Picosecond time resolved infrared absorption measurements reporting on the structural events in the photocycle of Green Fluorescent Protein

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Introduction

The spectroscopic and dynamical properties of the wild type Green Fluorescent Protein (GFP) from Aequorea victoria have been investigated[1]. The p-hydroxybenzylidene-imidazolinone chromophore which is formed from the Ser65-Tyr66-Gly67 sequence is shown in Figure 1. The chromophore exists mostly in the neutral ground state A, with the phenolic oxygen protonated. GFP absorbs maximally at 398 nm, which is the neutral A state. The absorption spectrum of wt-GFP shows an additional minor band at 478 nm which corresponds to the anionic B ground state[2,3]. A central question addresses the suppression of the non-radiative pathways: GFP fluoresces efficiently with blue excitation despite major electrostatic rearrangements resulting from photoionization of the chromophore and neutralization of Glu 222. Excited state proton transfer (ESPT) follows excitation of the neutral, phenolic chromophore and the resulting excited phenolate state I* shows high quantum yield red-shifted emission at 508 nm, with a 3.0 ns lifetime[2,3].

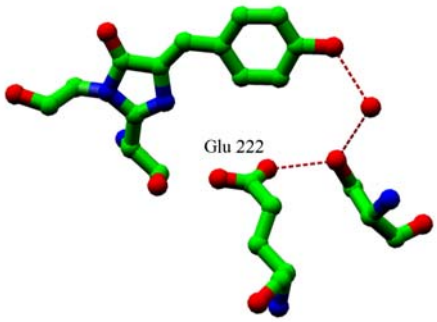

Figure 1. Structure of the p-hydroxybenzylidene-imidazolinone chromophore of the wild type Green Fluorescent Protein in the X-ray geometry. H-bonding via an internal water and Ser 205, to Glu 222 is indicated.

We have performed picosecond transient absorption measurements to address the reaction dynamics and vibrational changes during the photocycle of GFP. Time-resolved difference spectroscopy in the mid-infrared region probed the photocycle transitions that correspond to the excited state proton transfer reactions and the decay of the deprotonated radiative state which transiently populated a ground state intermediate. In addition, for the first time we have performed a pump-dump-probe experiment incorporating an infrared probe with optical pump and dump pulses. These measurements provided the difference spectrum which corresponds to the decay of the radiative intermediate directly.

Methods

A 12 ml, 1.7 mM GFP sample in 5mM Tris/HCl pD 7.8 in D_2O was flowed in a closed circulating system and was raster scanned in both x- and y- directions to avoid photodegradation[4]. The pathlength was 24 μm. This sample arrangement allowed data collection in several spectral windows which could be interleaved without scaling. Importantly, it also avoided significant accumulation of a phototransformed product, which irreversibly forms with low quantum efficiency and does not show excited state proton transfer[4].

Picosecond transient absorption measurements were performed using the PIRATE facility at the Central Laser Facility, Rutherford Appleton Laboratory. For a description of the instrument, see[5]. Briefly, the sample was excited with 400 nm, 2 μJ pulses, and probed with 150 cm^{-1} fwhm white infrared pulses. The signal was dispersed and detected using a 64 element MCT detector, providing an 11.6 nm spectral dispersion between channels. Time-resolved data was collected at 500 Hz in overlapping 200 cm^{-1} windows and interleaved. Spectral calibration was done by matching the probe arm spectrum to an atmospheric FTIR spectrum in the same region.

Pump-dump-probe spectroscopy was performed by including 532 nm pulses resonant with the deprotonated I* radiate state from the SHG from an ACE AOT_YVO_2QSP/MOPA Nd:YVO_4 laser operated at 1000 Hz, delivering 0.5 ns pulses with 5-10 μJ/pulse which were electronically timed to arrive approximately 500 ps after the 400 nm pump. The 532 nm dump pulses were chopped to 500 Hz and the delay was scanned in 100 ps steps to maximise the overlap with transient population of the I* state by maximizing the dump-on minus dump-off pump-probe difference spectrum with the probe delay line at 1000 ps.

Results

Transient absorption data was collected with delays ranging between 2 ps and 2000 ps after excitation in spectral windows centered at 1666, 1515, 1408 and 1328 cm^{-1}. After interleaving, this provided a data set for the mid-infrared region on time scales reporting on early and late intermediate states in the photocycle (Figure 2).

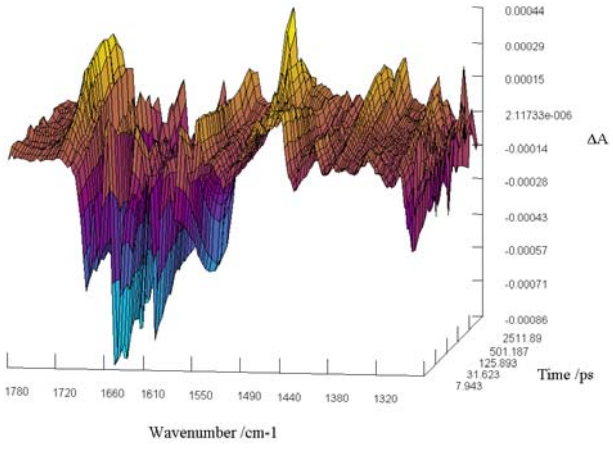

Figure 2. Interleaved picosecond time resolved difference absorption spectra between 1800 and 1250 cm^{-1} with delays between 2 ps and 2000 ps after excitation of the A state at 400 nm.

