Research Notes

Title:

Photoactivatable blue fluorescent protein (PA-BFP)

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Abstract

The mutation Y66H on the chromophore of the parental violet fluorescent protein SumireF generated a photoactivatable blue fluorescent protein (PA-BFP) that was irreversibly activated in seconds over ordinary UV transilluminators at 302 nm or 365 nm. The maximum excitation and emission wavelengths of this protein, centered at 358 nm and 445 nm, respectively, resemble the values of DAPI—the blue stain widely used in fluorescence microscopy to visualize nucleic acids in cells. Therefore, the immediate use of PA-BFP in cellular biology is clear because the technology required to analyze this new genetically encoded reporter at the microscopic level has already been established. To our knowledge, PA-BFP is the first photoactivatable protein in the blue range of the visible spectrum.

Running title: PA-BFP: Photoactivatable blue fluorescent protein

Keywords: Photoactivatable proteins, blue fluorescent proteins, genetic reporters, super-resolution microscopy, protein engineering

Report

Fluorescent proteins are important molecular tools that help illuminate many biological processes and answer a plethora of questions at the microscopic level [1]. These genetic reporters may be used to study gene expression, protein localization, protein traffic, protein fate, protein–protein interactions, sensing of metabolites, sensing of physiological conditions, and many other cellular events [2-6]. Their application is limited only by human imagination. The ocean contains fantastic fluorescent and chemiluminescent organisms whose fluorescent proteins or luciferases await discovery. Meanwhile, we can perform artificial evolution of already known genes to seek protein variants with new fluorescent properties [7-10].

In this context, we report on an interesting blue fluorescent protein derived from the recently published violet protein Sumire [11]. Sumire exhibits maximum excitation and emission wavelengths at 340 nm and 414 nm, respectively, and was derived from super folder GFP (sfGFP) [12] via nine site-directed mutations, as described in the supplementary material. However, when the gene was assembled, a fortuitous variant became violet at a faster rate than the target Sumire protein (Supplementary Fig. 1). This improved variant contained the original phenylalanine amino acid in position 165 instead of the expected tyrosine residue. It was named SumireF.

Directed evolution of the SumireF gene by error-prone PCR mutagenesis [13] and screening of the library over a UV transilluminator at 302 nm revealed the presence of a colony displaying a brilliant sky-blue phenotype (Fig. 1A). DNA sequencing of the plasmid recovered from this colony confirmed that the encoded protein contained the mutation Y66H in the center of the chromophore, as inferred by sequence comparison with the parental proteins Sumire, SumireF, and sfGFP (Supplementary Fig. 2). Many engineered blue fluorescent proteins contain histidine in this position [14-17]. However, when *E. coli* cells were transfected with this plasmid, the resultant colonies required some seconds over the 302 nm or 365 nm UV transilluminator to fluoresce blue, as shown in the companion videos (https://clipchamp.com/watch/MzZX0qKhsP3 and https://clipchamp.com/watch/T1LI7O1M4Gb). Photoactivation was also confirmed by fluorescence spectroscopy. When *E. coli* cells producing the "dark" SumireF-Y66H heterologous protein were scraped from a Petri dish, resuspended in PBS buffer, and subjected to reiterative acquisition of emission spectra every two minutes, with excitation at 360 nm, the fluorescence grew in each cycle due to the cumulative fraction of activated protein, as shown in Fig. 2. Kinetic maturation, followed by fluorescence spectroscopy, demonstrated that activation of the protein at 302 nm was faster than activation at 365 nm, as shown in Supplementary Fig. 3: Under 302 nm excitation, 50% of the protein was activated in 17.0 min, whereas 360 nm excitation produced the same ratio in 23.1 min (302 nm light is more energetic than 360 nm).

