A Chemically Modified Green-Fluorescent Protein that Responds to Cleavage of an Engineered Disulphide Bond by Fluorescence Resonance Energy Transfer (FRET)-Based Changes

Wei Zhang,[†] Miho Suzuki,[††,†††] Yoichiro Ito,[††††] and Kenneth T. Douglas*[†]

[†]*Wolfson Centre for Structure-Based Rational Design of Molecular Diagnostics, School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, M13 9PL U.K.*

[††]*Department of Functional Materials Science, Faculty of Engineering, Saitama University, 255 Shimo-okubo, Saitama 338-8570*

[†††]*REDS Group (Saitama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence, JST), Saitama Small Enterprise Promotion Corporation, 3-12-18 Kamiaoki, Kawaguchi, Saitama 333-0844*

[††††]*Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1, Yamadaoka, Suita, Osaka 565-0871*

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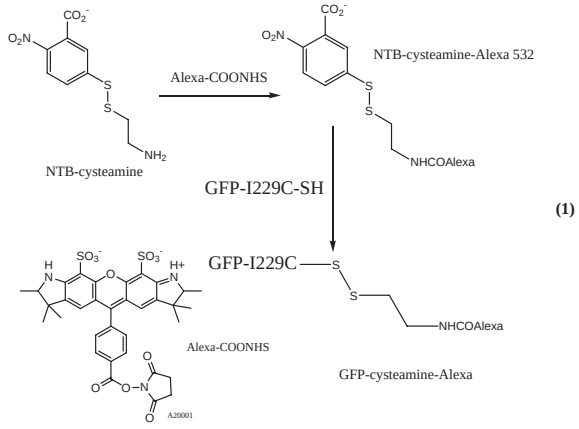
The mixed disulfide of a Green-Fluorescent Protein with an Alexa 532 fluorescent label showed FRET, with quenching of the GFP emission. Reductive cleavage of this disulfide bond destroyed the FRET, giving a change in the ratios of fluorescence intensity at the wavelengths corresponding to the GFP and the Alexa dye. Cleavage by glutathione (second order rate constant at pH 7.4 was $1.18 \pm 0.02 \times 10^2\ M^{-1}\ min^{-1}$) was faster than for sodium cyanoborohydride.

Green-fluorescent protein (GFP) from *Aequorea victoria* and its mutants[1–4] provides fluorescing markers that have served to probe just about every cellular organelle. GFP mutants with various emission maxima are often useful as pairs of partners for fluorescence resonance energy transfer (FRET).[5] GFP variants with altered fluorescence maxima are often linked by a spacer corresponding to a ligand-binding domain to give FRET, which can be disrupted by addition of that ligand, e.g. calcium-signalling.[6,7] Alternatively the switching of the FRET interaction can be elicited by a cleavage trigger such as proteolysis (e.g. references[5,8–14]). A lifetime-based approach with variants of GFP (fluorescence lifetime imaging, FLIM) was used to deal with the disadvantage of low brightness and autofluorescence of Blue-Fluorescent Protein and Cyan-Fluorescent Protein.[15]

FRET efficiency between two variants of GFP linked via an enzyme-sensitive sequence is limited. For example, as the fluorophores of the component GFPs cannot get closer than the sum of the distances that they are buried in the GFP barrel structure. It is also difficult to control the angle between the two GFP-type chromophores. We recently described a system for intramolecular FRET between the fluorophore of a GFP variant and a chemical FRET partner conjugated to a specific cysteine residue introduced ≈20 Å away.[16] Other constructs also make use of intramolecular FRET between the GFP fluorophore and a chemical dye.[17–19] We have used chemical constructs of GFP conjugated specifically with fluorescent dyes with triggers responsive to trypsin,[14] caspase 3.[20] The caspase 3 system can be delivered into cells and has been used for in vivo single cell demonstration that the action of caspase 3 during apoptosis in HeLa cells has different time profiles in the nucleus and cytosol.[20]

