J|A|C|S COMMUNICATIONS

Published on Web 11/16/2009

Ultrafast Dynamics of Protein Proton Transfer on Short Hydrogen Bond Potential Energy Surfaces: S65T/H148D GFP.

Minako Kondo,[†] Ismael A. Heisler,[†] Deborah Stoner-Ma,[‡] Peter J. Tonge,*[,‡] and Stephen R. Meech*[,†]

School of Chemistry, University of East Anglia, Norwich NR4 7TJ, U.K., and Department of Chemistry, Stony Brook University, Stony Brook, New York 11794-3400

Received September 10, 2009; E-mail: ptonge@notes.cc.sunysb.edu; s.meech@uea.ac.uk

With their unique intrinsic fluorescence, green fluorescent protein (GFP) and its mutants are firmly established as vital tools for imaging living systems.[1] As a result, their photophysics have been widely investigated.[2] One of the most intriguing facts to arise from these studies is that the GFP chromophore exists in neutral and anionic states (A and B) and in an additional anionic state (I) coupled to A by a reversible excited-state proton transfer (ESPT) reaction.[3] Within a few picoseconds of excitation of the neutral A state, the anionic I* state of the chromophore is generated, and this state emits the characteristic green fluorescence. Simultaneously, a glutamate residue (E222) is protonated in a three-step proton transfer (PT) reaction along a short proton wire.[4,5] The combination of this ESPT reaction, which is unique in proteins, with the ability to create and structurally characterize mutants of GFP suggests the possibility that GFP can be used as a model system with which to investigate PT reactions in proteins.[6]

PT reactions play important roles in the functioning of many proteins and can occur over relatively long distances, leading to the idea of transport via proton wires.[7,8] Experimental studies of PT dynamics in proteins are hampered by the large number of protons present and the facile nature of the reaction. ESPT in GFP permits photoinitiation of a specific PT reaction, and ultrafast spectroscopy allows the progress of that reaction to be monitored. Thus, ultrafast spectroscopy of GFP and its mutants allows detailed characterization of PT reactions in proteins, just as it has for PT reactions in molecules[9,10] and aqueous solution.[11]

Here we report a time-resolved fluorescence study of ESPT in S65T/H148D GFP. This mutant possesses a short (2.4 Å) hydrogen bond between the chromophore's hydroxyl group and the introduced aspartate, allowing it to serve as a model for short-H-bond-based PT dynamics in other proteins.[12,13] Short H bonds are a common feature in the structures of several important enzyme catalytic centers,[14] although their exact role has been a matter of controversy.[15] Recent high-resolution structural studies of photoactive yellow protein (PYP) suggest a role for short, low-barrier H bonds in the PYP photocycle.[16] The present study of S65T/H148D GFP provides new insights into the dynamics on short H bond potential energy surfaces (PESs).

The S65T/H148D mutant was first characterized by Remington and co-workers, who discussed the structure and photophysics in detail.[12,13,17] The observed short H bond resulted in a strongly perturbed, red-shifted A-state absorption relative to that for the pH dependent, single mutant S65T GFP. In contrast to the neutral form of S65T GFP, excitation of S65T/H148D generates the I state with high efficiency that, while not resolved, was shown to be faster than 175 fs.[12,13] In addition, the deuterium isotope effect observed in wild-type (wt) GFP was absent. As the proton wire to E222 is re-formed in S65T/H148D, it is possible that E222 could again serve as the proton acceptor.[13] However, Stoner-Ma et al.[18] reported transient IR spectroscopy of S65T/H148D GFP in which protonation of E222 was not observed. In fact, within the 400 fs time resolution of that experiment, only the formation of a perturbed (compared to S65T GFP) excited state was seen, with no further dynamics other than relaxation to the ground state.

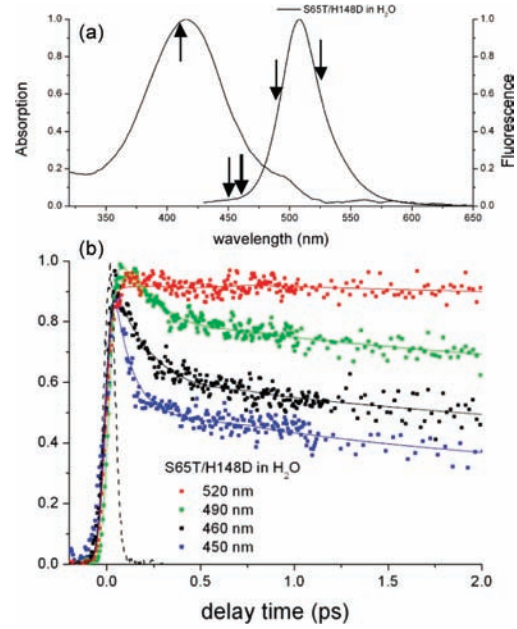

Figure 1. (a) Electronic spectra of S65T/H148D GFP. Arrows indicate excitation (415 nm) and emission wavelengths. (b) Time-resolved fluorescence data with time resolution indicated (dashed line). Time constants are presented in the Supporting Information.

