Arginine Grafting to Endow Cell-Permeability

Stephen M. Fuchs[†,‡] and Ronald T. Raines[†,§,*]

Page	Contents
S1	Table of Contents
S2–S3	Materials and Methods
S4	References
S5	Figure 1: Fluorescence Properties of cpGFP
S5	Figure 2: Conformational Stability of cpGFP
S6	Figure 3: Cellular Internalization of cpGFP at high concentration

METHODS

Materials. Plasmid pRSET$_B$, which contains a cDNA for eGFP, was a gift from S. J. Remington (University of Oregon). Primers for making mutations in the eGFP cDNA were obtained from Integrated DNA Technologies (Coralville, IA) and had the sequences 5′ CACTGGAGTTGTCCCAATTCTTGTTCGTTTACGTGGTCGTGTTAATGGGCACAAATTTTC TGTCAGTGG 3′ and its reverse complement (in which sites of mutation are underlined for the E17R, D19R, D21R substitutions), 5′ CGGGAACTACAAGACACGTGCTCGTGTCAAGTTTGAA GGTGAT ACCC 3′ and its reverse complement (for the E111R substitution), and 5′ CCCTTGTTAATAGA ATCCGTTTAAAAGGTATTGATTTTAAAG 3′ and its reverse complement (for the E124R substitution).

DH5α and BL21(DE3) competent cells were from Stratagene (La Jolla, CA).

Site-Directed Mutagenesis. cDNA encoding eGFP variants were produced by the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) using the primer pairs described above. Three successive rounds of mutagenesis yielded cpGFP, which is an eGFP variant with five substitutions: E17R, D19R, D21R, E111R, and E124R.

Protein Production. Plasmids containing the sequences for eGFP and cpGFP were transformed into BL21(DE3) cells, and colonies were selected for on Luria–Bertani (LB) agar plates by their ampicillin (Amp) resistance. Small cultures (25 mL of LB medium containing 200 μg/mL Amp) were started from a single colony and grown at 37 °C with shaking at 200 rpm to an optical density of *OD* = 0.6 at 600 nm. One-liter cultures of the same medium were inoculated with 4 mL of the starter culture and grown at 37 °C with shaking at 300 rpm to *OD* = 0.6. Cultures were then cooled to 15 °C, and GFP cDNA expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (final concentration: 1 mM). Cultures were grown at 15 °C with shaking at 300 rpm for 18 h and harvested by centrifugation (5,000 rpm for 10 min) in a Beckman Coulter Avant J-20 XPI centrifuge using a JLA 8.1 rotor. Cell pellets were either frozen or used immediately in protein purification.

Protein Purification. Cell pellets from 1 L of cell culture were resuspended in ~10 mL of ice-cold cell lysis buffer (50 mM sodium phosphate buffer, pH 7.2, containing 500 mM NaCl and 1 mM PMSF). Cells were lysed by sonication (50% duty/50% output) 5× for 30 s. Cell debris was removed by centrifugation at 22,000*g* for 60 min at 4 °C in a Beckman Optima XL-80K ultracentrifuge using a 60Ti rotor. Clarified cell lysate was dialyzed for at least 2 h against phosphate-buffered saline containing 500 mM NaCl (PBS+) (50 mM sodium phosphate buffer, pH 7.2, containing 636 mM NaCl) before loading onto a Ni–NTA agarose (Qiagen, Germany). The column was washed with the same buffer containing 20 mM imidazole before eluting with 50 mM sodium phosphate buffer, pH 7.2, containing NaCl (636 mM) and imidazole (500 mM). The fractions containing green-colored protein were pooled and diluted $^1/_{10}$ with water to lower the salt concentration. cpGFP was then loaded onto a 5-mL HiTrap SP FF Sepharose column (Amersham Biosciences, Piscataway NJ). Protein was eluted with a 100-mL linear gradient (50 + 50 mL) of 50 mM sodium phosphate buffer, pH 7.5, containing NaCl (0–1.00 M). Fractions containing green-colored protein were pooled and dialyzed against 50 mM sodium phosphate buffer, pH 7.5, containing NaCl (652 mM). The N-terminal histidine tag was removed as described previously (*1*). Briefly, protein was incubated with 1:50 (w/w) α-chymotrypsin for 20 h at room temperature. Chymotrypsin degrades the N-terminal tag but does not cleave the GFP protein (*1*). Protein was concentrated using Vivascience 5000 MW spin columns and protein concentration was determined by optical absorbance at 280 nm (ε_{280} = 19890 $M^{-1}cm^{-1}$) or by the BioRad protein assay.

Fluorescence Spectroscopy. Fluorescence measurements were performed with a QuantaMaster 1 photon-counting spectrofluorimeter equipped with sample stirring (Photon

Technology International, South Brunswick, NJ). Fluorescence excitation and emission spectra were obtained in PBS+ buffer using a 2-nm slit width and scanning at a rate of 1 nm/s.

