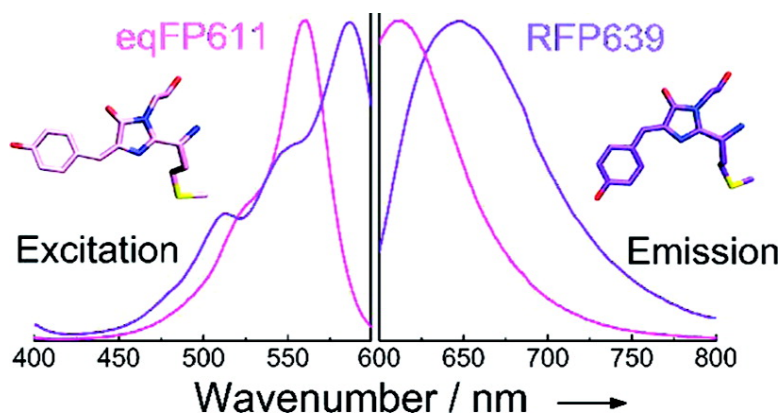Communication

Trans#Cis Isomerization is Responsible for the Red-Shifted Fluorescence in Variants of the Red Fluorescent Protein eqFP611

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Trans−Cis Isomerization is Responsible for the Red-Shifted Fluorescence in Variants of the Red Fluorescent Protein eqFP611

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Received June 18, 2008; E-mail: uli@uiuc.edu

In recent years, fluorescent proteins (FPs) have become key tools in cellular biology.[1,2] By fusing them to proteins of interest at the DNA level, they are utilized as specific markers in fluorescence microscopy studies of live cells and tissue in real time. Naturally occurring FPs have been optimized to yield bright fluorescence emission over a wide spectral range, and photoactivatable FPs have been developed that enable localized protein tagging[3] and play an important role in ultrahigh-resolution microscopy.[4]

Red fluorescent proteins (RFPs) have received particular attention, as their emission is well separated from the green-yellow autofluorescence of cells and, moreover, the reduced light scattering at longer wavelengths facilitates imaging of thick tissues. The tetrameric RFP of the sea anemone *Entacmaea quadricolor*, eqFP611, displays the most red-shifted fluorescence emission of any unmodified FP characterized so far, with its emission peak at 611 nm Stokes-shifted by 52 nm from its excitation peak at 559 nm.[5,6] Functional dimers d1eqFP611 and d2eqFP611 were obtained by the single mutations Val124Thr and Thr122Arg in the A/B interface.[7] The chromophore, 2-iminomethyl-5-(4-hydroxybenzylidene)-imidazolinone, forms autocatalytically from the tripeptide Met63-Tyr64-Gly65 and assumes a coplanar trans configuration in eqFP611,[8,9] whereas it is in the cis configuration in other RFPs including DsRed.[10] In the photoactivatable protein asFP595 from *Anemonia sulcata*, the red chromophore can be switched between a nonfluorescent trans and a fluorescent cis state by irradiation with green light.[11]

For the dimeric variant d1eqFP611, we had previously observed that a red-shifted species appeared upon irradiation with pulsed 532-nm light, and concomitant changes in the Raman spectrum were interpreted as evidence of a trans−cis isomerization of the chromophore.[12] Here we have revisited this problem by using X-ray crystallography in combination with site-directed mutagenesis to assess whether we can create a fluorescent, red-shifted eqFP611 variant with a cis chromophore. The X-ray structures of the dimeric eqFP611 variants are essentially identical, and we focus here on d1eqFP611 because of its excellent resolution of 1.1 Å (see Supporting Information (SI) Table 1). The electron density map in Figure 1a shows the chromophore of d1eqFP611 in the trans isomeric state, stabilized by hydrogen bonds from Ser158 and Asn143 to the oxygen atom of the phenolate moiety. A large hydrogen-bonded cluster resides underneath the chromophore, involving Lys67, Glu145, Tyr178, His197, Glu215, and structural water molecules (see SI Figure S1).
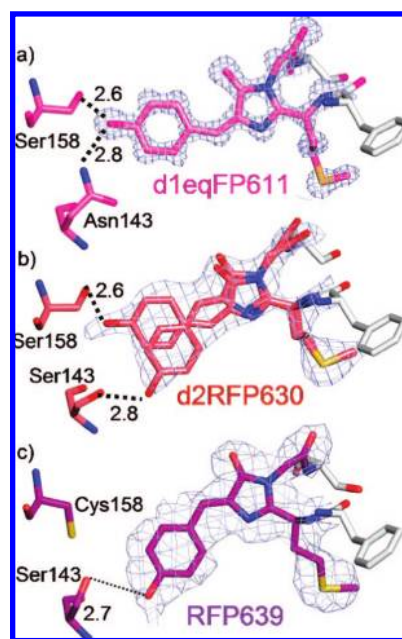
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Figure 1. 2Fo−Fc electron density difference maps of the chromophores of (a) d1eqFP611, (b) d2RFP630, and (c) RFP639, contoured at 2.0, 1.3, and 1σ, respectively, with the final models superimposed. The d2RFP630 map is a "cis omit" map, calculated with the phases of the protein containing the modeled trans chromophore. Hydrogen bond distances in Å.

