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# Ultrafast Dynamics of a GFP Chromophore

## Analogue: Competition between Excited-State Proton Transfer and Torsional Relaxation

*Tanmay Chatterjee,<sup>†</sup> Fabien Lacombat,<sup>‡,§</sup> Dheerendra Yadav,<sup>‡,§</sup> Mrinal Mandal,<sup>†</sup> Pascal Plaza,<sup>‡,§</sup> Agathe Espagne<sup>\*,‡,§</sup> and Prasun K. Mandal<sup>\*,†</sup>*

<sup>†</sup>Department of Chemical Sciences, Indian Institute of Science Education and Research (IISER) - Kolkata, Mohanpur, West-Bengal, 741246, India

<sup>‡</sup>Ecole normale supérieure, PSL Research University, UPMC Univ Paris 06, CNRS, Département de Chimie, PASTEUR, 24, rue Lhomond, 75005 Paris, France

<sup>§</sup>Sorbonne Universités, UPMC Univ Paris 06, ENS, CNRS, PASTEUR, 75005 Paris, France

ABSTRACT.

The competition between excited-state proton transfer (ESPT) and torsion plays a central role in the photophysics of fluorescent proteins of the Green Fluorescent Protein (GFP) family and their chromophores. Here, it was investigated in a single GFP chromophore analogue bearing o-

hydroxy and p-diethylamino substituents, OHIM. The light-induced dynamics of OHIM was studied by femtosecond transient absorption spectroscopy, at different pH. We found that the photophysics of OHIM is determined by the electron-donating character of the diethylamino group: torsional relaxation dominates when the diethylamino group is neutral, while ultrafast ESPT followed by cis/trans isomerization and ground-state reprotonation are observed when the diethylamino group is protonated and therefore inactive as an electron donor.

TEXT.

## ■ INTRODUCTION

Green Fluorescent Protein (GFP) from the jellyfish *Aequoria victoria* and its numerous variants are widely used as genetically encodable fluorescent markers in biological imaging.<sup>1,2</sup> The fluorescence of GFP ( $\Phi_{\text{fluo}} = 0.79$ ) is due to a p-hydroxybenzylidene-imidazolinone (p-HBDI) chromophore.<sup>3</sup> In GFP at physiological pH, p-HBDI exists in both neutral and anionic forms at the hydroxyl group.<sup>4</sup> The emissive species is however the anion, independently of the form initially excited, owing to fast deprotonation of the neutral form in the excited-state.<sup>5,6</sup> In solution, free p-HBDI is non-fluorescent ( $\Phi_{\text{fluo}} \sim 2 \times 10^{-4}$  in water<sup>7</sup>), with an excited-state lifetime of  $\sim 1$  ps.<sup>8,9</sup> Fluorescence quenching is ascribed to torsional motions at the ethylene bridge, which are no longer hindered by the protein. High fluorescence quantum yields may indeed be recovered by locking the ethylene bridge in a ring,<sup>10</sup> or encapsulating the whole molecule in an RNA aptamer.<sup>11</sup> The exact torsion coordinate of p-HBDI is however still under debate. Voliani et al. reported efficient cis/trans photoisomerization in water ( $\Phi_{\text{ct}} = 0.21$ ).<sup>12</sup> Based on the weak viscosity dependence of the excited-state dynamics of p-HBDI, Meech and coworkers proposed

a volume-conserving, "hula-twist" coordinate (concerted torsion of adjacent single and double bonds).<sup>8</sup> In contrast, theoretical studies suggest that hula-twist is energetically disfavored and that p-HBDI and related molecules decay by torsion about either the single or the double bond, depending on protonation state, substituents and environment.<sup>13-15</sup> When going from GFP to solution, the photophysics of p-HBDI therefore switches from excited-state proton transfer (ESPT) to torsional relaxation. p-HBDI torsion is however observed in some fluorescent proteins other than GFP, due to different chromophore-protein interactions. ON-OFF photoswitching in photochromic proteins such as Dronpa is for instance based on chromophore cis/trans photoisomerization.<sup>16,17</sup> The competition between ESPT and torsion is hence at the heart of the photophysics of GFP-related fluorescent proteins and their chromophores.

