FLUORESCENT PROTEINS

Electrostatics affect the glow

Chromophore twisting is probed with unnatural amino acids

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The chromophores of fluorescent proteins (FPs) form through self-catalyzed posttranslational modifications (*1*). In the original green FP (GFP) isolated from the jellyfish *Aequorea victoria*, Ser[65], Tyr[66], and Gly[67] residues form the 4-(*p*-hydroxybenzylidene)-5-imidazolinone (HBI) chromophore that contains a phenolate ring (P-ring), an imidazoline ring (I-ring), and a monomethine bridge (*1*). The protein cage excludes water that can quench fluorescence, but also enhances the fluorescence quantum yield (FQY) by restricting bond-twisting photo-isomerization of the HBI chromophore. However, the protein could also improve FQY through electrostatic effects. As reported on page 76 of this issue, Romei *et al.* (*2*) studied the effect of introducing groups that donate or withdraw chromophore electrons on the FQY of the photoswitchable FP Dronpa2 (*3*).

Mutations of residues near the chromophore can fine-tune the bond-rotation energy barrier to create photoswitchable variants. In Dronpa2, green light produces the protonated fluorescent cis form, and blue light produces the nonprotonated nonfluorescent trans form. This switching enables applications in super-resolution imaging (*4*) and optogenetics (*5*). In this protein, the chromophore is formed from Cys[62], Tyr[63], and Gly[64]. Investigation of the contribution of the electrostatics to FQY requires fine-tuning electrostatic parameters in the complex protein environment. Genetic code expansion (*6*) allows unnatural amino acids (UAAs) to be introduced into FPs. Yu *et al.* (*7*) and others established methods for the genetic incorporation of large numbers of Tyr analogs to modify the chromophore.

Upon photon absorption, the chromophore enters the S_1 state, in which negative charge flows from the P-ring to the I-ring (see the figure). Romei *et al.* found that replacing Tyr[63] with analogs bearing an electron-withdrawing group required more energy to transfer the electron, which blue-shifted the absorption maximum, and substitution with an electron-donating group red-shifted the absorption maximum. Both substitutions decreased FQY and likely lowered the energy barrier for bond rotation.

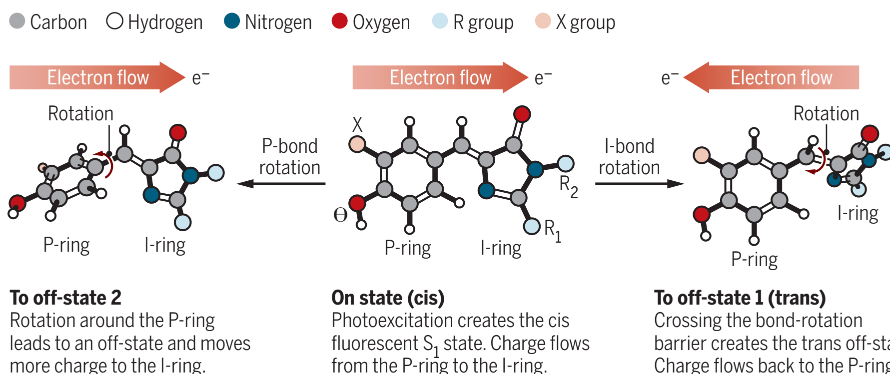
The next challenge was to decipher the mechanism of how bond twisting is modulated by electrostatics (*8*). In the transformation from the S_1 state to the fluorescence off-state 1, a cis-trans isomerization of the double bond connecting the P-ring and I-ring is required. Previous studies of the photoswitchable FP rsEGFP2 showed that in the transition state, the P-ring and I-ring are nearly perpendicular (*9*). This geometry reduces the amount of π-π orbital overlap between the P-ring and I-ring, which results in negative charge flowing back from the I-ring to the P-ring. Alternatively, in the S_1 state, the P-ring can rotate to reach another fluorescence off-state 2, and this process causes more negative charge flows from the P-ring to the I-ring. Electron-donating group substitution on Tyr[63] can lower the excited-state barrier for P-bond rotation and decreases FQY.

Romei *et al.* show that the electric field exerted by the chromophore's environment can either promote or hinder charge transfer, and thereby could effectively control the choice of bond rotation and isomerization pathway after photoexcitation. For optogenetic applications, photoinduced rotation around a specific bond of the FP chromophore or a retinal chromophore triggers a distinct conformational change of the protein, which results in specific kinase activation (*5*) or ion conductance (*10*). Engineering the electrostatic and steric environment of the chromophore by introducing charged, hydrogen-bonding, or unnatural amino acids in the protein scaffold could lead to more precise control of chromophore twisting and downstream signaling pathways, or create photoswitchable FPs with higher total photon number and faster on-off state switching for super-resolution imaging (*4*).

Recent advances in time-resolved serial femtosecond crystallography with x-ray free electron lasers (XFELs) have visualized bond-rotation events in proteins (*9*). Transient absorption spectroscopy could probe how genetically encoded UAAs influence the I-bond rotation and P-bond rotation pathways. These insights could in turn inform new mutagenesis efforts to develop improved FPs. ■

Excited-state outcomes

Romei *et al.* used genetic code expansion of a Tyr residue to change the H atom (denoted X) of a protein chromophore to groups that withdraw electrons (such as Cl) or donate electrons (such as OCH_3) to explore the effects of electrostatics on fluorescence (R_1 and R_2 are contacts to the protein).



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