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Articles

Concerted Asynchronous Proton Transfer in H-Bonding Relay Model: An Implication of Green Fluorescent Protein

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Theoretical investigations have been performed for the ground state (S_0) and the first excited state (S_1) of the hydrogen bonded green fluorescent protein (GFP) model. The potential energy surface (PESs) of S_0 was obtained by B3LYP method and that of S_1 was obtained by CIS method. Based on the relative stabilities of species and the energy barriers for the proton transfer, it was found that proton transfer could take place both under the ground state and the first excited state. As determined by the proton motions along the reaction coordinate, both the ground state proton transfer (GSPT) and the excited state proton transfer (ESPT) are considered as a concerted and asynchronous process.

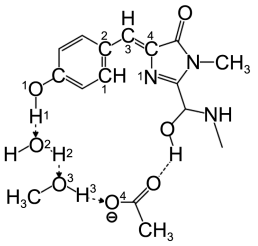
Key Words : Green fluorescent protein, Proton transfer, Concerted, Asynchronous, Theoretical

Introduction

The transportation of a proton from one atom to another in the electronic ground and excited states of molecules is a fundamentally important process that plays a crucial role in lots of reactions, especially chemical and biological processes.[1-3] There are many proteins that have H-bonded networks to serve the function of proton transport, where green fluorescent protein is included. In recent decades, the green fluorescent protein (GFP) has been widely used in imaging studies of protein folding, gene expression, protein trafficking and cell development from a nearly unknown protein.[4,5] Consequently, the 2008 Nobel Chemistry Prize was awarded to Shiomura, Chalfie and Tsien for the discovery and development of GFP.[6] However, due to the structural complexity and the massive size of proteins, direct investigation of proton transfer dynamics is very difficult experimentally. Thus, it is necessary to establish a simplified model *via* quantum simulation.

The *wt*-GFP has two absorption bands at 398 nm (band A, neutral form)[7] and 475 nm (band B, anionic form).[8] Excitation at 398 nm results in an emission centered at 508 nm, while that at 475 nm produces an emission centered at 503 nm.[9] For the conversion from A to B, a mechanism was proposed which is well-known as a three state photoisomerization model with an intermediate state (I).[10] It was confirmed that the change from A to I is simply a change of protonation state, while the change from I to B is a sterical conformational change involving the residues.[11-13] It is well recognized that the process from A to I adopts a multiple proton transfer both in the ground state and the excited state. It undergoes triple proton hopping through chromophore, water 22, Ser 205, Glu 222 hydrogen bonded 'bridge'. Much theoretical work has been undertaken on this issue. Lill and Helms[14] gave the first simulation on the 'three-step' excited state proton transfer and concluded that this reaction was triggered by the transfer of phenolic proton from chromophore to water, and then the other two proton transfer events proceed without barrier. However, in their simulation the first proton transfer from chromophore to water was enforced excluding a fully concerted mechanism (The H1 was shifted to chromophore and the back transfer was forbidden, then simulation was started). Vendrell *et al.*[15] reported that it is a rough concerted process by the treatment of freezing heavy atoms in the proton wires. As well, it was pointed out that the starting point of the ESPT process is the Ser25 proton transfer to Glu222, rather than the phenolic proton of chromophore, and the GSPT should take place *via* the same order. However, Smith *et al.*[16] reported that the ground state proton transfer (GSPT) in GFP is a blend of concerted and stepwise mechanism. The purpose of the present paper is to figure out the proton transfer mechanism, stepwise or concerted, synchronous or asynchronous, which may be helpful to produce GFP variants with desirable properties.

It is necessary to emphasize the difference between concerted/stepwise and synchronous/asynchronous. Concerted/stepwise and synchronous/asynchronous are two different concepts. Concerted/stepwise means that the reaction takes place *via* single/multiple barrier without/with stable intermediates. However, synchronous process means that a chemical process takes place in a synchronous fashion. A concerted reaction is defined by Dewar as "a reaction that



Scheme 1

takes place in a single kinetic step without necessarily being synchronous."[17] For example, if the reaction has a single potential barrier but the movement of the protons is not synchronous, *e.g.*, one moves earlier than the others, it is called as concerted and asynchronous process.

To better understand the reaction mechanism in this study, the reaction center (Scheme 1) in GFP was selected to study the proton transfer reaction. In the present paper, we focused on the protonation state conversion from A to I. The potential energy surfaces (PESs) for both the ground state and the first excited states were investigated.