Interestingly, the excited-state dynamics of o- and m-hydroxy derivatives of HBDI in solution were reported to be governed by ESPT,<sup>18,19</sup> offering the opportunity to study this reaction on simpler models than GFP. In order to investigate the competition between ESPT and torsion in a single GFP chromophore analogue, we synthesized OHIM (4-(2-hydroxy-4-N,N-diethylaminobenzylidene)-1,2-dimethyl-1H-imidazol-5(4H)-one; Scheme 1). In addition to an o-hydroxyl group, OHIM bears in place of the p-hydroxyl group of HBDI a diethylamino substituent, known to promote torsional relaxation in GFP chromophore analogs.<sup>20</sup> The Tolbert group recently reported that OHIM may be used as a fluorescent turn-on probe for human serum albumin (HSA).<sup>21</sup> Another interest of OHIM is that one expects its photophysics –in particular the efficiency of ESPT and torsion– to drastically depend on pH, due to the acid-base character of its substituents. Note that OHIM was synthesized in cis configuration, as the chromophore of GFP. We report here a comprehensive study of the light-induced dynamics of OHIM in water, in the pH range 1-12, by femtosecond transient absorption spectroscopy.

## ■ RESULTS AND DISCUSSION

**Spectrophotometric acid-base titration.** OHIM may exist in several acid-base forms depending on pH, due to three protonatable sites : the hydroxyl group, the diethylamino group and the imidazolinone ring. In order to determine the absorption spectra of the different forms, we performed a spectrophotometric acid-base titration of OHIM (see Supporting Information (SI), Fig. S1). The pH-dependent spectra were globally fitted with a three-equilibria model, resulting in the four spectra shown in Fig. 1, and three  $pK_a$ 's (see Scheme 1 for the values and corresponding acid-base forms).  $pK_1$  being typical of a phenol,<sup>22</sup> the black and red spectra of Fig. 1 may be attributed to species **1** (all sites deprotonated) and **2** (hydroxyl protonated) of Scheme 1, respectively. On the other hand, the low-pH, blue spectrum may be ascribed to species **4**, in which all sites are protonated.

The assignment of the green spectrum (Fig. 1) is less straightforward. It consists of two bands peaking at 375 and 495 nm. The emission spectra measured upon excitation of the two bands are very different (see SI, Fig. S2), suggesting two different species rather than two transitions of the same species. The  $pK_a$  of the diethylamino group is 6.6 in N,N-diethylaniline. Increased electronic conjugation of the base is however expected to lead to a lower value in OHIM. On the other hand, the  $pK_a$  of the imidazolinone ring is 1.8 in p-HBDI,<sup>23</sup> but values up to 3.6-3.9 have been reported in some other imidazolinone derivatives.<sup>24</sup> Consequently, we propose that the  $pK_a$ 's of diethylamino and imidazolinone are very close in OHIM, so that both sites protonate almost simultaneously, leading to a mixture of species **3** and **3'** (Scheme 1). Protonation of the second site occurs at lower pH, due to the repulsion of the positive charges. Diethylamino

protonation is expected to result in a pronounced hypsochromic shift of the absorption spectrum, as recently reported for other amino derivatives of HBDI,<sup>25</sup> because it significantly reduces the length of the conjugated  $\pi$ -system by deactivating the nitrogen lone pair. We therefore assign the 375-nm band of the green spectrum to **3'** (protonated diethylamino) and the 495-nm band to **3** (protonated imidazolinone).

Although **3'** and **4** have similar absorption spectra, their emission spectra are shifted by  $\sim 25$  nm with respect to each other (Fig. S2), which unambiguously indicates that they are different species. They nevertheless share very strong Stokes shifts (of more than 200 nm) of the fluorescence with respect to the absorption, suggesting some important structural rearrangement in the excited state.

**Excited-state behavior of the neutral diethylamino forms.** The light-induced dynamics of the different acid-base forms of OHIM were investigated by broadband UV-visible femtosecond transient absorption spectroscopy. Fig. 2 shows the spectra measured for **1** (A), **2** (B) and **3** (C and D) in water. The initial spectra (at 80-150 fs) of the three species share the same structure : a positive excited-state absorption (ESA) band below 430-460 nm and a negative band at longer wavelengths due to overlapping ground-state bleaching (GSB) and stimulated emission (SE) contributions. These initial spectra decay on similarly fast, femto-picosecond timescales. The details of spectral evolution are however different.

The initial spectrum of **1** (Fig. 2A) decays in an essentially homothetic manner, within  $\sim 20$  ps. A small residual negative signal at  $\sim 500$  nm is nevertheless observed after this decay, for at least 3 ns (insert of Fig. 2A). In contrast, the transient spectra of **2** and **3** exhibit significant changes of shape with time. In the case of **2** (Fig. 2B), SE decays much faster than other contributions

during the first  $\sim 6$  ps, especially near its maximum at  $\sim 500$  nm. A faster decay is also observed at the red edge of ESA ( $\sim 420$  nm). For pump-probe delays longer than 6 ps, the decay becomes homothetic. Like for **1**, a small long-lived signal persists after complete decay of the excited state (insert of Fig. 2B). As far as **3** is concerned, we observed a partial decay of the whole spectrum during the first  $\sim 5$  ps (Fig. 2C). The decay is however more pronounced at the red edge of ESA ( $\sim 420$  nm) and in the negative band around 535-550 nm, where a dip appears and evolves into a small positive transient absorption band. This band likely corresponds to the absorption of an excited-state intermediate, rather than a ground state, because the 5.6-ps spectrum still contains some emission beyond 560 nm. All bands, including the intermediate, decay to zero within the next  $\sim 50$  ps (insert of Fig. 2D).

