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RESEARCH PAPER

Single Amino Acid Replacement Transforms mCherry to a Far-red Fluorescent Protein

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Abstract Far-red fluorescent proteins are beneficial for imaging in mammals. Here, starting from mCherry, the most commonly used among the different types of red fluorescent proteins (RFP), not having a H-bond network in its original form, we sought to recover the hydrogen bond network in mCherry. By comparing the structure of wtGFP and mCherry, we focused on a few key residues involved in a proton wire, and discovered an I197T mutant that showed a more red-shifted fluorescence. The detailed optical and photo-switching properties of related engineered RFPs are described. This study will guide further development of monomeric far-red FPs.

Keywords: far-red fluorescent proteins, mCherry, mutagenesis

1. Introduction

Far-red fluorescent proteins (FPs) are desirable for deep-tissue imaging in animals since mammalian tissues absorb most wavelengths of visible light below 600 nm due to the strong absorption of hemoglobin [1]. In addition, reliable far-red FPs are also required for multi-color imaging combined with orange or red FP labeling. Efforts to produce stable far-red FPs have not yet met with success. An ideal FP for imaging in mammals would excite maximally above 600 nm to be compatible with the standard 633 ~ 640 nm lasers and be monomeric to prevent mistargeting or aggregation of fused constructs.

Several far-red FPs, such as mPlum [2], mNeptune [3] and E2-Crimson [4] have been developed. The DsRed derivative, mPlum, with excitation and emission peaks at 593 and 644 nm (Table 1), is the most popular monomeric far-red FP. However, its spectral property is complicated due to the presence of an immature green intermediate [5]. E2-Crimson would have the most red-shifted excitation/emission maxima at 611/646 nm but is in a tetrameric state, which makes it generally incompatible as a fusion tag for studying the interaction, localization, and diffusion of proteins of interest.

mCherry is one of the most widely used and best understood monomeric RFPs [6]. We set out to identify critical amino acid residues that would make mCherry redder while keeping its monomeric character. Tuning of excitation/emission wavelengths in FPs can be manipulated by altering the covalent structure of the chromophore and is determined by hydrogen bonding and electronic interactions of the chromophore with adjacent functional groups. We focused on the disconnected H-bond network around the chromophore of mCherry (Fig. 1) and tried to reconnect the proton wire by site-directed mutagenesis of residues Ile197 and Leu199 to protic amino acids such as serine or threonine. Here we describe one of the resulting proteins, mCherry-I197T, which shows a red-shifted emission peak at 615 nm, with only one residue change. Our analysis of mCherry-I197T suggested a simple mechanism contributing to the long wavelengths of mCherry-I197T, including the protonation state of the chromophore.

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Table 1. Properties of mCherry-I197T in comparison with mCherry and mPlum

Protein	mCherry	I197T	mPlum
Excitation max (nm)	587	593	593
Emission max (nm)	611	617	644
Quantum yield	0.22	0.03	0.1
$t_{1/2}$ for bleach (sec)	880	960	730
Extinction coefficient (/M/cm)	72,000	66,000	41,000