Computational Method

To reduce the computational cost, we simplified the Ser205 as methanol and Glu222 as acetate, as shown in Scheme 1. It has been pointed out that the ground state structures optimized by Density Functional Theory (DFT) method are reliable compared with those by CASSCF method.[18] Thus, DFT with Becke's three parameterized Lee-Yang-Parr exchange functional (B3LYP), which has been proven to be successful in describing free radicals and intermolecular complexes,[19-21] was chosen to investigate the ground state properties. The ground state structure was fully optimized by B3LYP/6-311+G* level. Then, the first excited state (S_1) structures were fully optimized by Configuration Interactions-Singles (CIS) method with 6-311+G* basis set, using the ground state geometry obtained by HF/6-311+G* as an initial guess. To study the multiple proton transfer, the potential energy surfaces (PESs) of S_0 and S_1 were obtained. Frequency calculations were performed for all the stationary points to verify that they are local minima or transition states as well as to get the zero point energy (ZPE) correction. Intrinsic reaction coordinate (IRC) calculations were carried out to elaborate the pathway connecting the TS to the reactant and the product geometries. All the calculations were performed using a suite of Gaussian 09 programs.[22]

Results and Discussion

Depending on the protonation states of O1 and N1, the isolated GFP chromophore can be denoted as a neutral monomer (NM), anionic monomer (AM), cationic monomer (CM) and zwitterionic monomer (ZM). For all these chromophore, there are two conformers, cis and trans, according to the rotation along the C3-C4 bond (depicted in Scheme 1). The cis neutral chromophore (NM_{cis}) and cis anionic one (AM_{cis}) can undergo proton relay through the hydrogen bonding network. Thus, only NM_{cis} and AM_{cis} were taken into account here. For the ground state, it was found that the cis conformers are more stable than the trans ones for NM and AM by 1.88 and 2.39 kcal/mol, respectively.

In NM_{cis}, the O-H bond length ($R_{O1\text{-}H1}$) of the chromophore was calculated to be 0.965 Å and the C-O bond length ($R_{C\text{-}O}$) is 1.363 Å with a coplanar geometry, as reflected by the dihedral angle C1-C2-C3-C4 (φ) and C2-C3-C4-N1 (τ) with corresponding values of –0.29° and –0.22°. When the phenolic proton was removed, the coplanar AM_{cis} was formed with $R_{C\text{-}O}$ of 1.251 Å and φ/τ of –0.23/–0.59°, respectively. The NM_{cis} forms a complex with water, methanol and acetate through hydrogen bonding network as shown in Scheme 1. The PES of S_0 was obtained by B3LYP/6-311+G* level. From the S_0 PES, two local minima were denoted by neutral complex (NC) and intermediate complex (IC) according to the protonation state of the chromophore, and the transition state was denoted by TS. The corresponding structural parameters are shown in Table 1. By the frequency calculations, NC and IC were confirmed to be local minima without any imaginary frequency, and the TS is a transition state because it has only one imaginary frequency (768i cm^{-1}). The relative energies and energy barrier with ZPE correction are shown in Figure 1. The vibrational motion of the only imaginary frequency includes the stretching mode of H1, H2 and H3 between the donor and the acceptor O atoms, with the most dominant one of H2 as shown in the inset of Figure 1. The vibrational coordinate of this mode is available in Table S1. It was further verified that the TS connects the reactant (NC) and product (IC) by the IRC calculations, no other stable intermediates. That is, GSPT can take place *via* single barrier. As seen in Figure 1, the relative energy difference (ΔE) was calculated to be 0.44 kcal/mol, where NC is slightly more stable than IC. The small energy difference implies an almost 50/50 equilibrium state, which is little derived from the spectrum data. The sensitivity of GFP spectrum to the environment is well known.[23] For instance, the effect of Threonine 203 was reported.[24] It was found that T203F and T203Y could stabilize the deprotonated form, while T203V, T203I and

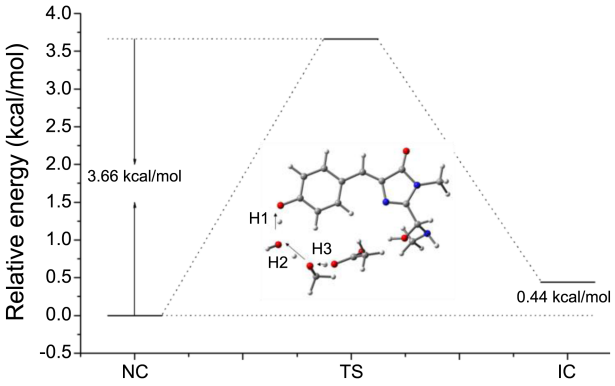


Figure 1. Energy profile for the ground state (inner: the imaginary frequency vibration mode of TS).

