# Mechanisms and Dynamics of Reversible Photoswitching in Fluorescent Proteins: Insights from Transient Absorption Spectroscopy

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Light-induced reactions in fluorescent proteins of the Green Fluorescent Protein (GFP) family are a central issue for their applications in fluorescence imaging. First considered as harmful, they have more recently been optimized to generate photoactive variants that are increasingly used in advanced fluorescence imaging (nanoscopy, fluorescence modulation microscopy...). The molecular mechanisms underlying fluorescent protein photochemistry are however still poorly understood, in particular from the viewpoint of chemical dynamics.

Reversibly photoswitchable fluorescent proteins (RSFPs) are a subfamily of photoactive GFP homologues that can be switched back and forth between a fluorescent (ON) state and a non-fluorescent (OFF) state by irradiation at two different wavelengths. X-ray crystallography studies revealed two types of photoswitching mechanisms. The most common one is chromophore cis/trans isomerization coupled to proton transfer, as for instance in Dronpa[1] (Fig. 1a). Dreiklang[2] is an exception, for which photoswitching relies on the reversible addition of a water molecule to the chromophore (Fig. 1b). X-ray crystallography however essentially provides static information. We will present our recent studies of the photoswitching dynamics of Dronpa and Dreiklang by femtosecond transient absorption spectroscopy (TAS).

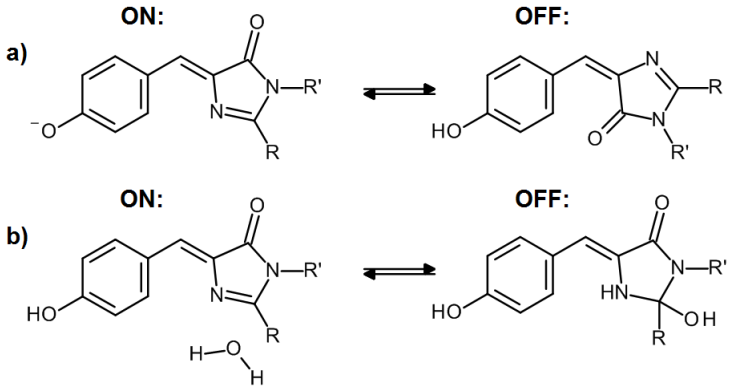


Figure 1. Chromophore structures in the ON and OFF states of a) Dronpa and b) Dreiklang.

We first reexamined the OFF→ON photoswitching of Dronpa, which had been the object of a few preceding studies[3,4,5] failing to provide a clear spectral signature of the trans→cis isomerization and a convergent timescale for chromophore deprotonation. By carefully controlling the excitation energy to avoid multiphoton ionization, we could measure both the spectrum and the anisotropy of the first photoswitching intermediate at ~300 ps and assign it unambiguously to a neutral cis chromophore.[6] We also used nanosecond TAS to monitor chromophore deprotonation, which was found to occur in ~10 μs.[6] Altogether, these results draw a clear picture of chromophore transformations involved in the OFF→ON photoswitching of Dronpa: the first step consists of ps trans→cis isomerization in the excited-state, and proton transfer follows much later in the ground-state, on the μs time scale.

We then investigated the ON→OFF photoswitching dynamics of Dreiklang, which was so far completely unknown, by fs TAS. We found that the reaction is triggered by an ultrafast deprotonation of the chromophore phenol group in the excited state, in 100 fs. This primary step is accompanied by coherent oscillations that we assign to its coupling with a low-frequency mode, possibly a deformation of the hydrogen bond network. A ground-state intermediate is formed in the ps-ns regime that we tentatively assign to the deprotonated water adduct. We suggest that proton ejection from the phenol group leads to a charge transfer from the phenol to the imidazolinone ring, which triggers imidazolinone protonation by a nearby glutamic acid and catalyzes the addition of the water molecule.

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