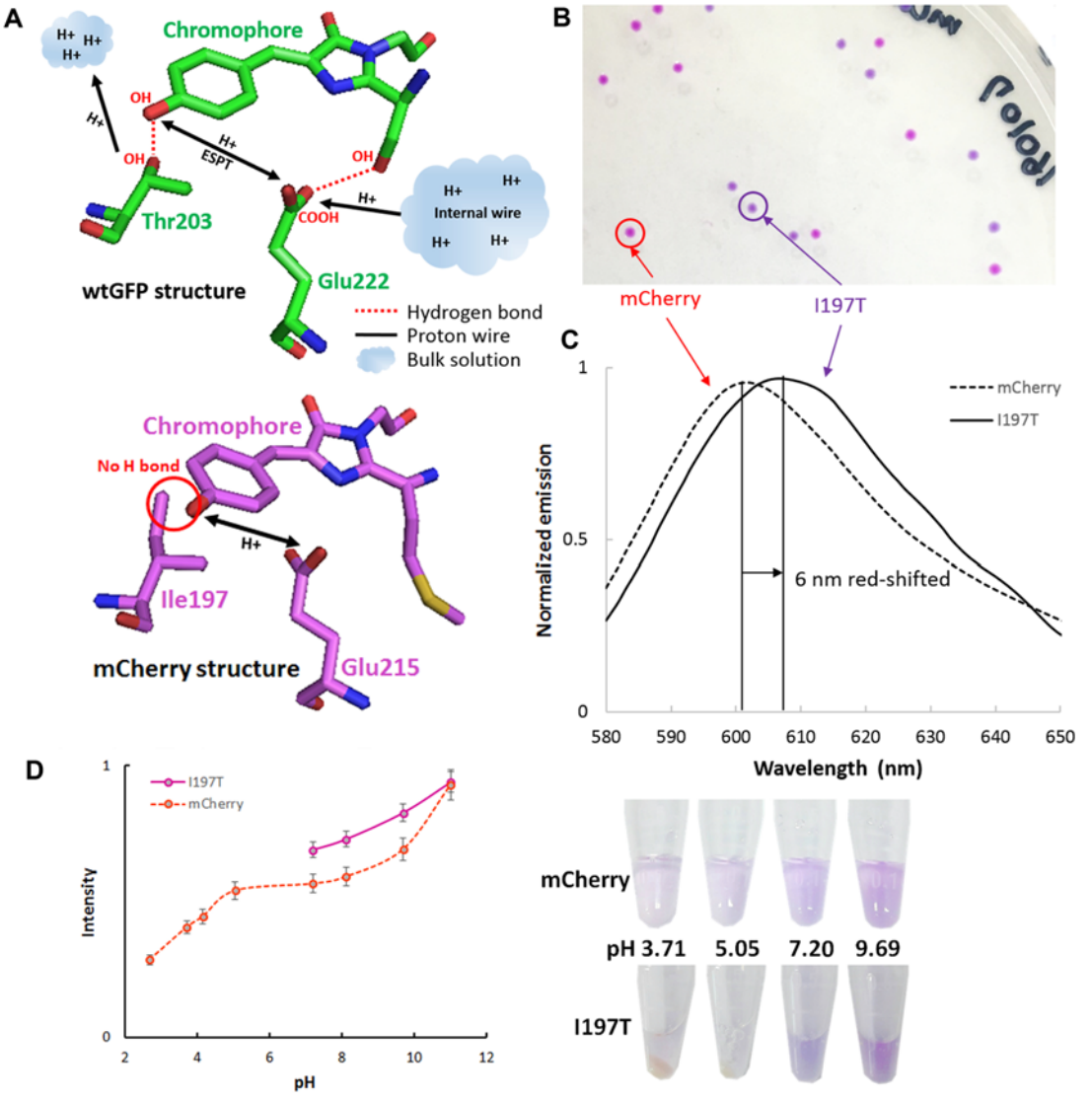

Fig. 1. (A) Comparison of the structure around the chromophores of avGFP and mCherry. The red circle indicates the disconnected point of the hydrogen bond network. (B) The color difference between mCherry and I197T mutant colonies. (C) Normalized emission spectra of mCherry (dotted) and I197T mutant (solid). The emission maxima of I197T is red-shifted by about 6 nm. (D) pH dependence (left) and aggregation pattern by pH (right) of mCherry and I197T mutant. Because the I197T aggregated in acidic condition, fluorescence measurement was impossible below pH 6.

2. Materials and Methods

2.1. Mutagenesis and cloning

First, the mCherry gene, codon optimized for *E. coli*, was cloned (Novagen) between the NcoI and BamHI sites in the pET15b vector so that the protein could be expressed in its original form without a His tag and trypsin cleavage site. The lac operator was deleted as in Fig. 2 to facilitate

Fig. 2. Alignment of the promoter sequence of the original pET15b vector and our modified mCherry vector. The important domains are underlined. The matched bases are highlighted in black.

screening of colonies by the color of expressed proteins. Without the lac operator, the FPs are produced in colonies without IPTG induction. Thus, the mutated violet colonies *i.e.,* I197T, (and not mutated red mCherry colonies) can be easily distinguishable as in Fig. 1B. After mCherry cloning, site-directed mutagenesis was performed with PCR amplification, using Pfu DNA Polymerase (Bioneer, Daejeon, Korea) and the PCR products were digested with Dpn I (Takara, Shiga, Japan). *Escherichia coli* strain BL21(DE3) was transformed with the plasmids for mCherry or its mutant by heat shock. Transformed bacteria were grown overnight (16 h) at 37ºC on Luria-Bertani (LB) -agar plates with ampicillin and the transformed bacterial colonies had a red color.