T203H shifted the equilibrium state toward the protonated form. In addition, π-π stacking interaction[24] and others[25] also affect the ground state equilibrium conformation. Considering the only small energy barrier (E_b) of 3.66 kcal/mol, the forward ground state proton transfer (GSPT) from NC to IC might happen. However, when the forward GSPT takes place, the reverse process from IC to NC should occur as well because of the smaller E_b of 3.22 kcal/mol. Our calculated energy barrier for GSPT of this GFP model is quite comparable to the experimental value (5.5 kcal/mol).[26] The small energy barrier and stability difference should be a reason for that the GSPT process deeply depends on the system environment such as PH,[27] temperature[28] and so on.

Due to the formation of hydrogen bond, $R_{O1\text{-}H1}$ of NC increases by 0.025 Å to 0.990 Å and $R_{O1\text{-}C}$ decreases by 0.015 Å to 1.348 Å, respectively. The chromophore roughly keeps its coplanarity with φ and τ slightly increasing to 6.34° and 3.56°, then decreasing to 0.15° and 0.30° upon proton transfer to IC. For NC, the negative charge is localized on the acetate moiety, thus, the hydrogen bonding between the methanol and acetate should be the strongest as evidenced by the shorten interatomic distances. Interatomic distance of O3-O4 ($R_{O3\text{-}O4}$) is 2.622 Å, which is shorter than that of O1-O2 ($R_{O1\text{-}O2}$) of 2.708 Å and O2-O3 ($R_{O2\text{-}O3}$) of 2.646 Å. Due to the strong hydrogen bonding, the O-H bond length of methanol ($R_{O3\text{-}H3}$) is much elongated to 1.010 Å. Thus, we expected that H3 might be the first proton to move rather than H1 in GSPT. For IC, on the other hand, the negative charge is localized on the phenoxyl group of chromophore. The hydrogen bond between chromophore and water is the strongest as evidenced by the interatomic distances. Interatomic distances $R_{O1\text{-}O2}$, $R_{O2\text{-}O3}$ and $R_{O3\text{-}O4}$ of IC are 2.621 Å, 2.689 Å and 2.661 Å, respectively. Opposite to the forward GSPT, we propose that H1 might be the first proton to move for the reverse GSPT reaction.

For multiple proton transfer reaction, it is argued whether the process is stepwise or concerted. Because of the single barrier along the S_0 PES, it must be a concerted pathway, even though not all protons move at exactly the same rate or time, which is called a concerted and asynchronous pathway. As shown in Figure 1, the TS geometry shows a largely sequential proton motion. In TS, H3 has migrated from O3 and almost bonded to O4 of acetate with $R_{H3\text{-}O4}$ of 1.003 Å, while H1 barely moves and still resides on the chromophore with $R_{O1\text{-}H1}$ of 1.012 Å. At the same time, H2 is in the process of moving across from O2 to O3, with $R_{O2\text{-}H2}$/$R_{H2\text{-}O3}$ of 1.245/1.178 Å, respectively. The OH bond lengths and OO distances variation along the reaction coordinate are shown in Figure 2. As it is clearly shown, the protons in the chain do not move simultaneously and display a distinct ordering. To further explore it, the relative displacement of proton (D_{OH}) at given point along reaction coordinate was investigated, which was defined by Wang *et al.*.[29] As one can see from the left picture in Figure 2, the proton shows clearly sequential motion. And H3 is the first proton to move and H1 is the last one. However, Wang *et al.* defined this motion as a blend of stepwise and concerted model. As stated in introduction, concerted/stepwise means that the reaction takes place *via* single/multiple barrier with/without stable intermediates. The GSPT reaction can take place *via* single barrier without any stable intermediates. Thus, it must be a concerted process. Considering the sequential proton motion, the GSPT reaction should be a concerted and asynchronous process. For the reverse GSPT process, the proton movement is completely in opposite order; the first proton to transfer is H1 and the last one is H3. During the GSPT process, the hydrogen bridge shows a breathing movement as reflected by the OO distances. In TS, O2 and O3 are negative charged, thus, the hydrogen bond between water and methanol is stronger than other two as evidenced by the interatomic distances. $R_{O2\text{-}O3}$ has the smallest value of 2.422 Å, while $R_{O1\text{-}O2}$ and $R_{O3\text{-}O4}$ are 2.525 Å and 2.501 Å, respectively.