The data were globally fitted with sums of exponentials, complemented for **1** and **2** with a small plateau. Depending on the species, three or four exponentials were needed to achieve a good fit. The lifetimes, gathered in Table 1, are all very short, within the 100 fs to 15 ps range. For species **2** (neutral OHIM), they are in good agreement with time-resolved fluorescence data recently reported by some of us.<sup>26</sup> The corresponding amplitude spectra (also called DADS, for decay associated difference spectra) are shown in Fig. S3 and support the spectral changes described above.

To learn more about the excited-state decay processes and, more specifically, about the possible involvement of molecular torsions, we investigated the sensitivity of the kinetics to viscosity. We repeated all experiments in buffers containing 60% glycerol, the viscosity of which is  $\sim 10$  times higher than that of pure water (10.7 cP instead of 1 cP<sup>27</sup>). This data is shown in Fig. S4. It could satisfactorily be fitted using the same multiexponential models as in water. The obtained lifetimes are given in Table 1, and the DADS are presented in Fig. S5. While the shortest

component ( $\tau_1$ ) is only modestly sensitive to viscosity, a pronounced slowing down of the other components ( $\tau_2$ ,  $\tau_3$  and  $\tau_4$ ) is observed for all three species (up to factors of 5 to 10). Such a large viscosity effect on the dynamics of a reaction is usually interpreted as a sign that large-amplitude molecular motion is involved. In particular, the light-induced torsion of single or double bonds bearing bulky substituents may be very sensitive to solvent viscosity.<sup>28,29</sup> The weaker effect on the short component suggests that it is associated to smaller-amplitude motion, such as the very start of a torsion or some other small structural rearrangement in the molecule or the solvent triggered by the electronic excitation.

In OHIM, torsion could occur at three different bonds : the central single and double C-C bonds forming the bridge between the two rings, as also proposed for p-HBDI,<sup>8,12-15</sup> and the C-N single bond linking the diethylamino substituent to the phenyl ring. Single-bond torsion in the excited-state most often results in a fast recovery of the initial ground state (no long-lived transient signal).<sup>28</sup> In contrast, cis/trans photoisomerization of a double-bond is expected to lead to a long-lived trans photoproduct, the barrier to torsion being usually high in the ground state, as for instance in stilbene.<sup>29</sup> Slight modifications of the absorption spectra have been reported to occur upon cis/trans isomerization of HBDI and related compounds, such as small wavelength shifts and/or intensity changes.<sup>12,17</sup> The small long-lived signals reported in this work for **1** and **2** very likely originate from such spectral modifications, hence from trans photoproducts. Conversely, the absence of any long-lived photoproduct for **3** indicates that double-bond torsion is not a dominant pathway in that particular case. It is of note that OHIM torsions are much more sensitive to viscosity than those of p-HBDI and its p-dimethylamino analogue DMPI, which were both suggested to undergo hula-twist, that is simultaneous rotations about adjacent single and double bonds.<sup>8,9,20</sup> We propose that the torsions of forms **1**, **2** and **3** involve independent



rotations about either the double bond (**1** and **2**) or one of the single bonds (**3**). Such "one-bond-flip" motions are expected to displace larger volumes of solvent and hence to be more strongly slowed down in viscous media.

Theoretical studies on molecules undergoing single or double-bond torsion in the excited-state usually predict the existence of  $S_1$  energy minima corresponding to twisted structures, with close-lying  $S_1/S_0$  conical intersections mediating internal conversion to the ground state.<sup>13-15</sup> Depending on the barrier height in  $S_1$ , these twisted minima may be populated and give rise to detectable excited-state intermediates in time-resolved spectroscopy experiments.<sup>28,29</sup> We propose that the 5.6-ps, ~535-nm intermediate of **3** (Fig. 2, C and D) corresponds to such a species. Although no net delayed positive band is seen for **2**, the strong reshaping of the spectra during the first picoseconds (Fig. 2B) could also be due to the formation of a twisted excited-state intermediate absorbing around 500-550 nm. Stimulated emission remains however dominant in this case, leading to a negative total differential signal.

