Intramolecular Fluorescent Resonance Energy Transfer (FRET) by BODIPY Chemical Modification of Cysteine-engineered Mutants of Green Fluorescent Protein

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Green Fluorescent Proteins (GFP) bearing an intramolecular BODIPY fluorophore partner were designed by molecular modeling based on GFP's three-dimensional structure, and prepared by mutating out native Cys-48 found at the GFP surface (replaced by Ala or Ser) and introducing new specific, surface cysteine residues for chemical modification by a thiol-directed fluorescent BODIPY 507/545 label. This gave derivatives which exhibited strong intramolecular fluorescence resonance energy transfer with quenching of the emission peak of GFP at 508 nm and the appearance of a new FRET emission from the conjugated BODIPY 507/545 around 530 nm.

Mutants of GFP with altered excitation and emission properties provide major tools for biology,[1,2] for example as FRET (fluorescence resonance energy transfer) tags where GFP mutants with variously shifted emission maxima serve as FRET pairs. Alternatively, a GFP variant can be combined with a chemical FRET partner in bulk solution such as FlAsH based on fluorescein[3] or ReAsH using rhodamine.[4] In this study we have engineered GFP proteins to remove an indigenous Cys residue (C48) and introduced a single surface Cys residue, at a precise location determined by molecular modelling based on the GFP X-ray coordinates.[5] This facilitates its ready derivatisation to bear a BODIPY moiety as a potential intramolecular FRET partner. By this strategy the second chemical fluorophore can be suitably located to ensure efficient intramolecular FRET with the GFP natural fluorophore.

WT GFP's X-ray structure[6] shows two Cys residues (Cys-48 and Cys-70). The starting GFP was GFPuv4 with 6 point mutations relative to WT GFP. Modeling GFPuv4 showed the surface-located Cys-48 as a potential complication in chemical derivatization using Cys reactivity (Cys-70 is buried and unlikely to be reactive to bulky reagents). A suitable distance for an intramolecular FRET partner would be provided by a Cys residue, for example, at residue 6 (Glu in WT: distance 23 Å) or at residue 229 (Ile in WT; 20.9 Å).

GFPuv4 was obtained as described[7] by introducing 3 point mutations into GFPuv (Clontech). Cys-48 was replaced by Ala or Ser by inverse PCR[8] using pGFPgcn4 with forward primer (5′CGCGACTACTGGAAAACTACCTGT) and reverse primer (5′ATAAATTTAAGGGTAAGTTT) for Ala substitution, forward primer (5′CAGTACTACTGGAAAACTACCTGT) and reverse primer (5′ATAAATTTAAGGGTAAGTTT) for Ser substitution. A new Cys residue was then introduced into each Ala- or Ser-substituted mutant at the position indicated by molecular modelling. The mutants constructed were: GFPuv4 (GFPuv plus F64L, S65T, S208L); UV4C48A (GFPuv4 mutations plus C48A); UV4C48S (GFPuv4 mutations plus C48S); UV4C48AE6C (UV4C48A mutations plus E6C); UV4C48SE6C (UV4C48S mutations plus E6C); UV4C48AI229C (UV4C48A mutations plus I229C); UV4C48SI229C (UV4C48S mutations plus I229C).

All plasmids were expressed in DH5 α *E.coli* strain and purified following Yakhanin et al[9] with slight modification. The GFPuv4 mutants with the various Cys residues introduced (or removed in control cases), were then exposed to chemical modification by iodoacetamide-BODIPY 507/545 (D-6004, Molecular Probes. Inc., Oregon, Figure 1). An aliquot of purified GFPuv4 mutant (200 μL of 3 μmol in phosphate buffered saline at pH 7.20 containing 250 μmol DTT) was incubated for 30 min and the DTT removed by gel filtration. An aliquot of eluate (400 μL) was immediately mixed with D-6004 (10 μL of 2.5 mmol in dimethyl sulphoxide) and the mixture incubated at 37 °C for 3 h. Excess reagent was removed by gel filtration. Fluorescence spectra of emission at 488 nm.

