non-linear regression function (Eq. 4) was used again. T_m values derived from these fits are listed in Table S3.

SUPPLEMENTARY FIGURES AND TABLES:

Table S1. Parameters and Statistics for apparent T_M curve fit.

	W76Y	E50X	K257X
Best-fit values			
Bottom	-31.28 mDeg	-28.96 mDeg	-29.57 mDeg
Top	-7.831 mDeg	-3.147 mDeg	-4.744 mDeg
T_M	96.02 ºC	94.19 ºC	93.66 ºC
HillSlope	48.53	48.28	45.16
log T_M	1.982	1.974	1.972
Span	23.45 mDeg	25.82 mDeg	24.83 mDeg
95% CI (profile likelihood)			
Bottom	-31.40 to -31.16 mDeg	-29.35 to -28.60 mDeg	-30.02 to -29.14 mDeg
Top	-8.549 to -7.017 mDeg	-4.208 to -1.871 mDeg	-5.832 to -3.409 mDeg
T_M	95.86 to 96.19 ºC	93.95 to 94.47 ºC	93.39 to 93.98 ºC
HillSlope	45.96 to 51.21	42.96 to 54.17	39.60 to 51.34
log T_M	1.982 to 1.983	1.973 to 1.975	1.970 to 1.973
Goodness of Fit			
Degrees of Freedom	17	17	17
R squared	0.9996	0.9979	0.9974
Sum of Squares	0.4544	3.523	4.150
Sy.x	0.1635	0.4552	0.4941
Number of points			
# of X values	63	63	63
# Y values	21	21	21

Table S2. Fluorescence anisotropy of HexY mutants using 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG).

Free Dye / Construct	Fluorescence Polarization
2-NBDG	0.065 +/- 0.007
HexY	0.090 +/- 0.007
E50X	0.078 +/- 0.007
K257X	0.087 +/- 0.010

Table S3. Parameters and Statistics for apparent K_d curve fit.

	E50X - pH 6.0	E50X - pH 7.5	E50X - pH 9.0
Best-fit values			
Bottom	2132 RFU	1659 RFU	1409 RFU
Top	2611 RFU	2320 RFU	2490 RFU
K_d	76.99 μM	48.76 μM	653.2 μM
HillSlope	0.8409	0.8631	0.7717
log K_d	-4.114	-4.312	-3.123
Span	479.3	661.3	1081
95% CI (profile likelihood)			
Bottom	2116 to 2146 RFU	1645 to 1672 RFU	1401 to 1417 RFU
Top	2587 to 2640 RFU	2302 to 2341 RFU	2454 to 2533 RFU
K_d	58.75 to 102.7 μM	41.37 to 57.48 μM	664.2 to 870.4 μM
HillSlope	0.6794 to 1.056	0.7618 to 0.9778	0.7147 to 0.8314
log K_d	-4.231 to -3.988	-4.383 to -4.240	-3.178 to -3.060
Goodness of Fit			
Degrees of Freedom	17	17	17
R squared	0.9922	0.9971	0.9991
Sum of Squares	6058	4517	2282
Sy.x	18.88	16.30	11.58
Number of points			
# of X values	21	21	21
# Y values	21	21	21

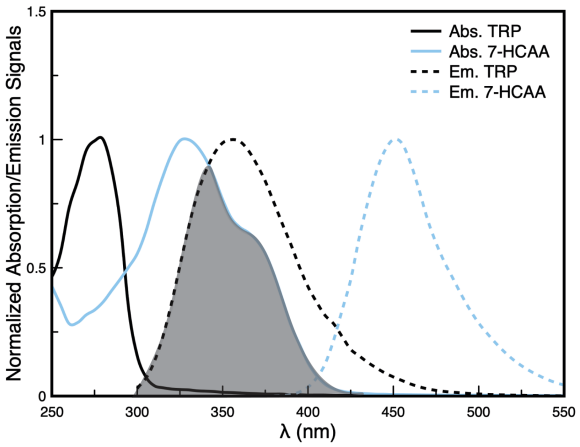

Figure S1. Absorption (solid lines) and emission (dashed lines) spectra for tryptophan (black lines) and 7-HCAA (light blue lines) at pH 7.5 showing the overlap integral in gray. Spectra are normalized to the λ_{max} for each individual spectrum.

