Biophysical Journal, Volume 98

Supporting Material

Distinguishing between protein dynamics and dye photophysics in single molecule FRET experiments

Hoi Sung Chung, John M. Louis, and William A. Eaton

Supporting material

Distinguishing between protein dynamics and dye photophysics in single molecule FRET experiments

Hoi Sung Chung*, John M. Louis, and William A. Eaton*

Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases
National Institutes of Health, Bethesda, MD, 20892-0520

*To whom correspondence may be addressed: E-mail: chunghoi@niddk.nih.gov or eaton@helix.nih.gov

Protein engineering, dye labeling, and immobilization

DNA insert encoding 56 amino acids of the immunoglobulin-binding domain B1 of streptococcal protein G (GB1; PDB code 3GB1) was subcloned in pET15b vector (Novagen, San Diego, CA) between the Nde1 and BamH1 sites. The Quik-Change mutagenesis protocol (Stratagene, La Jolla, CA) with the appropriate forward and reverse primers and the DNA template were used to generate the mutant construct, His-GB1^{C57}. The His-GB1^{C57} plasmid DNA was then used to generate the double mutant His-GB1$^{K10C/C57}$. Both constructs were verified by DNA sequencing and mass spectrometry. In these constructs, GB1 is preceded by a spacer sequence and a His-tag as illustrated in Fig. 1.

The GB1 expression constructs, His-GB1$^{K10C/C57}$ or His-GB1^{C57}, were transformed into *E.coli* BL-21(DE3; Stratagene). Cells were grown in Luria-Bertani medium, and expression was induced at 0.7 OD_{600nm} with a final concentration of 1 mM IPTG for a period of 3-4 h. Typically, cells harvested from a 400-mL culture were suspended and lysed in 70 mL of bactertial protein extraction reagent (B-PER, Thermo Scientific, Rockford, IL) in the presence of 2 mM dithiothreitol (DTT) and 5 mM benzamindine, sonicated, and then centrifuged at 16,000 rpm (SS-34 rotor, Thermo Fisher Scientific, Asheville, NC) for 30 min at 4ºC. The supernatant was subjected to affinity chromatography on a 5ml HisTrap HP column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The column was washed in 1x PBS, 1 mM DTT and 20 mM imidazole and bound his-tagged protein was eluted in the same buffer containing 0.22 M imidazole. The protein was concentrated using centriprep YM-3 devices (Millipore Corp, Bedford, MA), and ~ 30 mg was loaded onto a Superdex-75 column (2.6 cm x 60 cm; GE HealthCare, Piscataway, NJ) equilibrated in 0.5x PBS, 2 mM DTT at a flow rate of 3 mL/min at room temperature. Peak fractions were analyzed by SDS-PAGE, combined and subjected to reverse-phase HPLC on POROS 20 R2 resin (Perceptive Biosystems, Framington, MA) and eluted using a linear gradient from 99.95% water (v/v) and 0.05% TFA to 60% acetonitrile (v/v), 0.05% TFA (v/v) and 39.95% water (v/v) over a period of 16 min at a flow rate of 4 ml/min. Aliquots (0.5 mg) of the peak fraction were lyophilized and stored at -70ºC. The mass of His-GB1$^{K10C/C57}$ and His-GB1^{C57} was determined to be 8333 and 8358, respectively.

For dye labeling, typically, 0.5 mg of lyophilized His-GB1$^{K10C/C57}$ was dissolved in 0.15 mL of degassed 4M guanidine hydrochloride (GdHCl), 50 mM Tris-HCl, pH 7.5, in an oxygen-free chamber and then added to a tube containing an excess of Alexa Fluor 488 C_5-maleimide (Alexa 488) and Alexa Fluor 594 C_5-maleimide (Alexa 594) dyes (Invitrogen, Carlsbad, CA) dissolved in a ratio of 2:3 in DMSO. The reaction was carried out in dark for 1 h at room temperature. The protein was then incubated with 20 mM 2-mercapto-ethanol for 1-2 h at room temperature to prevent any unreacted dye from binding to the resin in the subsequent column steps. The reaction mixture was first loaded onto a Superdex-peptide column (1 cm x 30 cm; GE HealthCare) equilibrated in 0.5x PBS to separate the unreacted dye from the labeled protein. Peak fractions were then fractionated on a Mono-Q column (1 mL, GE HealthCare) using a 0 to 1 M NaCl gradient in 25 mM Tris-HCl, pH 8, over a period of 30 min at a flow rate of 1 mL/min at room temperature monitored at three specific wavelengths, two specific to the dyes and one at 280 nm. The peak fraction corresponding to the protein having nearly equimolar ratio of the acceptor and donor Alexa dyes was used for the experiments described. Alternatively, His-GB1^{C57} was used for donor only labeling similar to that described above and purified in a single step using Superdex-peptide column.

