Methodology article

Efficiently folding and circularly permuted variants of the Sapphire mutant of GFP

Otilia Zapata-Hommer and Oliver Griesbeck*

Address: Max-Planck-Institut für Neurobiologie Am Klopferspitz 18a 82152 Martinsried, Germany

Email: Otilia Zapata-Hommer - zapata@neuro.mpg.de; Oliver Griesbeck* - griesbeck@neuro.mpg.de

* Corresponding author

Abstract

Background: The green fluorescent protein (GFP) has been widely used in cell biology as a marker of gene expression, label of cellular structures, fusion tag or as a crucial constituent of genetically encoded biosensors. Mutagenesis of the wildtype gene has yielded a number of improved variants such as EGFP or colour variants suitable for fluorescence resonance energy transfer (FRET). However, folding of some of these mutants is still a problem when targeted to certain organelles or fused to other proteins.

Results: By directed rational mutagenesis, we have produced a new variant of the Sapphire mutant of GFP with improved folding properties that turns out to be especially beneficial when expressed within organelles or as a fusion tag. Its absorption spectrum is pH-stable and the pKa of its emission is 4.9, making it very resistant to pH perturbation inside cells.

Conclusion: "T-Sapphire" and its circular permutations can be used as labels of proteins or cellular structures and as FRET donors in combination with red-fluorescent acceptor proteins such as DsRed, making it possible to completely separate donor and acceptor excitation and emission in intensity-based FRET experiments.

Background

Mutants of the green fluorescent protein (GFP) have been exploited for various applications in biochemistry and cell biology, serving as reporters of gene expression, protein labels and since recently also as active indicators of physiological signals [1]. Especially fluorescence resonance energy transfer (FRET) between suitable GFPs has received widespread attention as it allows, in principle, to monitor protein conformations and protein-protein interactions inside living cells [2]. Currently the most preferred donor-acceptor combination is CFP-YFP (Cyan Fluorescent Protein-Yellow Fluorescent Protein). One drawback of this combination, however, is the long emission tail of the donor CFP that overlaps with the YFP emission. While this is

tolerable for genetic probes in which donor and acceptor are concatenated to each other within a single gene construct, this poses a serious problem when studying interactions between two different proteins labelled with the corresponding donor and acceptor GFP. Acceptor channel emission will be contaminated with donor emission even under conditions in which no FRET occurs, depending on transfection efficiencies and expression levels of the constructs that may vary significantly. Therefore alternative fluorescent labels with reduced overlap are desirable.

Sapphire, also termed H9-40 [1], is a mutant of GFP in which the T203I mutation abolishes the second excitation peak at 475 nm that can be found in wildtype GFP. As a

result the mutant protein exhibits a huge Stoke's shift, with an excitation peak at 399 nm and an emission peak at 511 nm [3,4]. Sapphire so far has not been much considered for intensity-based FRET (fluorescence resonance energy transfer) applications because its emission was too much overlapping with that of other GFP mutants used as acceptors. Also, so far no attempts have been made to improve its folding and expression properties inside cells, a prerequisite to allow precise targetings and successful fusions to proteins of interest. We therefore examined the effects of a series of folding mutations on Sapphire expression and characterized the resulting proteins spectroscopically. We also assessed the folding properties of one resulting variant when expressed in the endoplasmic reticulum of HEK293 cells and explored possibilities of using Sapphire as a donor protein in FRET-based genetically encoded indicators.