2.2. Protein production and characterization

E. coli containing original mCherry gene [6] or its mutant I197T were grown overnight at 37ºC in 10 mL of LB media with ampicillin and then scaled up in 400 mL LB medium with ampicillin. When the optical density reached 0.4 ~ 0.6, the shaking incubator was cooled to 24ºC for 20 h. Proteins were purified using fast-protein liquid chromatography (FPLC, GE Healthcare, Buckinghamshire, UK) with a size exclusion column (Superdex™ 200 Increase 10/300 GL, GE Healthcare, Buckinghamshire, UK) in 25 mM Tris and 150 mM NaCl pH 7.4 buffer, followed by anion exchange chromatography with a Hitrap Q HP column (GE Healthcare). Purified proteins were exchanged into 25 mM Tris pH 7.4 buffer by ultrafiltration with Vivaspin (GE Healthcare, Buckinghamshire, UK). Absorbance measurements were performed using a UV-vis spectrophotometer (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer, Thermo Fisher Scientific, Massachusetts, USA). Fluorescence measurements were performed using a fluorescence spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer, California, USA) for excitation and emission spectra. Quantum yield was determined by comparing fluorescence of the FPs with equally absorbing mCherry (quantum yield 0.22) [7].

2.3. Photobleaching measurement

An aqueous solution of purified RFPs in 25 mM Tris-HCl (pH 7.4) was mixed with mineral oil (Samchun, Korea) in a chamber gasket (Cover well perfusion chamber, VWR, USA) on a sliding glass. Vigorous pipetting formed micron-scale droplets that can be observed under the fluorescence microscope (Ti-u, Nikon, Japan). The high-pressure mercury lamp equipped with the microscope was used for bleaching and imaging. The illumination light intensity for photo-bleaching through a ND2 filter, 540/25 bandpass filter, and the 10x objective was measured by a LaserCheck light meter (Coherent, USA). The intensity versus time was obtained from a monochrome camera (Edge gold 5.5, PCO, Germany) and the time to bleach 50% of initial emission intensity was measured.

3. Results and Discussion

3.1. Recovery of H-bond network in mCherry and redshift of the emission spectrum

The hydrogen-bonding network around the chromophore of the green fluorescence protein from *Aequorea victoria* (avGFP) permits the transfer of protons, the direction of which determines the ionization property of the chromophore [8]. The H-bond network of mCherry, derived from DsRed, is different from that of avGFP. The H-bonds around the chromophore of avGFP and mCherry are shown in Fig. 1A. Both avGFP and mCherry share a phenolic chromophore and an H-bond with glutamic acid. But the most prominent difference is that the H-bond of Thr203 connected to the chromophore of GFP is absent in mCherry because the counterpart of Thr203 in mCherry is Ile197, which is unable to form any H-bond. We assumed that replacement of Ile197 with a protic amino acid such as serine, threonine or histidine could recover the proton wire.

As a result, the introduction of Thr showed interesting properties. The colony of the I197T mutant showed a dark violet color and its maximum emission was 6 nm red-shifted relative to mCherry (Figs. 1B and 1C). This color difference is more distinct in the side-by-side comparison picture with mCherry, the I197T mutant and mPlum (Fig. 3B). As the proton wire was connected, the I197T mutant became more susceptible to the pH of the solution. The

