Supplementary Information

An improved miniprotein host for fluorogenic supramolecular assembly on the surface of living cells

Bi Xu, Xinqi Zhou, and Cliff I. Stains*

Department of Chemistry, University of Nebraska-Lincoln, Lincoln, Nebraska 68588, United States

E-mail: cstains2@unl.edu

Table of Contents

1. Cloning

The pCT-Fn3 vectors carrying wild-type Fn3 and 6.2.22 genes were extracted from yeast cells, as previously described.[1] The DNA sequence for 6.2.22 was PCR amplified using primers containing NdeI and HindIII restriction sites and then cloned into the pMAL-c5X-His vector (NEB).

Forward Primer: 5'-GCGCATATGGTTTCTGATGTTCCG-3'

Reverse Primer: 5'-GCGAAGCTTTTACTGGGATGGTTTGTC-3'

2. Protein expression and purification

pMAL-c5X-His-wtFn3, pMAL-c5X-His-6.2.18 and pMAL-c5X-His-6.2.22 were transformed into BL21-Gold (DE3) competent cells (Stratagene). Transformed cells were grown in 1L of Terrific Broth media (Research Products International Corp., T15000) with ampicillin and 0.2% (w/v) glucose at 37 °C to an OD_{600} of ~0.5. Cells were then induced with 0.3 mM of IPTG at 16 °C overnight. After induction, cells were collected by centrifugation at 3000 g for 20 min. The resulting pellets were resuspended in 50 mL of cell lysis buffer (50 mM sodium phosphate (pH = 8.0), 0.5 M NaCl, 5% glycerol (v/v), 5 mM CHAPS and 1x complete EDTA-free protease inhibitor cocktail (Thermo Scientific, 1862209)) and sonicated at 40% amplitude using 1 sec on and 1 sec off cycles for 90 s. Lysates were centrifuged at 18,000 g for 40 min. The soluble fraction was incubated with pre-equilibrated amylose resin at 4 °C for 1 h. The resin was then washed with 50 mL of lysis buffer followed by 50 mL of 1x PBS buffer (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 (pH = 7.4)). Proteins were eluted with 5 mL of 1x PBS containing 10 mM maltose. Proteins were concentrated and further purified by a FPLC (AKTAprimePlus, GE Healthcare) with a HiLoad 16/600 Superdex 200pg column (GE Healthcare)). Protein absorbance was monitored at 280 nm.

Protein concentrations were determined by absorbance at 280 nm (MBP-wtFn3 molar extinction coefficient = 80,790 $M^{-1}cm^{-1}$; MBP-6.2.18 molar extinction coefficient = 86,750 $M^{-1}cm^{-1}$; MBP-6.2.22 molar extinction coefficient = 90,760 $M^{-1}cm^{-1}$).

3. Circular dichroism (CD) spectrometry

MBP-6.2.22 or MBP-wtFn3 (2 μM in 10 mM sodium phosphate, 300 μL) was added to a 0.1 cm quartz cuvette and scanned from 190 nm to 260 nm at 20 nm/min with an interval of 1 nm and a data integration time of 2 seconds, using a Jasco J-815 CD Spectrometer.

4. EC_{50} determination

The EC_{50} was determined by measuring the fluorescence change of 500 nM sI-Pht in the presence of different concentrations of MBP-6.2.22 (from 30 nM to 60 μM) in a 384-well plate (Corning, black with 0.2 mm glass bottom) using a plate-reader (BioTek SynergyH1 hybrid reader). In each well, 30 μL of MBP-6.2.22 solution (in 1x PBS) was incubated with 500 nM sI-Pht at room temperature for 30 min. In the background well, 30 μL of 500 nM sI-Pht in 1x PBS was incubated at room temperature for 30 min. Each sample was prepared in triplicate. Fluorescence from each sample was measured in a plate-reader with the excitation wavelength at 575 nm. Arbitrary fluorescence of each well at 619 nm was background subtracted using the sI-Pht only sample and plotted against the log of the concentration of MBP-6.2.22. The EC_{50} was determined by fitting the resulting data to a four-parameter logistic function in KaleidaGraph.

5. Quantum yield determination

The quantum yield (QY) value was determined using a Fluoromax-4 Spectrofluorometer (HORIBA Scientific) with a 1 cm quartz cuvette. Sulforhodamine 101 in ethanol was used as the standard.[2] sI-Pht was added to 50 μM MBP-6.2.22 in 1x PBS to give an absorbance of ~0.05. QY was calculated as reported.[3]

6. Molar absorptivity

Experiments were done using a BIOMATE 3S (Thermo Scientific) with a 1 cm quartz cuvette. For the MBP-6.2.22-sI-Pht complex, sI-Pht (500 nM) was added to MBP-6.2.22 (50 μM) in 1x PBS. The molar absorptivity was determined using Beer's Law.

7. Job's plot

MBP-6.2.22 was incubated with sI-Pht in 30 μL of 1x PBS in a 384-well plate at varying mole fractions for 30 min at room temperature. The total concentration of species (MBP-6.2.22 plus sI-Pht) was kept at 10 μM. Each sample was prepared in triplicate. Fluorescence was measured by a plate-reader using excitation at 575 nm. Fluorescence of each sample at 619 nm was plotted against the mole fraction of MBP-6.2.22.

