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Protonic Gating of Excited-State Twisting and Charge Localization in GFP Chromophores: A Mechanistic Hypothesis for Reversible Photoswitching

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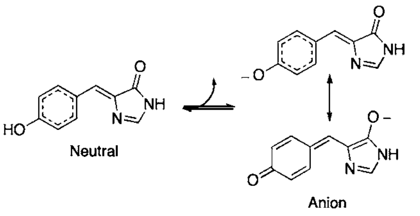
Fluorescent proteins[1] (FPs, homologues of green fluorescent protein,[2] GFP) are powerful tools.[3] Variants which can be reversibly photoswitched between fluorescent (ON) and nonfluorescent (OFF) states (RSFPs, reversibly photoswitchable fluorescent proteins[4]) have potential utility in biooptical technologies.[5] FP chromophores are derivatives of a *p-hydroxybenzylideneimidazolinone* motif[1] (HBI, Scheme 1). We investigated the protonation and photoisomerization photochemistry of HBI using *ab initio* multiple spawning[6] (AIMS) dynamics. *We found that photoisomerization occurs about alternate bonds of the (methine) bridge in a protonation-dependent way (Figure 1).*

Scheme 1. Titration States of the HBI Chromophore



HBI anion is a monomethine dye, defined by the charge-transfer resonance shown in Scheme 1. As the ground state double bond is delocalized, so also is the excited state biradical. Consequently, twisting about *either* of the phenoxy (P) or imidazolinone (I) bonds of the bridge is possible in the excited state of the anion.[7−10] Protonation of the phenoxy group (as in Scheme 1) detunes the resonance, yielding definite bond alternation and a localized excited-state biradical. Excited-state twisting in neutral HBI is favorable *only* about the I-bond (see Figure 2 and Supporting Information (SI)). AIMS data (Figure 1) and electronic structure calculations suggest there is a small residual detuning in the anion, so that I-bond twisting is weakly activated in isolated HBI, while P-bond twisting is barrierless (Figure 2, SI, and ref 9). This preference can be altered by substitution[8,11] and reaction field effects.[10]

Negative-mode RSFPs (e.g., Dronpa[12−16] and others[4,17,18]) are fluorescent (ON) in their native state.[4] Using Dronpa as a specific example, absorbance of ON is dominated by a feature like the GFP “B” band[19] consistent with an anionic chromophore ($\lambda_{abs}^{ON} = 503$ nm[15]).[20] Illumination near the maximum ($\lambda_{exc}^{ON} = 488$ nm[15]) yields fluorescence ($\phi_{fl}^{ON} = 0.85$[15]) with a low yield ($\phi_{sw}^{ON-OFF} \approx 0.00032$[15]) of photoswitching to a nonfluorescent state (OFF).[14,15] Increased intensity drives bulk population to OFF. Absorbance of OFF is similar to the GFP “A” band,[19] suggesting a protonated chromophore ($\lambda_{abs}^{OFF} = 388$ nm[15]).[20] Excitation of OFF ($\lambda_{exc}^{OFF} = 405$ nm[15]) yields reversion to ON ($\phi_{sw}^{OFF-ON} = 0.37$[15]) with negligible fluorescence ($\phi_{fl}^{OFF} = 0.02$).[15] The absorption wavelengths suggest that the ON−OFF transition involves chromophore protonation,[16] but X-ray structures implicate *cis*−*trans* isomerism about the I-bond.[12,18] Apparently, a *correlated* change in chromophore titration and isomerization state underlies the switch.

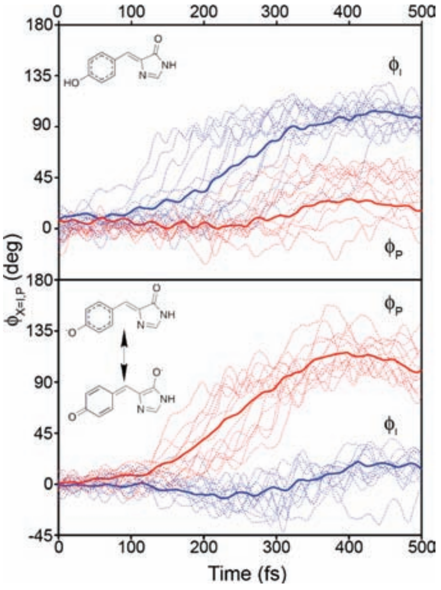


Figure 1. Phenoxy-bridge (ϕ_P) and imidazolinone-bridge (ϕ_I) dihedral angles for neutral (top) and anionic (bottom) S_1-excited HBI along 15 AIMS trajectories with SA2-CAS(2,2)/6-31 g. Individual trajectories (dotted) and averages (solid) are shown. The neutral form twists about the I-bond, and the anion twists about the P-bond.