Our objective was to design and prepare a chemically modified GFP that is able to register the reduction of a disulfide bond introduced into it by means of a change in the fluorescence spectrum of the GFP–fluorescence conjugate. We now describe a chemical modification of a GFP mutant that responds to reductive action, such as increased levels of GSH, or by the presence of chemical or other reducing agents. These GFP probes of reduction status were designed as follows. From the UV5 GFP mutant with the two native Cys residues (C48, C70) mutated to remove potentially reactive SH sites, and a his-tagged variant with a unique, surface cysteine site (at an I229C mutation) constructed. This was isolated as its mixed disulfide with cysteine in a modification of a published procedure.[14] To activate (i.e. release the –SH group of C229) of the purified cysteine-mixed disulfide GFPuv5tag I229C mutant, an aliquot (200 μL of 0.11 mM in 10-fold diluted phosphate-buffered saline (PBS) containing 0.5 mM DTT) was incubated for 30 min. and the DTT removed under nitrogen using a PD-10 column (Amersham Bioscience). This was chemically converted to the required disulfide, one terminus of which carried a fluorophore suitable as a FRET partner for the native fluorophore of GFP (Eq 1) by using a mixed-disulfide reagent based on an *N*-fluorophore-modified cysteamine.[21,22] For chemical derivatization, an aliquot of eluate (200 μL of 25 μM in PBS) was immediately mixed with dried NTB-cysteamine-Alexa 532 (final concentration, 27.5 μM) and the mixture incubated at 37 °C for 4 h. Excess NTB-cysteamine-Alexa 532 was removed (PD-10 column equilibrated with PBS) to provide GFP-cysteamine-Alexa (≈2–3 μM).



(1)

To synthesise NTB-cysteamine-Alexa 532, a mixture of NTB-cysteamine (400 μL of 0.672 mM in 100 mM MOPS buffer, pH 8.5) and Alexa Fluor 532 (200 μL of 1.38 mM in the same buffer; A-20001, Molecular Probes Inc., Oregon) was incubated at 37 °C for 3 h, and aliquots (200 μL) submitted to re-

verse-phase HPLC.[22] NTB-cysteamine-Alexa 532 eluted at 35–40% acetonitrile (concentration was estimated spectrophotometrically using e_{525} of 81,000 M^{-1} cm^{-1} for Alexa 532 (Handbook of Fluorescent Probes and Research Products, Molecular Probes)). Dried (Speed Vacuum) pooled fractions were stored at −20 °C.

Treatment of GFPuv5I229C protein with NTB-cysteamine-Alexa532 decreased the 511 nm fluorescence intensity and a new peak appeared at ≈554 nm, consistent with intramolecular FRET between the GFP fluorophore and the appended Alexa dye. Addition of reduced glutathione (GSH) caused time-dependent spectral changes for GFP-S-S-Alexa 532 (Figure 1). The native fluorophore emission at 508 nm recovered as the FRET was destroyed by cleavage of the disulfide linking the fluorophore to the GFP structure.

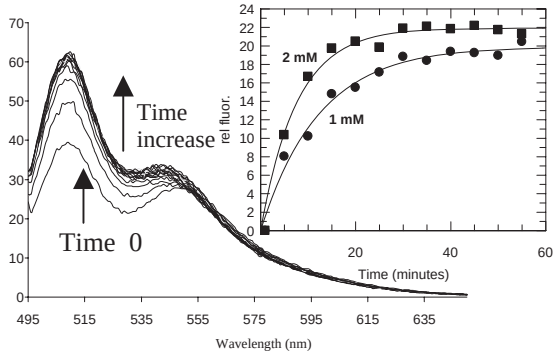
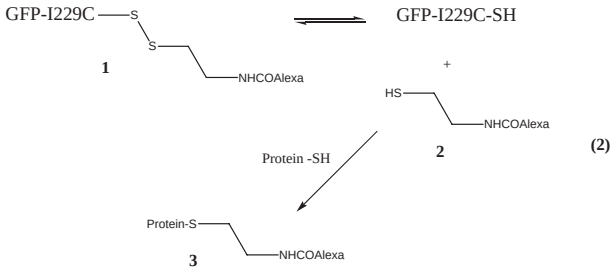


Figure 1. Time course for disulfide cleavage of GFP-S-S-Alexa 532 (made from GFPuv5I229Ctag[14] in pH 7.4 PBS at 10 °C containing 2 mM GSH (spectra taken every 3 minutes). Reaction was initiated by addition of a small aliquot of glutathione (1 M stock in PBS buffer) to the GFP and recording fluorescence emission spectra as a function of time. The inset shows first-order kinetic plots of the fluorescence emission intensities at 508 nm: lines are theoretical for rate constants of $0.082 \pm 0.007\ min^{-1}$ for 1 mM GSH and $0.131 \pm 0.013\ min^{-1}$ for 2 mM GSH.

