

Photoisomerization of the green fluorescence protein chromophore and the *meta*- and *para*-amino analogues†

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The $Z \rightarrow E$ photoisomerization and fluorescence quantum yields for the wild-type green fluorescence protein (GFP) chromophore (*p*-HBDI) and its *meta*- and *para*-amino analogues (*m*-ABDI and *p*-ABDI) in aprotic solvents (hexane, THF, and acetonitrile) and protic solvents (methanol and 10–20% H₂O in THF) are reported. The dramatic decrease in the quantum yields on going from aprotic to protic solvents indicates the important role of solvent–solute hydrogen bonding in the nonradiative decay pathways. The enhanced fluorescence of *m*-ABDI is also discussed.

The green fluorescence protein (GFP) discovered in the Pacific jellyfish *Aequorea victoria* has been widely used as a biological marker.¹ The green fluorescence (508 nm) is known to result from the anionic form of the chromophore, *p*-hydroxybenzylideneimidazolinone (*p*-HBI), through excited-state proton transfer (ESPT) from the phenolic oxygen to the protein matrix.¹ However, the dramatic difference between the fluorescence quantum yield (Φ_f) for *p*-HBI in the protein β -barrel ($\Phi_f \sim 0.8$) and that in fluid solutions ($\Phi_f < 10^{-3}$) has puzzled scientists for many years.^{1–10} More specifically, a unified answer for the questions as to: (1) what the ultrafast non-radiative decay pathway is for both the neutral and anionic forms of the free chromophore, and (2) how the protein matrix suppresses such a decay route so efficiently, remained to be established. In this context, the photodynamics of the dimethyl derivative of the GFP chromophore (*p*-HBDI)^{2–6} and many other related model systems^{7,8} and GFP mutants^{7,9} has been extensively investigated.

The currently discussed nonradiative decay mechanisms for *p*-HBDI in fluid solutions are all associated with its torsional motions, including (a) the torsion of the exocyclic C–C bond (φ) (see Chart 1) that leads to the formation of a twisted intramolecular charge transfer (TICT) state, and either (b) the C=C bond torsion (τ) or (c) a concerted torsion of both the C–C and C=C bonds (*i.e.*, the hula twist) that leads to $Z \rightarrow E$ photoisomerization. However, none of these mechanisms can satisfactorily account for all the key experimental and computational results. For example, the C=C bond torsion^{3,4} conflicts with the volume-conserving nature of the decay process and with the fast ground-state recovery of *p*-HBDI in alcohols (*i.e.*, little *E*-isomer formation).² In the case of the C–C bond

torsion,⁵ not only its disagreement with a volume-conserving path but also the absence of any explicit conical intersection near the twisted intermediate is the deficiency.³ Although a concerted torsion of the C–C and C=C bond^{2,6} is volume-conserving, it encounters an unfavorable uphill coordinate.^{3,4} Another common problem for the TICT and hula-twist mechanisms is the lower possibility of these torsions being effectively inhibited by the protein matrix.¹⁰

We report herein a new approach toward understanding the nonradiative pathways of *p*-HBDI through quantitative determination of the $Z \rightarrow E$ photoisomerization quantum yields (Φ_{ZE}) for *p*-HBDI and its *para*- and *meta*-amino analogues *p*-ABDI and *m*-ABDI in different solvents. Our results have led to a new excited-decay mechanism proposed for *p*-HBDI in fluid solutions.

Our design concepts for the investigation of ABDIs are manifold. First, the excited-state behaviour of the anionic form of *p*-HBDI (a phenolate) might be correlated to that of *p*-ABDI (an aniline), because the degree of photoinduced intramolecular charge transfer (ICT) might be similar, as reflected by the same pK_a changes ($pK_a = 6$) on going from S_0 to S_1 for 2-naphthol (with a phenolate-like conjugate base) and the conjugated acid of 2-aminonaphthalene.¹¹ This correlation might resolve the solubility problem upon measuring Φ_f and Φ_{ZE} for the anionic form of *p*-HBDI in nonpolar solvents. Second, a reliable measurement of Φ_{ZE} requires a stable *E* isomer of *p*-HBDI either in the neutral or anionic forms. However, it has been reported that *p*-HBDI undergoes efficient thermal $E \rightarrow Z$ isomerization in D₂O and CD₃OD at room temperature.^{12,13} Whereas Falk and coworkers have attributed the facile thermal isomerization to a weak double-bond character of the exocyclic C=C bond due to significant delocalization of the oxygen lone-pair electrons,¹³ an alternative interpretation is the presence of an acidic phenol group (the pK_a value for phenol is 10.0 in H₂O and 18.0 in DMSO),¹⁴ which self-catalyzes the isomerization reaction. Along this line, the thermal isomerization would become even more efficient upon irradiation because of the enhanced acidity of *p*-HBDI in S_1 .^{8c} Since the ABDIs are free of acidic groups (the