However, the cis isomer is known to be energetically preferred by the isolated chromophore,[13] so that weakening of hydrogen bonding interactions to the phenolate oxygen is expected to shift the equilibrium toward cis. Consequently, we introduced the Asn143Ser substitution into d2eqFP611 to remove one of the two hydrogen bonds. This variant, denoted as d2RFP630, is highly fluorescent, and its absorption (emission) maximum is shifted by 24 (19) nm to 583 (630) nm.[14] The spectral bands of d2RFP630 are broader than those of eqFP611 (Figure 2d−f), which could result from a mixture of spectrally distinct species (see below). Further support for this hypothesis is provided by the observation that exposure to mild alkaline conditions (pH 12) causes a smooth transition of both excitation and emission spectra toward those of eqFP611, and a crisp isosbestic point in the corresponding absorption spectra reveals that this spectral change is due to a pH-induced transition between two conformations.[14]

In Figure 1b, we have plotted the electron density map of the chromophore in d2RFP630. Here, in contrast to dimeric eqFP611, the electron density cannot be adequately modeled with a single chromophore configuration. The majority (∼80%) of chromophores
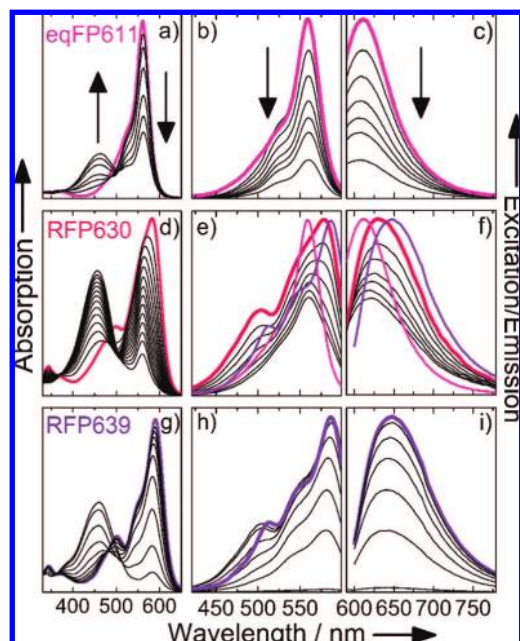
Figure 2. (a, d, g) Absorption, (b, e, h) excitation, and (c, f, i) emission spectra of (a–c) eqFP611, (d–f) d2RFP630, and (g–i) RFP639. Initial spectra are scaled to equal amplitudes (colored lines). Black lines depict a time series during 1 h after a jump from pH 7 to 12. Arrows point toward increasing time.

is found in the cis form, which is stabilized by a hydrogen bond between Ser143 and the phenolate oxygen. The ratio of cis and trans chromophores agrees with the ratio of the two species found in the optical spectra (Figure 2d–f), which strongly suggests that the additional red shift (~20 nm) of d2RFP630 is caused by the presence of the cis isomer. This finding is also consistent with recent experiments and theoretical calculations on another RFP, asFP595.[15,16] In contrast to asFP595, however, the chromophore in eqFP611 is coplanar and hence highly fluorescent in both isomeric states. As shown in Figure 2e and f, a transition to a blue-shifted form occurs over the course of 1 h after a pH jump from 7 to 12. This transition is induced by deprotonation of a nearby residue, possibly from the hydrogen-bonded cluster (SI); the altered hydrogen bonding then triggers cis–trans isomerization of the chromophore.

Interactions between the chromophore and the protein are similar in both isomeric forms, so that the spectral shift between eqFP611 and RFP630 is largely based on the intrinsic electronic properties of the chromophore. As RFP630 is a mixture of cis and trans forms, a further red shift of the excitation (emission) to 588 (639) nm is introduced by the additional mutation Ser158Cys, which weakens the potential hydrogen bond (Figure 1) and, thereby, further destabilizes the trans form. The optical spectra of this variant, RFP639, suggest that it consists of a single chromophore species, presumably the cis form (Figure 2g–i). And indeed, the electron density map of RFP639 shows the chromophore only in the cis configuration (Figure 1c). The distance between the Ser143 hydroxyl group and the phenolate oxygen of the chromophore is 2.7 Å. Moreover, there is an additional hydrogen bond to a water molecule at 2.7 Å.

Additional red shifts of the chromophore bands may still be possible by further modification of its environment. As the electron density is known to shift from the phenolate ring toward the heterocycle upon electronic excitation,[17,18] changes in the polarity can have significant effects, and excited-state stabilization and hence a large Stokes shift will be essential. We believe that the highly polar, hydrogen-bonded network of eqFP611 (Figure S1) is responsible for the large Stokes shift of ~50 nm. The DsRed variant mPlum has an even larger Stokes shift (59 nm),[19] which develops on the picosecond time scale; a glutamate side chain coupled to the iminomethyl moiety has been implicated in this effect.[20]

By combining X-ray crystallography with optical (and vibrational[12]) spectroscopy, we have related isomeric states and protein interactions of the chromophore to its optical properties in eqFP611 variants. We expect our results to be helpful for the further rational engineering of FPs with optimized or even entirely novel properties.

Supporting Information Available: Crystal growth, data collection, structure determination, and illustration. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA8046443