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Advances in engineering of fluorescent proteins and photoactivatable proteins with red emission

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Abstract

Monomeric fluorescent proteins of different colors are widely used to study behavior and targeting of proteins in living cells. Fluorescent proteins that irreversibly change their spectral properties in response to light irradiation of a specific wavelength, or photoactivate, have become increasingly popular to image intracellular dynamics and super-resolution protein localization. Until recently, however, no optimized monomeric red fluorescent proteins and red photoactivatable proteins have been available. Furthermore, monomeric fluorescent proteins, which change emission from blue to red simply with time, so-called fluorescent timers, were developed to study protein age and turnover. Understanding of chemical mechanisms of the chromophore maturation or photoactivation into a red form will further advance engineering of fluorescent timers and photoactivatable proteins with enhanced and novel properties.

Introduction

Since the discovery that green fluorescent protein (GFP) from jellyfish is encoded by a single gene and its fluorescence requires no enzymes or cofactors except molecular oxygen, the fluorescent proteins (FPs) became invaluable tools in biomedical sciences. Cloning of the first FP with red-shifted excitation and emission spectra, DsRed, led to the discovery of many new orange and red fluorescent proteins (RFPs) in non-bioluminescent organisms [1]. Further directed evolution of wild-type FPs allowed the creation of a wide palette of enhanced FPs, which span the visible spectrum from 420 nm to almost 650 nm [2]. Development of the monomeric RFPs allowed extending possibilities of a Förster resonance energy transfer (FRET) approach to three and four colors in a single cell [3].

The RFPs, whose chromophores are formed by induction with light, are known as the photoactivatable FPs (PA-RFPs). Two different groups of PA-RFPs are presently being distinguished. Members of the first group exhibit an irreversible photoconversion from the non-fluorescent or green fluorescent state to the red fluorescent state. Members of the second group undergo reversible photoswitching between the non-fluorescent and fluorescent states. Introduction of photoactivatable FPs into cell biology greatly extended the spatio-temporal limits of *in vivo* biological dynamics [4] and have become useful tools for the super-resolution microscopy approaches such as a photoactivation localization microscopy (PALM) [5].

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Therefore, recently developed monomeric irreversible PA-RFPs are of a particular interest for tracking individual intracellular molecules.

In RFPs, the fluorescence shift toward the red is a result of the expansion in the π-system of the conventional GFP-like chromophore. Currently known RFPs share two types of chromophores, called a DsRed-like chromophore [6] and a Kaede-like chromophore [7] after the first proteins where they have been found. The DsRed-like chromophore may form either through autocatalytic post-translational modifications or via induction by irradiation with violet light. There are also some proteins containing derivatives of the DsRed-like structure, which form due to chemical modifications of the N-acylimine group in the DsRed-like chromophore [8,9,10]. The Kaede-like chromophore is characteristic for the green-to-red photoconvertible fluorescent proteins. Initially, the proteins of this group mature to a green-emitting state with the GFP-like chromophore. However, UV-violet light at approximately 350–450 nm efficiently converts them into the red fluorescent state.

Here, we provide a brief overview of RFPs and irreversible PA-RFPs published within the last few years.

Irreversibly photoactivatable red fluorescent proteins

Monomeric PA-RFP Dendra2 [11] has already found wide application for protein [12•] and cell tracking [13]. Dendra2 exhibits a high contrast photoconversion from the green to the red fluorescent state (Table 1). The unique feature of Dendra2 is that a low phototoxic 488 nm laser line can be used for its photoactivation. Furthermore, Dendra2 is simultaneously monomeric and efficiently matures at 37°C. Dendra2 performs well in sensitive fusions and possesses low cytotoxicity. The only disadvantage that should be mentioned is a relatively low pH stability of the activated red form.

In order to develop a monomeric version of KikGR [14], 21 amino acids mutations were introduced in 15 rounds of mutagenesis. mKikGR [15] has almost the same spectroscopic characteristics and kinetics of photoswitching as its parental protein KikGR [16]. High photostability and brightness of mKikGR activated red form allow for high resolution in photoactivation localization microscopy, as well as single-molecule tracking.

