Communication

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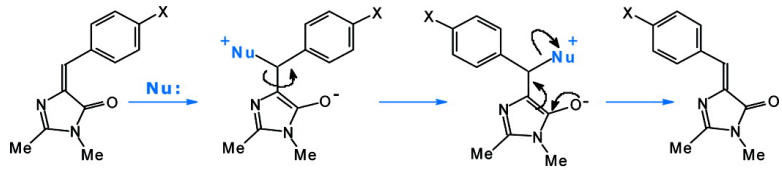

E-XBDI *Z*-XBDI

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Isomerization in Fluorescent Protein Chromophores Involves Addition/Elimination

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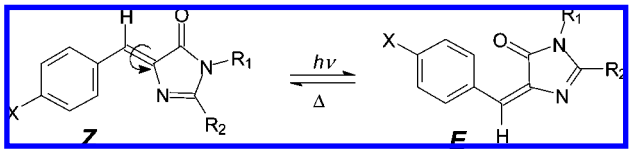
Fluorescent proteins related to the green fluorescent protein (GFP) are thought to undergo Z/E photoisomerization between fluorescent and dark states. The Z form ("cis") is the resting fluorescent form, while the E ("trans") form is nonfluorescent, although exceptions are known.[1]

Such proteins are also characterized by "blinking", that is, temporary conversion to a nonfluorescent form, which has been variously attributed to triplet formation[2] or proton transfer.[3] Additionally, strong support for cis/trans isomerization is provided by the behavior of kindling fluorescent proteins,[4] in which the resting nonfluorescent form has trans stereochemistry. Upon irradiation into the long-wavelength band, the protein is "kindled" to the fluorescent cis form. That kindling is the result of photoisomerization is given strong support by recent single crystal X-ray determinations of both forms of two kindling proteins, *dronpa* and *mTFP0.7*, which differ in the stereochemistry about the benzylidene bond of the chromophores.[5] A key unresolved issue in the photophysics of the fluorescent proteins is whether the cis/trans isomerization is related to the blinking phenomenon, which, in addition to isomerization, has been ascribed to protonation and triplet formation mentioned earlier. We have observed that, in solution, the isolated UV-excited GFP chromophore undergoes a fast relaxation to yield a resting state which shows considerable twisting[6] at the same time that the excited-state is quenched.[7] In the protein, however, the common view is that the protein prohibits twisting about the double bond. Nonetheless, both calculations[8] and the aforementioned kindling behavior require that, in at least some instances, formal isomerization, that is, decay from the twisted intermediate onto the trans hypersurface, must be permitted. Moreover, the quite wide variation in blinking behavior as a function of protein structure—conditions which either facilitate or inhibit such isomerization—suggest that blinking and isomerization are intimately involved.

Scheme 1. Isomerization in the GFP Chromophores



While the photoisomerization mechanism has been the subject of several studies[9] and corresponds in unexceptional ways to the mechanisms of other arylidene chromophores, the mechanism of the thermal reverse isomerization is more problematic. The blinking phenomenon requires that isomerization, if involved, be thermally reversible. Tonge has recently measured the rates of thermal isomerization of the representative E protein chromophore *p*-hydroxybenzylidenedimethylimidazolinone (**HOBDI**, X = OH)[10] following photoisomerization from the Z form and obtained a barrier of 13.1 kcal/mol for the isomerization from an Arrhenius plot.[11] Surprisingly, little account has been taken of the observation that a high level *ab initio* calculation from Weber, et al., produces a barrier of 57 kcal/mol,[12] a value more typical of double-bond isomerization barriers for unexceptional benzylidene molecules such as **HOBDI**. Alternatively, tautomerization to a zwitterionic intermediate via an uncalculated transition structure, which then rotates with a 7.3 kcal/mol[12] barrier, presents another facile mechanism for isomerization. This poses a conundrum: how does one resolve the discrepancies between highly credible experimental and theoretical determinations?