For free diffusion experiments dye-labeled proteins were diluted to 80 pM in pH 7.5, 50 mM phosphate buffer with urea and 0.01% Tween-20 (Thermo Scientific) to prevent sticking of proteins on

the glass surface. 25 μL of protein solution was loaded on a 1-inch glass cover slip and covered with CoverWell (PCI-0.5, Grace Bio-Labs) to prevent evaporation.

For immobilization experiments Cu^{2+} - iminodiacetic acid - multi-armed polyethyleneglycol-surfaces (1) were purchased from Microsurfaces Inc. (Cu_01, low density) and used after being cleaned with DI water and dried with N_2 stream. The surface was covered with Coverwell (PC8R-0.5) having two 2 mm holes. For the immobilization, 20 μL of protein solution (40 pM protein in 50 mM, pH 7.5 phosphate with the same urea concentration with that of the working buffer) was applied and removed by a pipet through a hole of the cover after ~ 5 min. Then, 20 μL of the working buffer was added and the holes were sealed with parafilm to prevent evaporation. The working buffer (50 mM phosphate, pH 7.5, 3 – 7 M urea) contains 10 nM protocatechuate 3,4-dioxygenase (PCD, P8279-25UN, Sigma) and 2.5 mM 3,4-dihydroxybenzoic acid (PCA, 37580-25G-F, Sigma) as an oxygen scavenging system, and 1 mM Trolox (238813, Sigma) for triplet quencher (2). No sticking of dye or protein molecules was found on PEG surface without Cu^{2+}.

For the given protein concentration (40 pM), 50 – 100 molecules were immobilized in an area of 12 × 12 μm^2. We have previously estimated that the probability for two randomly distributed molecules to be located in the diffraction limited spot is ~ 4% (3).

Stopped flow ensemble kinetics

The ensemble folding/unfolding kinetics of dye-labeled proteins were measured at 23°C with a XS20 stopped flow instrument (Applied Photophysics). A pH 7.5, 50 mM phosphate buffered solution of 70-400 nM dye-labeled protein was mixed in 1:1 ratio with the same buffer at a different urea concentrations. The donor was excited at 488 nm and changes in donor fluorescence intensity were selectively measured by blocking acceptor fluorescence with a band pass filter (545AF75, Omega). Mixing was repeated 4 – 7 times and the relaxation time was obtained for each kinetic progress curve by a single exponential fit and averaged.

Effect of acceptor dye quantum yield on FRET efficiency *E*.

The FRET efficiency, E, is defined in this work as

$$E = \left(1 + \frac{n_D}{n_A}\right)^{-1} \tag{S1}$$

where the n_D and n_A are the number of donor and acceptor photons after background subtraction. Background counting rates for donor and acceptor photons in immobilization experiments were obtained from the segment after the donor dye was bleached for each trajectory. In the free diffusion experiments, the mean background photon counting rates were obtained from the total number of photons divided by the total data collection time. These mean background counting rates were used to generate background donor and acceptor photons (by the Poissonian statistics) for each burst to correct the number of donor and acceptor photons. It is an apparent FRET efficiency, because no correction is made for differences in quantum yields or detection efficiencies for the two dyes. The true FRET efficiency, E_t, can be obtained by introducing a factor γ to account for these differences

$$E_t = \left(1 + \gamma \frac{n_D}{n_A}\right)^{-1} = \left(1 + \gamma' \frac{\varphi_A n_D}{\varphi_D n_A}\right)^{-1} \quad \text{(S2)}$$

Here, γ is divided into three terms: the quantum yields of the donor (φ_D) and the acceptor (φ_A) and γ', which depends on the ratio of the detector sensitivities in the donor and acceptor channels of the instrument.