Several PA-mCherry variants, including PA-mCherry1, [17••] enable two-color diffraction-limited photoactivation imaging and super-resolution techniques, such as PALM. Irreversibly photoactivatable monomeric derivatives of mCherry, PA-mCherrys, are potentially less disruptive to tagged fusion partners. Before photoactivation these proteins have an absorbance maximum at about 400 nm and practically do not fluoresce. However, they can be easily photoactivated with a 405 nm laser line, achieving contrast up to 3,000–5,000-fold. In the photoactivated state, PA-mCherrys exhibit red fluorescence, which is stable in time and doesn't relax back to the dark state. All variants have fast maturation time and perform excellent in protein fusions in live cells.

The green-to-red photoconvertible tandem dimeric tdEosFP protein [18] paired with the photoswitchable protein Dronpa was also applied to two-color PALM imaging [19], but tdEosFP still does not localize accurately in fusions, and its monomeric version mEosFP does not mature at 37°C [18]. Recently, McKinney *et al.* [20•] developed a true monomeric version of EosFP, called mEos2, which efficiently matures at 37°C. The spectral properties, brightness, pK_a, photoconversion and contrast of the improved mEos2 are similar or better to those of tdEosFP, but there is a much better maturation at 37°C. Despite the dimerization tendency *in vitro*, mEos2 performs well even in difficult protein fusions in cells.

The only example of protein that displays both a reversible photoswitching and an irreversible photoactivation is the EosFP-derived protein, IrisFP [21], which has a single Phe181Ser amino acid substitution (here and below we use an amino acid numbering according to the alignment with wild-type GFP). IrisFP exhibits the irreversible green-to-red photoconversion under violet light, like its parental protein, and, in addition, both green and red fluorescent states can be turned off and on over again independently.

Novel monomeric red fluorescent proteins

Despite the growing role of photoactivatable FPs in advanced cell imaging approaches, common RFPs are still the proteins of choice for many standard biological applications. Although monomeric RFPs of the first generation are well-suited for protein labeling and exhibit efficient chromophore formation [2], they still have many drawbacks compared to common enhanced GFP (EGFP) and its derivatives.

Recently, several new RFPs with an enhanced brightness, rapid chromophore maturation and high photostability have been developed. Two wild-type RFPs, eqFP583 [22] and eqFP611 [23], were used to design a whole series of enhanced RFPs. Chudakov and coworkers subjected eqFP578 to a combination of site-specific and random mutagenesis to generate red and far-red monomeric FPs named TagRFP [22] and mKate [24], respectively. Crystallographic analysis of mKate [25] allowed for the substantial improvement of its pH-stability, brightness and photostability, resulting in mKate2 and tandem dimeric tdKatushka2 [26•].

The emission spectra of mKate2 and tdKatushka2 extend into a near-infrared “optical window” (650–900 nm), which is advantageous for light penetration in living tissues [27]. This feature makes them the RFPs of choice for visualizing fusion tags in tissues and whole animals. Further development of proteins with emission beyond 650 nm will possibly require extension of a conjugated π-electron system of the red chromophore or increasing Stokes shifts [28]. Alternatively, monomeric infrared FPs can be engineered on a basis of phytochromes, which incorporate an exogenous low-molecular weight chromophore [29].

Tsien and coworkers developed highly photostable FPs, named mOrange2 and TagRFP-T [30], which maintain most of the beneficial qualities of the original proteins and perform excellently for long-term imaging in fusion constructs. However, mOrange2 has a decreased brightness and chromophore maturation efficiency compared to parental mOrange. Tsutsui *et al*. generated a fast-maturating version of orange-emitting mKO, named mKOk, by introducing seven mutations [31•]. mKOk is 2-fold brighter than its precursor and fairly pH-resistant.