The AIMS results in Figure 1 show that the neutral chromophore leads exclusively to twisting about the I-bond after photoexcitation, while the anionic chromophore leads exclusively to twisting about the P-bond. The origin of this behavior for the neutral chromophore is readily apparent from cuts of the excited state potential energy surface as shown in the lower panel of Figure 2. However, both I-bond and P-bond torsion are available channels for the anionic chromophore as shown previously[7,9] and in the upper panel of Figure 2. This conclusion holds both at the state-averaged complete active space with two electrons in two orbitals, i.e. SA-2-CAS(2/2), level of electronic structure theory used in the AIMS simulations and also when dynamic electron correlation effects are included with multireference perturbation theory, i.e. SA-2-CAS(2/2)-MRPT2. A slight bias toward P-bond torsion may be seen in Figure 2, and this is in accord with previous PES calculations by Olivucci and co-workers[9] using CAS with a larger active space. Thus, the present result may be a combination of effects from dynamics and the shape of the PES. The isolated anion chromophore lies on the “razor’s edge” between I-bond and P-bond torsion, allowing

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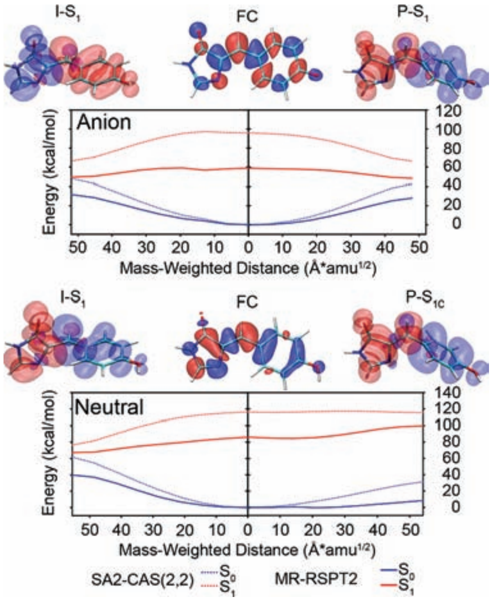

Figure 2. Potential surface slices through the CAS(2/2)/6-31 g (on which AIMS simulations were run, dotted line) and CAS(2/2)-MRPT2 (solid line) S_0 and S_1 PESs of anionic (top) and neutral (bottom) HBI. Slices are linear interpolations between Franck−Condon (FC) and S_1-relaxed geometries twisted about the P (P−S_1) or I (I−S_1) bond. Neutral HBI has no P-twisted S_1 minimum, so a constrained S_1-relaxed geometry (P−S_{1C}) was used. S_1−S_0 difference density isosurfaces at anchor points are shown (isodensity ± 0.001; red is positive; blue is negative). Twisted HBI anion shows charge localization with stereospecific polarity. Only one polarity is accessed in the neutral form. Inversion of polarity between neutral and anion at I−S_1 suggests protonation coupled to photoisomerization.

relatively minor environmental and/or dynamical effects to tune between the two outcomes.

HBI[7] and derivatives[8,11] have low-lying conical intersections near twisted geometries. In isolated HBI, these are higher in energy than the twisted S_1 minima. Accordingly, little population decay is seen in the AIMS simulations (see SI).

Because similar photoswitching is observed in a variety of RSFPs from different sources but with identical chromophores,[12,18,21] it is likely that the switching behavior emanates from the properties of the chromophore itself. We suggest that RFSP-like switching can be generated from the HBI chromophore with only two environmental effects. First, there should be a means for arresting P-bond torsion and directing this channel toward fluorescence. This could be accomplished by H-bonds that limit the extent of P-bond torsion, and such coordination is known to occur in RSFPs in the ON state.[12,18] Fluorescence yields are higher than the ON−OFF switching yield in RSFPs,[15] as would be expected from the AIMS results shown here if fluorescence and switching are associated with P-bond and I-bond torsion, respectively. A conserved arginine residue coordinating the imidazolinone in RSFPs[12,18] could act to further maintain the bias toward P-bond torsion. The flatness of the PES toward I- and P-bond torsion suggests that higher energies will result in a more equitable branching between the two channels, amounting to an increase in the I-bond torsion identified with switching. Such increased energy is likely to arise from excitation with increased intensity (the experimental means for promoting switching) through either direct excitation of a higher-lying dark state (which would rapidly convert to vibrationally excited S_1) or local temperature increases resulting from repeated excitation and fluorescence of the chromophore.

Second, protonation state and isomerization need to be coupled for I-bond torsion. Figure 2 shows the S_0/S_1 electron density differences at the FC point and the I/P-twisted geometries. Neutral and anionic forms of the chromophore behave similarly on P-bond twisting but oppositely on I-bond twisting. I-Bond twisting in the anionic excited state leads to electron accumulation on the phenoxy (Figure 2 and ref 9), suggesting increased *basicity*. I-bond twisting in the neutral excited state leads to electron depletion on the phenoxy (Figure 2), suggesting increased *acidity*. This suggests that the anionic excited/neutral ground and neutral excited/anionic ground states are *diabatically correlated and the diabats have a distinguishable* pK_A.[22] Given a reservoir with an intermediate pK_A, acid−base chemistry can be coupled to photoisomerization, as seen in RSFPs.[4,12,15−18]

A more detailed sketch can be found in the SI. Importantly, the mechanism predicts that if P-bond torsion is unhindered in an RSFP, photochromic switching could be preserved without fluorescence. Since fluorescence is of primary technological interest,[5] such variants may exist, having received little attention.

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Supporting Information Available: Illustration of mechanism and computational details. This material is available free of charge via the Internet at http://pubs.acs.org.

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