From this point, the protein was named photoactivatable blue fluorescent protein (PA-BFP). There are many photoactivatable fluorescent proteins of different colors, including cyan [18], green [19], yellow [20], orange [21], and red [22, 23], but PA-BFP

seems to be the first photoactivatable protein in the blue range. Manual purification of the heterologous protein by immobilized nickel affinity chromatography yielded a “dark” protein whose maximum absorption was centered at 329 nm (Fig. 1B). Excitation at this wavelength produced an emission peak at 397 nm in the violet region of the visible spectrum (Fig. 1C). These blue-shifted values compared to the parental protein suggest an intermediate structure, such as Sumire, in which the phenol moiety of the chromophore is replaced by an imidazole ring, and the cyclized imidazolone ring remains open in the hydrated form, as proposed in the mechanism shown in Fig. 3. The exposition of the intermediate protein after 3 min at 365 nm over a UV transilluminator produced a new protein version whose maximum absorbance was centered at 360 nm (Fig. 1B). Excitation at this wavelength produced an emission peak at 445 nm, as shown in Fig. 1C. Contrary to PA-BFP, the exposition of SumireF to UV did not produce any phenotypical changes to the protein. The Sumire protein was rationally designed with the photoswitchable protein dreiklang—a protein that is activated with UV at 365 nm to produce a green fluorescent protein [24]. Therefore, we hypothesize that PA-BFP is being irreversibly activated via a photolytic dehydration process, as proposed in Fig. 3, which is the last step in the maturation process, because fluorescence emerges immediately after UV exposition. The final chromophore of PA-BFP is the same as that present in many classical BFPs derived from the original Aequorea Victoria GFP. The difference is the photoactivation step in the maturation process. Electrospray ionization mass spectrometry of the activated protein indicated a molecular weight of 27,523 Da, which accounts for the loss of a water molecule and two hydrogens with respect to the expected value of the complete polypeptide. This confirms the final structure of the

chromophore shown in Fig. 3. SDS-PAGE analysis of PA-BFP revealed the presence of only one band around 28 kDa after boiling the sample under denaturing conditions (Supplementary Fig. 4). This ruled out breakage of the polypeptide backbone, as occurs in the activation of kaede [25], EosFP [26], KiKGR [27], Dendra [28] and further supported photolytic dehydration as the final step in the activation of the protein. Size exclusion chromatography of PA-BFP demonstrated that it is a monomer like its parental precursors sfGFP and SumireF (Supplementary Fig. 5); therefore, it can be used to label other proteins of biological interest.

A comparison of the fluorescence properties determined under the same experimental conditions demonstrated that PA-BFP is more brilliant than SumireF, as reported in Table 1. PA-BFP exhibits a higher quantum yield than SumireF and a lower molar extinction coefficient. PA-BFP was found in the first round of random mutagenesis. Perhaps more mutation rounds will improve its maturation efficiency and, consequently, its molar extinction coefficient after the UV activation process.

The fluorescent properties of PA-BFP resemble those exhibited by DAPI (Ex_{max}: 358nm, Em_{max}: 461 nm)—the universal dye employed to stain nucleic acids in cells. Most fluorescence microscopes are equipped with filters for DAPI; therefore, the technology has already been established for use with this new protein reporter.

In summary, we have added a new tool to the palette of photoactivated fluorescent proteins that can be used in multiple-labeling experiments, analyzed by photoactivated localization microscopy [29], and other super-resolution microscopy techniques [30].