Results and Discussion

Sapphire variants with improved folding properties and circular permutations

No attempts had been made so far to improve folding of Sapphire. We therefore set out to test combinations of the common folding mutations to improve Sapphire folding. In brief, the combination that was found to work best was Q69M/C70V/V163A/S175G. This variant was called "T-Sapphire" (T for Turbo). V163A and S175G are common folding mutations [1,5]. The effects of Q69M on YFP had been reported before [6]. C70V was an unexpected mutation that resulted from a PCR error but turned out to be crucial for the Q69M effect on Sapphire as without it SapphireQ69M remained non-fluorescent. Other mutations tried included F99S and M153T [5]. They, however, resulted in no detectable folding improvement and were therefore omitted from the final construct. The mutation F46L that was recently describe to accelerate the oxidation step in YFP [7] was also tested and found to have no effect on Sapphire maturation (data not shown), indicating that its effects are rather specific to YFP. These variants had identical excitation and emission maxima and quantum yields and extinction coefficients in a comparable range (Table 1). As pH-changes within cells can cause severe artefacts in FRET measurements it was important to characterize the pH-sensitivity of T-Sapphire. Absorbance was completely stable between pH 4–7 (Fig. 1C), and fluorescence had a pKa of 4.9 (Fig. 1B), making this protein very resistant to pH changes within the cytosol of live cells that are in the range of pH 6.8–7.3. Also applications within slightly acidic compartments such as the Golgi or secretory vesicles can be envisaged with this protein. The folding efficiencies of Sapphire and T-Sapphire were compared in an assay that involves denaturing the completely folded and matured protein in 8 M urea. As the chromophore is exposed to the solvent, fluorescence is lost. Refolding can be studied by following the development of fluorescence as the unfolded non-fluorescent protein is added to a suitable refolding buffer [8] As can be seen in Figure 1A, fluorescence of T-Sapphire developed significantly faster than that of Sapphire. Also, total fluorescence recovery was much higher, with 65% for T-Sapphire compared to slightly less than 20% for Sapphire. Thus, the mutations Q69M\C70V\V163A\S175G were effective in facilitating folding of Sapphire. We also constructed circular permutations [9,10] of the T-Sapphire sequence. Circular permutations of fluorescent proteins are of interest because they provide alternative versions of a given protein with identical excitation and emission maximum, but with slightly different orientation of the chromophore. As orientation is an important factor in tuning FRET efficiency of two given fluorophores it is desirable to have a collection of permuted proteins available. Circularly permuted T-Sapphire variants became fluorescent at 37°C, in contrast to versions made from the original Sapphire, which were temperature-sensitive. The first construct made was cpT-Sapphire145-144, which however had a comparably low quantum yield and extinction coefficient (Table 1) and with a pKa of 5.6 also sacrificed some of the good pH-properties of T-Sapphire. A better permutation turned out to be cpT-Sapphire174-173, with a suitable quantum yield, extinction coefficient and pKa that came close to the values of the non-permuted variant (Table 1).

Targeted expression in HEK293 cells

Addition of targeting sequences to GFPs or fusions to proteins of interest can exert a considerable strain on the folding of a given fluorescent protein that often results in incomplete targetings or unsuccessful protein fusions. We examined the folding properties of T-Sapphire under these conditions by adding the N-terminal signal peptide of calreticulin and a C-terminal KDEL-motive to either T-Sapphire or Sapphire (Fig. 2A), thereby effectively targeting the proteins to the endoplasmic reticulum. These constructs were transfected into 293 cells. Reticular patterns of fluorescence typical of the endoplasmic reticulum were visible in 293 cells and the nucleus was found excluded of fluorescence (Fig. 2C, top), unlike the cytosolic protein that, with a molecular weight of about 23 kD, completely fills the cytosol and nucleus of transfected cells (Fig. 2C, bottom). In order to verify localization within the ER we co-transfected 293 cells with ER-T-Sapphire and the genetically encoded calcium probe Yellow Cameleon3-ER, whose localization had been verified by immunogold labelling combined with electron microscopy [11]. Constructs could be specifically excited at 400 nm or 488 nm, respectively. As can be seen in Figure 2C specific excitation of either ER-T-Sapphire or YC3ER co-expressed within one cell resulted in identical fluorescence patterns indicating correct localization of ER-T-Sapphire to the endoplasmic reticulum. When expressed in the cytosol without any tags
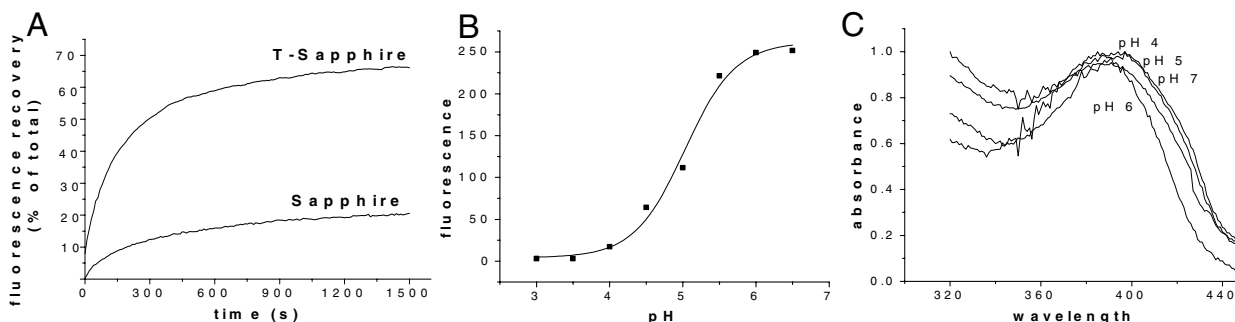
Figure 1
Characterization of T-Sapphire A: Refolding of Sapphire and T-Sapphire from their denatured states prepared by incubation in denaturation buffer (8M urea, 1 mM DTT) at 95°C for 2 min. Recovery of fluorescence (emission at 511 nm and excitation at 399 nm) was initiated by 100fold dilution into renaturation buffer (35 mM Kcl, 2 mM MgCl2, 1 mM DTT, 50 mM Tris pH 7.5). B: pH-titration of fluorescence of T-Sapphire. Excitation was at 399 nm and emission at 511 nm. D: Absorbance of equal amounts of T-Sapphire protein at pH 4, 5, 6 and 7. No significant differences were detected.

