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Solvatochromism of the Green Fluorescence Protein Chromophore and Its Derivatives

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Green fluorescent protein (GFP) and its mutants have revolutionized molecular biology.[1] Despite the effort toward expanding the class of GFP through gene shuffling techniques, successful examples invariably incorporate the β-barrel to isolate the chromophore. The β-barrel, presumably by restricting the conformational space of the chromophore, reduces the rate of radiationless decay, leading to ca. 10^4 higher fluorescence quantum yields. At the same time, the β-barrel can apparently tolerate a high degree of disorder within the protein, as judged by analysis of the numerous crystal structures, as well as a variety of π systems. The β-barrel obviously plays an enormous role in the photophysics of GFP; its most obvious effect is to solvate both the chromophore and its conjugate base. In this work we consider the effect of the β-barrel on the optical properties of the GFP chromophore (*p*-hydroxybenzylideneimidazolone, *p*-HBDI) experimentally by selective variation of the protonation state of the latter and its microenviroment.

It is known that a careful pH titration indicates three forms of *p*-HBDI: a (presumably nitrogen-) protonated cationic form C below pH = 2, the neutral form N, and the anionic form A above pH = 10.[2] The absorbance spectrum of wild-type (wt)-GFP demonstrates the presence of the two forms of the chromophore with absorbance bands at 398 (A_p band) and 477 nm (B_p band, Figure 1), their ratio very weakly depending on pH. It has been noted that the high-energy wt-GFP absorbance band corresponds to the absorbance of the *p*-HBDI cation in water (Figure 1). Nevertheless, most of the current literature assigns band A_p to the absorbance of the neutral species, not the cation, because no evidence of the latter was found in the Raman and resonance Raman spectra of wt-GFP and its S65T mutant,[3] and the imidazolone nitrogen atom is not sufficiently basic to be protonated in the protein. As a reference point, the analogous spectra of *p*-HBDI at different pH were used.[4,5] Despite this observation, the possibility of cationic involvement in the absorbance spectra of the wt-GFP cannot be excluded.[6,7]

Only scattered experimental treatments of solvatochromism in *p*-HBDI and its derivatives are known.[2,8−10] A very weak solvent dependence of the N form absorption was reported,[8] while the absorbance of the C form exhibited a blue shift with polarity increase.[9] The hypsochromic shift of the A form was correlated with either solvent polarity[8] or acidity[2,10] increase. At the same time, it was found that mutations influence the position of the absorption bands of the chromophore to a much greater extent than simple solvent variations.[11] In all cases, no *quantitative* analysis was made. To more clearly understand the role of the solvent and protein in mediating the photophysics, and to correlate the absorbance bands of the chromophore in bulk solvents and in protein, we have undertaken a careful evaluation of the solvent effects on the various acid/base forms of *p*-HBDI and its new derivatives, *O*- and *N*-methylated analogues. The selective blocking of the acid−base groups in the chromophore may give insight into the relative

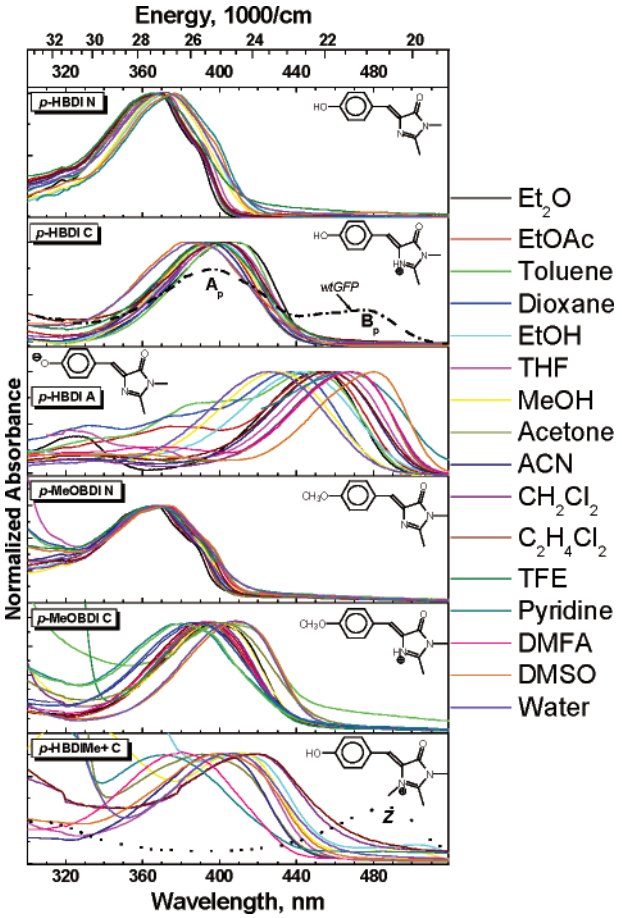

Figure 1. Absorption spectra of different protonation states of *p*-HBDI, *p*-MeOBDI, and *p*-HBDIMe+ in various solvents. For comparison we present the absorption spectra of wt-GFP at pH 7 in the "p-HBDI C" graph as a black dash−dotted line. Absorbance of *p*-HBDIMe+ in water at pH 7.6 is presented in the bottom graph as a black dotted line; the absorption peak at 486 nm is due to the zwitterion (Z) absorption. The intensity of the latter is scaled to fit the graph.

contribution of the various protonated form of the chromophore to the ground-state behavior of the protein.

The GFP chromophore *p*-HBDI was synthesized according to ref 12. Its *O*-methylated derivative, *p*-MeOBDI, was synthesized by a slightly modified procedure using *p*-methoxybenzaldehyde. The *N*-methylated derivative, *p*-HBDIMe+, was synthesized by methylation of *p*-HBDI with methyl trifluoromethanesulfonate.[13] Similar to *p*-HBDI, both new compounds had negligible fluorescence in bulk solvents at room temperature.