Finally, let us recall that the torsional dynamics of **1**, **2** and **3** is both ultrafast and multiexponential (see Table 1). Such characteristic features strongly evoke the diffusion over a barrierless or quasi-barrierless excited-state potential, as modeled by Bagchi et al.<sup>30</sup> and reported for p-HBDI,<sup>8,13-15</sup> cis-stilbene<sup>29</sup> and other molecules subject to torsion.<sup>28,31-33</sup> The model we propose for forms **1**, **2** and **3** of OHIM is shown in Scheme 2A. Single or double-bond torsion along a quasi-barrierless potential leads to a twisted excited-state, which may be observed or not, and from which internal conversion occurs to the ground state. In the case of double-bond torsion, a mixture of cis and trans isomers is obtained.

**Excited-state behavior of the protonated diethylamino forms.** The light-induced dynamics of species **3'** and **4**, bearing protonated diethylamino groups, are markedly different from those of **1**, **2** and **3**. Fig. 3 shows the transient absorption spectra measured for **4** in water. Their temporal evolution comprises four steps, highlighted in sections A to D. The initial spectrum at 120 fs (Fig. 3A, black spectrum) consists of GSB below 410 nm, ESA between 410 and 550 nm and SE beyond 550 nm. GSB remains essentially constant during the first two steps (120 fs to 3 ps ; Fig. 3, A and B), which may therefore be ascribed to excited-state reactions. The first step (120 fs to 580 fs ; Fig. 3A) is characterized by a delayed rise of both the ESA and SE bands. Let us note that this growing SE band corresponds well to the steady-state fluorescence (dotted grey spectrum in Fig. 3A), strongly red-shifted (to ~610 nm) with respect to the absorption spectrum. This means that the fluorescent state of **4** is not the locally excited state but rather a photoproduct. The second step (580 fs to 3 ps ; Fig. 3B) consists of a partial decay of the ESA and SE bands, accompanied by a continuous red-shift of the SE, from ~615 nm to ~635 nm. The third step (3 ps to 30 ps ; Fig. 3C) is characterized by a complete decay of SE, indicating return of the excited molecules to the ground state. GSB decay is however only partial on this timescale, due to the formation of a ground-state photoproduct (we call **X**) showing absorption in the 400-565 nm region (peak at ~470 nm). Finally, during the fourth step (30 ps to 1 ns ; Fig. 3D), this first photoproduct evolves into a second, blue-shifted photoproduct (**Y**) peaking at ~415 nm. The transient absorption spectra of **3'**, displayed in Fig. S6, exhibit essentially the same initial structure and temporal evolution. The fourth step is however much slower than in **4**, so that we could only observe its beginning in our probe temporal window of ~2.5 ns (Fig. S6D). The spectrokinetic data of **4** were best fitted using four exponentials, corresponding to the four steps described above, and a plateau. In the case of **3'**, five exponentials were needed because

ground-state recovery (third step ; Fig. S6C) was found to be biexponential. The lifetimes of the components obtained for species **4** and **3'** are given in Table 1, and their DADS are shown in Figs. S7A and S7B, respectively.

The 610-nm SE that develops in the transient spectra of **4** on the timescale of  $\tau_1 = 125$  fs (Fig. 3A) resembles that of a tautomer of some o-HBDI derivatives observed in aprotic solvents.<sup>18,26</sup> This tautomer was proposed to be formed by ultrafast ESPT along an intramolecular hydrogen-bond connecting the hydroxyl group with the imine nitrogen of the imidazolinone ring. It seems very unlikely that such a hydrogen bond exists in **4**, due to the protonated state of the imidazolinone group. The species resulting from simple deprotonation of the hydroxyl group of **4** (i.e. proton transfer to the solvent) is however analogous to the o-HBDI tautomer from the viewpoint of proton distribution, and is expected to display a similar emission spectrum. We therefore propose that species **4** behaves like a photoacid and that the first light-induced process is the excited-state deprotonation of the hydroxyl group, with transfer of the proton to water. As can be seen in Table 1, the deprotonation kinetics ( $\tau_1$ ) of **4** is moderately slowed down by deuteration (factor of 1.4) but significantly more by viscosity (factor of 2.2). This could be an indication that proton dissociation is controlled by solvent dynamics rather than proton tunneling.<sup>34</sup>

The charge of the molecule and its spatial distribution change very fast upon excited-state deprotonation and the solvent is expected to adapt with some delay (dynamic solvation). The shift of SE to the red on the timescale of  $\tau_2 = 730$  fs (Fig. 3B) is in good agreement with this expectation. We therefore suggest that the second step of spectral evolution corresponds to solvent relaxation following ESPT.