Table 1: Spectroskopic properties and pKa of Sapphire mutants.

	λ (ex)	λ (em)	QY	ε	pKa
Sapphire	399	511	0.64	25000	4.9
T-Sapphire	399	511	0.6	44000	4.9
cpT-Sapphire 145-144	399	511	0.14	32000	5.6
cpT-Sapphire 174-173	399	511	0.53	44000	5.2

L(ex) is the peak of the excitation spectrum in units of nm. L (em) is the peak of the emission in units of nm. QY is the fluorescence quantum yield, which is dimensionless. E is the extinction coefficient in units of $M^{-1}cm^{-1}$.

no significant differences in fluorescence intensities between the two Sapphire variants were detectable (data not shown). In Fig. 2B fluorescence intensities from 293 cells transfected either with ER-Sapphire or ER-T-Sapphire are compared, documenting the superiority of T-Sapphire over Sapphire under such conditions inside cells.

Long emission wavelength genetic indicators of protease activity
In order to test whether T-Sapphire can be used as a donor molecule in FRET-based types of indicators we constructed fusion molecules consisting of T-Sapphire and DsRed, linked by a 25 residue cleavable sequence containing a

mammalian enterokinase recognition site specifically proteolyzed by enteropeptidase. Previously, Sapphire had been used once as a donor in genetically encoded calcium indicators [12]. Unfortunately, no spectra of these indicators had been displayed in this study leaving some of the properties of these probes unclear. Purified fusion proteins were examined spectroscopically. Figure 3A for clarity shows the excitation and emission spectra of T-Sapphire and DsRed as single molecules. In Figure 3B the emission spectrum of the T-Sapphire\DsRed fusion molecule is depicted before (solid line) and at various time points after the start of digestion with enteropeptidase (dotted lines). The T-Sapphire/DsRed fusion protein
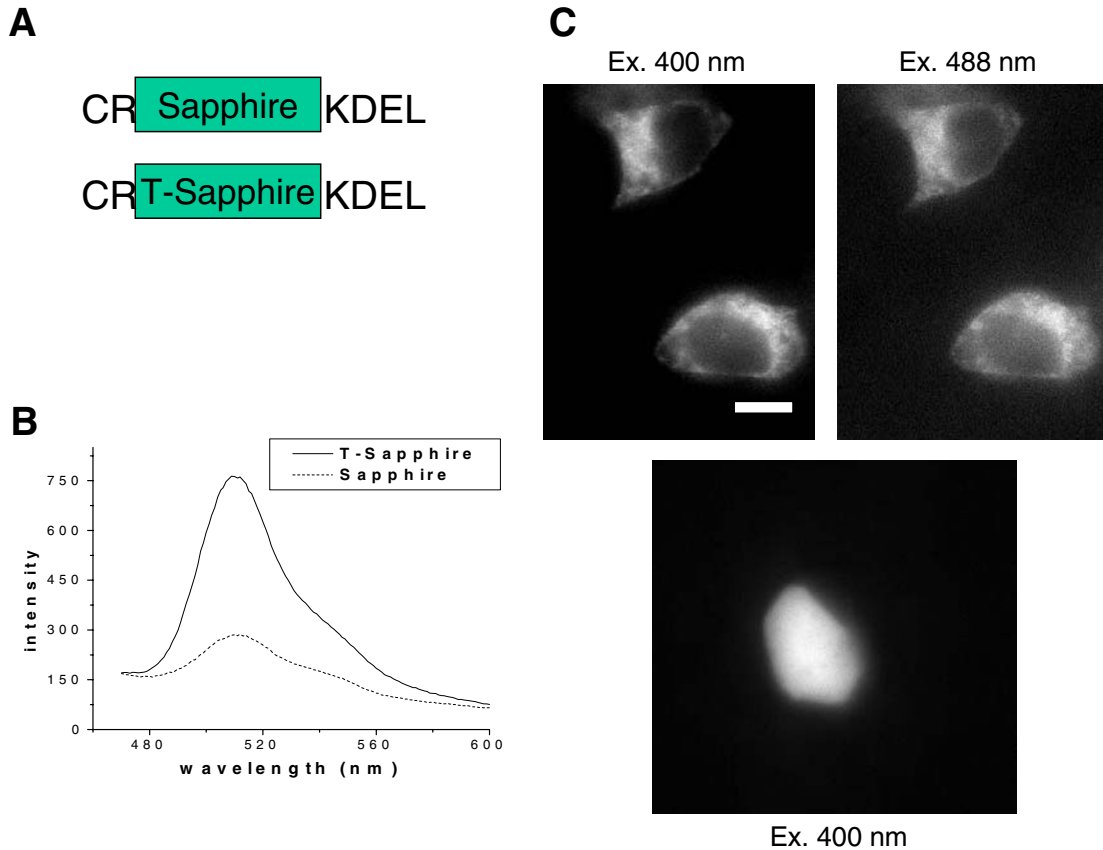
Figure 2
Targeted expression in HEK293 cells A: Schematic representation of the constructs used for targeting. CR is the signal peptide of calreticulin, KDEL is the ER retention motive. B: Fluorescence emission spectrum of dissociated HEK293 cells transfected for 2 days either with ER-Sapphire or ER-T-Sapphire. Excitation was at 399 nm. C: Individual 293 cell co-expressing ER-T-Sapphire and Yellow Cameleon 3ER. Excitation was either at 400 nm to specifically excite T-Sapphire or 488 to excite the YFP component of YC3ER. Note the identical fluorescence pattern. For comparison, T-Sapphire expressed in the cytosol fills the whole cell including the nucleus (bottom). The emission filter was 535/25. Scale bar 10 μM.