8. Scatchard plot

The background subtracted emission/[MBP-6.2.22] from the EC_{50} assay was plotted against emission.

9. MBP-6.2.22 selectivity

MBP-6.2.22 was diluted in 1x PBS to a concentration of 10 μM and incubated with 500 nM of 4DMN,[4-6] NBD[7,8] or sI-Pht in 40 μL of 1x PBS in a 384-well plate at room temperature for 30 min. As controls, dyes alone (500 nM) in 40 μL of 1x PBS were incubated at room temperature for 30 min. Samples were prepared in triplicate. 4DMN, NBD and sI-Pht samples were excited at 460 nm, 485 nm and 575 nm, respectively. Fluorescence was measured using a plate-reader at the emission wavelength of 535 nm, 565 nm and 619 nm for 4DMN, NBD and sI-Pht, respectively. Fluorescence of each protein-with-dye sample was normalized to the fluorescence of the corresponding dye-only sample. Experiments were performed in triplicate.

10. Sedimentation velocity

MBP-6.2.22 was diluted in 1x PBS to a concentration of 50 **μM. A blank sample (400 μL of 1x PBS) was used as the reference for ultracentrifuge calibration. The protein only sample (400 μL of 50 μM MBP-6.2.22) or the protein with dye sample (400 μL of 50 μM MBP-6.2.22 plus 5 μM sI-Pht) were incubated at room temperature for 30 min and then loaded into ultracentrifuge cells for analysis. Experiments were performed at 35,000 g at 20 °C. The absorbance was set at 295 nm or 590 nm where indicated. Experiments were performed on a Beckman Coulter ProteomeLab XL-I Protein Characterization System with an An-50 Ti rotor.**

11. Isothermal titration calorimetry

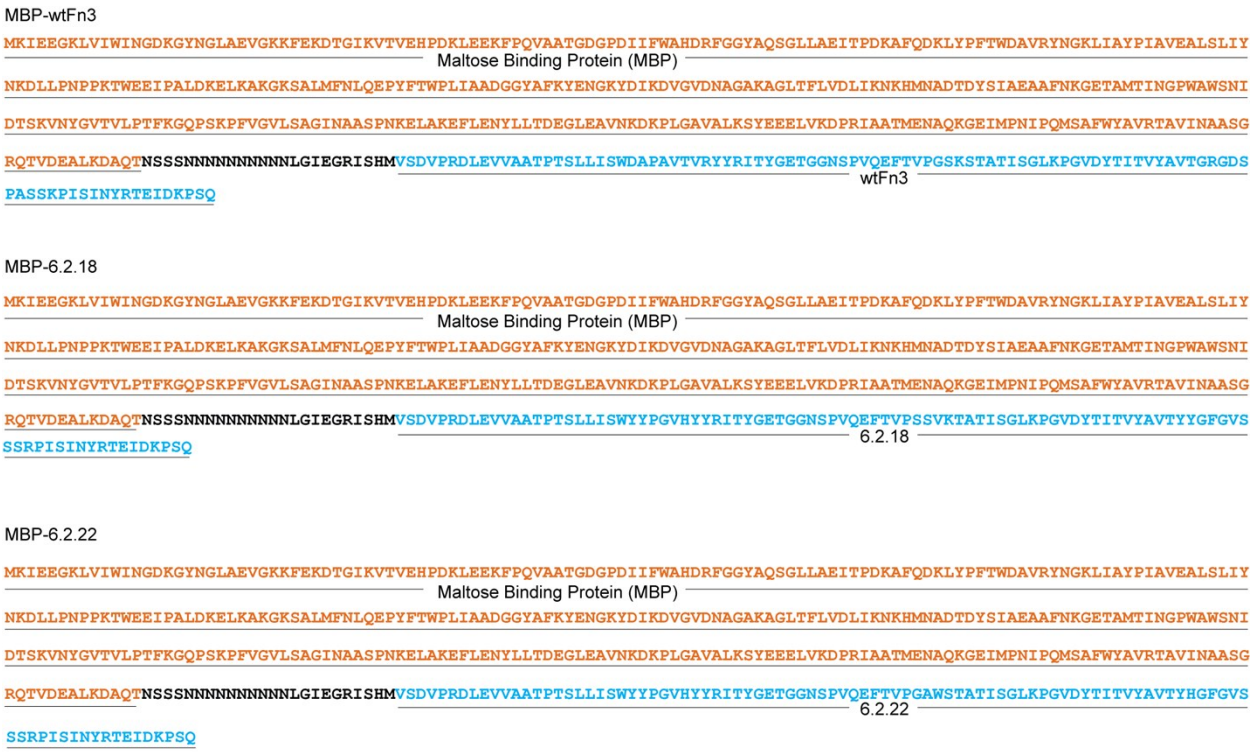
MBP-6.2.22 was diluted in 1x PBS to a final concentration of 80 μM. sI-Pht was dissolved in 1x PBS to a final concertation of 400 μM. sI-Pht was titrated into the cell containing protein (1.5 mL total volume of cell). A total of 50 injections were made at 25 °C. The volume of each injection was 5 μL. The resulting data was fit using a one-site binding model in order to determine an EC_{50} for supramolecular complexation. ITC assays were carried out using a Microcal GE Healthcare VP-ITC.