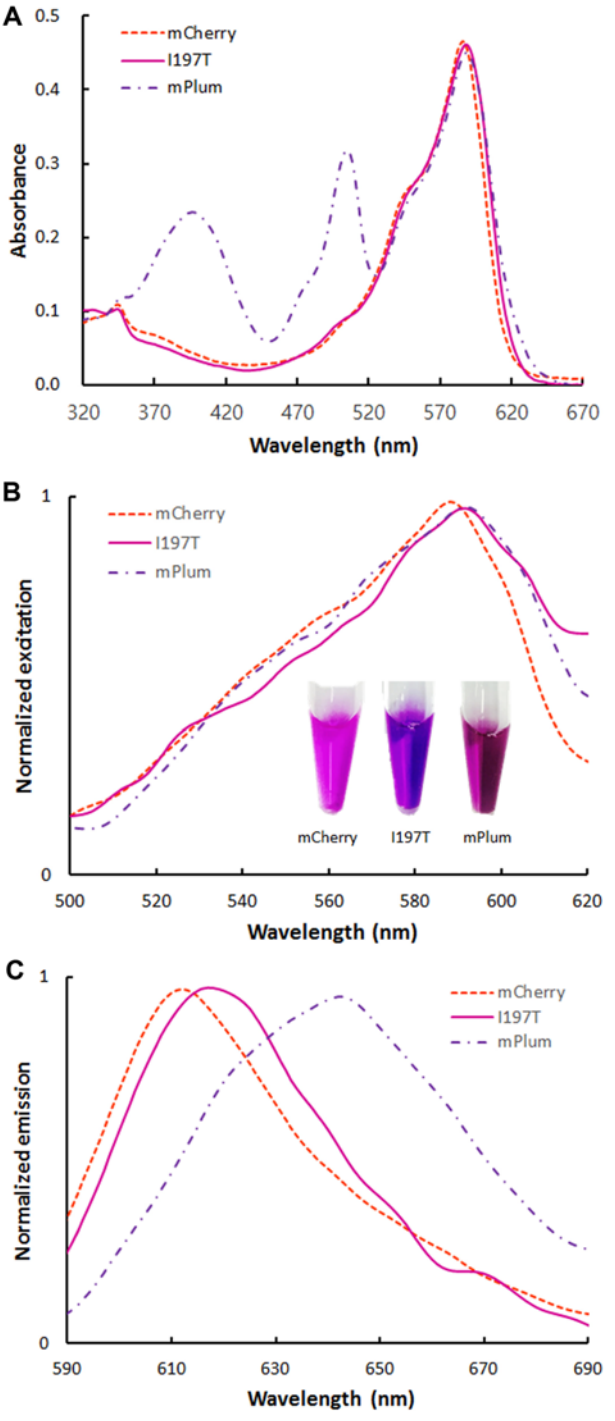

Fig. 3. Normalized absorbance (A), excitation (B), and emission (C) spectra of mCherry (dotted orange), I197T (pink), and mPlum (dot-dashed violet). Inset: purified mCherry, I197T, and mPlum proteins at 0.5 mg/mL in visible light.

I197T mutant was aggregated below pH 5, while mCherry was not affected (Fig. 1D). The fluorescence emission of mCherry change little with pH in the range between 5 and 10 in contrast to most avGFP variants [9], probably due to the disconnected proton wire. However, that of I197T was less steeply changed in basic range (Fig. 1D), though measurement below pH 6 was not possible due to the aggregation. This pH sensitivity, different from the original mCherry, clearly proves the recovery of an H-bond network in the bulk solution. This result identifies the origin of the pH resistance of mCherry and gives an important clue to the mechanism of protein aggregation in acidic conditions.

In comparison, the I197S mutant showed a pale violet color and very low absorption, probably due to impaired chromophore maturation. Although threonine and serine share a hydroxyl group, the extra methyl group of threonine brings it closer to the Ile and causes a smaller impact on the overall protein structure than Ser. On the other hand, L199S and L199T mutant was normally expressed but showed no distinguishable spectral change from the parent mCherry.

3.2. Spectroscopic properties of mCherry-I197T

This red-shift of I197T by an H-bond resembles the red-shifting mechanism of mPlum, where a hydrogen bond between Glu-13 and the chromophore extends the conjugated bond system of the chromophore, thus inducing a red-shift in the emission [5]. The H-bond of Thr197 to the terminal phenolate of the conjugated bond system of the chromophore would cause similar effects. The almost completely overlapping absorption spectra of mCherry and the I197T mutant (Fig. 3A) also support the H-bond-induced red-shifting mechanism since the H-bond primarily affects the emission spectrum [5]. Based on the absorption spectra, the chromophores of both mCherry and I197T appear to be in a deprotonated phenolate form that is available for hydrogen bonding with a nearby hydroxide group. Interestingly, the three absorption spectra of mCherry, I197T and mPlum are closely overlapping in the 530 ~ 620 nm range (Fig. 3A). However, their emission spectra are largely different, as the emission maxima are 611 nm for mCherry, 617 nm for I197T and 644 for mPlum (Fig. 3C and Table 1).

