Resolution of the Complete Proton Relay Cycle in the Green Fluorescent Protein with Femtosecond to Microsecond Transient Infra-red

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Introduction

The green fluorescent protein from *Aequorea victoria* (avGFP) exhibits excited state proton transfer (ESPT), in which a proton is transported *ca* 10 Å down a 'proton wire' (the structure and proton wire are illustrated in Figure 1). This unique property has established GFP as a model system in which to study proton transport in biology. The vast majority of time domain data on avGFP concerns only the ultrafast ESPT step. In this work we characterise the complete proton transfer cycle in real time using femtosecond to microsecond time resolved infra-red spectroscopy developed at the Harwell Research Complex. Measurements on avGFP are extended to two mutants, T203V and S205V which modify the structure of the proton wire and the proton transfer dynamics. We observe very different effect in ground and excited state proton transfer, and find evidence for a giant kinetic isotope effect. The full report on these data is published elsewhere [1].

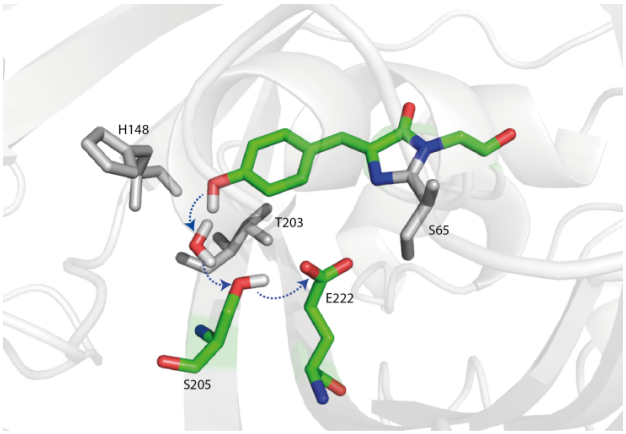


Figure 1. Structure of avGFP and its mutants, with the proton wires highlighted

Experiments

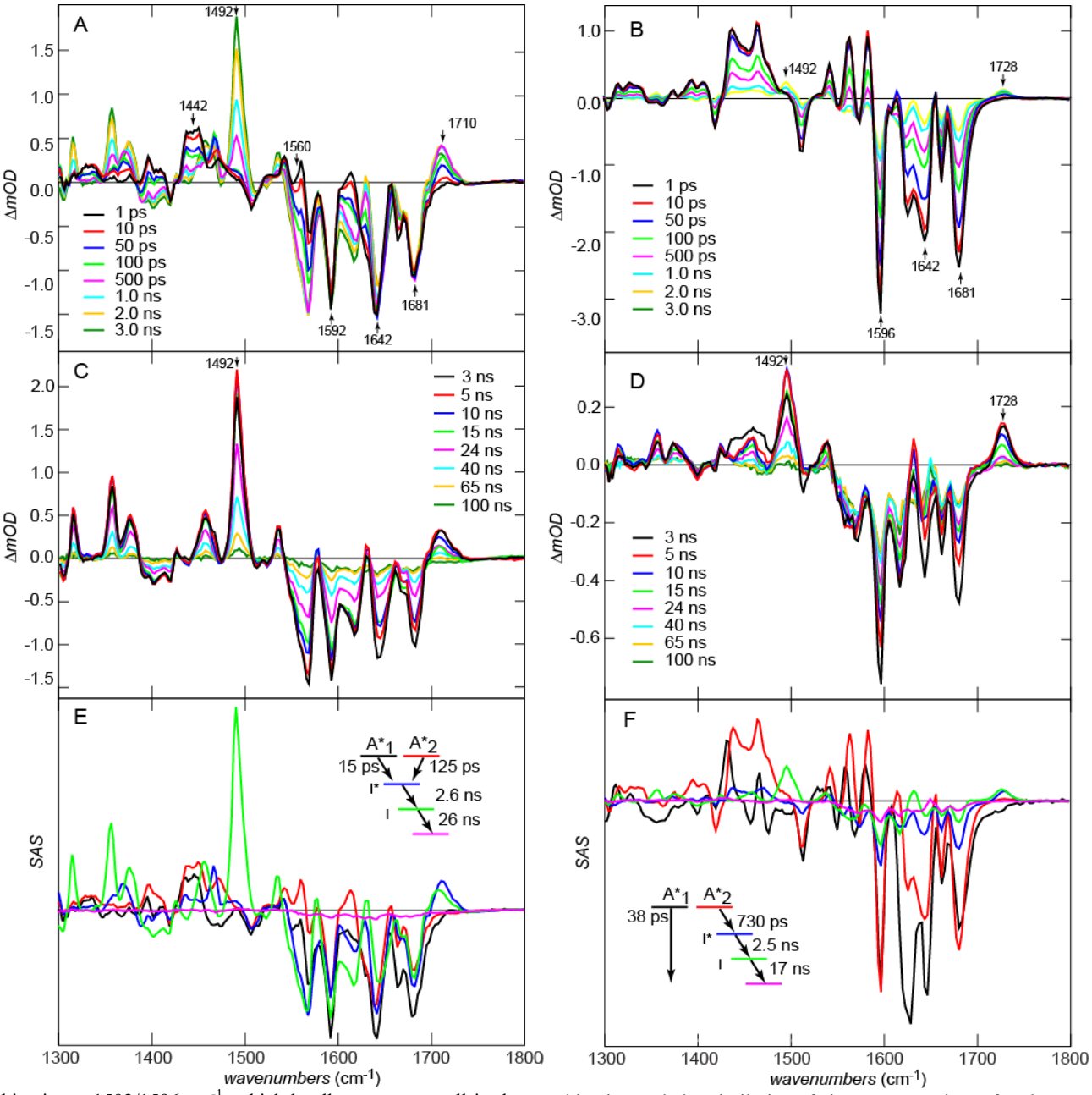
The TRMPS experiment developed at the LSF combines the vibrational and temporal resolution of TRIR with greatly extended observation times. Here we apply that method to record the complete photocycle of avGFP, T203V and S205V. The TRIR data for T203V and S205V are shown in Figure 2 on the picosecond to 0.1 microsecond timescale. The neutral (A) state was excited at 400 nm with a 100 fs pulse and the response probed between 1300 and 1800 cm^{-1}. Sub-nanosecond TRIR data for T203V (Figure 2A) are very similar to those for avGFP. The negative signals (bleaches) appearing at time zero are due to vibrational modes of the chromophore ground state, removed or