E_t can also be expressed in terms of Förster radius R_0, the sixth power of which is proportional to φ_D ($R_0^6 = \varphi_D \cdot (R_0')^6$).

$$E_t = \left(1 + \left(\frac{r}{R_0}\right)^6\right)^{-1} = \left(1 + \frac{1}{\varphi_D}\left(\frac{r}{R_0'}\right)^6\right)^{-1} \quad \text{(S3)}$$

Rearranging Eqs. (S1) , (S2), and (S3) results in

$$E = \left(1 + \frac{1}{\gamma}\left(\frac{1}{E_t} - 1\right)\right)^{-1} = \left(1 + \frac{1}{\varphi_A}\frac{1}{\gamma'}\left(\frac{r}{R_0'}\right)^6\right)^{-1} \quad \text{(S4)}$$

which shows that, while the true FRET efficiency, E_t, obtained from counting donor and acceptor photons depends on the quantum yields of both donor and acceptor, the apparent FRET efficiency, E, depends only on the acceptor quantum yield. Since the acceptor quantum yield is given by the ratio of the measured and radiative lifetimes: $\varphi_A = \tau_A / \tau_A^{rad}$ the apparent FRET efficiency is related to the measured acceptor lifetime by:

$$E = \left(1 + \frac{\tau_A^{rad}}{\tau_A}\frac{1}{\gamma'}\left(\frac{r}{R_0'}\right)^6\right)^{-1} \quad \text{(S5)}$$

Eq. (S5) can then be used to predict the expected variation in E from the measurements of the variation in τ_A, assuming that τ_A^{rad} does not also vary.

Theoretical calculation of amplitude of donor-acceptor cross-correlation function

The amplitude of the donor-acceptor, cross-correlation function as $t \rightarrow 0$ is given by

$$C_{DA}(0) = \frac{\langle n_D n_A \rangle}{\langle n_D \rangle \langle n_A \rangle} - 1 = -\frac{\langle E_t^2 \rangle - \langle E_t \rangle^2}{\langle E_t \rangle - \langle E_t \rangle^2} \quad \text{(S6)}$$

where E_t is the true FRET efficiency, so that

$$\left\langle E_t \right\rangle = \frac{\int_a^L E_t(r)P(r)dr}{\int_a^L P(r)dr} \quad \text{and} \quad \left\langle E_t^2 \right\rangle = \frac{\int_a^L E_t(r)^2 P(r)dr}{\int_a^L P(r)dr} \tag{S7}$$

where $P(r)$ is the radial probability distribution for the inter-dye distance of dyes attached to a Gaussian chain and is given by

$$P(r) = 4\pi r^2 \left(\frac{3}{2\pi \left\langle r^2 \right\rangle} \right)^{3/2} \exp\left(-\frac{3r^2}{2\left\langle r^2 \right\rangle} \right) \tag{S8}$$

Since the inter-dye distance cannot be longer than the length of the fully extended polypeptide L (= 0.36 H N nm), $N \approx 51$ (47 residues + dye linkers), the integration is performed from the distance of closest approach of the dyes (a = 0.4 nm) to L. The true FRET efficiency, E_t, was calculated from the donor emission from:

$$\left\langle E_t \right\rangle = 1 - \frac{\int_0^\infty I_{DA}(t)dt}{\tau_D} \tag{S9}$$

where τ_D is the donor lifetime obtained from a single exponential fit to the singly labeled protein, and I_{DA} is the normalized intensity of the donor in the presence of acceptor, which exhibits a non-exponential decay because the motion of the polypeptide chain is slower than the donor lifetime (4, 5). Using $\langle r^2 \rangle^{1/2}$ = 8 nm, which is larger than disordered peptides of the same length, presumably because of the large (-5) net negative charge (6), yields the experimental value of $\langle E_t \rangle$ = 0.32 from Eq. (S9) ($\langle E_t^2 \rangle$ = 0.22). The computed correlation amplitude using Eqs. (S6) - (S9) is then $C_{DA}(0)$ = - 0.53.

Quenching of acceptor dye by copper ions.

To show that that Cu^{2+} reduces the apparent FRET efficiency by quenching the acceptor dye, we measured the lifetime of Alexa 594 in the doubly-labeled protein in a free diffusion experiment and found a reduction from 4.5 ns to 4.0 ns in the presence of 2 μM $CuSO_4$ (Fig. S1). At this concentration, the major mechanism of quenching is presumably intramolecular from Cu^{2+} ions bound to the his-tag.