Guanidine–HCl-Induced Equilibrium Unfolding. The conformational stability of GFP variants was determined by following the change in fluorescence as a function of denaturant concentration (*2*). GFP proteins (1–5 nM) were incubated in 96-well flat-bottom plates (total volume: 100 μL) in 50 mM sodium phosphate buffer, pH 7.5, containing NaCl (500 mM) and guanidine–hydrochloride (Gdn–HCl) (0–6.30 M) for 24 h at room temperature. Fluorescence intensity was determined using a Tecan Ultra 384 fluorescence plate reader. Data were fitted to a two-state unfolding mechanism and could be used to calculate the standard free energy of denaturation: $\Delta G°$ ($= -RT\ln K$), where R is the gas constant, T is the absolute temperature, and K is the equilibrium constant calculated from the experimental data with the equation (*3, 4*): $K = [y_N - y]/[y - y_D]$. The value of y is the observed fluorescence value, and y_N and y_D are the y values for the native and denatured states, respectively.

Cell Internalization. HeLa cells, Chinese hamster ovary cells (CHO-K1), and glycosaminoglycan-deficient cell lines (CHO-677 and CHO-745) were obtained from the American Type Culture Collection (ATCC) and maintained according to recommended instructions. The day before protein incubation, cells were seeded onto 4- or 8-well Lab-Tek II Chambered Coverglass tissue culture dishes (Nalge Nunc International, Naperville, IL) to yield 75% confluency on the next day. The following day, protein solutions (in PBS containing 500 mM NaCl) were added to cells in 200 μL (protein volume added was less than $^{1}/_{20}$ of the total volume) of medium or PBS containing $MgCl_2$ (1 mM) and $CaCl_2$ (1 mM). Protein was incubated with cells for known times and the cells were then washed with PBS containing magnesium and calcium three times prior to visualization. In some samples, cell nuclei were counterstained with Hoechst 33342 for 5 min prior to washing. Internalization was visualized with a Nikon C1 laser scanning confocal microscope equipped with 20×, 60×, and 100× lenses.

Images were acquired as 512 × 512 pixel images representing a 636.5 μM (20×) or 127.3 μM (100×) window. All images were taken on the same day using the same laser intensity (~10% output). Images were taken as 3 × 3 in sections from the original file and reduced by 50% for publication.

Electrostatic Potential Diagrams. Electrostatic potential diagrams were made by using the atomic coordinates for F64L/S65T/Y66L GFP (Protein Data Bank entry 1S6Z(*5*)) and the program MacPyMol (DeLano Scientific, South San Francisco, CA). Default settings were used except that the Coulomb dielectric was set to be 80. A model of cpGFP was created and likewise modeled by using the program MacPyMol.

REFERENCES

1. Hanson, G. T., Aggeler, R., Oglesbee, D., Cannon, M., Capaldi, R. A., Tsien, R. Y., and Remington, S. J. (2004) Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators, *J. Biol. Chem. 279*, 13044-13053.
2. Stepanenko, O. V., Verkhusha, V. V., Kazakov, V. I., Shavlovsky, M. M., Kuznetsova, I. M., Uversky, V. N., and Turoverov, K. K. (2004) Comparative studies on the structure and stability of fluorescent proteins EGFP, zFP506, mRFP1, “dimer2”, and DsRed1, *Biochemistry 43*, 14913-14923.
3. Tanford, C. (1968) Protein denaturation, *Adv. Protein Chem. 23*, 121-282.
4. Pace, C. N., and Scholtz, J. M. (1997) Measuring the conformational stability of a protein, in *Protein Structure* (Creighton, T. E., Ed.) pp 299-321, Oxford University Press, New York.
5. Rosenow, M. A., Huffman, H. A., Phail, M. E., and Wachter, R. M. (2004) The crystal structure of the Y66L variant of green fluorescent protein supports a cyclization–oxidation–dehydration mechanism for chromophore maturation, *Biochemistry 43*, 4464-4472.