Table 1. Some important structural parameters of the hydrogen bonded complexes under the ground state and the first excited (denoted with an asterisk)

	Ground state			Excited state		
	NC	TS	IC	NC*	TS*	IC*
$R_{C\text{-}O1}$	1.348	1.325	1.276	1.328	1.304	1.243
$R_{C2\text{-}C3}$	1.443	1.434	1.417	1.402	1.401	1.409
$R_{C3\text{-}C4}$	1.360	1.366	1.378	1.420	1.414	1.407
$R_{O1\text{-}H1}$	0.990	1.047	1.610	0.955	0.988	1.772
$R_{O2\text{-}H1}$	1.720	1.478	1.012	1.833	1.578	0.962
$R_{O1\text{-}O2}$	2.708	2.525	2.621	2.787	2.566	2.729
$R_{O2\text{-}H2}$	0.997	1.245	1.700	0.957	1.183	1.854
$R_{O3\text{-}H2}$	1.650	1.178	0.990	1.791	1.199	0.951
$R_{O2\text{-}O3}$	2.646	2.422	2.689	2.743	2.380	2.803
$R_{O3\text{-}H3}$	1.010	1.449	1.665	0.963	1.575	1.823
$R_{O4\text{-}H3}$	1.613	1.058	1.003	1.737	0.992	0.958
$R_{O3\text{-}O4}$	2.622	2.501	2.661	2.700	2.561	2.773
φ	6.34	2.00	0.15	1.17	0.25	0.35
τ	3.56	1.52	0.30	33.78	15.16	14.28
φ: C1-C2-C3-C4; τ: C2-C3-C4-N1						

The overall PES of the first excited state was once obtained *via* CASSCF and CASPT2 method by Vendrell *et al.*.[15] All the three protons were placed at 1.0 Å from either the donor or the acceptor O atoms and the heavy atoms in the proton chain were restricted during calculations. A reasonable argument was reported that why complete relaxation of heavy atom may not be meaningful on the ESPT time scale. However, to better understand the proton motion during ESPT, full relaxation simulation was performed here, which is kinetically and dynamically necessary. The S_1 PES was obtained by CIS/6-311+G* method and NC*, IC* and TS* were abstracted. The relative energy and barrier energy with ZPE correction are shown in Figure 3. From the frequency calculations, NC* and IC* were confirmed to be local minima without any imaginary frequency, and TS* to be a transition state with only one imaginary frequency at 1463i cm^{-1} which has a similar vibration mode to that of TS. As well, the TS* was confirmed to connect to NC* and IC*

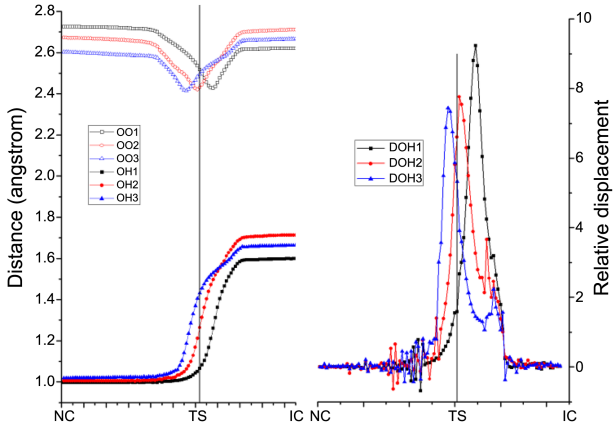


Figure 2. The OH bond length, OO distance (left) and the relative displacement of protons (right) variations along the reaction coordinate under the ground state.