It should also be noted that mPlum exhibited a significant fraction of residual green fluorescent component [5], which can cause problems in multicolor labeling experiments. This is observed as a strong band at 395 nm and at 500 nm in the absorption spectrum (Fig. 3A). It has been shown that both the red and green chromophores can be formed as dead-end products during the maturation process [10]. Recently, it was shown that replacement of Glu-16 by Pro improves the maturation problem [11]. In contrast, mCherry and the I197T mutant did not show such a fraction of the green fluorescent state.

In Table 1, we have compiled parameters of mCherry, I197T, and mPlum for comparison. Both the wavelength of the absorption maximum and the extinction coefficient of the I197T mutant are nearly identical with mCherry and mPlum. In contrast to mCherry, which emits mainly at

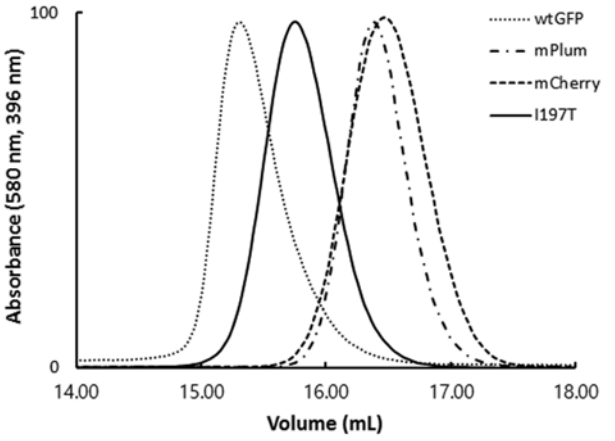

Fig. 4. Normalized elution profiles on size exclusion FPLC are shown for I197T (solid), mCherry (dashed), mPlum (dot-dashed), and dimeric avGFP (dotted). mCherry and avGFP serve as monomeric and dimeric controls, respectively.

611 nm, I197T shows an emission maximum at 617 nm. Though there was a 6 nm difference in the excitation maximum between mCherry and I197T, they showed the same Stokes shift. This reflects that the introduced H-bond caused a red-shift in excitation, which resulted in the color change. The fluorescence quantum yields of I197T of 0.03 is significantly lower compared to the value of 0.22 for mCherry, while their extinction coefficients are almost identical. This can be interpreted as a large fraction of absorbed energy being wasted by a radiationless decay, normally due to relaxation of chromophore rotation. The slightly smaller size of Thr than Ile could be responsible for giving more room to the chromophore, albeit its crystal structure is required for showing the structural basis of possible mechanisms.

3.3. Comparison of oligomerization tendencies

Oligomerization of fluorescent proteins for labeling can be detrimental in the study of protein tracking for spatial distribution or the analysis of protein interactions. We performed size-exclusion chromatography to estimate the oligomerization tendency of the I197T mutant with mCherry as a monomeric standard and avGFP as a dimeric standard. Surprisingly, the elution peak of I197T was faster than mPlum co-migrated with mCherry and slower than avGFP (Fig. 4), suggesting slight dimerization. Oligomerization of FPs is generally known to be due to hydrophobic patches or electrostatic interactions on the protein surface. Because Ile197 is located near the chromophore, inside the mCherry structure, the effect of mutagenesis of Ile197 on the oligomerization property should be negligible. One possibility is that changing interior hydrophobic Ile too hydrophilic Thr rearranged the whole protein structure. However, the normal chromophore maturation of the I197T, which requires every participating residue in place, indicates the overall structure is well-retained as of mCherry. The result of aggregation by low pH (Fig. 1D) would suggest a possibility that the I197T partially aggregated in the condition of size-exclusion chromatography (pH 7), as the aggregation was started just below pH 7.2. The crystal structure of it would give more clue for this observation.

4. Conclusion

Our work details the red-shift effect of a single amino acid change in mCherry, which is probably the most understood RFP. It is characterized by a monomeric nature, high pH resistance, and high maturation rate [6]. Based on the spectroscopic properties of the I197T mutant, this red-shift can be attributed to the formation of new hydrogen bonding with the chromophore phenolate and by a similar mechanism as mPlum. Furthermore, the replaced 197Thr reconnected the proton wire to the outside solution and recovered the pH sensitivity as in GFP [12]. Our findings raise the possibility of developing advanced far-red FPs originating from mCherry. Furthermore, optimization by directed evolution and tuning of electrostatic interactions around the chromophore could further improve its properties as a fluorescent marker protein.

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