The third step ( $\tau_3 = 6.9$  ps ; Fig. 3C), which corresponds to ground-state recovery, is slowed down by a factor 5.5 in the presence of 60% glycerol (see Table 1). Such a strong effect suggests the involvement of a large-amplitude motion, hence single or double-bond torsion, as for species **1**, **2** and **3**. Starting from a cis configuration, ESPT followed by double-bond torsion would result after ground-state recovery in some trans, deprotonated **4**, while single-bond torsion would exclusively lead to the corresponding cis form. Since photoproduct **X** could equally well correspond to one or the other isomer, it is not possible to discriminate between the different possible torsion coordinates at this stage. Additional elements will be brought by the discussion below.

In photoacids, excited-state deprotonation is followed by ground-state reprotonation, the apparent unimolecular rate constant of which is expected to be proportional to the concentration of protons. In order to identify the reprotonation step, we lowered the pH from 1 to 0.5. At pH = 0.5, species **4** remains the major acid-base form of OHIM and the proton concentration is  $\sim 3$  times higher (0.32 M) than at pH 1 (0.10 M). Interestingly, we observed an acceleration of the fourth step ( $\tau_4 = 103$  ps), by a factor of 3.6 (Table 1). The kinetics of this step was in addition found to be inversely proportional to viscosity : it was slowed down by a factor of 11.7 upon addition of 60% glycerol, by which solvent viscosity is multiplied by  $\sim 11$ . These effects suggest that step 4 (Fig. 3D) corresponds to a diffusion-controlled bimolecular reaction of reprotonation of the hydroxyl group (ground-state proton transfer, GSPT). For such a reaction, the rate constant is indeed expected to be a fraction of the diffusion rate constant, which is in turn proportional to the reactants mutual diffusion coefficient and, according to the Stokes-Einstein relation, inversely proportional to viscosity.<sup>35</sup>

Importantly, the reprotonated product (**Y**) is spectrally different from **4**, as indicated by the non-zero differential signal at 1 ns (Fig. 3D). This retrospectively means that the previous step of the reaction (step 3) does not consist in single-bond torsion. Had this occurred, the initial species **4** would have been restored after reprotonation. We conclude that the coordinate responsible for excited-state decay during step 3 is the torsion of the double bond. Photoproduct **X** thus corresponds to the trans isomer of the hydroxyl-deprotonated **4** species and photoproduct **Y** to trans **4**.

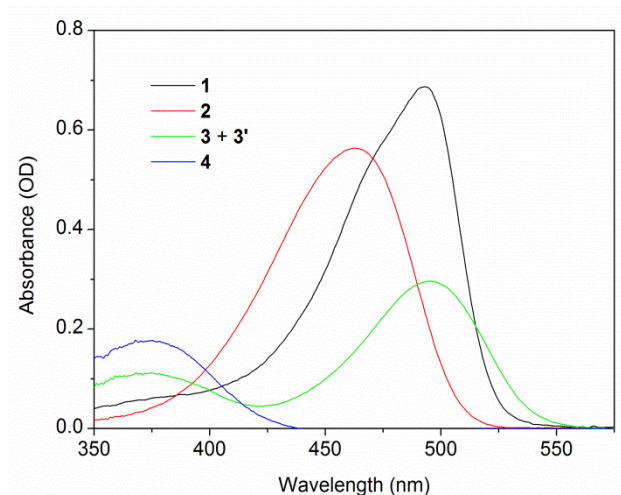
The same conclusions are applied to species **3'** and summarized in Scheme 2B. Let us however note that the proton concentration at pH 2.7 (pH at which the measurements on **3'** were done) is only  $2 \times 10^{-3}$  M, that is, 50 times less than at pH 1. Under these conditions the characteristic time for hydroxyl reprotonation is expected to be on the order of 5 ns, which probably explains why this step is barely observed for species **3'**, in our probe time window of  $\sim 2.5$  ns (Fig. S6D).

