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Resonantly Enhanced Multiphoton Ionization Spectrum of the Neutral Green Fluorescent Protein Chromophore

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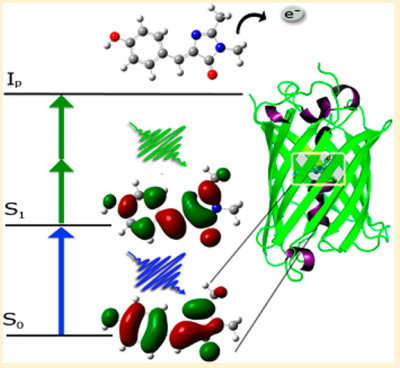
Supporting Information

ABSTRACT: The photophysics of the green fluorescent protein is governed by the electronic structure of the chromophore at the heart of its β-barrel protein structure. We present the first two-color, resonance-enhanced, multiphoton ionization spectrum of the isolated neutral chromophore in vacuo with supporting electronic structure calculations. We find the absorption maximum to be 3.65 ± 0.05 eV (340 ± 5 nm), which is blue-shifted by 0.5 eV (55 nm) from the absorption maximum of the protein in its neutral form. Our results show that interactions between the chromophore and the protein have a significant influence on the electronic structure of the neutral chromophore during photoabsorption and provide a benchmark for the rational design of novel chromophores as fluorescent markers or photomanipulators.



SECTION: Spectroscopy, Photochemistry, and Excited States

Green fluorescent protein (GFP), first discovered in the jellyfish *Aequorea victoria*,[1] is used extensively as a genetically encoded fluorescent marker in cell biology.[2] In wild-type GFP, the *para*-hydroxybenzilidene-imidazolinone (*p*-HBDI) chromophore is formed autocatalytically from serine, tyrosine, and glycine residues in the polypeptide. It is covalently bound to the protein and held in place at the heart of a β-barrel structure by a network of hydrogen bonds and ionic interactions.[3] The absorption spectrum of wild-type GFP has maxima around 395 and 480 nm, which are attributed to the neutral and deprotonated anionic forms of the chromophore, respectively.[4] The neutral form of the chromophore in the protein undergoes excited-state deprotonation, thus photoexcitation of either form of the protein results in fluorescence from the excited anionic state at 520 nm with a quantum efficiency of $\Phi \approx 0.8$. The absorption spectrum of the isolated deprotonated HBDI anion in the gas phase has been shown to be remarkably similar to that of the anionic form of GFP.[5−9] This led us to ask whether the absorption spectrum of the neutral *p*-HBDI molecule in the gas phase is similar to that of the protein with its chromophore in a neutral form.

The intrinsic absorption properties of the anion have been studied in ion storage devices through fragmentation induced by photon absorption, known as action spectroscopy.[5−11] Several of these measurements have shown that the deprotonated chromophore anion in the gas-phase anion has the same absorption maximum as that of the protein in its anionic form, whereas in solution the spectrum is blue-shifted. This has led to the suggestion that the environment of the chromophore in the protein is similar to that of an isolated chromophore in the gas phase; however, this is a subject of debate. The observed action (dissociation) can involve absorption of more than one photon, and there is also a competing electron detachment process that has been studied by photoelectron spectroscopy[12−18] and could be important in the role of GFP as a light-induced electron donor in redox processes in cells.[19] Some theoretical and experimental studies advocate that the spectrum for the isolated anion is blue-shifted by 0.23 eV relative to the protein.[9,20,21] Moreover, the fluorescence quantum yield of the isolated deprotonated *p*-HBDI anion is negligible in the gas phase, as it is in solution,[22−27] and recent femtosecond studies have shown that the time scale for ultrafast relaxation back to the electronic ground state for the isolated *p*-HBDI anion in the gas phase is remarkably similar to that in solution.[12,22] Systematic theoretical investigations of the effect of the protein on the anionic form of the chromophore have also shown that individual protein interactions affect the absorption spectrum but collectively they cancel one another out.[28−30]

There are no direct experimental measurements of the absorption spectrum of the neutral chromophore, only action spectroscopy measurements for model systems with substituted

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charged groups, from which an absorption maximum of 370 nm was inferred (a blue shift of 25 nm or 0.22 eV relative to the protein).[31,32] Interestingly, the challenges of modeling the neutral molecule accurately can be seen in the range of vertical excitation energies (VEEs) obtained using different computational methods (3.1 to 4.1 eV corresponding to 400−300 nm, see Supporting Information).[28,33−36]

We report an alternative method for measuring the in vacuo absorption spectrum of the neutral GFP chromophore, *p*-HBDI, together with variants 3,5-dimethoxy-4-hydroxybenzylidene-1,2-dimethylimidazolinone (DMHBDI) and 3,5-difluoro-4-hydroxybenzylidene-1,2-dimethylimidazolinone (DFHBDI) to investigate the effect of substituting electron-donating and electron-withdrawing groups on the phenolic moiety. Gas-phase absorption spectra are compared with those in 1/1 (v/v) methanol−water solution and, for *p*-HBDI, they are compared with the absorption spectrum of the protein. The experimental measurements are supported by computational chemistry calculations.