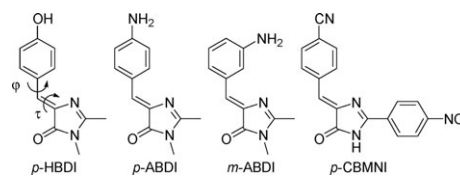


Chart 1

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† Electronic Supplementary Information (ESI) available: Experimental methods, synthetic details, and spectroscopic data for *p*-ABDI and *m*-ABDI; crystal structure data for *m*-ABDI (CCDC number 667977).

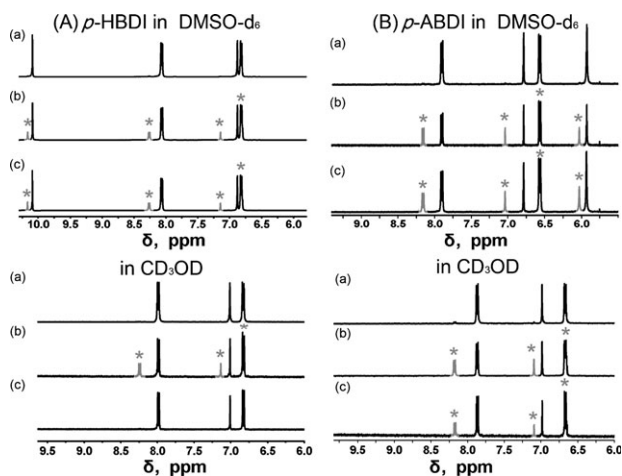


Fig. 1 The ^1H NMR spectra for (A) *p*-HBDI and (B) *p*-ABDI (~ 2 mM) in $\text{DMSO-}d_6$ and CD_3OD (a) before and (b) after 20 min of irradiation with 350 nm light and (c) after 24 h at room temperature in the dark. The *E/Z* ratio is the same for the conditions (b) and (c) for all cases except for *p*-HBDI in CD_3OD , where the *Z* form completely isomerized back to the *E* form.

$\text{p}K_a$ value for aniline is 30.7 in DMSO),¹⁵ complication of the thermal $E \rightarrow Z$ isomerization for the determined Φ_{ZE} would be reduced. Indeed, as shown in Fig. 1, the $E \rightarrow Z$ thermal isomerization of *p*-HBDI is negligible in $\text{DMSO-}d_6$ but efficient in CD_3OD , consistent with the larger $\text{p}K_a$ value of phenol in aprotic vs. protic solvents. In addition, the *E* isomers of *p*-ABDI and *m*-ABDI are rather stable in both $\text{DMSO-}d_6$ and CD_3OD . Third, comparison of the amino-modified GFP chromophores and aminostilbenes is inherently interesting, because the issue of TICT formation vs. photoisomerization has also been extensively discussed for the latter systems.¹⁶ Our recent studies on *trans*-aminostilbenes have shown that the single-bond torsion that leads to the TICT state does not couple with the *trans* \rightarrow *cis* double-bond torsion.¹⁶ In other words, the formation of a TICT state competes with the *trans* \rightarrow *cis* photoisomerization, leading to low photoisomerization quantum yields (Φ_{is}). Thus, the Φ_{ZE} values might allow us to elucidate the excited-decay mechanism for ABDIs and thus for *p*-HBDI, provided that the C–C and C=C bond torsions in the latter are also decoupled.

The ABDIs were synthesized using our modification of Niwa's procedure described in the ESI†.¹⁷ As is the case for *p*-HBDI, the *Z* isomers of the ABDIs were obtained. One piece of solid evidence is provided by the X-ray crystal structure of *m*-ABDI†.

