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Photoconversion in the Red Fluorescent Protein from the Sea Anemone *Entacmaea quadricolor*: Is Cis−Trans Isomerization Involved?

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Proteins from the family of the green fluorescent protein (GFP) are presently extensively used in molecular and cellular biology.[1] During the past decade, GFP-like proteins have been engineered to achieve brighter fluorescence as well as an extended spectral range.[2] Furthermore, photoswitchable proteins, in which a bright and dark state can be switched reversibly upon irradiation, have been developed recently.[3] X-ray crystallographic studies suggest that the brightness of GFP-like proteins is related to the conformation of the chromophore. While the chromophore of wild-type GFP has a coplanar cis configuration,[4] the non fluorescent chromoprotein Rtms5[5] and the dim state of the far-red fluorescent protein HcRed[6] have their chromophore mainly in a noncoplanar trans configuration. More recently, the structure of the dark and the bright state of a photoswitchable protein asFP595 was identified.[7] Although the chromophore in the dark state has a trans configuration, the bright state has a cis configuration. Clearly, the conformation of the chromophore seems to be one of the key factors for the brightness of GFP-like proteins.[9] However, the evidence is mainly derived from protein crystals via X-ray studies. This technique is not applicable for proteins in solution undergoing small conformational changes on irradiation with intense visible light. Using stationary and time-resolved fluorescence spectroscopy as well as Raman spectroscopy, we report on photoinduced changes in the dimeric variant V124T of the red fluorescent protein eqFP611 from the sea anemone *Entacmaea quadricolor*,[8] which we interpret as conformational changes of the chromophore. A rationale is presented, trying to link the observed results to the recently available crystallographic data of red fluorescent proteins.[6]

The crystal structure of eqFP611 shows that the chemical structure of the chromophore (Met[63]-Tyr[64]-Gly[65]), *p*-hydroxybenzylidene-imidazolinone with an extended π-conjugated system, is nearly identical to the chromophore of another red fluorescent protein, DsRed (Gln[66]-Tyr[67]-Gly[68], see Supporting Information).[9,10] However, the chromophore of eqFP611 is mainly present in a coplanar trans configuration, whereas the chromophore of DsRed has a coplanar cis configuration (see Figure 1). Therefore, some of the differences in the Raman spectra of eqFP611 and DsRed should be directly related to the conformation of the chromophore.

Photoconversion experiments of the chromophore of eqFP611 were performed with pulsed 532-nm light.[11] Upon prolonged irradiation, the absorption peak at 559 nm diminishes, and concomitantly, the shoulder at ~600 nm gets more pronounced (see Figure 2a). The corresponding fluorescence spectra, recorded with

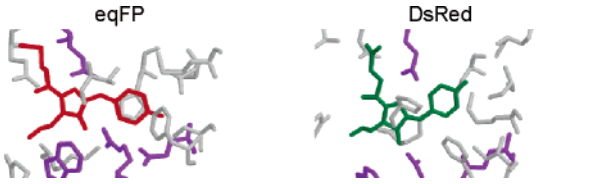

Figure 1. Crystal structure of the chromophore of eqFP611 (left, red) and DsRed (right, green). Conserved (purple) and nonconserved (gray) amino acid residues are also depicted.

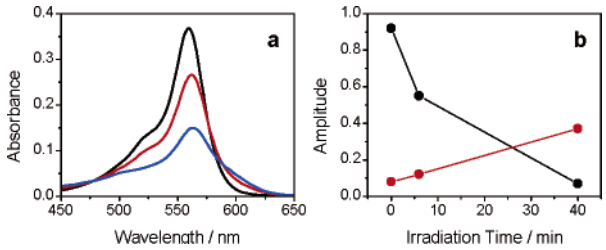

Figure 2. Photoconversion of eqFP611 in PBS (pH = 7.4) upon irradiation with a 532-nm pulsed laser at an excitation power of 100 mW/cm^2. (a) Time evolution of the absorption spectrum. (b) Relative amplitudes of the 2.6 ns and 1.1 ns components in the fluorescence decay excited at 543 nm and detected at 610 nm.

540-nm excitation, show a drastic decrease in the quantum yield of fluorescence. Fluorescence decays of the nonirradiated eqFP611 show a double-exponential behavior with a major contribution of 2.6 ns (92%) and a minor contribution of 1.1 ns (8%) (see Figure 2b). As expected from the steady-state measurement, the fluorescence of the irradiated eqFP611 decays much faster.

Interestingly, the decay components observed in the nonirradiated sample are also present in the irradiated sample, but with different contributions. While the relative amplitude of the 2.6 ns component decreases to 7% on irradiation, the amplitude of the 1.1 ns component increases to 30% (see Figure 2b) and two components in the picosecond range appear.