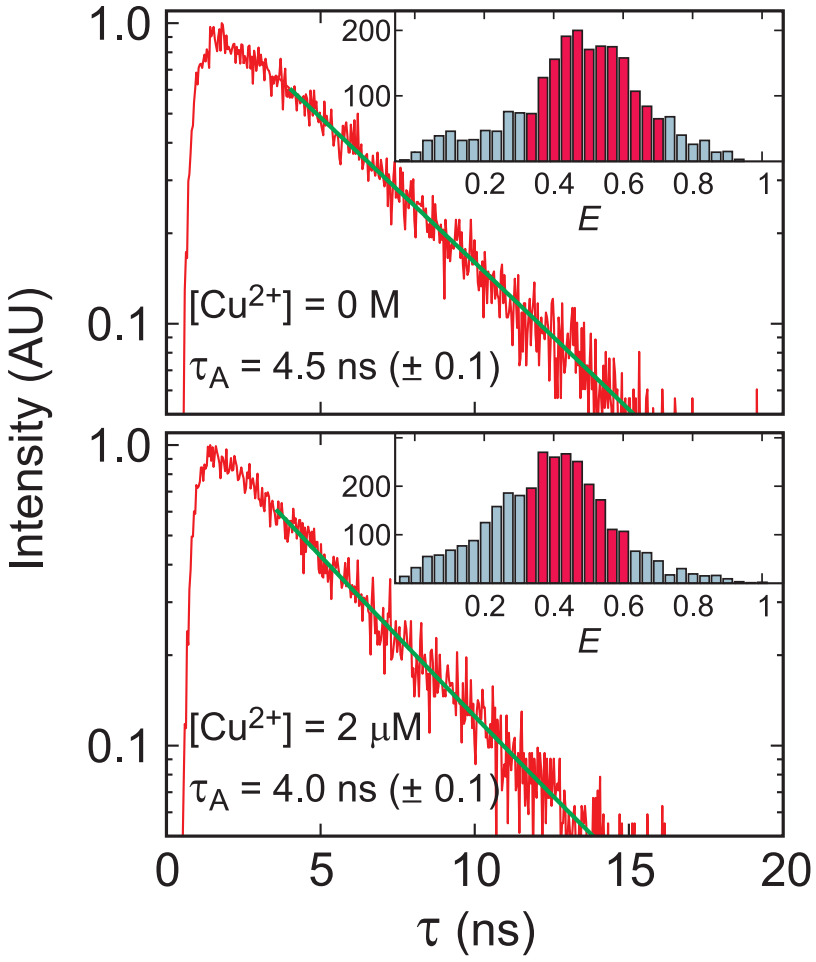

Fig. S1. Quenching of the acceptor (Alexa 594) fluorescence by 2 μM free Cu^{2+} ($CuSO_4$). The lifetime was calculated by collecting the acceptor photons from bursts for which $0.3 < E < 0.7$ (without ($CuSO_4$)) and $0.3 < E < 0.6$ (with ($CuSO_4$)) in the FRET efficiency histogram (insert), calculated with a threshold of 30 photons.

REFERENCES

1. Cha, T., A. Guo, Y. Jun, D. Q. Pei, and X. Y. Zhu. 2004. Immobilization of oriented protein molecules on poly(ethylene glycol)-coated Si(111). *Proteomics* 4:1965-1976.
2. Aitken, C. E., R. A. Marshall, and J. D. Pulglisi. 2008. An oxygen scavenging system for improvement of dye stability in single-molecule fluorescence experiments. *Biophys. J.* 94:1826-1835.
3. Chung, H. S., J. M. Louis, and W. A. Eaton. 2009. Experimental determination of upper bound for transition path times in protein folding from single-molecule photon-by-photon trajectories. *Proc. Natl. Acad. Sci. USA* 106:11837-11844.
4. Merchant, K. A., R. B. Best, J. M. Louis, I. V. Gopich, and W. A. Eaton. 2007. Characterizing the unfolded states of proteins using single-molecule FRET spectroscopy and molecular simulations. *Proc. Natl. Acad. Sci. USA* 104:1528-1533.
5. Nettels, D., I. V. Gopich, A. Hoffmann, and B. Schuler. 2007. Ultrafast dynamics of protein collapse from single-molecule photon statistics. *Proc. Natl. Acad. Sci. USA* 104:2655-2660.
6. Buscaglia, M., L. J. Lapidus, W. A. Eaton, and J. Hofrichter. 2006. Effects of denaturants on the dynamics of loop formation in polypeptides. *Biophys. J.* 91:276-288.