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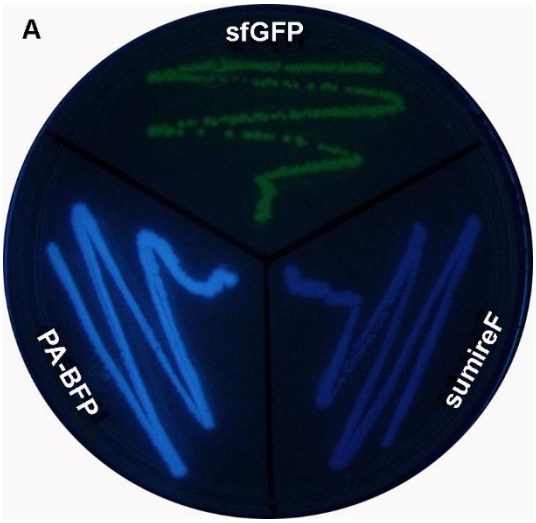
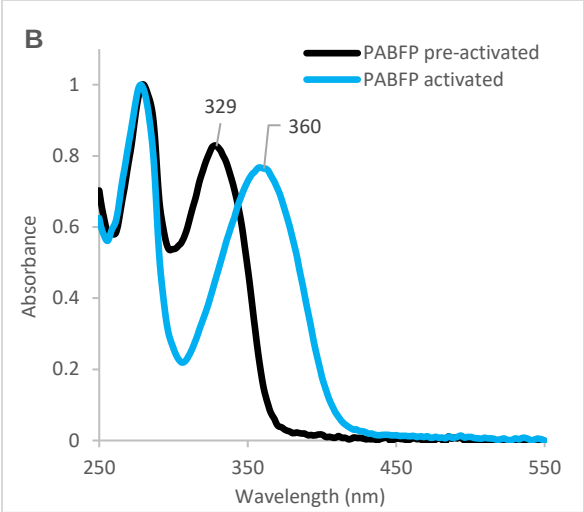
Table 1. Optical properties of PA-BFP and its parental protein, SumireF. These properties were determined under the same experimental conditions. λ_{Ab}: maximum absorbance wavelength, λ_{Ex}: maximum excitation wavelength, λ_{Em}: maximum emission wavelength, ε_{max}: molar extinction coefficient in units $uM^{-1}cm^{-1}$, φ_{max}: quantum yield. Brightness: $(\varepsilon_{max} * \varphi_{max})/1000$. φ_{max} was determined using EGFP as a reference, with a value of 0.60.

Sample	λ_{Ab}	λ_{Ex}	λ_{Em}	ε_{max}	φ_{max}	Brightness
SumireF	341	342	413	23065	0.191	4.4
PA-BFP	358	362	445	13983	0.516	7.2


A
sfGFP
PA-BFP
sumireF


B
PABFP pre-activated
PABFP activated
329
360
Absorbance
Wavelength (nm)
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0.2
0.4
0.6
0.8
1
250
350
450
550

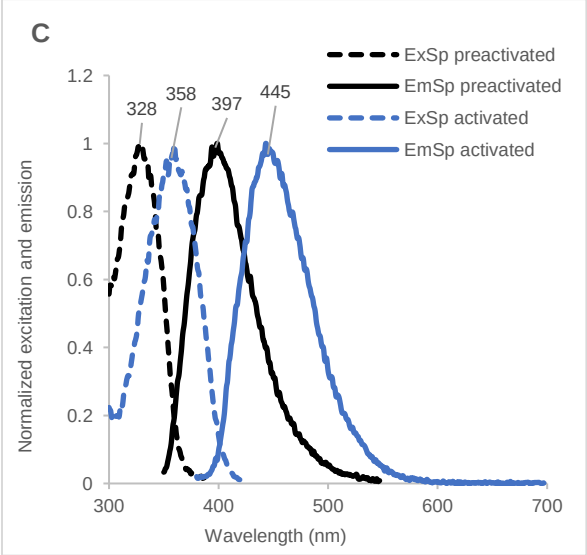
C
ExSp preactivated
EmSp preactivated
ExSp activated
EmSp activated
328
358
397
445
Normalized excitation and emission
Wavelength (nm)
0
0.2
0.4
0.6
0.8
1
1.2
300
400
500
600
700

Figure 1. Analysis of the photoactivatable blue fluorescent protein (PA-BFP). A) Comparison of *E. coli* streaks expressing the protein SumireF-Y66H (PA-BFP) and its precursor proteins SumireF and sfGFP, visualized at 302 nm over a UV transilluminator. B) UV-Vis absorbance of the pure protein PA-BFP before and after UV activation. C) Excitation and emission spectra of the pure protein PA-BFP before and after UV activation. ExSp: excitation spectrum; EmSp: emission spectrum