Both the values of Φ_{f} and Φ_{ZE} for *p*-HBDI, *p*-ABDI, and *m*-ABDI in protic (MeOH and $\text{H}_2\text{O-THF}$) and aprotic (hexane , THF , and MeCN) solvents are shown in Table 1. Since the calculated potential energy profiles for *p*-HBDI^{6a} resemble those for stilbenes, where the conical intersection locates at a twisted angle of 90° , it is reasonable to assume that the probability of product formation for the *E* vs. *Z* isomer from such a perpendicularly twisted geometry is *ca.* 50%, in analogy to the case of stilbenes.¹⁸ As a result, the quantum yield for the C=C bond torsion (Φ_{d}) can be obtained as $2\Phi_{ZE}$. In all the investigated aprotic solvents, the

sum of $\Phi_{\text{f}} + \Phi_{\text{d}}$ ($= \Phi_{\text{f}} + 2\Phi_{ZE}$) is close to 1.0 (0.90–1.08) within experimental error for both *p*-ABDI and *m*-ABDI. We can thus conclude that in aprotic solvents the nonradiative decay channel is mainly the C=C bond torsion. The absence of solvent-polarity dependence of the $\Phi_{\text{f}} + 2\Phi_{ZE}$ values and the observation of an increase of Φ_{f} at the expense of Φ_{ZE} on going from *p*-ABDI to *m*-ABDI support the assumption that $\Phi_{\text{d}} = 2\Phi_{ZE}$ and the conclusion. It should also be noted that the much larger Φ_{f} values for *m*-ABDI vs. *p*-ABDI in aprotic solvents resembles the case of the *m*- vs. *p*-aminostilbene.¹⁹ Evidently, there exists a close photochemical correlation between the GFP chromophore and aminostilbenes. To our knowledge, the fluorescence quantum yield for *m*-ABDI in hexane is the largest among the various derivatives of the GFP chromophore.^{7,20}

In contrast, the C=C bond torsion no longer dominates the nonradiative decay of ABDIs in protic solvents on the basis of the fact that the value of $\Phi_{\text{f}} + 2\Phi_{ZE}$ is much lower than 1.0 (Table 1) and solvent-dependent. Although the Φ_{ZE} values in pure water were unavailable due to the solubility problem, low values of Φ_{ZE} for both ABDIs in pure water can be readily predicted on the basis of the dramatic reduction of Φ_{ZE} on going from pure THF to that containing only 10–20% water. The lower Φ_{ZE} values for *m*-ABDI vs. *p*-ABDI is consistent with the slower photoisomerization process and thus the lower ability to compete with the new nonradiative decay channel. Regarding the new nonradiative decay channel, the possibility of TICT state formation through the C–C bond torsion can be excluded, because the marked difference in acetonitrile vs. methanol could hardly be interpreted in terms of the comparable solvent polarity.¹⁶ Furthermore, the related chromophore *p*-CBMNI (see Chart 1) also displays a dramatic decrease in Φ_{f} on going from aprotic dioxane (0.22) to protic glycerol (0.023),⁷ but it cannot be a consequence of TICT formation due to the lack of an explicit electron donor. Therefore, hydrogen bonding-induced deactivation of the excited ABDIs is proposed, since the phenomenon of H-bond-mediated vibronic coupling to the ground state is known to be an important radiationless decay channel for both inter- and intramolecular H-bonded species.^{21,22} In particular, it has been well demonstrated by studies of several aryethylenes that the ultrafast $Z \rightarrow E$ photoisomerization can be completely inhibited by intramolecular H-bonding.²¹

Table 1 Quantum yields for fluorescence (Φ_{f}) and $Z \rightarrow E$ photoisomerization (Φ_{ZE}) for *p*-HBDI, *p*-ABDI, and *m*-ABDI in protic and aprotic solvents