In this work, the time resolution of the ultrafast fluorescence up-conversion experiment was improved, allowing us to probe in real time the PT dynamics on short H bond PESs in a protein environment. The apparatus has been described in detail elsewhere.[19] The resolution was improved by using reflective optics and thin nonlinear crystals and by the addition of dispersive mirrors to recompress both excitation and up-conversion beams. Up-conversion of Raman scattering from heptane revealed a time resolution better than 70 fs. Figure 1b shows the time-resolved fluorescence spectra of S65T/H148D GFP recorded at the wavelengths indicated on the electronic spectra with corresponding fits to a three-exponential function (solid lines in Figure 1b). Care was taken to eliminate contributions to the signal from Raman scattering (see the Supporting Information).

[†] University of East Anglia.
[‡] Stony Brook University.

10.1021/ja907690g

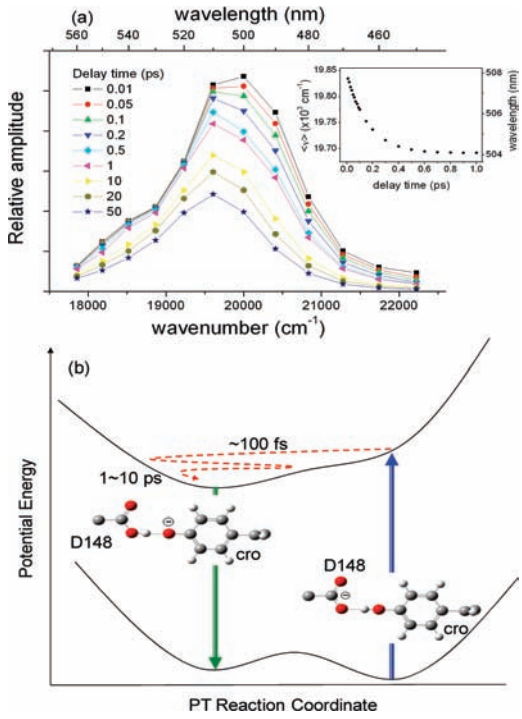

Figure 2. (a) Time-dependent emission spectra and (inset) mean frequencies. (b) Model PES illustrating ultrafast PT and subsequent relaxation.

The dynamics are very different from those expected for an excited-state equilibrium between protonated and deprotonated forms of the chromophore. At all wavelengths, the decay was multiexponential. On the extreme blue edge of the emission, a sub-100 fs time constant with a weight greater than 50% was clearly resolved. In addition, decay times of a few picoseconds and >100 ps were found (see the Supporting Information). The long lifetime is associated with the relaxed I* state. No rising component of the emission was detected even on the red edge. Identical results were obtained in protonated and deuterated solutions. These data suggest an ultrafast evolution along the ESPT reaction coordinate following electronic excitation, with a fraction of the long-lived deprotonated I* state being formed within the 50 fs time resolution. This behavior is quite different from the picosecond-time-scale, deuterium-isotope-dependent establishment of the proton donor−acceptor equilibrium observed following electronic excitation of wtGFP.[3]

Evolution on a reactive PES is best represented by time-dependent emission spectra obtained from a three-dimensional intensity−wavenumber−time surface calculated using fluorescence decay data recorded across the emission spectrum (Figure 2).[19] The time-resolved spectra show a subpicosecond collapse of emission on the high-energy side followed by a smaller gradual red shift on picosecond and longer time scales. We assign the fastest process to the ultrafast PT reaction and the picosecond relaxation, which involves both a time-dependent Stokes shift and significant spectral narrowing, to a combination of vibrational cooling in the I* state of the chromophore and relaxation of the protein environment to accommodate the new charge distribution.

Approximate PESs can be drawn for this process (Figure 2b). The solution pK_a of the aspartate acceptor is ∼3.9 (and probably higher in the hydrophobic protein environment), whereas that of the chromophore's phenolic hydroxyl is 8.1.[20] Thus, even though the aspartate perturbs the spectrum and can form a short H bond with the chromophore, the proton is localized closer to the phenolic oxygen rather than shared equally (as in a low-barrier H bond).[21] This is consistent with the absence of a downfield proton resonance in the NMR spectrum.[13] The situation in the excited state is quite different, as the chromophore is a strong photoacid with a pK_a* calculated to be as low as 0.5 in solution[22] (the calculation does not apply exactly to the protein, where the energy levels and chromophore structure differ from those in solution). However, it is likely that the decreased hydroxyl pK_a on excitation drops below that of the aspartate. Therefore, at equilibrium, the proton is located on the aspartate (Figure 2b). The short distance and relative pK_a ensure that the PT coordinate is barrierless, consistent with the observed ultrafast reaction. Thus, upon Franck−Condon excitation, the system is created far from equilibrium, and the proton translates rapidly (sub-100 fs) along the PT coordinate. On this time scale, no other structural changes occur in the protein. The ps component observed in the time-resolved spectra reflects vibrational cooling in the PT coordinate and reorganization of the environment (Figure 2b).