showed detectable FRET as evaluated from the emisson spectrum of the intact protein because excitation of T-Sapphire at 399 nm resulted in substantial red emission (Fig. 3B). Digesting the cleavable linker between the two fluorescent proteins separated the two fluorophores from each other and disrupted FRET. After 1hr 30 min of digestion, no further changes in spectra could be observed indicating that the cleavage had proceeded to completion, as verified by PAGE (data not shown). Interestingly, the DsRed emission was completely abolished after separation of the two fluorophores demonstrating that it is possible to specifically excite the donor and separate the emissions of the donor and acceptor molecules completely using this combination of fluorescent proteins. In an independent exper-

iment, digestion with enterokinase was not found to affect fluorescence of DsRed or T-Sapphire directly. For comparison, identical fusion proteins were constructed using CFP or EGFP as donors (data not shown). With these donors spectra could not be separated completely. Small emission maxima at 583 nm still seen after complete cleavage of CFP-DsRed and EGFP-DsRed are due to cross-excitation of DsRed at the given wavelengths of 432 nm and 488 nm, respectively, which was unavoidable due to the complex excitation spectrum of DsRed.

In general, red-shifted indicators are useful because excitation occurs at longer wavelengths, emission is further removed from auto-fluorescence and detection instruments
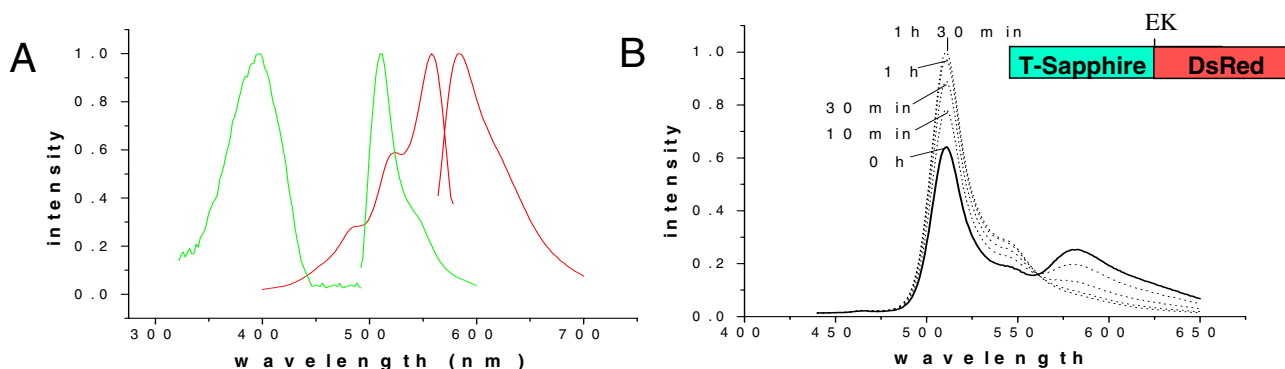
Figure 3
Long emission wavelength genetic indicator of protease activity Fusion of T-Sapphire to DsRed. The GFPs are linked by a protease cleavage site specific for enterokinase (EK). A shows the excitation and emission spectra of the donor T-Sapphire (green) and DsRed (red) individually. B shows the emission spectrum of the fusion protein before (thick solid line) and at 10 min, 30 min, 1h and 1h 30 min (dotted lines) after digestion with Enterokinase. Digestion by enterokinase completely separates the fluorophores from each other after 1hr 30mins. Excitation of the fusion protein was at 399 nm. Note the complete separation of the donor and acceptor spectra after digestion with enterokinase.