Solvent	<i>p</i> -HBDI		<i>p</i> -ABDI		<i>m</i> -ABDI	
	Φ_{f}	Φ_{ZE}	Φ_{f}	Φ_{ZE}	Φ_{f}	Φ_{ZE}
Hex	$<10^{-3}$	0.53 ^a	$<10^{-3}$	0.45 ^a	0.34	0.37 ^a
THF	$<10^{-3}$	0.46	$<10^{-3}$	0.49	0.28	0.35
MeCN	$<10^{-3}$	0.48 ^a	$<10^{-3}$	0.50 ^a	0.16	0.45 ^a
MeOH	$<10^{-3}$	~ 0.1	$<10^{-3}$	0.17	$<10^{-3}$	0.08
$\text{H}_2\text{O-THF}^b$	$<10^{-3}$	~ 0.2	$<10^{-3}$	0.37	$<10^{-3}$	0.08
$\text{H}_2\text{O-THF}^c$	$<10^{-3}$	~ 0.1	$<10^{-3}$	0.28	$<10^{-3}$	0.06

^a For the purpose of solubility, Hex and MeCN contain 20% THF for the measurement of Φ_{ZE} . ^b $\text{H}_2\text{O-THF} = 1 : 9$ (v/v). ^c $\text{H}_2\text{O-THF} = 2 : 8$ (v/v).

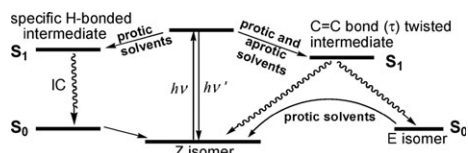


Fig. 2 Simplified scheme for the radiationless decay channels for *p*-HBDI and its amino analogues *p*-ABDI and *m*-ABDI in bulk protic vs. aprotic solvents.

The photochemical behaviour of *p*-HBDI essentially parallels that of *p*-ABDI (Table 1), although attempts to obtain more accurate Φ_{ZE} values for *p*-HBDI in protic solvents are hampered by the unstable *E* isomer in protic solvents.^{12,13} The similar behaviour of *p*-HBDI and *p*-ABDI is reminiscent of the common photodynamic scheme for the neutral and anionic forms of *p*-HBDI.^{1,2} Thus, the correlation between *p*-ABDI and the anionic *p*-HBDI appears to be appropriate.

Accordingly, we might conclude that *p*-HBDI simultaneously possesses two different ultrafast radiationless decay channels (Fig. 2). Whereas the decay in protic solvents involves both the solute–solvent H-bond-mediated internal conversion (major) and C=C bond torsion (minor), the C=C bond torsion alone accounts for the decay in aprotic solvents. It is important to note that H-bonding-induced deactivation is consistent with the volume-conserving photodynamic scheme observed for *p*-HBDI in alcohols.² In addition, the deactivating H-bonded intermediate appears to be different from the Franck–Condon excited state, and its formation requires the reorganization of the solvent H-bonding network and vibrational relaxations of the excited *p*-HBDI in order to account for the previously proposed two-state two-mode model.^{2c} Although the exact deactivating H-bonding modes remain to be determined, the most possible candidates are the H-bonds between the solvent molecules (H-bond donor) and the imidazolinone group (H-bond acceptor) of *p*-HBDI. One reason is that the imidazolinone group becomes a better H-bond acceptor in the lowest excited state (S_1) than in the ground state (S_0) as a result of ICT. Another reason is that the imino nitrogen of the imidazolinone group is not H-bonded to any water molecules or neighbouring residues inside the protein β -barrel.¹ It appears that the protein matrix not only suppresses the C=C bond torsion^{3,10} but also prevents the formation of the specific H-bonding modes in the excited state, which accounts for the high fluorescence quantum yield of GFP. It should be pointed out that a general H-bonding effect in the excited-state decay of *p*-HBDI and its derivatives has been noted.^{2,7} However, the argument of the presence of certain H-bonding modes that dominate the nonradiative decays of *p*-HBDI in protic solvents had not been recognized. Meech *et al.* had considered solvent–chromophore H-bonding as a possible nonradiative mechanism for *p*-HBDI, but it was finally ruled out in view of the fact that this channel alone cannot interpret their data in acetonitrile (aprotic) vs. methanol (protic).^{2c}

In summary, we have provided for the first time the $Z \rightarrow E$ photoisomerization quantum yields for *p*-HBDI and its amino analogues *p*-ABDI and *m*-ABDI in both aprotic and protic solvents. In addition, *m*-ABDI displays record-high fluorescence in aprotic solvents at room temperature. The solvent-dependent fluorescence and photoisomerization behaviour has

led to a new photodynamic picture for the GFP chromophore. Further studies toward the identification of the deactivating H-bonding modes for *p*-HBDI in protic solvents are in progress in our laboratory.

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