The C forms of *p*-HBDI and *p*-MeOBDI were generated by adding trifluoromethanesulfonic acid to their neutral solutions, while the A forms of *p*-HBDI were formed by adding KOH/crown ether complex. In aqueous solutions, the pH was controlled to ensure

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Table 1. Solvatochromic Parameters (in 10^3/cm) Used in Multivariable Regression Fits of Absorption Data for Neutral, Cationic, and Anionic Forms of Various Chromophores According to Eq 1; pK_a Values Are Also Presented[13]

compound	ν_0	p	a	b	R^a	p$K_a{}^b$
p-HBDI N	28.3	−0.71	−0.17	−0.7	0.94	8.53
p-HBDI C	24.5	1.2	0	0.5	0.90	2.36
p-HBDI A	22.9	−1.4	1.7	0.53	0.89	
p-MeOBDI N	27.9	−0.6	0	−0.18	0.87	
p-MeOBDI C	24.0	0.8	0	1.8	0.95	2.76
p-HBDIMe + C	23.0	2.2	−1.3	1.9	0.92	6.4

[a] Correlation coefficient. [b] Measured in MeOH/H_2O 1:1 v/v.

complete (de)protonation.[13] The basicity of the imidazole nitrogen increased slightly upon methylation of the hydroxy group in *p*-HBDI, while the acidity of the hydroxy group increased by more than 2 pK_a units upon methylation of the imidazole nitrogen (Table 1). Titration of *p*-HBDIMe+ in the region pH = 4−7.4 demonstrated[13] a clear equilibrium between C and its conjugate base, the zwitterion Z that absorbs at 486 nm in water (Figure 1). This is the first experimental observation of the ground-state GFP chromophore zwitterion that allows the investigation of the long-debated *p*-HBDI tautomer.[6,7,14,15] In solutions with pH > 8.0, the Z form decomposed rapidly,[13] probably by hydrolysis of the imidazolone ring. For the same reason, we could not detect absorbance of the Z in nonaqueous solvents in the presence of base; however, trace amounts of Z were observed in several neutral solvents.

Each of these forms showed a complex solvatochromic behavior (see Figure 1). The absorption maxima of the N forms of the molecules had the weakest solvent dependence, while those of C and especially A forms varied over a very broad range. To analyze the solvatochromic behavior of the N, C, and A forms of the chromophores, we used the Kamlet−Taft multivariant approach,[16] which correlates the spectral shift ν of the solute with the solvent parameters that are responsible for its acidic (α), basic (β), and polar solvating (π^*) properties:

$$\nu\ (1000/\mathrm{cm}) = \nu_0 + p\pi^* + a\alpha + b\beta \qquad (1)$$

We[17] and others[18] have successfully used this approach for various hydroxyaromatic compounds. It allows a straightforward separation of selective (H-bonding) and nonselective (dipole−dipole interaction) solvation. Here we report that the solvatochromic behavior of *p*-HBDI and its derivatives is governed by *both* polar and acid/base properties of the solvents. The magnitudes and directions of the solvatochromic shifts strongly depend on the protonation state of the solute (Table 1). For N forms of *p*-HBDI and *p*-MeOBDI, an increase in all solvent parameters induced a bathochromic shift. As with the naphthols,[17] the presence of a hydroxy group in *p*-HBDI makes the solvent basicity (selective solvation) as important as the polar solvation factor. The *p*-HBDI cationic form blue-shifts as solvent polarity and basicity are increased, while for the anionic form the polarity and acidity of the solvent work in opposite directions! The magnitude of the *solute* parameters p can be related to the relative dipole moments of the molecules, while proton susceptibility parameters a and b reflect the relative proton basicity and acidity of the chromophore. Our analysis clearly demonstrates an increase in dipole moment of the chromophores upon ionization and shows their amphoteric behavior. *p*-HBDIMe+ C shows the same absorption pattern for the *p*-HBDI C, validating the protonation site in *p*-HBDI.

We note, however, that at no polarity/acidity extreme did the absorption of the *p*-HBDI neutral correspond to those in the protein. We are tempted to conclude that the wt-GFP absorption was misassigned and the A_p band corresponds to the cation, leaving the 477 nm absorption to the zwitterion.[7] However, as we have discovered, the red-shifted absorbance maximum of the Z form does not match this value in any solvent, while the *p*-HBDI A absorption in some basic solvents fits the 477 nm wt-GFP absorption quite well (Figure 1). Thus, there is no reason to discount the common assignment of Ap as the neutral form. To explain the existing discrepancy between the experimental data on neutral species absorption in *p*-HBDI and red-shifted wt-GFP, a combination of mainly structural factors (small breakdown of planarity), with a small contribution from polarization effects of the environment,[19,20] must be taken into account. Equally important could be the presence in GFP and all its fluorescent mutants of the obligatory positively charged Arg96. Calculations indicate[20] that Arg96 red-shifts the absorption spectrum of the chromophore by ca. 40 nm, presumably by lowering the energy of the charge-transfer excited state. Nevertheless, this is the first experimental verification of this rationale.

The complex solvatochromic behavior of the GFP chromophore and its derivatives requires both polar and acid/basic properties of the environment to be taken into account. However, no solvent effect serves to "turn on" the fluorescence, which remains subject to stereoelectronic effects. This stark contrast between solution and protein-constrained behavior suggests that there is something truly unusual about this chromophore, which is the subject of continued investigation in many laboratories.

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Supporting Information Available: Synthesis of *p*-MeOBDI and *p*-HBDIMe+, experimental data, and fitting procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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