often are more effective at red wavelengths. They are complementary to indicators based on the CFP-YFP pair and can therefore be used for double-labelings or imaging of several parameters within the same cell. It has to be mentioned that first generation DsRed has several disadvantages for use in FRET experiments. It is a tetramer, forms aggregates within cells and matures slowly and incompletely [13]. However, most of these problems have been overcome recently and even monomeric versions have been generated [14,15] so that that the combination Sapphire/DsRed should become the pair of choice for FRET experiments in the future. Therefore the development of an efficiently folding variant of Sapphire is com-

plementary to the current evolution of red fluorescent proteins.

Conclusions
By directed rational mutagenesis, we have produced a new variant of the Sapphire mutant of GFP and permutations thereof with improved folding properties that turns out to be especially beneficial when expressed within organelles or as a fusion tag. Its absorption spectrum is pH-stable and the pKa of its emission is 4.9, making it very resistant to pH perturbation inside cells. We believe T-Sapphire will be useful in a number of applications ranging from labelling of cellular structures to FRET where the combination of T-Sapphire as donor and a red fluorescent

protein such as DsRed as acceptor allows the complete separation of donor and acceptor emissions in intensity-based FRET experiments.

Methods

Molecular biology

Gene clonings were done into the bacterial expression plasmid pRSETB (Invitrogen). Circular permutations with new N-termini were made by two separate PCRs and corresponding 5' and 3' fragments were linked with the peptide sequence GGTGGS that included a KpnI site. Site-directed mutagenesis was performed using the QuickChange kit (Stratagene). Fusion proteins between different GFP mutants and DsRed were done by first cloning DsRed into the BamHI-site of pRSETB, then inserting the corresponding GFP mutant in frame into the Nhe-site after the polyhistidine tag, thereby creating a short linker with an enterokinase cleavage site between the two fluorescent proteins.

Protein expression and spectroscopy

All constructs of interests were cloned into pRSETB (Invitrogen) and transformed into the bacterial strain E. coli BL-21. Purification of the His-tagged proteins followed via Nickel-chelate column chromatography according to previous procedures [10]. Spectroscopy of purified protein was usually performed in 100 mM KCl, 10 mM K-MOPS, pH 7.25, in a fluorescence spectrometer (Cary Eclipse, Varian). pH-titrations were performed as described [10]. Extinction coefficients were determined according to the "base denatured chromophore" method [16]. Proteolysis of fusion constructs was done at room temperature with recombinant enterokinase (Invitrogen).

Cell culture and microscopy

In order to evaluate targeted expression of Sapphire mutants, identical amounts of DNA (30 μg) of ER-Sapphire or ER-T-Sapphire in pcDNA3 were transfected into HEK293 cells (500,000 per 50 mm dish). After 2 days of expression cells were suspended in Hanks-buffered saline solution (HBSS), normalized at OD 600 and measured in the fluorescence spectrometer. Pictures of 293 cells were taken with a charge-coupled device camera (CoolSnap, Roper Scientific). Ilumination was done with a DeltaRam monochromator (Photon Technology International) at 400 nm or 488 nm, emission filter was 535/25.

Authors' contributions

OZH did most of the mutagenesis, protein work and spectroscopic characterization. OG did the cell culture work and protease experiments. Both authors read and approved the final version.

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