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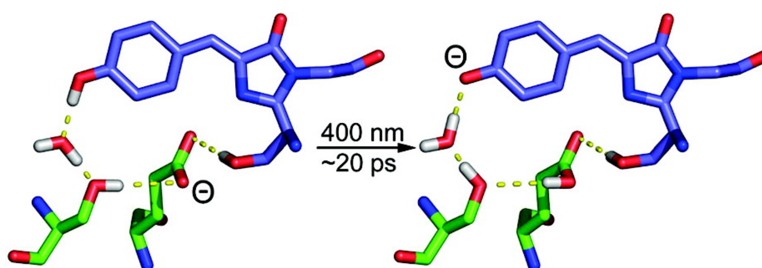

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Observation of Excited-State Proton Transfer in Green Fluorescent Protein using Ultrafast Vibrational Spectroscopy

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Green fluorescent protein (GFP) has had a major impact on our ability to image events in living cells since autocatalytic chromophore formation renders GFP intrinsically fluorescent.[1] Due to the importance of GFP as a bioimaging reagent, there is substantial interest in understanding the photophysics of the embedded chromophore and, in particular, in determining the structural changes in and around the chromophore following photoexcitation.[1] While techniques such as absorption and fluorescence spectroscopy, provide critical data on the nature and lifetimes of photogenerated species, these methods do not yield information on the precise structures that are present. In the current study, we have used ultrafast transient infrared (TIR) spectroscopy to illuminate structural changes that occur on the picosecond (ps) time scale following photoexcitation. In particular, we provide direct evidence that E222 is the terminal proton acceptor following excited-state proton transfer (ESPT).

The absorption spectrum of wild-type GFP (wtGFP) is characterized by two bands at 395 and 475 nm.[2] Excitation into either band results in highly efficient generation of green fluorescence (508 nm). The excited-state dynamics of wtGFP have been studied using ultrafast fluorescence and absorption spectroscopies.[3−5] Boxer and co-workers concluded that the light-driven conversion between the neutral (A-form) and anionic (B-form) chromophore proceeds via ESPT and passes through an intermediate state I (Figure 1).[3] Using hole-burning spectroscopy, Creemers et al. characterized the A, B, and I states in wtGFP and demonstrated that they had distinct photophysical properties.[6]

On the basis of X-ray crystallographic data, Remingtion, Tsien, and co-workers proposed that a hydrogen bonding network links the chromophore hydroxyl group and E222.[7] Photoexcitation of the A-form is then proposed to result in A* which transfers a proton to E222 via a water molecule and S205. While initial FTIR studies found no evidence that E222 was the ultimate proton acceptor,[8] it was later shown that photoconversion had caused E222 to decarboxylate, preventing the appearance of a protonated E222 IR band.[9] We have now used TIR spectroscopy to analyze structural changes following photoexcitation on the picosecond time scale using power densities that avoid decarboxylation.

Previous Raman studies[10−13] have resulted in a detailed understanding of the chromophore structure[10] and form the basis for our analysis of the TIR spectra. The wtGFP data are compared with spectra of the model chromophore, 4′-hydroxybenzylidene-2,3-dimethylimidazolinone (HBDI), as well as S65T, a GFP mutant in which ESPT has been disabled,[14] and T203V/E222Q,[15] a mutant which is believed to exist solely in the I ground state.

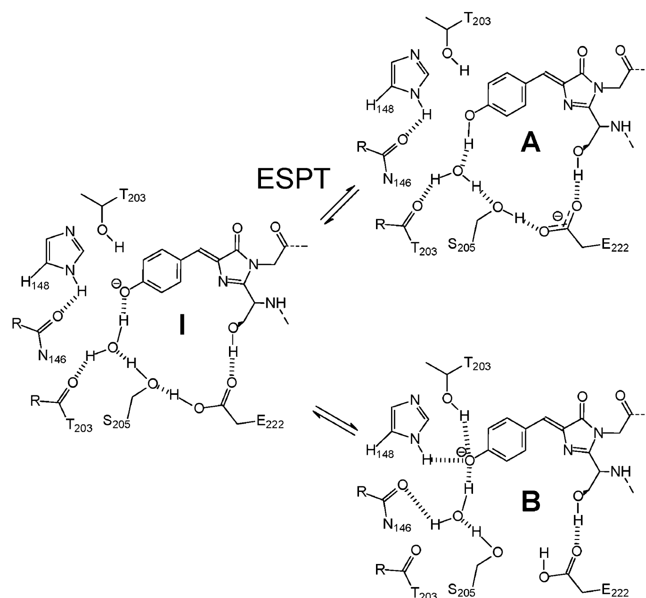

Figure 1. Model for the interconversion of the A and B forms of the chromophore via state I. Adapted from Brejc et al.[7]

The TIR spectra were obtained using an instrument with picosecond time resolution.[16] TIR spectra of neutral HBDI excited at 400 nm (Figure 2A) show an immediate bleach and ultrafast ground-state recovery accompanied by corresponding transient absorption, consistent with the known ultrafast relaxation of the isolated chromophore.[17] Specifically, bands at 1604, 1640, and 1693 cm^{-1} arising from the phenol ring mode, exocyclic C=C, and C=O modes, respectively,[12] are rapidly bleached. Transient absorption at the slightly lower frequencies of 1595, 1625, and 1680 cm^{-1}, appearing within the time resolution, can be assigned to the corresponding modes in the excited state. In addition, there is a transient absorption at 1577 cm^{-1} arising from the excited state. Similarly, on excitation, wtGFP shows instantaneous bleaching in ground-state modes associated with the A-form of the chromophore at 1596, 1637, and 1680 cm^{-1} (Figure 2B) that can be assigned to the corresponding modes identified for HBDI. In addition, a bleach develops at 1565 cm^{-1} with a rise time on the order of 50 ps. Significantly, there is no corresponding band in HBDI, so this mode is assigned to a structural change in a protein residue. The complex appearance of the 1565 cm^{-1} band is due to overlap of the excited-state absorption of A* (1570 cm^{-1} in S65T) with the 1565 cm^{-1} bleach. Significantly, the bleach at 1565 cm^{-1} is accompanied by the appearance of absorption at 1706 cm^{-1} on the same time scale. Bleach and absorption persist for at least 500 ps, but the original ground state is recovered in <1 ms. The 1706 cm^{-1} band could, in