12. Microscopy

Wild-type Fn3, 6.2.18 and 6.2.22 were expressed on the yeast surface as described previously.[1] For each sample, 10^7 cells were washed with 1x PBSA (1x PBS with 0.1% (w/v) bovine serum albumin) and resuspended in 50 µL of 1x PBSA. Cells were incubated with 0.5 µL of mouse anti-c-myc (BioLegend) at room temperature for 20 min. The resulting cells were then washed with 1x PBSA and resuspended in 50 µL 1x PBSA. A secondary goat, anti-mouse Alexa Fluor 488 conjugated antibody (Invitrogen) was added as a 1:100 dilution and incubated on ice for 15 min. Cells were again washed with 1x PBSA and incubated with 500 nM sI-Pht in 50 µL of 1x PBSA at room temperature for 30 min. The resulting cells were imaged under a confocal microscope for green and red fluorescence. Images were taken on a Nikon A1 LSCM on a Nikon ECLIPS 90i microscope. Software: NIS Element 4.20. Laser lines were: 488 nm (green); 561 nm (red). Emission filters: 500-550 nm (green); 570-620 nm (red). Images for the green and red channels were taken sequentially. Exposure time: 0.5 frame/sec.

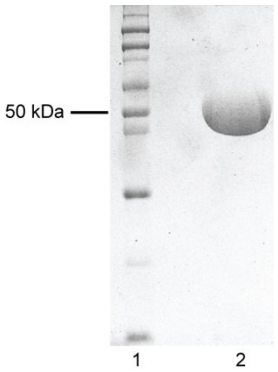
13. Figures

Figure S1



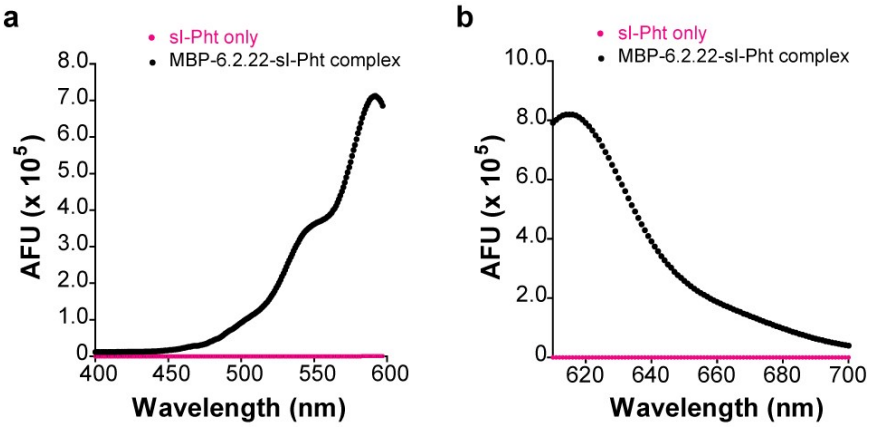
Amino acid sequences for MBP-wtFn3, MBP-6.2.18 and MBP-6.2.22 constructs.

Figure S2



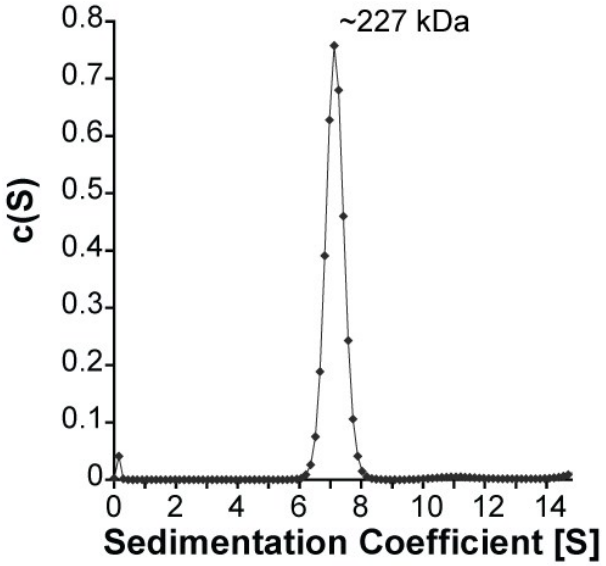
SDS-PAGE of purified MBP-6.2.22. Lane 1: protein ladder; Lane 2: MBP-6.2.22. MBP-6.2.22 calculated molecular weight = 53.8 kDa. The purification of MBP-wtFn3 and MBP-6.2.18 has been described previously.[9]

Figure S3



Excitation (a) and emission (b) spectra for sI-Pht (500 nM) only versus sI-Pht (500 nM) complexed with MBP-6.2.22 (50 μM) in 1x PBS.

Figure S4



Sedimentation velocity data of MBP-6.2.22 (50 μM) with sI-Pht (5 μM) monitored at 590 nm.

14. References

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