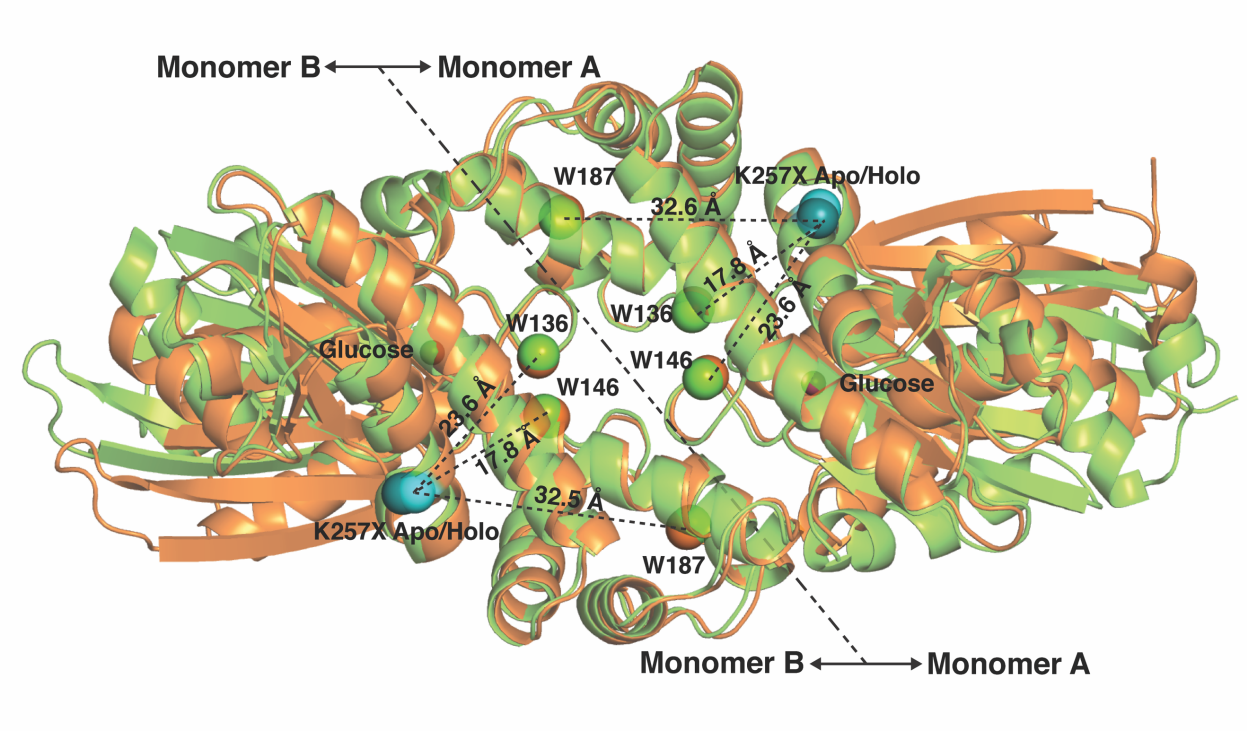

Figure S2. Models of the K257X hexokinase mutant dimer in the apo (green; PDB 2e2n) and holo (orange; PDB 2e2o) are shown superimposed with one another.[2] Residue K257, is shown as a dark teal or cyan sphere in the apo and holo forms, respectively. Three native tryptophan residues (W136, W146, and W187) are identified with orange and green spheres that correspond to their positions in the apo and holo structures, respectively. Distances between residue 257 and each tryptophan are displayed to indicate that residue 257 does not move in relation to any tryptophan upon glucose binding.

