Time-Resolved Impulsive Raman Study of Excited State Structures of Green Fluorescent Protein

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Abstract: Structural dynamics of green fluorescent protein was studied by femtosecond time-resolved impulsive Raman spectroscopy. The excited-state vibrational spectra of the protein with three different chromophore forms were obtained, revealing their structural differences and excited-state deprotonation.
OCIS codes: (300.6450) Spectroscopy, Raman; (300.6500) Spectroscopy, time-resolved

1. Introduction

Green fluorescent protein (GFP, from *Aequorea victoria*) is the most famous fluorescent protein that is widely used in bio-imaging application. The photophysics of GFP attracts much attention to understand how the bright green fluorescence is emitted. So far, spectroscopic measurements revealed that the intrinsic chromophore, *p*-hydroxybenzylideneimidazolinone, stays at the equilibrium between protonated state (A state) and deprotonated state (B state) inside GFP. The A state exhibits a major absorption band at ~398 nm while the B state gives another minor band at ~475 nm. When photoexcited, the A state undergoes excited state proton transfer (ESPT) and is converted to the highly emissive deprotonated form of the chromophore, which is origin of the green fluorescence of GFP. This deprotonated state after ESPT is called I state, not B state, because the fluorescence after ESPT displays a different spectrum from that of B state (Figure 1). This difference has been attributed to the distinct chromophore pockets of the I and B states. Although the photochemistry of GFP and involvement of multiple excited states are the bases of the widely-utilized GFP fluorescence, the structures of the A, B, and I states, especially those of their excited states (A^{ex}, B^{ex}, and I^{ex} states), have not been clearly characterized yet by experiments. In this study, we used time-resolved impulsive stimulated Raman spectroscopy (TR-ISRS) [1] to investigate excited-state structures of GFP.

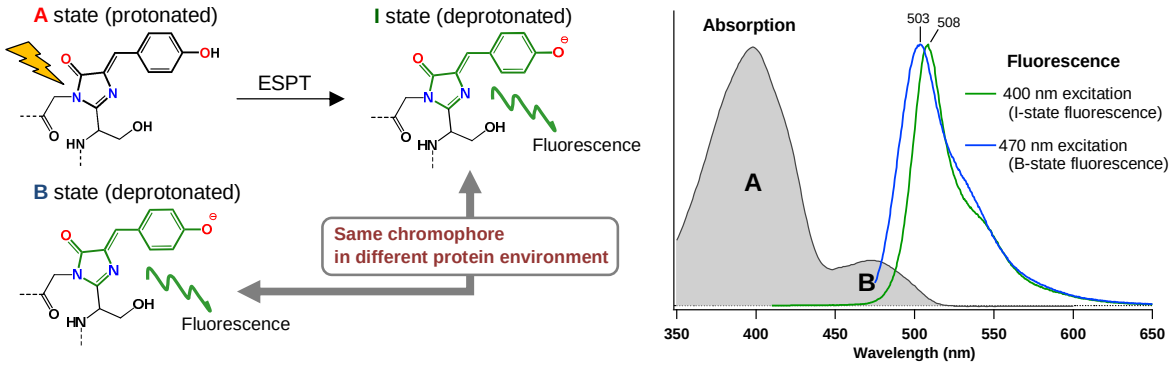

Figure 1. Three states of GFP chromophore (left) and absorption/fluorescence spectra (right)

2. Experimental method

The experimental scheme of TR-ISRS is illustrated in Figure 2. TR-ISRS uses three pulses (P1, P2 and P3). The first P1 pulse of ~150 fs duration prepares excited state population. The wavelength of P1 pulse is set at 390 nm (or 470 nm) to excite A state (or B state). The second P2 pulse interacts with the excited state at ΔT and induces the vibrational coherence in the excited state. Then, the induced vibrational coherence is probed by the third P3 pulse. The P2 and P3 pulses cover the spectral range of 550-650 nm and are compressed in time down to ~10 fs. The signal from the excited state is almost selectively observed by tuning the wavelength of the P2 and P3 pulses to be resonant with the stimulated emission of the excited state.

3. Results and Discussion

Figure 3 shows the TR-ISRS signals obtained after A-state excitation at selected P1-P2 delays (ΔT), together with that obtained after B-state excitation for comparison. The TR-ISRS signal, i.e. the absorbance change induced by

the P2 pulse, consists of the component arising from the excited-state population change and the oscillatory component due to impulsively excited Raman-active vibrations.