A monomeric RFP, mRuby, with emission maximum at 607 nm was recently shown to be a promising marker for peroxisomes in live mammalian cells [32]. In addition, Strack *et al.* [33,34] engineered several rapidly maturating tetrameric fluorescent proteins, called DsRed-Express2, DsRed-Max, E2-Orange and E2-Red/Green, with different spectral properties and low cytotoxicity. These proteins should facilitate production of transgenic organisms and stable cell lines.

Random mutagenesis of a chromoprotein derived from *Montipora* stony coral led to RFP named Keima, which exhibits a Stokes shift of 180 nm [35,36]. Structural and spectroscopic studies of mKeima revealed an excited-state proton transfer (ESPT) pathway from the chromophore hydroxyl to Asp165 acceptor, causing the large Stokes shift and pH-induced *cis-trans* chromophore isomerization [37]. Subach *et al.* [38•] showed that RFPs can be converted into the blue FPs using amino acids substitutions at limited number of positions. This strategy was applied to five RFPs, including mKeima, TagRFP, mCherry, HcRed1 and M355NA, which all were engineered into blue probes.

Chromophore photochemistry in fluorescent proteins

A structural basis for the photoactivation of PA-mCherrys, and the similar but rather dim PA-mRFP1 protein [39], remains unknown, while the mechanism of the green-to-red conversion for the Kaede-like proteins has been investigated thoroughly. However, it was suggested that the pre-activated PA-mCherry1 protein contains an mTagBFP-like chromophore, which absorbs violet light but does not fluoresce (FV Subach, VV Verkhusha, unpublished data). The PA-mCherry1 photoactivation possibly involves a decarboxylation of its Glu222 residue and subsequent oxidation of the mTagBFP-like chromophore that results in formation of the fluorescent DsRed-like chromophore but in a *trans* configuration (Figure 1). The quantitative decarboxylation of Glu222 via a Kolbe-like mechanism was detected after the PA-GFP photoactivation [40], and likely occurs in the course of the PS-CFP photoconversion [41] (Figure 1).

The Kaede-like proteins share the same chromophore-forming tripeptide His65-Tyr66-Gly67, which autocatalytically forms the green-emitting chromophore. X-ray analysis of the original green and photo-converted red chromophore forms revealed a light-induced extension of the chromophore π-electron system, known to result from backbone cleavage between the N^{α} and C^{α} atoms of His65 and formation of a double bond between the C^{α} and C^{β} atoms in His65 [42,43] (Figure 2). This photo-induced process occurs only in the neutral state of the chromophore and requires no molecular oxygen. The structural basis for the β-elimination reaction is the unique positioning of His65 and the stereochemistry of the amino acid residues in chromophore environment. Glu222 is essential for the red chromophore formation, but it does not decarboxylate as it does in PA-mCherrys. Other Kaede-like proteins exhibit the similar chemical reaction during the photoconversion with minor variations, depending on the chromophore environment. In this way, a blue shift of absorption and emission peaks of Dendra2 can be explained by a local structural changes involving mainly Arg69 and neighboring water molecule [44]. It was shown that reversible photoswitching of IrisFP between the fluorescent and nonfluorescent states is based on the *cis–trans* chromophore isomerization, accompanied by protonation–deprotonation events [21].

Essentially distinctive green-to-red photocoversion mechanism has been recently revealed for EGFP and other green FPs, including aceGFP, TagGFP, zFP506, amFP486 and ppluGFP2 [45••]. In contrast to the previously described anaerobic EGFP redding, the authors found that a redding of green FPs also occurs in the presence of oxidants in common aerobic conditions. This oxidative redding occurred in solution as well as in live cells, when the protein was irradiated with a high-intensity 488 nm laser. A possible explanation is that the DsRed-like red chromophore is formed as the result of a two-electron oxidation (Figure 2). The same mechanism could also explain the photoconversion into a far-red state found in the mOrange variants [46•] and mKO [47]. Efficient photoconversion of mOrange occurred at 458 nm or 488 nm laser excitation. Excitation and emission maxima of the photoconverted state were approximately at 610 nm and 640 nm, respectively. In contrast to Kaede-like proteins, the mOrange photoconversion was not substantially affected by pH, however it required the 4–5-fold higher illumination power than that for Dendra2 photoconversion.