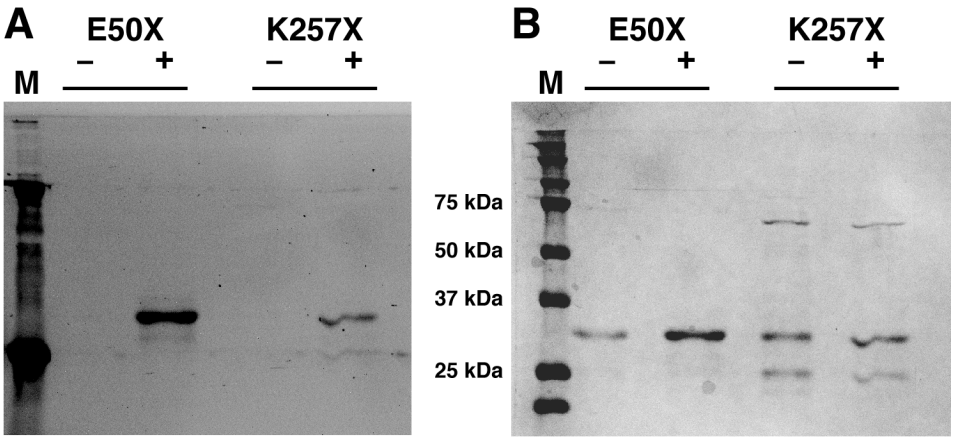

Figure S3. Reducing SDS-PAGE analysis of the expression of the E50X and K257X hexokinase mutants is shown. Visualization of the proteins was achieved by (A) UV light illumination and (B) coomassie stain. Protein expression of both mutants was carried out in the absence (-) and presence (+) of 1 mM 7-HCAA. Full-length protein expression is only expected in the presence of 7-HCAA. The presence of full-length protein in the (-) lanes in panel B can be attributed to amber suppression with a mis-acylated $tRNA_{CUA}$ and likely contain tyrosine at position 50 or 257 instead of 7-HCAA. In our experience, mis-acylation of the $tRNA_{CUA}$ with canonical amino acids occurs more frequently when NCAAs are not present during expression. Based on spectroscopic analysis (comparison of A_{280} and A_{325}) of the mutant proteins, the amber codons in E50X and K257X were suppressed by 7-HCAA at ~92% and 86% efficiencies, respectively.

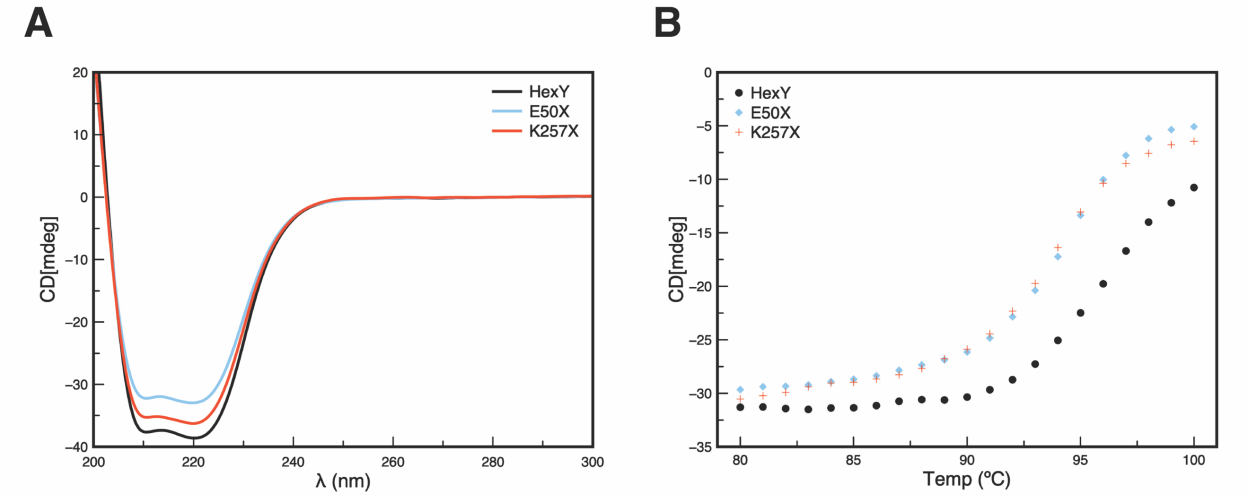

Figure S4. (A) CD spectra of HexY (black line), E50X (light blue line), and K257X (red line) (~10 μM) at pH 7.5. Spectra are the average of three readings. (B) Melting curve of HexY (black circles), E50X (light blue diamonds), and K257X (red crosses) at 222 nm. All data was processed and smoothed using the manufacturer's software.