## ■ SUMMARY AND CONCLUSIONS

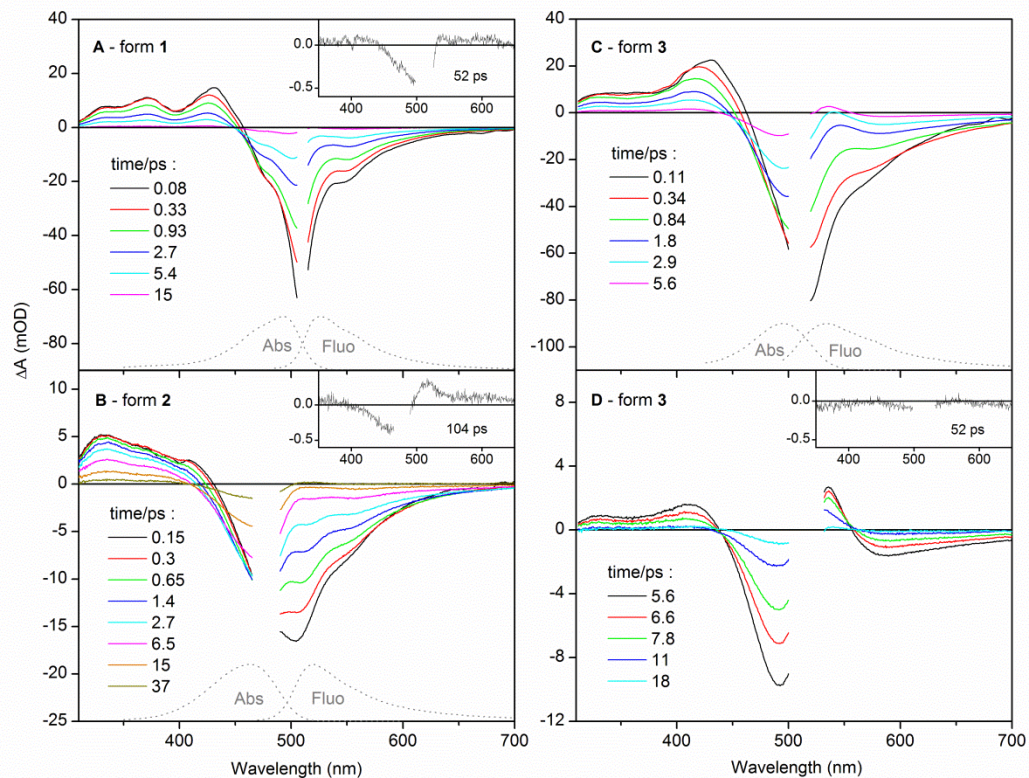
The present work shows that the light-induced reactivity of OHIM is controlled by the protonation state of the diethylamino group. When it is deprotonated (forms **1**, **2** and **3**), the excited-state dynamics is dominated by torsion (Scheme 2A). Conversely, when it is protonated (forms **3'** and **4**), ultrafast proton transfer to the solvent is observed, followed by cis/trans isomerization of the double-bond and ground-state reprotonation (Scheme 2B). This strong effect is likely due to the fact that protonation drastically reduces the electron donor character of the diethylamino group by deactivating the nitrogen lone pair. OHIM is in fact a "push-pull" conjugated system, in which the diethylamino nitrogen lone pair may be transferred toward the

imidazolinone group. Such a charge transfer is expected to weaken the central ethylenic double-bond and facilitate cis/trans isomerization. The dynamics of light-induced torsion has actually been reported to correlate to the electron donor-acceptor character of molecular structures in a series of derivatives of the PYP (Photoactive Yellow Protein) chromophore, a molecule closely related to p-HBDI.<sup>36,37</sup> As far as OHIM is concerned, we propose that protonation of the diethylamino group indirectly favors proton transfer by slowing down torsion, which can no longer compete efficiently. In conclusion, the present study on OHIM allowed us to explore the competition between ESPT and torsional relaxation for the first time in a single GFP chromophore analogue, just by varying the pH. This competition is central to the photophysics of GFP-related fluorescent proteins and their chromophores.