Interestingly, tdKatushka and mKate underwent a red-to-green photoconversion upon single-photon laser excitation at 405 and 561 nm, resulting in the green state with excitation and emission maxima at 495 nm and 518 nm, respectively [46•]. This red-to-green photoconversion suggests a reduction of the π-conjugated system of the DsRed-like chromophore. Monomeric state of mKate, low dependence of photoconversion from pH, and low phototoxicity of the converting blue light make mKate a promising template to design an efficient optical highlighter for live cell imaging. Furthermore, induction of a light-driven electron transfer in EGFP could be applied to monitor and manipulate with light the intracellular redox processes.

Spectral properties of proteins could be also affected simply with time, without any light irradiation. The first monomeric fluorescent timers (FTs) [48•], which exhibit distinctive fast, medium, and slow blue-to-red chromophore maturation rates, were developed on the basis of mCherry (Table 1). It was suggested that a blue-emitting form of FTs contain the mTagBFP-like chromophore, which is converted after oxidation to the red-emitting DsRed-like chromophore (SV Pletnev, VV Verkhusha, unpublished data) (Figure 1). The blue and red forms of FTs are bright enough to use FTs either alone in protein fusions or together with green FPs for multicolor imaging. FTs exhibit the similar timing behavior in bacteria, insect and mammalian cells. The predictable time course of changing fluorescent colors allows a quantitative analysis of temporal and spatial molecular events based on the ratio between the blue and red fluorescence intensities. Availability of three FTs with distinctive blue-to-red maturation times will be useful for studies of intracellular processes with substantially different time scales.

Conclusions

Recently developed monomeric RFPs and PA-RFPs extend the range of available probes and provide exciting new options in biotechnology, developmental and cell biology. We expect further broadening of the applications of these proteins in intact tissues and transgenic animals, using spatial-restricted deeper multi-photon laser excitation. Further studies generating the novel far-red and infra-red genetically encoded fluorescent markers and photoactivatable probes are of great practical interest because of high transparency of animal tissues in the 650–900 nm region.

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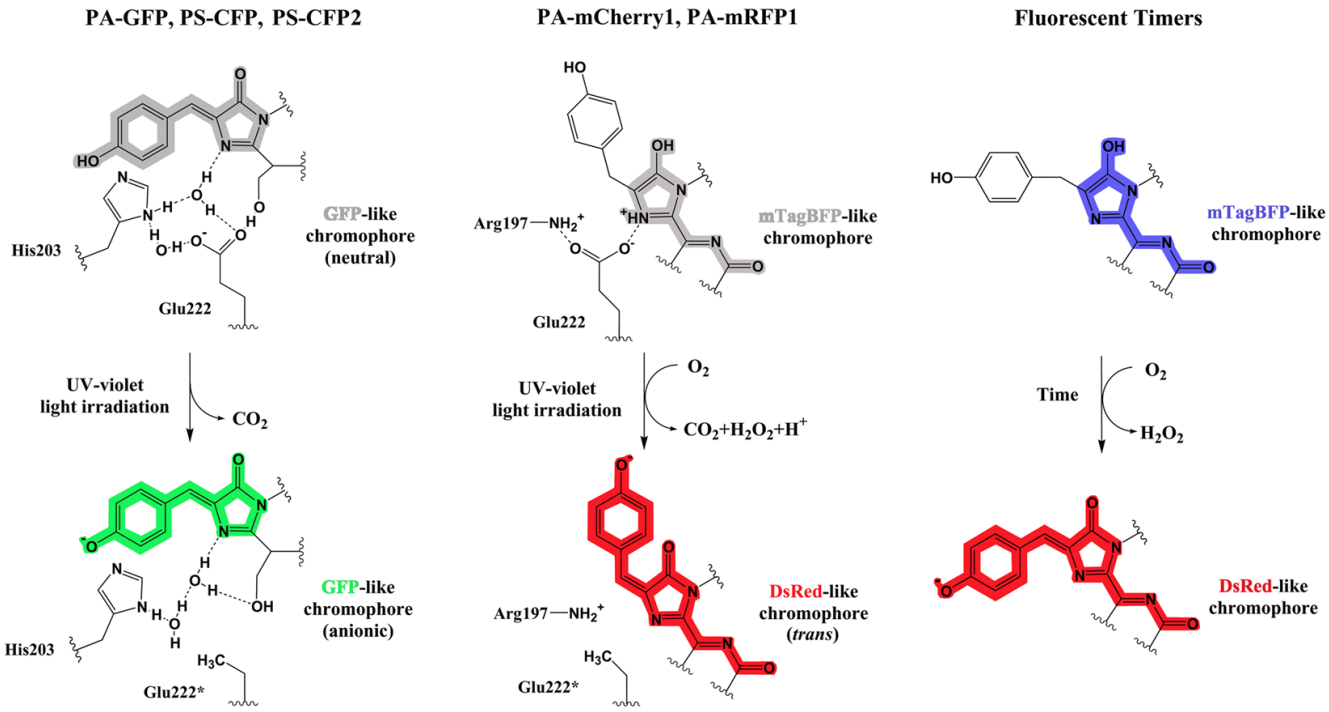