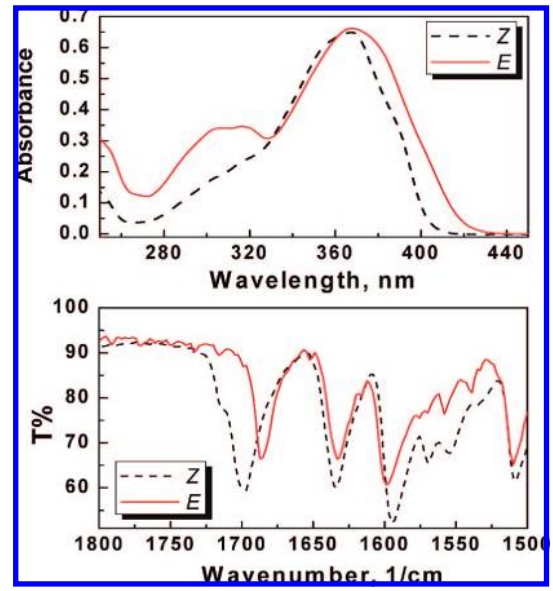


Figure 1. UV−vis (top) and IR (bottom) absorption spectra of E and Z isomers of ***p*-MeOBDI** in MeCN.

To develop further insight into this process, and to exclude possible proton-transfer processes in the isomerizations, we first examined the methyl ether of **HOBDI**, **MeOBDI**. In the process of exploring the optimal solvents for our physical studies, we encountered a surprise. That is, in benzene and acetonitrile, no thermal isomerization occurred, but isomerization was readily observed in methanol and, at slower rates, in Me_2SO-d_6.[13] Indeed, we were able to isolate the trans isomer of **MeOBDI** by silica gel chromatography and record its NMR, ir, and electronic absorption spectra (see Figure 1 and Supporting Information).[14] Again, this result is inconsistent with a facile thermal isomerization, and suggests that a more complex process is intervening.

These waters were further muddied by a recent claim[15] that an analogous 4-methylbenzylidene derivative does not isomerize under

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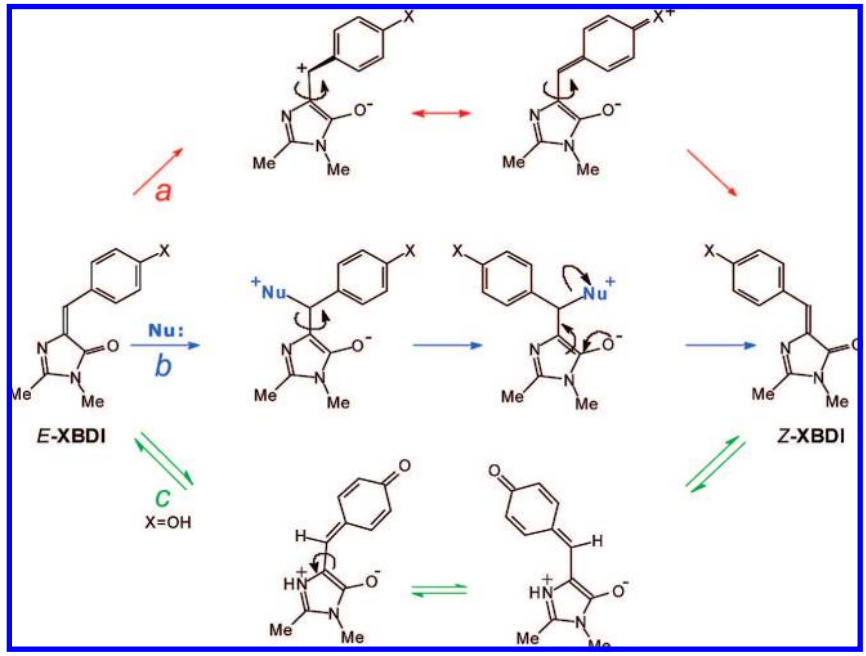

Figure 2. Pathways for the E-XBDI isomerization. (a) Direct; (b) addition/elimination; (c) isomerization by tautomerization.

similar conditions, which was rationalized as the result of a strong "push-pull" transition structure (see second resonance structure for Path a in Figure 2) in which a donating group such as hydroxyl weakens the double bond character. However, such a structure belies the strong electron donation effect of the methyl group, which would be inconsistent with a difference in rates of 3 or more orders of magnitude.