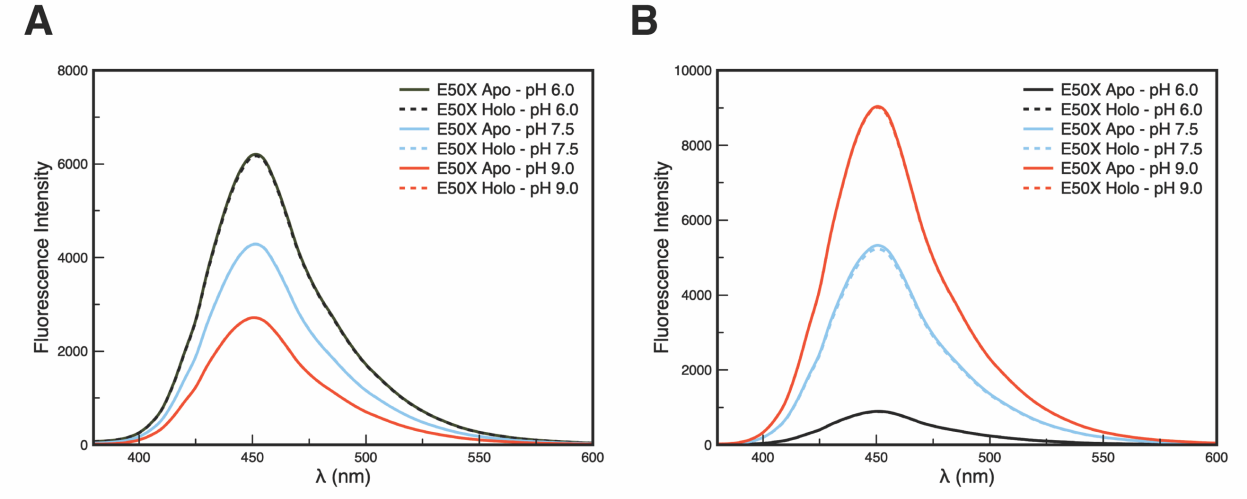

Figure S5. Fluorescence spectra for E50X in the absence (apo) and presence (holo) of 10 mM glucose for pH 6.0 (black lines), pH 7.5 (light blue lines), and pH 9.0 (red lines) excited with either (A) 325 nm or (B) 360 nm wavelength light.