Figure 1.

Mechanisms of chromophore conversion from a neutral (protonated) to anionic (deprotonated) forms are illustrated for three key subgroups of fluorescent proteins such as PA-GFP, PS-CFP and PS-CFP2 (left), PA-mCherry1 and PA-mRFP1 (middle), and Fluorescent Timers (right). Chemical structures are shown for chromophores of the representative proteins before (top) and after (bottom) the conversion reactions. Colors of the chemical structures correspond to the spectral range of the chromophore emission except for the gray color, which indicates the non-fluorescent chromophore. UV-violet light-induced decarboxylation of the Glu222 residue is followed by the reorganization of the hydrogen bond network around the GFP-like chromophore that results in the chromophore deprotonation (left). Photoactivation by UV-violet light involves decarboxylation of the Glu222 residue and oxidation of the mTagBFP-like chromophore by molecular oxygen to the DsRed-like chromophore in the *trans*-configuration (middle). Slowed down oxidation of the mTagBFP-like chromophore by molecular oxygen without any light irradiation results in the formation of the DsRed-like chromophore in Fluorescent Timers (right).

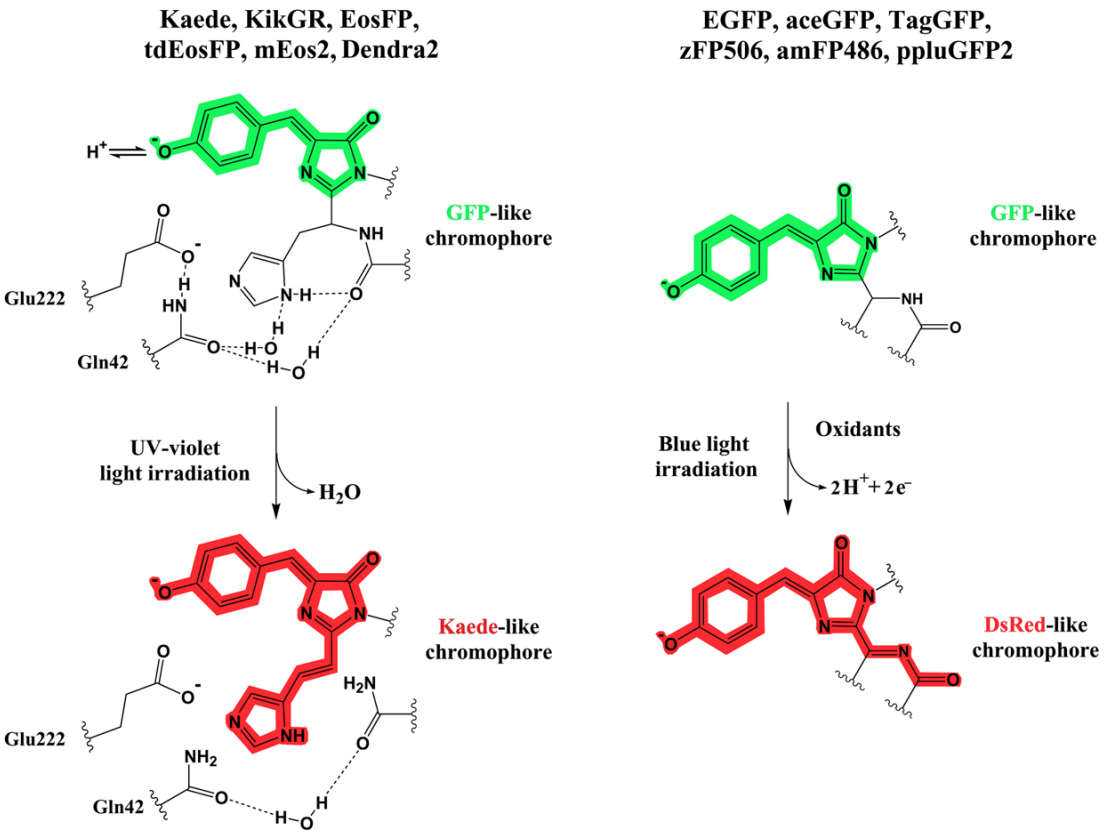

Figure 2.

Mechanisms of chromophore photoconversion from an anionic (deprotonated) green to anionic (deprotonated) red forms are illustrated for two key subgroups of fluorescent proteins such as Kaede, KikGR, EosFP, tdEosFP, mEos2 and Dendra2 (left), and EGFP, aceGFP, TagGFP, zFP506, amFP486 and ppluGFP2 (right). Chemical structures are shown for chromophores of the representative proteins before (top) and after (bottom) the photoconversion reactions. Colors of the chemical structures correspond