To resolve this conundrum, we embarked on the synthesis and rate study of the effect of substituents on the thermal isomerization of *cis*-**XBDI**, using substituents that are either donors or acceptors. We reasoned that use of a classical Hammett plot would reveal the nature of the isomerization and confirm or disprove the proposed mechanism. Thus we used or synthesized BDI derivatives with decreasing para-donating ability, HO, CH_3O, CH_3, H, and Cl, with the expectation that a Hammett plot would produce a negative ρ value if the "push-pull" zwitterionic nature of the transition state dominates as in Path a. In the process, we discovered that the reported[15a] ethyl derivative does not have the assigned structure, an observation we confirmed by single-crystal X-ray diffraction. The NMR of the reported derivative is characterized by a downfield doublet at 7.45 ppm, whereas every BDI derivative we have made has a doublet at 8.0−8.2 ppm for the alpha aryl proton. Because this product was obtained in 15% yield, we conclude that this was a byproduct of the reaction.

In our hands, all five analogs underwent ready photoisomerization in a variety of solvents, including MeOD, Me_2SO-d_6, and MeCN-d_3, allowing us to study the thermal reversion by proton NMR spectroscopy (see Supporting Information). In addition, all photoisomers, including **MeBDI**, underwent the reverse reaction at a convenient rate in Me_2SO-d_6. Ignoring **HOBDI** (see below), the plot of $\log(k_X/k_H)$ gave a positive correlation with σ, with the best results obtained in acetonitrile (see Figure 3), a result inconsistent with the previous mechanistic analysis.[15a,b]

What, then, is the correct mechanism? The key is provided by the positive ρ and by the observation that thermal isomerization occurs only in nucleophilic solvents such as Me_2SO-d_6. This result is reminiscent of that for S_NAr reaction, in which substitution by an electron-releasing group at the ipso position retards the reaction, producing a positive ρ.[16] Thus we conceived instead a similar mechanism, an addition/elimination mechanism (Path b in Figure 2).

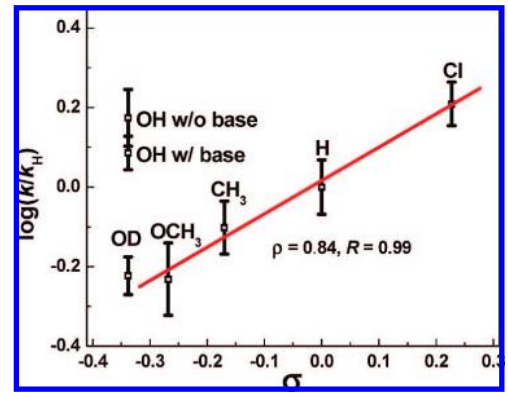

Figure 3. Hammett $\sigma\rho$ plot for *para*-**E-XBDI** thermal isomerization in CD_3CN in the presence of 0.089 M DABCO; $k_H = 1.4\ 10^{-5}\ s^{-1}$.

To test this mechanism, we examined the thermal reversion in a variety of solvents. As a test case, we again used **MeOBDI**, since this molecule is isoelectronic to **HOBDI** without allowing for the possibility of intra- or intermolecular proton transfer involving hydroxyl group. Unlike the results in Me_2SO-d_6, we observed no isomerization in the absence of added base in either acetonitrile or benzene. However, both exhibited facile isomerization in the presence of primary amines, for example, propyl amine. In the case of tertiary amines, either 1,4-diaza[2.2.2]bicyclooctane (DABCO) or 4-(dimethylamino)pyridine (DMAP) isomerization was observed only in acetonitrile solvent.