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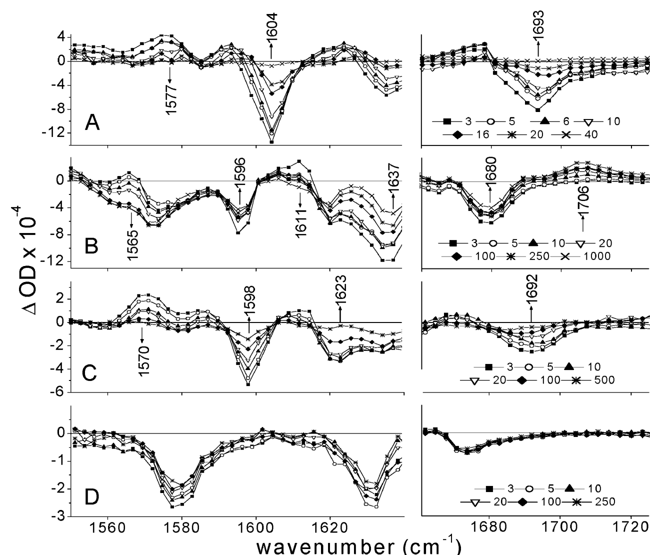

Figure 2. TIR data for (A) neutral HBDI (30 mM in DMSO-d_6), (B) wtGFP, (C) S65T, and (D) T203V/E222Q. IR spectra were acquired at the given times (picoseconds) following the pump pulse [<1 μJ; 400 nm (A−C) and 500 nm (D)]. Proteins (1−2 mM) were in D_2O at pH 7.5 (wtGFP and T203V/E222Q; 20 mM KH_2PO_4, 300 mM NaCl) or pH 5.5 (S65T; 30 mM MES, 300 mM NaCl). Arrows indicate the direction of absorbance with increasing time. The absence of an arrow shows that the band did not exhibit any time dependence beyond that associated with the excited-state lifetime.

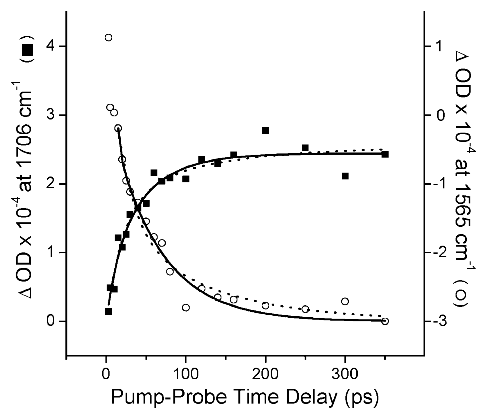

Figure 3. Transient absorption/bleach data for wtGFP at 1706 cm^{-1} (■) and 1565 cm^{-1} (○). Solid lines are free fits to the data with two exponential components (1706 cm^{-1}, 15 and 49 ps; 1565 cm^{-1}, 3 and 60 ps). Dashed lines use the time constants determined by Chattoraj et al. (22 and 116 ps) from the fluorescence decay of A*.[3] The data are statistically the same, as judged by χ^2 value. This indicates that the rate-determining step in the protonation of E222 is the initial proton transfer.

principle, arise from the formation of I*. To test this possibility, we measured the TIR of neutral S65T, which does not exhibit fast proton transfer, and T203V/E222Q, which exists as the I form (Figure 2C,D). Neither GFP mutant shows either the transient absorption at 1706 cm^{-1} or the corresponding bleach at 1565 cm^{-1}. Thus, we conclude that the 1706 cm^{-1} band is also a protein mode associated with the proton-transfer reaction.

The positions of the protein modes are consistent with titration of a carboxylate to carboxyl in the protein on the picosecond time scale.[18] Specifically, the 1565 cm^{-1} band is assigned to the antisymmetric stretch of the E222 carboxylate, while the 1706 cm^{-1} band arises from the protonated E222 carboxyl group. These assignments are consistent with static FTIR difference spectra of wtGFP in which E222 has decarboxylated. Irreversible photoconversion of wtGFP[9,13] results in the loss of a band at 1565 cm^{-1} assigned to the E222 carboxylate (data not shown).

Mechanistic information on the proton-transfer reaction can be obtained by comparing the dynamics of the 1565/1706 cm^{-1} modes with the excited-state dynamics reported previously.[3] In Figure 3, we compare the rate of bleaching/absorption with the fluorescence dynamics for A*.[3] Importantly, there is good correspondence between the TIR data and fluorescence decay. This is consistent with the initial proton-transfer step being rate determining and with subsequent steps being very much faster. The same conclusion was reached in a simulation of the subpicosecond proton transfer from W235 to E222 by Helms and co-workers.[19]

Further analysis of the TIR data, coupled with isotope editing, will provide finer detail on the dynamics of GFP. For example, an initial rapid partial recovery of the wtGFP signals at 1596 and 1680 cm^{-1} could result from rapid ground-state recovery (A* to A). This possibility will require a more complex model for the excited-state dynamics, such as that proposed by Vohringer and co-workers in which two forms of A* are populated.[5]

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