The data was analysed by Singular Value Decomposition, which provided three relevant spectral components (not shown). The scaled time traces for the three components could be fitted with three rate constants, τ_1= 10 ps, τ_2 = 75 ps and τ_3 = 1.7 ns. The first two components τ_1= 10 ps, τ_2 = 75 ps correspond to the excited state deuterium transfer reaction, similar to the 12 ps (42%) and 69 ps (58%) time-constants which were determined for this step in D_2O (and 2.2 ps (49%) and 11.1 ps (51%) in H_2O) from transient optical spectroscopy[3]. The third time-constant, τ_3 = 1.7 ns, corresponds to the decay of the deprotonated excited state intermediate called I*. Because the data extends only to 2.0 ns this fit is underdetermined and the actual life-time has been accurately determined to be 3.0 ns[2,3].

Global fitting of the data provided the time-independent difference spectra for the three kinetic components (not shown). The global fitting was done imposing the underlying reaction scheme for the two parallel excited state proton transfer reactions τ_1= 10 ps, τ_2 = 75 ps, followed by the subsequent formation of the I* and I_2 intermediates (Figure 5).

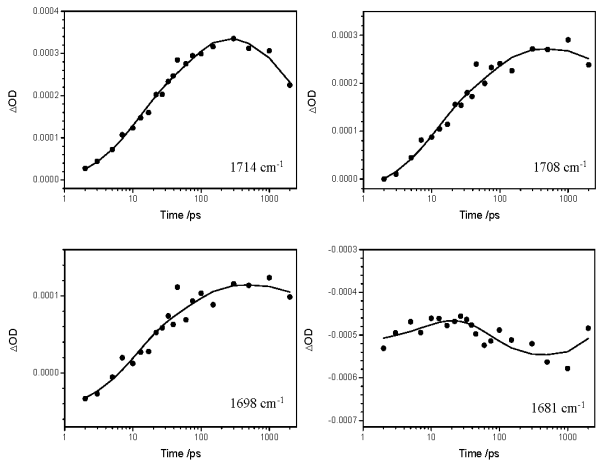

Figure 3. Global fitting of transient absorption traces of GFP in D_2O. 4 out of 202 traces globally fitted with the photocycle model using decay constants of τ1 = 10 ps, τ2 = 75 ps, τ3 = 3.0 ns and τ4 = 5.0 ns for the A_1*, A_2*, I* and I_2 states respectively, at 1714 cm^{-1}, 1708 cm^{-1}, 1698 cm^{-1} and 1681 cm^{-1}

Figure 3 shows as an example four globally fitted traces in the high frequency region, with contributions from the transient protonation of Glu 222 (COOH), structural perturbation of a protein sidechain carbonyl and the chromophore C=O bond vibration. Assignments for chromophore modes could be proposed for the A and I_2 ground states (Figure 5) on the basis of reported DFT calculations and isotope substitution studies of the chromophore model compound 4-hydroxybenzylidene-1,2-dimethylimidazolinone (HBDI)[6,7]. Assignments for the excited state intermediates A* and I* could be proposed on the basis of the expected bond order changes[1].

A Pump-Dump-Probe experiment was performed in order to measure directly the I_2 – I* difference spectrum (Figure 4). A nanosecond laser at 532 nm was used to dump the transiently accumulated I* intermediate to its ground state, I_1[3]. I_1 has a lifetime of 7 ps in D_2O[3], so may not be observed with ns dumping. Subsequently, the product state observed is the I_2 state which has a lifetime of 5.0 ns in D_2O[3].

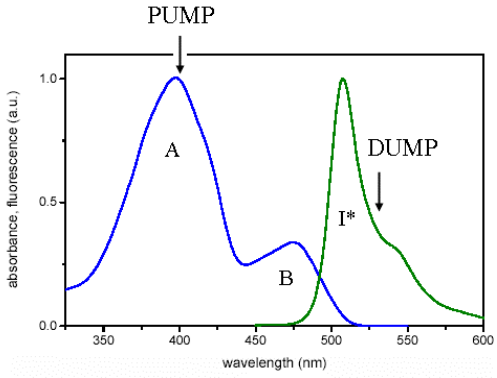

Figure 4. Absorption and fluorescence emission spectra of the wild type green fluorescent protein, showing the wavelengths used for pumping and dumping, together with the infrared probing.

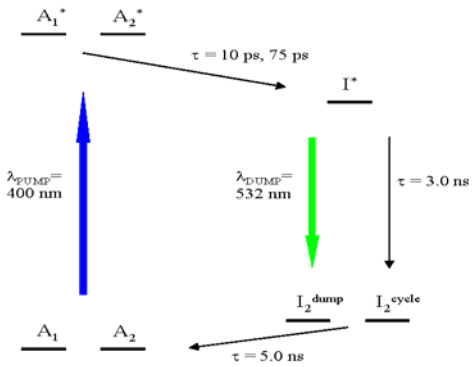

Figure 5. Photocycle scheme for wild type GFP including the energy level diagram and pump-dump-probe spectrum. The wavelengths used for pumping and dumping are given as well as the lifetimes for the intermediates in the absence of optical dumping. Part of the pump-dump-probe minus pump-probe difference spectrum shown directly corresponds to the I_2 – I* difference spectrum.

Conclusions

a. Comparison of the transient absorption data of wild type GFP with an E222D mutant definitively identifies Glu 222 as the excited state proton acceptor.

b. SVD and global analysis of 202 transient absorption traces strongly supports a biphasic excited state proton transfer reaction and provides the corresponding time-independent difference spectra.

c. Spectroscopic markers at 1695, 1539 and 1354 cm^{-1} indicate that GFP is unrelaxed and is in an intermediate environment even in the long-lived ground state intermediate I_2.

d. All major chromophore modes can be assigned in the A and I_2 ground state spectra and assignments can be proposed for the excited state intermediates A* and I*.

References

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