shifted on excitation, and to vibrations of the protein perturbed by excitation. The positive signals (transients) are due to the excited state of the chromophore and perturbed protein modes

Results and Discussion

The TRIR data for T203V and S205V are shown in Figure 2 on the picosecond to 0.1 microsecond timescale. The neutral A state was excited at 400 nm with a 100 fs pulse and the response probed between 1300 and 1800 cm^{-1}. The negative signals (bleaches) were assigned to C=O (1681 cm^{-1}), C=C (1642 cm^{-1}), phenyl ring localised modes (e.g. at 1592/1596 cm^{-1}) and to vibrations of the protein (described elsewhere, [1]). The time dependent growth of the transient at 1710 cm^{-1} arises from protonation of E222 via the proton wire. The increasingly negative features developing after t = 0 at 1560 and 1442 cm^{-1} on the same timescale as the growth at 1710 cm^{-1} are due to the disappearance of symmetric and anti-symmetric modes of the carboxylate form of E222. These data for T203V are spectroscopically and kinetically very similar to those for avGFP (not shown) and show protonation of E222 by proton transport along the proton wire of Figure 1 in tens of picoseconds.

Figure 2B reveals important similarities and differences between ESPT in T203V (or avGFP) and S205V, in which the proton wire has been rerouted via T203 (Figure 1). The most significant similarity is the rise at 1728 cm^{-1} in S205V, which is assigned to protonation of E222, providing spectroscopic evidence that the two proton wires in these mutants have a common terminus, even though the rate of ESPT is greatly reduced (the peak transient absorption is reached in ca 3 ns for S205V rather than < 500 ps in T203V). That this mode appears at higher wavenumber in S205V suggests that the H-bond between E222 and T203 is weaker than that between E222 and S205 in T203V. Significantly, in addition to the slower ESPT, S205V has a lower apparent yield of I* formation (compare the relative amplitudes of the t = 0 phenyl ring bleach at 1592/1596 cm^{-1} to the peak of the E222 transients after 1-3 ns). There are a number of possible reasons for a lower yield, including kinetics (slow formation of I* in S205V in competition with the *ca*. 3 ns I* decay) and spectroscopy (the transition moment may be lower). However, TRIR data for S205V also reveal a rapid ground state recovery not present in T203V; compare the



kinetics at 1592/1596 cm^{-1}, which hardly recovers at all in the first 100 ps in T203V, but show strong recovery in S205V (Figure 2A,B). We can thus assign the relatively low yield to a population of S205V where the proton wire is not properly formed, so the ESPT cannot occur; this population instead relaxes directly from A* to A. The

Figure 2. Picosecond to microsecond transient IR data. All samples in deuterated buffer. A. 0 - 3 ns IR difference spectra for T203V; B. As A for S205V; C. 3 – 100 ns IR difference spectra for T203V (the modes associated with I are highlighted); D. as C for S205; E. Species associated spectra for the T203V photocycle. The inset shows the kinetic scheme; F. as E but for S205V.

existence of such a channel is confirmed by time resolved fluorescence up-conversion, where a 9 ps component in the decay of A* which does not appear in the rise of I* was resolved (not shown). The fast quenching may be associated with intramolecular motion of the chromophore unconstrained by formation of the proton wire; such flexibility promotes rapid internal conversion in the FP chromophore. Figure 2C,D show the GSPT, which is clearly very much slower than ESPT. In particular the nanosecond growth and decay of the 1492 cm-1 mode is informative. This mode is associated with the anionic (I) state of the chromophore, which is being formed by fluorescence decay of I* (formed by ESPT), which subsequently repopulates the ground state. Note the tens of ns kinetics and the similarity of the recovery times for the two mutants.

These data are summarized in Figs 2E and F, which show species associated spectra (SAS) for all states in the photocycle. SAS are obtained from global analysis of the data assuming a specific kinetic scheme (also shown). All the features discussed above are evident in the SAS. The quality of the fit was good, suggesting that no other intermediates are detectable in the photocycle (e.g. from isomerization or electron transfer reactions). The SAS for S205V (Figure 2F) point to significant spectroscopic difference between ESPT active and inactive channels (A_1* and A_2*). The main bleach modes associated with the chromophore are similar, but the modes which arise from interaction with the protein are different; However, after the formation of I* in S205V its decay to A follows a similar pathway to T203V and avGFP.

Two key conclusions can be drawn. The very different ESPT kinetics of T203V and S205V are not reflected in ground state kinetics. This suggests a common rate determining step in the ground state, probably the first step, which is similar between mutants. Finally, comparison with literature data shows a huge KIE (ca 45) consistent with a tunnelling mechanism.

Acknowledgements

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References

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