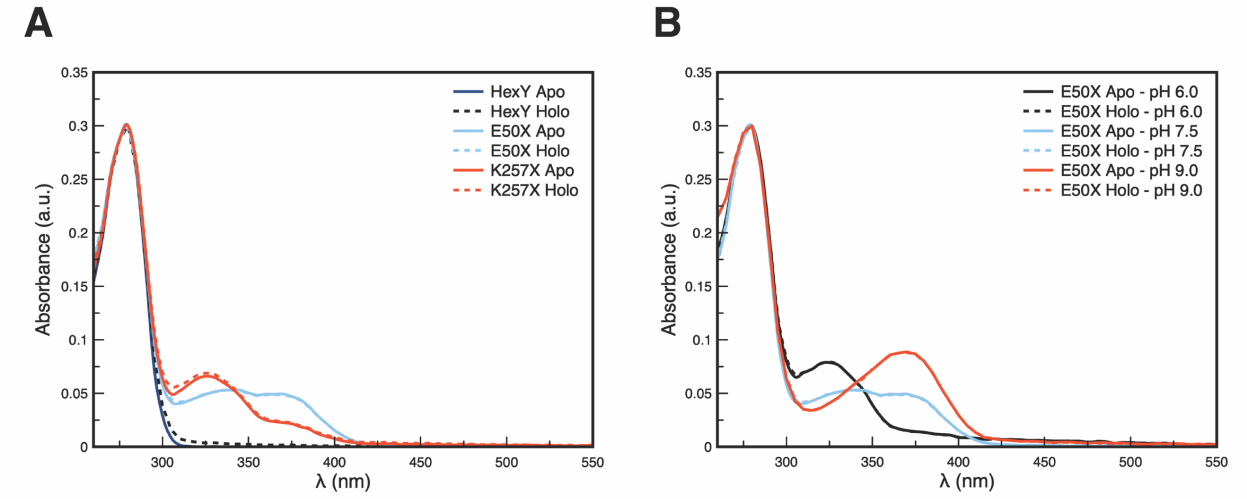

Figure S6. (A) Absorbance spectra of HexY (black lines) and the E50X (light blue lines) and K257X (red lines) mutants are shown in the absence (solid lines) and presence (dashed lines) of 10 mM glucose. (B) Absorbance spectra of the E50X mutant are shown at pH 6.0, 7.5, and 9.0 in the absence (solid lines) and presence (dashed lines) of 10 mM glucose. Spectra are scaled to absorbance at 280 nm.

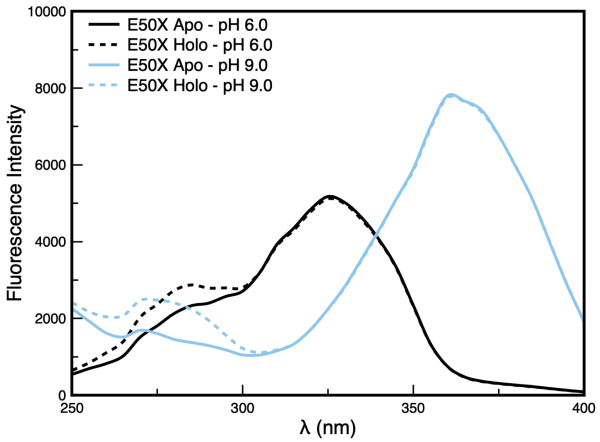

Figure S7. Excitation scan of 450 nm emission at pH 6.0 (black lines) and pH 9.0 (light blue lines). These data show that increases in emission at 450 nm are localized to 280 nm excitation and can be associated with a FRET event from tryptophan.

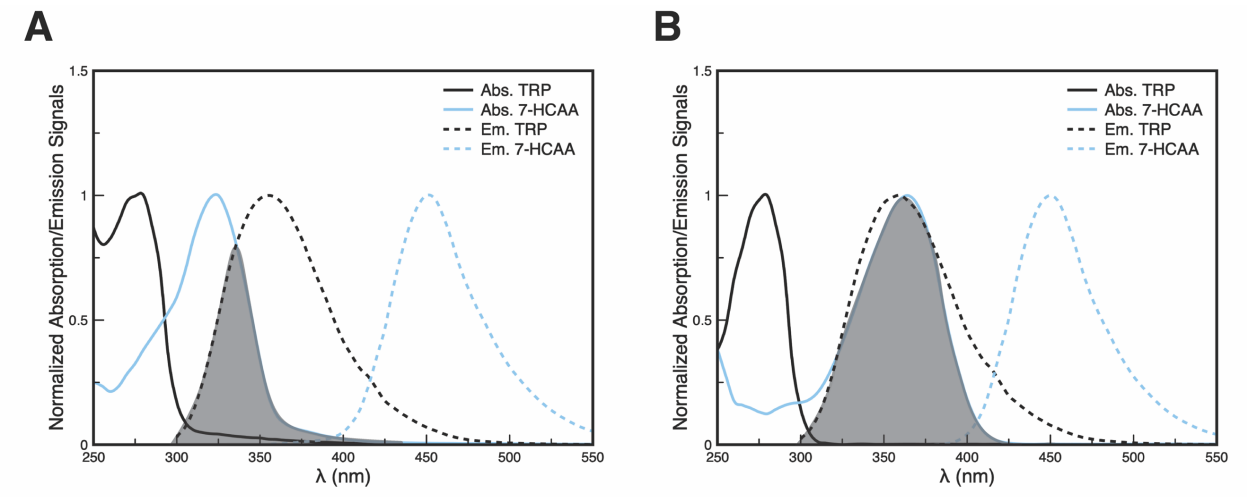

Figure S8. Absorption (solid lines) and emission (dashed lines) spectra for tryptophan (black lines) and 7-HCAA (light blue lines) at (A) pH 6.0 and (B) pH 9.0 showing the overlap integral in grey. Spectra are normalized to the λ_{max} for each individual spectrum.

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