## FIGURES.

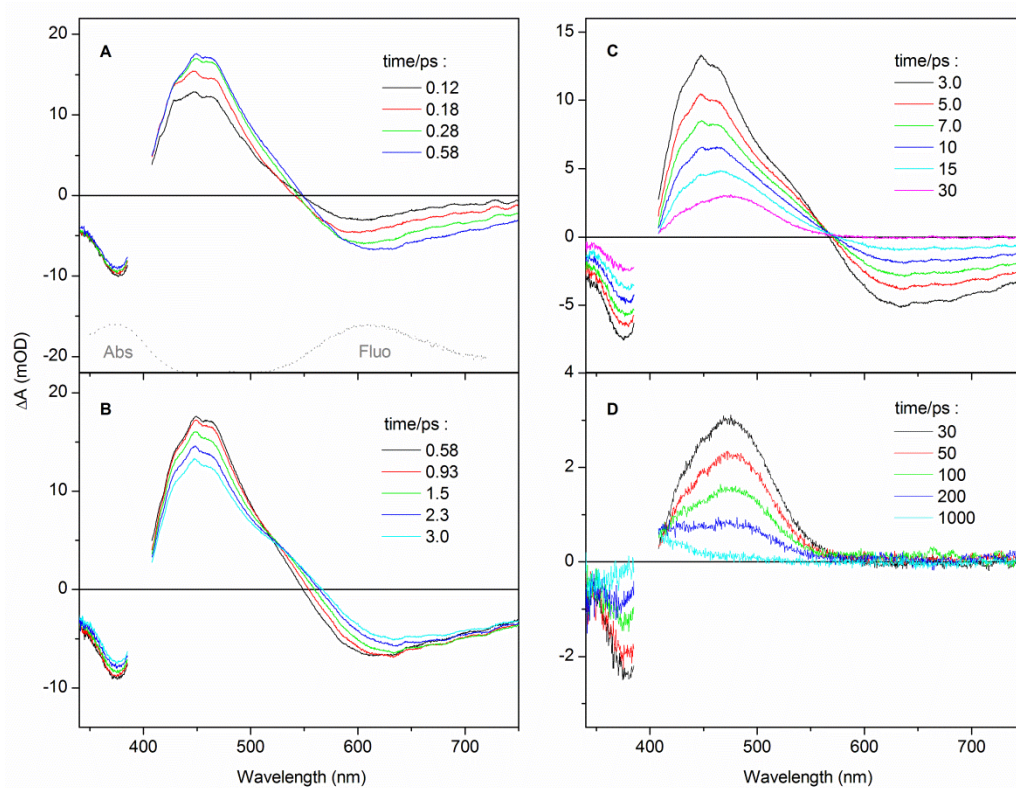


**Figure 1.** Steady-state absorption spectra of the different acid-base forms of OHIM. Black: form **1**, all sites deprotonated. Red: form **2**, protonated hydroxyl. Green, 495-nm band: form **3**, hydroxyl and imidazolinone protonated. Green, 375-nm band: form **3'**, hydroxyl and diethylamino protonated. Blue: form **4**, all sites protonated.



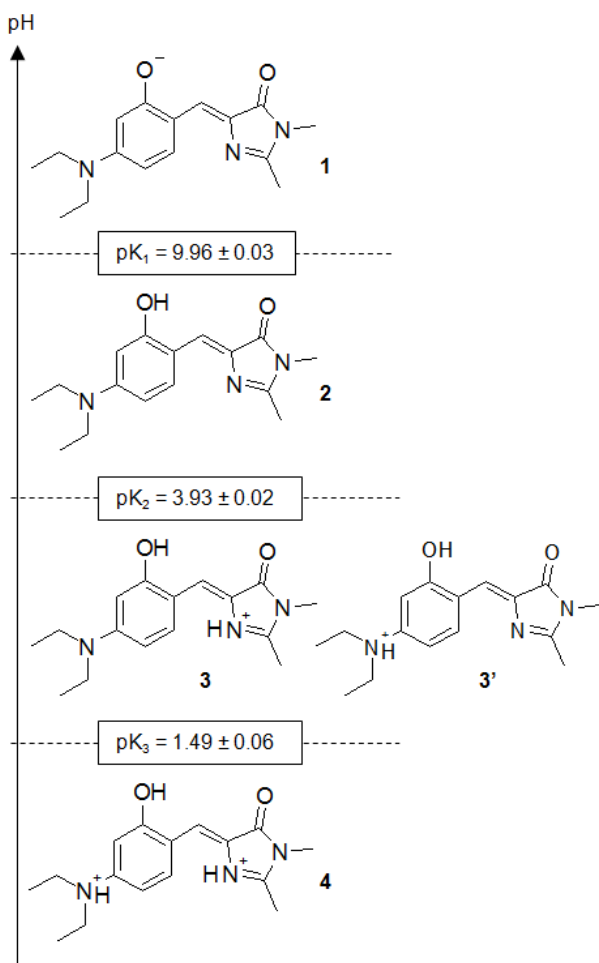
**Figure 2.** Femtosecond transient absorption spectra of forms **1** (A), **2** (B) and **3** (C and D) of OHIM in aqueous buffers. The data were corrected for the chirp of the probe. The insets show the small long-lived signals remaining after excited-state decay. The grey dashed lines are the steady-state absorption and emission spectra of the different forms.