Plots of the fluorescence intensity at 508 nm against time (Figure 1 inset) fitted the first-order equation with pseudo first-order rate constants (k_{obs}) of $0.082 \pm 0.007\ min^{-1}$ for 1 mM GSH and $0.131 \pm 0.013\ min^{-1}$ for 2 mM GSH, respectively. This gave a second-order rate constant for the cleavage by GSH of $1.18 \pm 0.02 \times 10^2\ M^{-1}\ min^{-1}$ in pH 7.4 PBS at 10 °C. Slower cleavage, effectively by a hydride ion species, was also observed for sodium cyanoborohydride (k_{obs} 0.029 ± 0.004 min^{-1} at 50 mM sodium cyanoborohydride, which corresponds to a second order rate constant of $0.58\ M^{-1}\ min^{-1}$) in pH 7.4 PBS at 10 °C.

On reductive cleavage the emitted color reverts from that of the conjugated Alexa dye (red, λ_{max} emitted 548 nm) to that of the native fluorophore of GFP (green, λ_{max} emitted 508 nm). Also two fragments of very different molecular dimensions are formed (a GFP-like protein plus an Alexa dye thiol of molecular weight ≈685 Daltons), so that each fragment will have substantially different correlation times also, and which are thus amenable in principle to correlation-based confocal microscopy.

Reductive cleavage of the GFP-I229C-Alexa species under conditions follows Eq 2. Thus, it produces an Alexa dye species with a free SH group that is also capable in principle of reacting with other cellular thiols of higher molecular weight, which also different correlation times. Thus, that the microscopy properties of the system are potentially expanded yet further to the correlation mode.



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References

1 G. N. Phillips, Jr., *Curr. Opin. Struct. Biol.*, **7**, 821 (1997).
2 R. Y. Tsien, *Annu. Rev. Biochem.*, **67**, 509 (1998).
3 M. Chalfie, *Photochem. Photobiol.*, **62**, 651 (1995).
4 R. Heim and R. Y. Tsien, *Curr. Biol.*, **6**, 178 (1996).
5 B. A. Pollok and R. Heim, *Trends Cell Biol.*, **9**, 57 (1999).
6 V. A. Romoser, P. M. Hinkle, and A. Persechini, *J. Biol. Chem.*, **272**, 13270 (1997).
7 A. Miyawaki, J. Llopis, R. Helm, J. M. McCaffery, J. A. Adams, M. Ikura, and R. Y. Tsien, *Nature*, **388**, 882 (1997).
8 Y. Ito, M. Suzuki, and Y. Husimi, *Biochem. Biophys. Res. Commun.*, **264**, 556 (1999).
9 R. D. Mitra, C. M. Silva, and D. C. Youvan, *Gene*, **173**, 13 (1996).
10 X. Xu, A. L. V. Gerard, B. C. B. Huang, D. C. Anderson, D. G. Payan, and Y. Luo, *Nucleic Acids Res.*, **26**, 2034 (1998).
11 L. Tyas, V. A. Brophy, A. Pope, A. J. Rivett, and J. M. Tavare, *EMBO Rep.*, **1**, 266 (2000).
12 N. P. Mahajan, D. C. Harrison-Shostak, J. Michaux, and B. Herman, *Chem. Biol.*, **6**, 401 (1999).
13 S. Karasawa, T. Araki, T. Nagai, H. Mizuno, and A. Miyawaki, *Biochem. J.*, **381**, 307 (2004).
14 M. Suzuki, Y. Ito, H. E. Savage, Y. Husimi, and K. T. Douglas, *Biochim. Biophys. Acta*, **1679**, 222 (2004).
15 A. G. Harpur, F. S. Wouters, and P. I. H. Bastiaens, *Nat. Biotechnol.*, **19**, 167 (2001).
16 M. Suzuki, Y. Ito, H. E. Savage, Y. Husimi, and K. T. Douglas, *Chem. Lett.*, **32**, 306 (2003).
17 B. A. Griffin, S. R. Adams, J. Jones, and R. Y. Tsien, *Methods Enzymol.*, **327**, 565 (2000).
18 B. A. Griffin, S. R. Adams, and R. Y. Tsien, *Science*, **281**, 269 (1998).
19 G. Gaietta, T. J. Deerinck, S. R. Adams, J. Bouwer, O. Tour, D. W. Laird, G. E. Sosinskyl, R. Y. Tsien, and M. H. Ellisman, *Science*, **296**, 503 (2002).
20 M. Suzuki, Y. Ito, I. Sakata, T. Sakai, Y. Husimi, and K. T. Douglas, *Biochem. Biophys. Res. Commun.*, **330**, 454 (2005).
21 R. Bertrand, J. P. Capony, J. Derancourt, and R. Kassab, *Biochemistry*, **38**, 11914 (1999).
22 R. Bertrand, J. Derancourt, and R. Kassab, *Biochemistry*, **39**, 14626 (2000).

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