by IRC calculation, implying a single barrier. As shown in Figure 3, this imaginary vibrational mode mostly includes the stretching motion of H2 between the donor (O2) and acceptor (O3). The relative energy difference (ΔE*) between NC* and IC* was calculated to be 4.96 kcal/mole, where IC* becomes more stable than NC*. Thus, in the excited state, the proton transferred structure is more stable. Upon excitation, the energy barrier (E_b*) from NC* to IC* was calculated to be 13.19 kcal/mol, that is, the ESPT might happen. The calculated value deviates from the experimentally estimated barrier (2.8 kcal/mol).[26] There are several reasons for this discrepancy. First, it should originate from the full relaxation considered here, which may lead larger barrier. Second, the real GFP matrix is quite complicated which can deform the planar chromophore by H-bonding, π-π stacking or other forces. This non-planar deformation can accelerate ESPT process.[30] Anyhow, the main purpose is to figure out the proton motion, rather than obtain accurate energy barrier. To take the surrounding environment into account or get correct reaction rate, higher level simulation is needed, such as quantum dynamics/molecular dynamics (QDs/MSs) or quantum mechanical/molecular mechanics (AM/MMs). For the reverse reaction from IC* to NC*, the barrier (18.15 kcal/mol) is significantly larger than the forward process. Thus, the reverse ESPT takes little possibility to happen. Our result is qualitatively in line with experiment.

Consequently, the orbital transition upon excitation was investigated as well. The most dominant contribution of the orbital transition for the first excitation of both NC and IC comes from the HOMO-LUMO transition as determined by the CIS calculations. The HOMOs and LUMOs of complexes are shown in Figure 4. Both the HOMOs and LUMOs of NC and IC show the electrons distributed over the chromophore. The HOMO of NC shows that C3-C4 has a bonding character, while C2-C3 an anti-bonding one, thus, $R_{C2\text{-}C3}$ is longer than $R_{C3\text{-}C4}$ in the ground state. Upon excitation to LUMO, C2-C3 changes into a bonding character, and C3-C4 an anti-bonding one, thus, $R_{C2\text{-}C3}$ (1.402 Å) becomes shorter than $R_{C3\text{-}C4}$ (1.420 Å). As well to IC, there is no electron cloud on the bridging CH group in HOMO, while it is covered by electron cloud with both C2-C3 and C3-C4 anti-bonding. This HOMO-LUMO transition could induce partial charge transfer to the CH group, which can cause the C2-C3 and C3-C4 bond length to change.[31] In addition, HOMO and LUMO of the complex are localized within the chromophore and therefore proton transfer would hardly be affected by the electronic transition, which is consistent with the fact that proton transfer in the system follows the same pathway in both ground and excited states.

Upon excitation from NC to NC*, $R_{C\text{-}O1}$ decreased to 1.328 Å and $R_{O1\text{-}H1}$ increased to 0.955 Å compared with that of S_0 by HF, which implies the enhanced acidity of phenol moeity. That is, it is easier to abstract the phenolic proton under the excited state. At the same time, $R_{O1\text{-}O2}$ decreased by 0.043 Å, while $R_{O2\text{-}O3}$ and $R_{O3\text{-}O4}$ slightly decreased by 0.015 Å and 0.004 Å, respectively. Due to the shorten OO distances, NC* is no longer coplanar as noted by the τ increasing to 33.78° and the φ decreasing to 1.17°. The much decreased $R_{O1\text{-}O2}$ provides strong abstraction force to the H1, which may induce H1 movement prior than other two protons. However, as check the TS* geometry, no significant proton motion change was observed compared to the TS, which can be confirmed by the proton motion in Figure 3 and 5. The TS* shows a similar proton motion to that of TS. For TS*, H3 has been transferred to acetate with $R_{O4\text{-}H3}$ of 0.992 Å, while H1 moves with $R_{O1\text{-}H1}$ increased only by 0.033 Å to 0.988 Å. At the same time, H2 is located near the center of O2 and O3, with $R_{O2\text{-}H2}$ and $R_{H2\text{-}O3}$ of 1.183 Å and

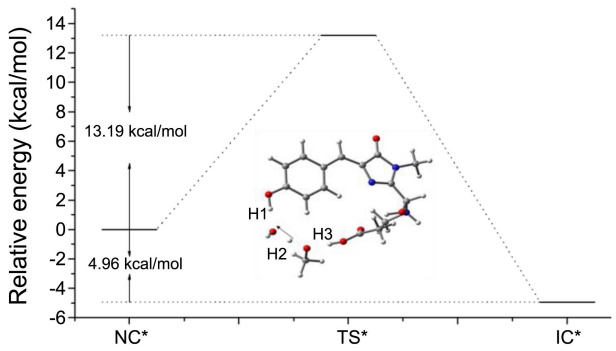


Figure 3. Energy profile for the first excited state (inner: the imaginary frequency vibration mode of TS*).