Fourier transform analysis of the oscillatory component yields the time-resolved vibrational spectra of the excited states, as is shown in Figure 4. The spectrum clearly changes with ΔT. Immediately after photoexcitation, the spectrum (red, at ΔT = 0.3 ps) shows vibrational bands at 214, 600, 821, 889, 1144, 1240 cm^{-1}. As ΔT increases, the vibrational band at 1240 cm^{-1} gradually disappears and a new band at 1301 cm^{-1} grows significantly within 30 ps. The rise time of the 1301 cm^{-1} band (6.6 ps), which is concurrent with ESPT (~6 ps), indicates that the spectrum immediately after excitation is attributable to A^{ex} state, while the spectrum at 30 ps is assigned to I^{ex} state. The major difference of the A^{ex} and I^{ex} spectra is found in the frequency of this phenolic C-O stretching mode. The ~60 cm^{-1} upshift on going from the A^{ex} to I^{ex} states (1240 cm^{-1} → 1301 cm^{-1}) is fully consistent with the deprotonation in the ESPT process. Our experiment further showed that the C-O stretching frequency of the I^{ex} state is equal to that of the B^{ex} state obtained by direct excitation of the B state. Although X-ray crystallography of GFP mutants proposed that B and I states have the quite different hydrogen bond lengths between Thr203 and C-O group of the chromophore [2], this argument is not supported because the C-O stretching frequency is the same between B^{ex} and I^{ex} states. Rather, the significant difference between I^{ex} and B^{ex} states is found for the relative intensities of imidazolinone ring vibrations at 599 and 636 cm^{-1}, which suggests that a distortion of imidazolinone ring is involved in the structural differences of the I^{ex} and B^{ex} states.

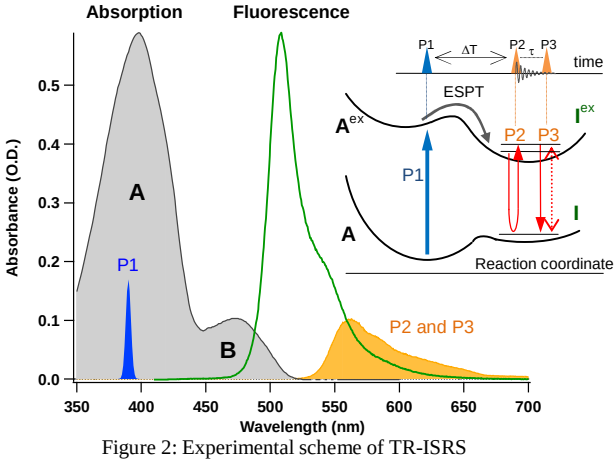

Figure 2: Experimental scheme of TR-ISRS

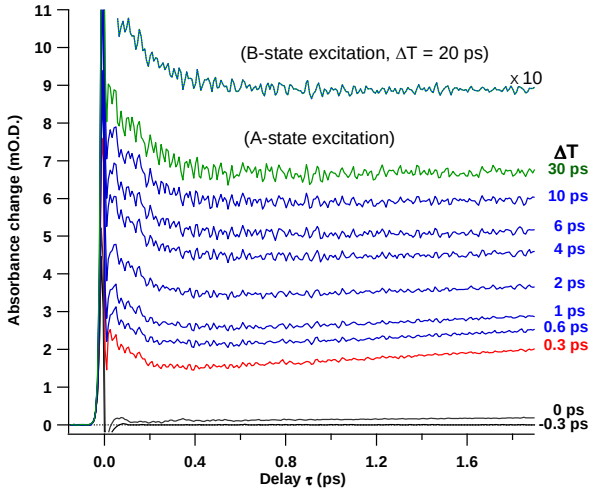

Figure 3: TR-ISRS signal after A-state excitation. The signal after B-state excitation is shown for comparison (top).

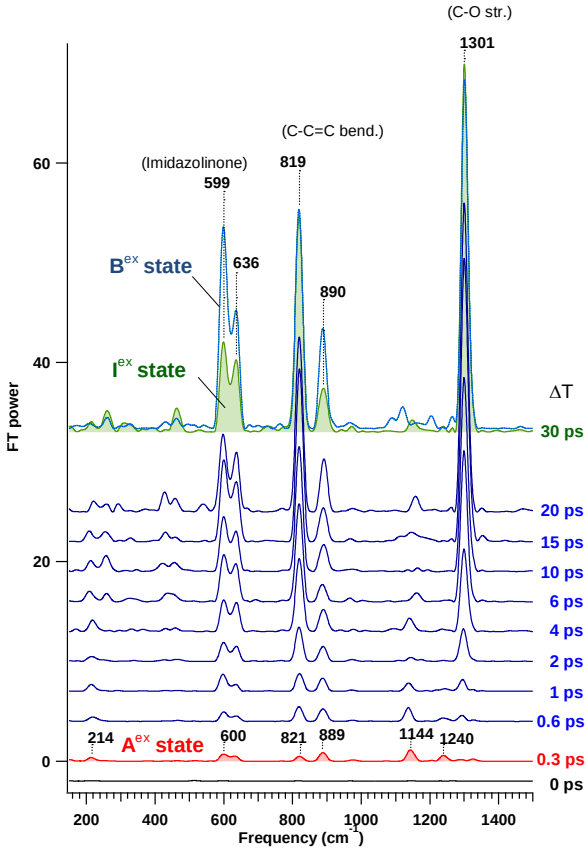

Figure 4: TR-ISRS spectra after photoexciation of A state. I^{ex} state spectrum at 30 ps is compared with B^{ex} state spectrum (top).

References

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