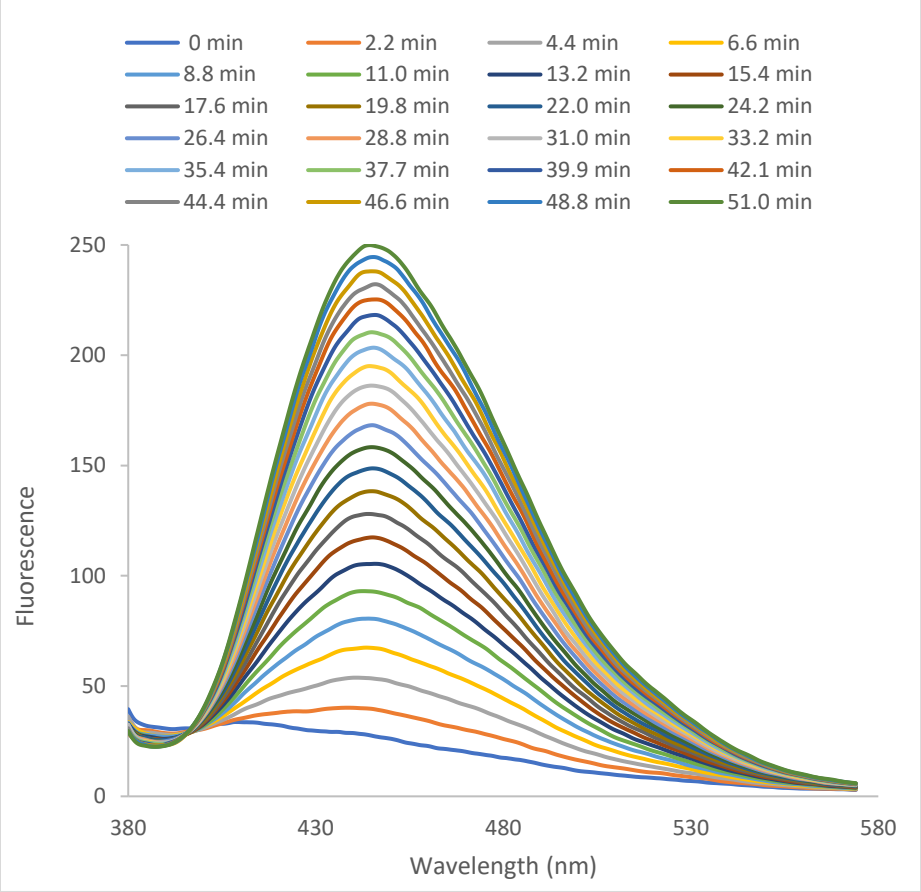

Figure 2. Reiterative acquisition of fluorescence spectra for every two minutes of *E. coli* cells resuspended in PBS buffer expressing the initially dark SumireF-Y66H protein (PA-BFP). Excitation: 360 nm; scanning rate: 100 nm/min; excitation slit: 15 nm (maximum allowed); emission slit: 5 nm

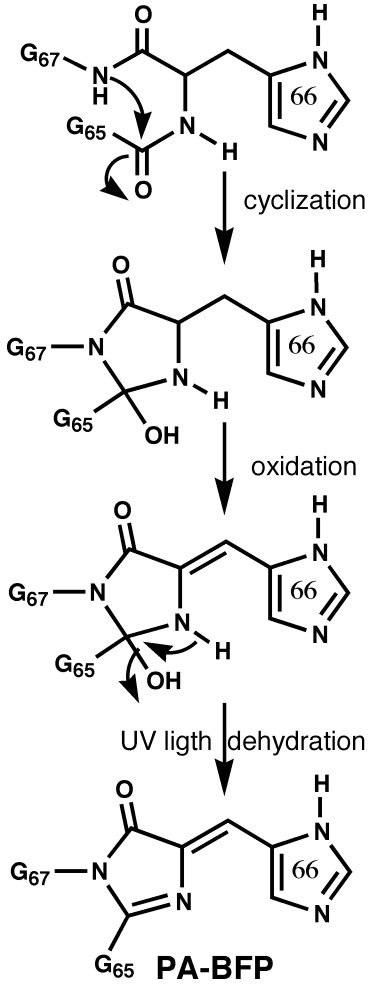

Figure 3. Proposed post-translational mechanism of maturation of the chromophore found in the photoactivatable blue fluorescent protein (PA-BFP). The three chromophore-forming amino acids are Gly65, His66, and Gly67.