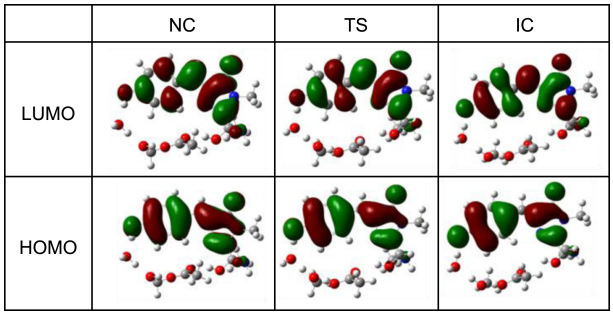


Figure 4. The HOMOs and LUMOs of NC, TS and IC obtained by HF method.

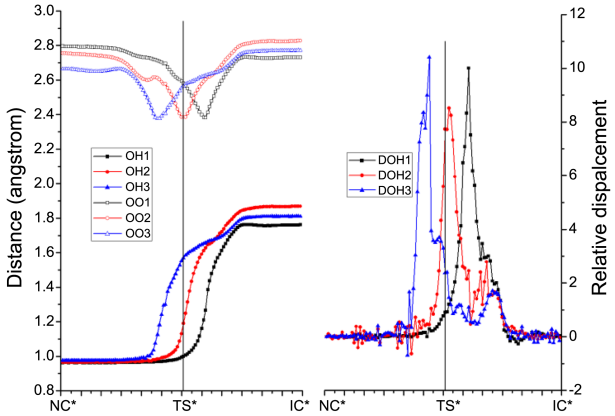


Figure 5. The OH bond length, OO distance (left) and the relative displacement of protons (right) variations along the reaction coordinate under the first excited state.

1.199 Å, respectively. Thus, the ESPT reaction is a concerted and asynchronous process, which is the same to GSPT. H3 is still the first proton to move, then H2 moves, and H1 is the last to move. Thus, our result rules out the proposition in an earlier work[14] that H1 moves first under the excited state. Upon proton transfer, methanol and water become negatively charged. Thus, hydrogen bond strength between O2 and O3 is the strongest with the shortest interatomic distance of 2.380 Å. After proton transfer, τ further decreases to 14.28°, while φ almost keeps the same.

As studied above, concerted and asynchronous triple-proton transfer can take place both under the ground state and the first excited state. However, the real protein environment is quite complicated. The GSPT can only change the protonation state of chromophore, while the ESPT induced by photon absorption may cause cis-trans isomerization.[18] And different proteins contain different residue matrixes which may provide different proton transfer pathway. S65T GFP, a mutation of *wt*-GFP, provides a single-proton transfer pathway from phenol moiety of chromophore to the residue.[32] A new ESPT mechanism that the proton transfer takes place from chromophore to Glu 222 through water and Ser 205 was proposed between anionic and zwitterionic chromophore which would quench the fluorescence of asFP595.[33] As a matter of fact, it was reported that the proton can further migrate through longer H-bonding networks exiting in the barrel.[34,35] Brakemann *et al.* reported a reversibly photoswitchable GFP-like protein with a hydration/dehydration.[36] Nevertheless, this result provides useful information in understanding of the functioning mechanism of GFP.

Conclusion

Multiple proton transfer of GFP model was investigated in the ground state and the first excited singlet state. It was found that both the forward and reverse GSPT reaction, which is affected by the protein environment, can take place due to the small energy (~3 kcal/mol) barrier and small difference (~0.5 kcal/mol) in relative stability between the reactant and the product. Upon excitation, the forward ESPT reaction can occur with an energy barrier of 13.19 kcal/mol. And IC* becomes more stable in energy than NC* upon proton transfer by 4.96 kcal/mol. Both of the GSPT and ESPT reactions take place *via* single barrier without stable intermediate, they must be concerted process. Meanwhile, the protons show asynchronous character. Thus, both GSPT and ESPT are considered as a concerted and asynchronous process. For the forward GSPT and ESPT, the first proton to transfer is the methanol proton (H3) to acetate, and the chromophore proton (H1) is the last one to transfer. While for the reverse GSPT, H1 should be the first, while H3 should be the last to move

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