Note that the fluorescence decay for DsRed, in which the chromophore is present only in the coplanar cis conformation, is monoexponential with a decay time of 3.6 ns.[11] On the other hand, the fluorescence of HcRed, having a nearly identical chromophore but present in a coplanar cis conformation and a nonplanar trans conformation,[6] decays biexponentially with decay times of 1.47 ns and 0.51 ns (results not shown). These results suggest that there are two different conformations of the chromophore present in eqFP611 as well. Note that the different decay times cannot be linked directly to the different species due to large spectral overlap and eventual (excited state) interconversion processes. This is further evidenced by the fact that the decay of the irradiated sample, excited at either 543 or 590 nm, contains identical decay components with nearly identical contributions (results not shown). As stated before,

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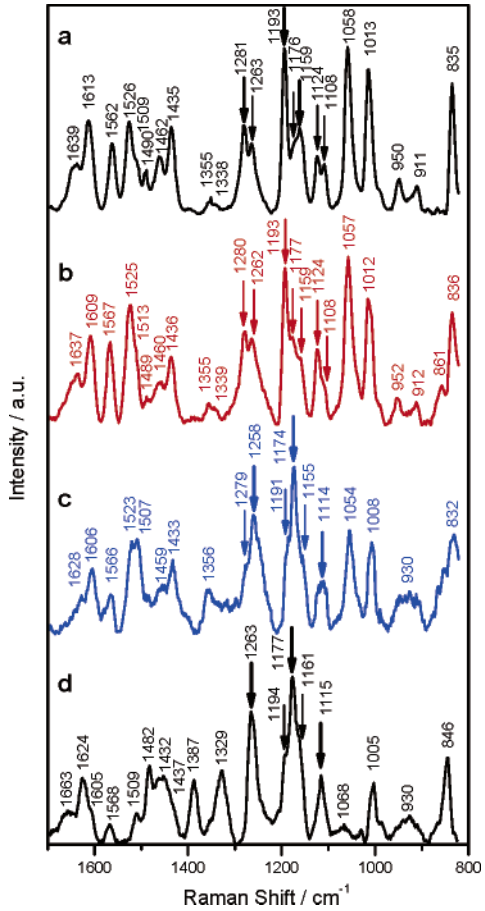

Figure 3. Time evolution of the preresonance Raman spectrum of eqFP611 in PBS (pH = 7.4) on irradiation; spectra are shown for (a) 0, (b) 6, and (c) 40 min of light exposure. (d) Preresonance spectrum of a DsRed sample without prior irradiation.

upon irradiation, additional fast components appeared in the fluorescence decay of the irradiated samples. Together with the drastic decrease in fluorescence quantum yield, this is an indication for new nonradiative pathways that are opened, probably related to deviation from planarity.[6,11]

Raman spectra were acquired using a confocal microscope.[11] The excitation source was the 752-nm line of a Ti:sapphire laser. Spectra were measured with a collection time of 30 min at a spectral resolution of ~5 cm^{-1}. Excitation at 752 nm results in preresonance-enhanced conditions which allow probing the chromophore site with minimal spectral interference from the surrounding protein environment.

Spectra a and d of Figure 3 show Raman spectra of the nonirradiated eqFP611 and DsRed, respectively. There are some similarities, but also clear differences. A doublet of bands is observed for the eqFP611 spectrum at around 1120 and 1270 cm^{-1}. In contrast, the DsRed spectrum shows only a single band for the corresponding vibrational mode. In the Raman spectrum of HcRed, the doublet at 1270 cm^{-1} is also observed (see Supporting Information). Since eqFP611 and HcRed have two different chromophore conformations and DsRed only one in its native state, these observations suggest that the doublet of bands in eqFP611 arises from the two species in the sample. Upon irradiation with a 532-nm pulsed laser, the relative intensities of the doublet changed (Figure 3b). After 40 min irradiation, only single bands can be seen in these regions (Figure 3c), consistent with essentially complete photoconversion at this stage (see Figure 2b). Spectral changes were also observed for the bands located at 1159, 1174, and 1193 cm^{-1}. After complete photoconversion, the relative intensities and peak positions show striking similarity with the those of the Raman spectrum of DsRed (Figure 3).

Raman spectra of wild-type GFP, of its mutants, and of a model compound of the chromohore have been reported,[12] and most of the bands have been assigned.[13] In contrast, Raman spectra of red fluorescent proteins are not yet well characterized. While a coherent anti-Stokes Raman scattering spectroscopy study on DsRed evidenced the extended conjugation system of the DsRed chromophore,[14] exact assignment of the bands is not yet available. Nevertheless, differences in the Raman spectra of eqFP611 and DsRed should arise from differences in the conformation of the chromophore since their chemical structures are nearly identical. Therefore, we interpret the observed changes in the Raman spectrum of eqFP611 upon irradiation tentatively as evidence for trans-to-cis isomerization of the chromophore.

Our study reveals the potential of Raman spectroscopy combined with fluorescence spectroscopy for studying minute structural changes in the chromophores of red fluorescent proteins. The study also strongly underlines the need of precise band assignment in Raman spectra of this class of compounds.

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Supporting Information Available: Chemical structures of chromophores of DsRed and eqFP61; Raman spectra of DsRed and HcRed. This material is available free of charge via the Internet at http://pubs.acs.org.

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