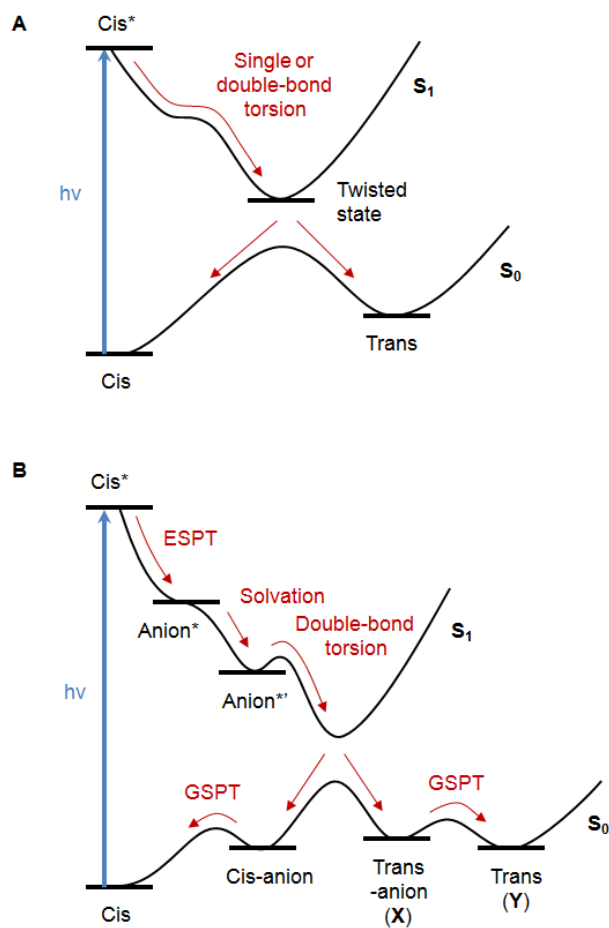


**Figure 3.** Femtosecond transient absorption spectra of form 4 of OHIM in phosphate buffer at pH = 1.0, measured after excitation at 388 nm for pump-probe delays ranging from (A) 120 fs to 580 fs, (B) 580 fs to 3 ps, (C) 3 to 30 ps and (D) 30 ps to 1 ns. The data were corrected for the chirp of the probe. The grey dashed lines in section A are the steady-state absorption and emission spectra of form 4.

SCHEMES.



**Scheme 1.** The different acid-base forms and pKa's of OHIM.



**Scheme 2.** Models of the light-induced reactivity of OHIM : (A) forms **1**, **2** and **3** and (B) forms **3'** and **4**.

TABLES.

		$\tau_1$ (ps)	$\tau_2$ (ps)	$\tau_3$ (ps)	$\tau_4$ (ps)	$\tau_5$ (ps)
<b>1</b>	pH 12	$0.250 \pm 0.015$	$1.30 \pm 0.06$	$5.7 \pm 0.1$	$\infty$	
	pH 12, 60 % glycerol	$0.330 \pm 0.025$	$3.1 \pm 0.2$	$30.4 \pm 0.6$	$\infty$	
<b>2</b>	pH 7	$0.305 \pm 0.015$	$2.18 \pm 0.05$	$15.6 \pm 0.2$	$\infty$	
	pH 7, 60 % glycerol	$0.560 \pm 0.020$	$11.8 \pm 0.7$	$77 \pm 2$	$\infty$	
<b>3</b>	pH 2.7	$0.110 \pm 0.006$	$0.29 \pm 0.02$	$1.31 \pm 0.02$	$3.28 \pm 0.06$	
	pH 2.7, 60 % glycerol	$0.125 \pm 0.005$	$1.6 \pm 0.2$	$12 \pm 1$	$38 \pm 2$	
<b>3'</b>	pH 2.7	$< 0.100$	$0.67 \pm 0.11$	$1.8 \pm 0.2$	$9.3 \pm 0.2$	$4400 \pm 450$
<b>4</b>	pH 1	$0.125 \pm 0.003$	$0.73 \pm 0.03$	$6.91 \pm 0.07$	$103 \pm 4$	$\infty$
	pD 1	$0.180 \pm 0.005$	$0.93 \pm 0.06$	$8.6 \pm 0.2$	$66 \pm 5$	$\infty$
	pH 1, 60 % glycerol	$0.280 \pm 0.020$	$3.2 \pm 0.3$	$39.1 \pm 0.7$	$1200 \pm 100$	
	pH 0.5	$0.155 \pm 0.005$	$0.85 \pm 0.03$	$6.2 \pm 0.1$	$29 \pm 1$	$1480 \pm 200$

**Table 1.** Lifetimes obtained from global fits of the transient spectra of the different forms of OHIM.

## ASSOCIATED CONTENT

**Supporting Information.** Experimental methods, steady-state absorption and emission spectra of OHIM at different pH, femtosecond transient absorption spectra of forms **1-3** in water-glycerol mixtures, femtosecond transient absorption spectra of form **3'**, DADS of the different forms. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## AUTHOR INFORMATION

### Corresponding Authors

Agathe Espagne: [agathe.espagne@ens.fr](mailto:agathe.espagne@ens.fr) ; +33-144322413

Prasun K. Mandal: [prasunchem@iiserkol.ac.in](mailto:prasunchem@iiserkol.ac.in) ; +91-9874306747

### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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