to the spectral range of the chromophore emission. The Glu222 residue stabilizes a transition state of the UV-violet light-induced polypeptide backbone cleavage by forming the hydrogen bond network with the Gln42 residue and chromophore forming His65 residue via water molecules; protonation-deprotonation equilibrium shown for the green chromophore is important for the photochemical behavior (left). The oxidative redding of the GFP-like chromophore is a one-photon process, which requires two equivalents of the oxidant per molecule of the fluorescent protein and possibly goes via formation of a radical of the chromophore, resulting in the DsRed-like chromophore (right).

Table 1

Properties of the new fluorescent proteins that become red fluorescent after light irradiation or with time.

Protein	Oligomeric state	Excitation, nm	Emission, nm	Extinction coefficient, $M^{-1}cm^{-1}$	Quantum yield	pK_a	Maturation half-time at 37°C, h	Photostability, sec	Reference
Dendra2	monomer	490	507	45,000	0.50	6.6	ND	45	[11]
		553	573	35,000	0.55	6.9		378	
IrisFP	tetramer	488	516	52,200	0.43	ND	ND	ND	[21]
		551	580	35,400	0.47	ND		ND	
tdEosFP	dimer	506	516	84,000	0.66	5.7	ND	47	[18]
		569	581	33,000	0.60	ND		380	
mEos2	monomer	506	519	56,000	0.84	5.6	ND	42	[20]
		573	584	46,000	0.66	6.4		323	
PA-mCherry1	monomer	404	466	6,500	<0.001	ND	0.38	ND	[17]
		564	594	18,000	0.46	6.3		18	
mKikGR	monomer	505	515	49,000	0.69	ND	ND	14	[15]
		580	591	28,000	0.63	ND		21	
Fast-FT	monomer	403	466	49,700	0.30	2.8	0.25	ND	[48]
		583	606	75,300	0.09	4.1	7.1	ND	
Medium-FT	monomer	401	464	44,800	0.41	2.7	1.2	ND	[48]
		579	600	73,100	0.08	4.7	3.9	ND	
Slow-FT	monomer	402	465	33,400	0.35	2.6	9.8	ND	[48]
		583	604	84,200	0.05	4.6	28	ND	

ND, not determined.