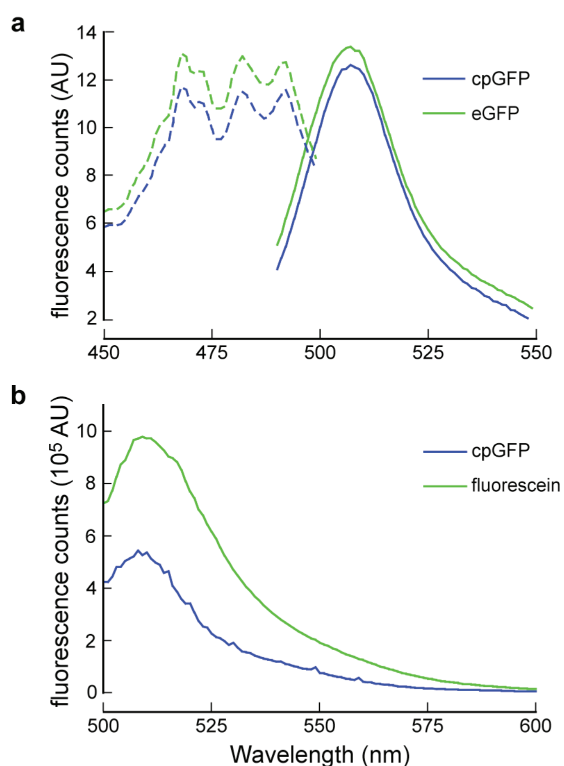

Figure S1 Fluorescence properties of cpGFP. (**a**) Fluorescence excitation (----) and fluorescence emission (—) spectra for cpGFP (blue) and eGFP (green). Data were collected in 1-nm increments with a scan rate of 5 nm/s. (**b**) Raw data for determination of the quantum yield (Φ) for cpGFP. Solutions of cpGFP (blue) and fluorescein (green) ($\Phi_{fluorescein}$ = 0.95 in 0.10 N NaOH) of equal absorbance at 490 nm were diluted in PBS+ (for cpGFP) or 0.10 N NaOH (for fluorescein), and the area under the emission spectrum curves from 500–700 nm was determined. The quantum yield of cpGFP was determined to be Φ_{cpGFP} = 0.44 with the equation: $\Phi_{cpGFP} = \Phi_{fluorescein}$(cpGFP area / fluorescein area).

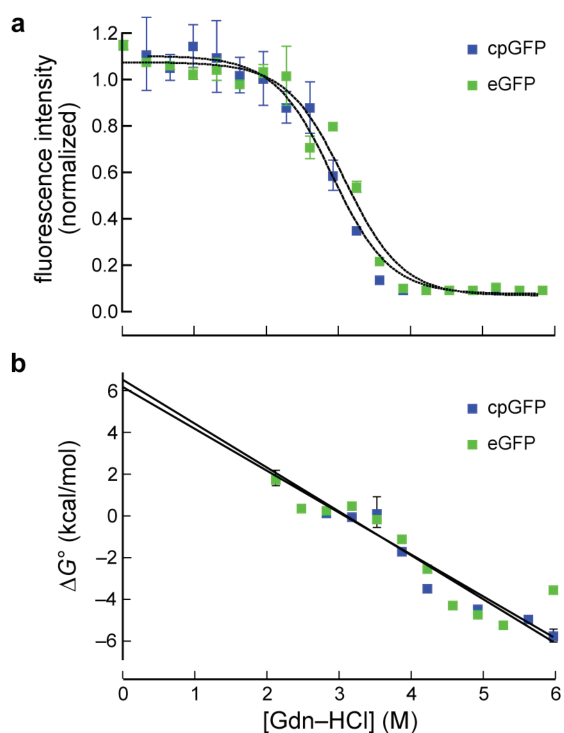

Figure S2 Conformational stability of cpGFP. The unfolding of cpGFP (blue) and eGFP (green) were induced by Gdn–HCl. (**a**) Dependence of normalized fluorescence intensity on denaturant concentration. The midpoint of the transformation was fitted to a sigmoidal dose–response curve with the program Prism (Graphpad Software, San Diego, CA). The midpoint of the transition corresponds to the value of $C_{1/2}$, which is the concentration of denaturant at which the protein is 50% unfolded at equilibrium. (**b**) Dependence of $\Delta G°$ on Gdn–HCl concentration. Data were fitted to a by linear regression such that the slope (m) is the dependence of $\Delta G°$ on denaturant and the y-intercept approximates the free energy of the protein in the absence of denaturant ($\Delta G° \sim \Delta G(H_2O)$).

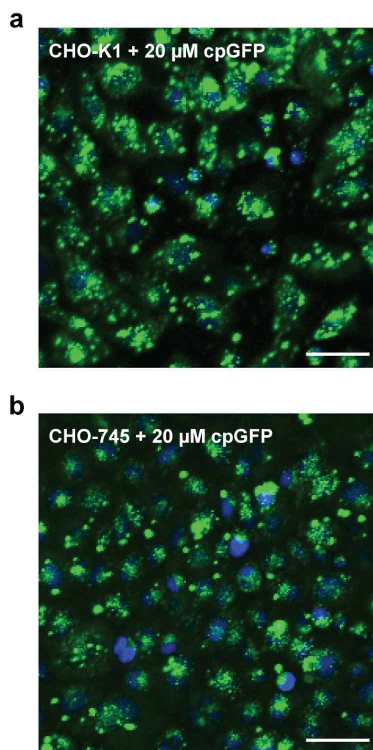

Figure S3 Cellular internalization of cpGFP in CHO-K1 and CHO-745 cells at high concentration. cpGFP (20 µM) was added to Opti-MEM medium containing CHO-K1 (**a**) and CHO-745 (**b**) cells, and incubated for 3 h at 37 °C. Cells were then placed in fresh medium for 1 h, and stained with Hoechst 33342 (blue) prior to visualization. Scale bars: 50 µm.