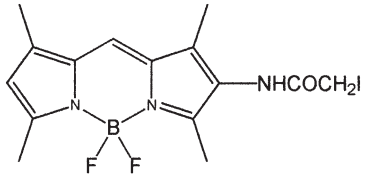

Figure 1. Structure of BODIPY 507/545-iodoacetamide.

The fluorescence spectra of GFPuv4 and its mutants were overall similar, with excitation maxima between 492 nm and 493 nm for 511 nm emission, and emission maxima between 508 nm and 510 nm when excited at 488 nm. Since all mutants had almost equivalent fluorescence intensities based on protein concentration, we can compare directly the changes in each emission at around 510 nm. Figure 2 shows that mutation of Cys-48 to Ala or Ser quenches the shoulder seen for GFPuv4 at around 525 nm and emission is characterised by a relatively sharp band at around 508 nm. This strongly suggests that these mutants did not react with the iodoacetamide-BODIPY 507/545 reagent (Molecular Probes, D-6004). However, introducing a Cys residue at either position 6 (to give E6C) or 229 (I229C) with the C48 mutated to Ala or Ser allowed reaction with this reagent and a new emission band around 530 nm appeared. This is consistent with intramolecular chemical FRET arising from excitation of the GFP native fluorophore undergoing FRET to the chemically-introduced BODIPY moiety on C6 or C229 in the mutants (D-6004 has

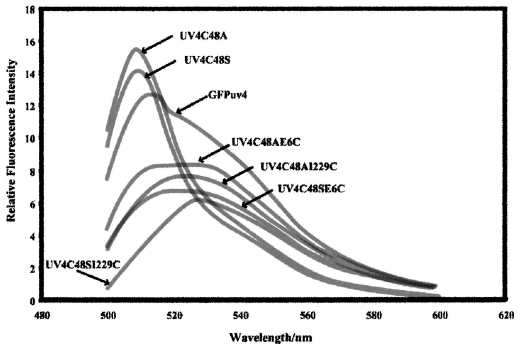

Figure 2. Fluorescence emission spectra in pH 7.20 phosphate buffered saline of GFPuv4 and its engineered mutants following exposure to chemical modification by BODIPY 507/545-iodoacetamide (D-6004) as described in the text: excitation was at 488 nm. The spectra are annotated with descriptors defining the composition of the GFPuv4 mutant.

its excitation maximum at 508 nm, corresponding to the observed emission maximum of mutated GFPuv4 and an emission maximum at 543 nm consistent with the quenched emission seen in Figure 2 around 508 nm and the new broad band around 530 nm). The strengths of FRET signals obtained here in each mutant were highly consistent with the location of the introduced Cys sites based on the X-ray structure of GFP with the potentially reactive Cys at position 48, far from native fluorophore compared to Cys at positions 6 or 229. However, at the stage of the present communication it is not possible to calculate R_0 distances between BODIPY and each Cys because of the need for full characterisation of the mutated and chemically derivatised proteins, but this aspect is now under.

In principle, this approach can effectively be extended to other fluorophores (we have preliminary data also for rhodamine and eosin modifications). The approach can also be applied to other colour variants of GFP. There are other sites in GFP that can also be regarded as suitable for mutational chemical derivatisation, but a primary requirement is that the ablation of native Cys residues and introduction of new ones do not compromise the fluorescence of the native GFP fluorophore , or affect protein folding and conformational maturation. In principle it is possible to design enzyme-switched GFP chemical intramolecluar FRET constructs by introducing a suitable sequence near the Cys site introduced. Action of, say, a protease at such a site can be arranged to cleave the GFP construct, destroying the intramolecular FRET as the donor and acceptor fluorophores will then reside on separate molecules. We have thus incorporated enzyme-sensitive sequences, and have preliminary data for trypsin and caspase. In this way we intend to provide switch sites for biological signalling studies in vivo when this type of GFP construct is used in combination with methods for protein introduction into cells.

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