In summary, we have measured time-resolved PT dynamics on a short H bond within a protein. PT reactions, which are common in proteins, can occur on a sub-100 fs time scale in response to changes to the local environment, such as pK_a or H-bond strength. The present data can test simulations and calculations of such protein PT reactions.

Acknowledgment. We are grateful to EPSRC, NSF (CHE-0822587 to P.J.T.), and U.S. Public Health Services (DK-007521) for financial support.

Supporting Information Available: Experimental methods and data analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Tsien, R. Y. *Annu. Rev. Biochem.* **1998**, *67*, 509.
(2) Tonge, P. J.; Meech, S. R. *J. Photochem. Photobiol., A* **2009**, *205*, 1.
(3) Chattoraj, M.; King, B. A.; Bublitz, G. U.; Boxer, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8362.
(4) Brejc, K.; Sixma, T. K.; Kitts, P. A.; Kain, S. R.; Tsien, R. Y.; Ormo, M.; Remington, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2306.
(5) Stoner-Ma, D.; Jaye, A. A.; Matousek, P.; Towrie, M.; Meech, S. R.; Tonge, P. J. *J. Am. Chem. Soc.* **2005**, *127*, 2864.
(6) Stoner-Ma, D.; Jaye, A. A.; Ronayne, K. L.; Nappa, J.; Tonge, P. J.; Meech, S. R. *Chem. Phys.* **2008**, *350*, 193.
(7) Cui, Q.; Karplus, M. *J. Phys. Chem. B* **2003**, *107*, 1071.
(8) Frank, R. A. W.; Titman, C. M.; Pratap, J. V.; Luisi, B. F.; Perham, R. N. *Science* **2004**, *306*, 872.
(9) Lochbrunner, S.; Wurzer, A. J.; Riedle, E. *J. Chem. Phys.* **2000**, *112*, 10699.
(10) Takeuchi, S.; Tahara, T. *J. Phys. Chem. A* **2005**, *109*, 10199.
(11) Mohammed, O. F.; Pines, D.; Dreyer, J.; Pines, E.; Nibbering, E. T. J. *Science* **2005**, *310*, 83.
(12) Shi, X.; Abbyad, P.; Shu, X.; Kallio, K.; Kanchanawong, P.; Childs, W.; Remington, S. J.; Boxer, S. G. *Biochemistry* **2007**, *46*, 12014.
(13) Shu, X.; Kallio, K.; Shi, X.; Abbyad, P.; Kanchanawong, P.; Childs, W.; Boxer, S. G.; Remington, S. J. *Biochemistry* **2007**, *46*, 12005.
(14) Gerlt, J. A.; Kreevoy, M. M.; Cleland, W. W.; Frey, P. A. *Chem. Biol.* **1997**, *4*, 259.
(15) Schutz, C. N.; Warshel, A. *Proteins: Struct., Funct., Bioinf.* **2004**, *55*, 711.
(16) Yamaguchi, S.; Kamikubo, H.; Kurihara, K.; Kuroki, R.; Niimura, N.; Shimizu, N.; Yamazaki, Y.; Kataoka, M. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 440.
(17) Elsliger, M. A.; Wachter, R. M.; Hanson, G. T.; Kallio, K.; Remington, S. J. *Biochemistry* **1999**, *38*, 5296.
(18) Stoner-Ma, D.; Jaye, A. A.; Ronayne, K. L.; Nappa, J.; Meech, S. R.; Tonge, P. J. *J. Am. Chem. Soc.* **2008**, *130*, 1227.
(19) Heisler, I. A.; Kondo, M.; Meech, S. R. *J. Phys. Chem. B* **2009**, *113*, 1623.
(20) Bell, A. F.; He, X.; Wachter, R. M.; Tonge, P. J. *Biochemistry* **2000**, *39*, 4423.
(21) Kumar, G. A.; McAllister, M. A. *J. Am. Chem. Soc.* **1998**, *120*, 3159.
(22) Scharnagl, C.; Raupp-Kossmann, R. A. *J. Phys. Chem. B* **2004**, *108*, 477.

JA907690G