If the proposed mechanism were correct, the rate of isomerization should be rate-limiting in formation of the adduct, which in term should be proportional to the concentration of nucleophile. Again we used DABCO with **MeOBDI** in acetonitrile. As anticipated for the proposed bimolecular mechanism, isomerization was linear with base concentration over 1 order of magnitude (see Figure S3, Supporting Information). Repeating the Hammett plot with 0.089 M DABCO, we obtained a ρ-value of 0.84, consistent with nucleophilic attack (see Figure 3). **HOBDI** itself isomerized without added base in MeCN-d_3, but the isomerization was inhibited by the presence of added amine.

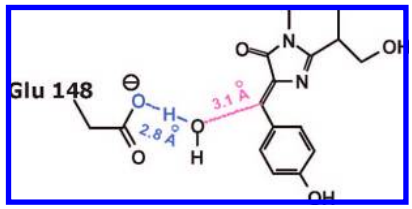

Figure 4. Nucleophile in *mTFP0.7*.

The results we have observed are congruent with the addition/elimination mechanism shown as Path b in Figure 2. Although this is a plausible mechanism, literature precedents are fairly rare. The isomerization of cinnamate anion has been shown to involve such processes.[17] Such mechanisms have been proposed as possible mechanisms in biological isomerizations.[18] A more immediate question is the relevance to the chemistry of fluorescent proteins, particularly to the blinking phenomenon. As noted earlier, if blinking is associated with cis/trans isomerization, blinking back "on" requires either reverse photoisomerization or a thermal process, and this work demonstrates that the unassisted process has too high a barrier to compete. Conversely, the addition/elimination mechanism, as a bimolecular diffusional process, is also relatively slow. In a protein, however, such diffusion is irrelevant; rather, the question is whether there is a competent nucleophile to initiate such a mechanism. In the particular case of *mTFP0.7*, fluorescence recovery through reisomerization occurs over a span of minutes.[5a] Is there a nucleophile available for this process? Indeed there is! The crystal structure of *trans-mTFP0.7* is characterized by the presence of a water molecule interposed between a glutamate and the benzylidene carbon of the chromophore such that the glutamate can promote addition of water to the double bond (see Figure 4).[19]

Finally, we consider the isomerism of **HOBDI** itself. This derivative, the closest analogy to the wild-type GFP chromophore, undergoes isomerization in the absence of a nucleophile, although its limited solubility in nonpolar solvents precluded studies in benzene. Curiously, the presence of DABCO depressed the isomerization rate (see Figure 3). On the one hand, if the Falk's mechanism were valid, the methoxy derivative **MeOBDI**, with similar resonance characteristics, should undergo equally facile isomerization. On the other hand, if deprotonation were required, then the base should accelerate the reaction. Furthermore, calculations are not consistent with the deprotonated form of **HOBDI** undergoing faster reaction. An alternative mechanism was intimated by Weber, and suggested by Yang, that is, tautomerization to a quinomethane derivative (see Path c in Figure 2).[9e] This mechanism is supported by a small but measurable deuterium isotope effect (see Figure 3 and Supporting Information). The depressive effect of DABCO thus may be the result of a reduced equilibrium concentration of tautomer. In DMSO, we observed measurable, if slow, rates for **HOBDI** and **MeOBDI**.[13] We note that the latter cannot undergo such a mechanism, suggesting that tautomerization is highly solvent dependent. Does this mechanism apply within the β-barrel? We can only speculate, although the crystal structure of *mTFP0.7* shows a favorable disposition for nucleophilic attack via Path b (see Figure 4).

Addition/elimination emerges as a compelling mechanism for isomerization of fluorescent protein chromophores. The presence of an internal nucleophile thus becomes an additional point of mutation of such chromophores that may mediate their photophysical properties. Such mutations are being explored.

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Supporting Information Available: Synthetic and kinetic details, and NMR spectra for all **BDI** derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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