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Unraveling the excited-state structural dynamics of LSSmOrange protein through time-resolved impulsive stimulated Raman spectroscopy

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[Abstract] LSSmOrange is a mutant of a coral fluorescent protein, DsRed. The absorption and emission maxima of LSSmOrange are separated by 5453 cm^{-1}, and this large energy difference has provided the capability of multicolor imaging with a single excitation laser line in living cells. The chromophore of LSSmOrange has a neutral form in the ground state. Upon photoexcitation, it undergoes ultrafast excited-state proton transfer (ESPT), resulting in the formation of the bright anionic state. This ESPT dynamics of the LSSmOrange is represented by the multi-exponential kinetics, however, its mechanism has not been fully understood yet. To clarify the ESPT mechanism from a structural viewpoint, we employed time-resolved impulsive stimulated Raman spectroscopy (TR-ISRS) using sub-10 fs pulses, and recorded the Raman spectra of the chromophore with femtosecond time resolution. The obtained time-resolved Raman data indicate that the chromophore in LSSmOrange is present in two different structural forms and thus explain the observed multi-exponential kinetics of the ESPT.

[Introduction] Studying biological processes in the living cells is a challenging task, however,

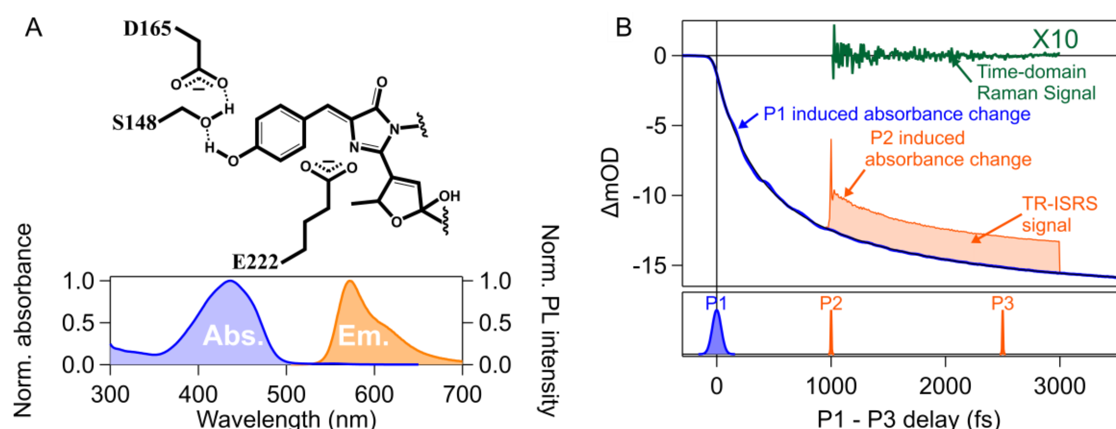

Fig. 1. (**A**) Chromophore structure with interactions with the nearest amino acids in LSSmOrange based on the crystallographic structure (PDB ID: 4Q7R). Blue and orange traces are the steady state absorption and fluorescence spectra of LSSmOrange (pH 7.4, 20 mM Tris-HCl buffer + 150 mM NaCl). (**B**) Measurement scheme and pulse sequence of TR-ISRS. Blue and orange traces denote a pump-probe signal measured with P1 and P3 pulses, and a TR-ISRS signal measured with P1, P2 and P3 pulses, respectively. Green trace is the time-domain Raman signal, which is extracted from the TR-ISRS signal by subtracting the slowly-varying population component.

fluorescent proteins have provided the capability of observing these biological processes under the physiological conditions. LSSmOrange is a fluorescent protein developed to fill the spectral gap between green-yellow and red fluorescent proteins. The absorption and emission maxima of LSSmOrange is separated with very large energy (Figure 1A). This large energy separation is rationalized with the mechanism of ultrafast excited-state proton transfer (ESPT)[1]. The initial photo-excited chromophore has a neutral form (Figure 1A), and it undergoes the ultrafast ESPT, resulting in the formation of the bright anionic state. However, the excited-state dynamics of LSSmOrange have not been clearly understood yet. To clarify the excited-state dynamics from a structural viewpoint, we employed time-resolved impulsive stimulated Raman spectroscopy (TR-ISRS)[2] using sub-10 fs pulses, and recorded the Raman spectra of the chromophore with femtosecond time resolution.

[Methods] Measurement scheme and pulse sequence of TR-ISRS are shown in Figure 1B. The actinic pump pulse (P1, 415 nm, 90 fs) was generated by frequency-doubling the output of the Ti:Sapphire amplifier. The Raman pump and probe pulses (P2 and P3, 600–800 nm) were generated using a non-collinear optical parametric amplifier (NOPA). The output of the NOPA was compressed to <8 fs using a folded prism pair and micro-machined membrane deformable mirror, and the obtained pulse was characterized by self-diffraction frequency-resolved optical gating (SD-FROG) at the sample position. These three pulses (P1, P2, and P3) were focused together onto a 0.3-mm thick flow cell.

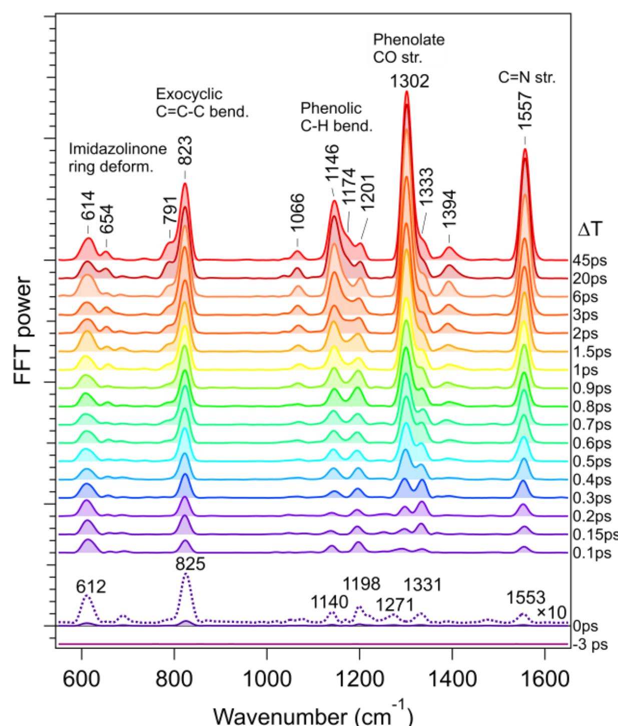

Fig. 2. Time-resolved Raman spectra of LSSmOrange (pH 7.4, 20 mM Tris-HCl buffer + 150 mM NaCl) at various P1-P2 delays (ΔT). Raman spectrum at 0 ps is 10 times magnified (dotted curve).

[Results and Discussion] Time-resolved Raman spectra of LSSmOrange, obtained by TR-ISRS, are shown in Figure 2. Immediately after photoexcitation (for example, ΔT = 0), weak Raman bands of the neutral form before the ESPT were observed. Subsequently, on the femto-to-picosecond time scale, we observed the growth of numbers of Raman bands, which is attributable to the formation of the anionic form chromophore through the ESPT. The kinetic analysis of these anionic form Raman bands reveals bi-exponential growth of its population. The analysis also indicates that this bi-exponential feature is associated with two parallel ESPT pathways producing two different anionic form species with different Raman spectra, suggesting the heterogeneity of the chromophore structure of LSSmOrange. On the basis of the previous X-ray crystallographic study of LSSmOrange[3], we attribute the origin of this heterogeneity to the different hydrogen bond environments around the chromophore.

[References]

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