The structures of the three chromophores are shown in Figure 1. The equilibrium geometries of each were calculated using density functional theory with the B3LYP/6-311++G(d,p) method.[37−41] To support the experimental measurements, in particular the comparison between the three molecules, we have calculated VEEs and oscillator strengths of the S_1-S_0 transitions for each molecule using time-dependent density functional theory (TDDFT) and the CAM-B3LYP/6-311++G(d,p) method.[42] This provides values that are in good agreement with our experimental data (see below) and other high-level computational methods (see Supporting Information). All calculations were performed using the Gaussian09 suite of programmes.[43]

For gas-phase absorption measurements of the neutral molecules, a molecular target was generated by gentle CW laser heating (50 °C) of solid samples deposited on a stainless-steel foil. The foil forms part of the repeller electrode of a time-of-flight (ToF) mass spectrometer.[44−46] To measure the absorption spectrum of the neutral molecules, the ion yield from a two color, resonantly enhanced, multiphoton ionization scheme was employed. A UV laser pulse, tunable between 315 and 420 nm and with an intensity of 2×10^8 W cm^{-2}, was used to excite the S_1 state of the chromophore. A second pulse at 514 nm and an intensity of 1.8×10^9 W cm^{-2} was spatially and temporally overlapped with the UV pulse to ionize an electron from the excited S_1 state by further absorption of two photons. Each laser pulse was ∼200 fs in duration. The intensities of both pulses were sufficiently low that only parent ions were produced in the ToF mass spectrum, and the ion yield obtained from each laser beam on its own was a small fraction of the two color yield. Further details of the experimental methods can be found in the Supporting Information.

Absorption spectra of *p*-HBDI, DMHBDI, and DFHBDI in solution were measured with a PerkinElmer Lambda 950 UV/vis spectrophotometer. The samples were prepared in 1/1 (v/v) methanol−water solutions.

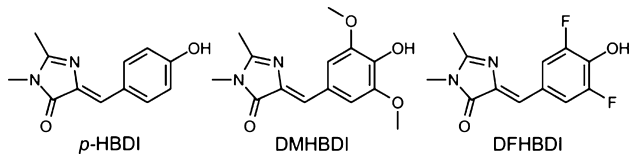

Figure 1. Structures of the chromophores.

The gas- and solution-phase absorption spectra of the three neutral chromophores are presented in Figure 2, together with the calculated VEEs. The CAM-B3LYP/6-311++G(d,p) orbitals involved are plotted for the *p*-HBDI chromophore in Figure 3. The highest occupied molecular orbital (HOMO) is delocalized across the whole molecule and has a π orbital on one of the ethylene groups of the CCC bridge between the phenol and imidazolinone moieties. The first excited singlet state is dominated by a transition from the HOMO to the lowest unoccupied molecular orbital (LUMO), which involves an ethylene-like $\pi^* \leftarrow \pi$ transition on the CCC bridge.

The absorption maxima obtained in solution and in vacuo are compared with the calculated VEEs. The absorption maximum in vacuo may not be exactly equivalent to the calculated VEE due to the high density of vibrational states, but the difference is expected to be small compared with the uncertainty in the peak maximum. We have ruled out the possibility of resonances at the two-photon level blue-shifting the absorption maxima measured in vacuo by comparing the two-color spectra (Figure 2) with one-color spectra (see Supporting Information). Table 1 and Figure 2 show that there is good agreement between experiment and theory for the isolated molecules. The π-electron-donating groups in the ortho positions on the phenol moiety in DMHBDI result in a substantial red shift of the absorption spectra of DMHBDI compared with *p*-HBDI both in the gas-phase and in solution. This can be rationalized by considering the destabilizing effect of adding π-electron density

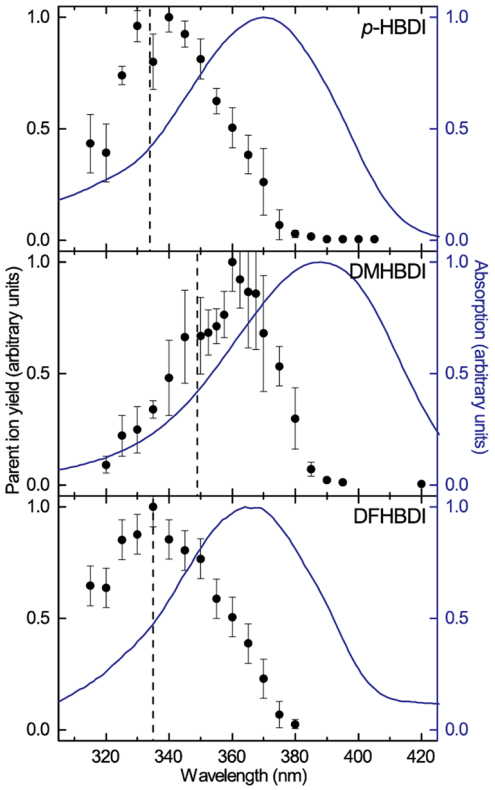

Figure 2. Absorption spectra of the neutral chromophores in vacuo (data points) and in 1/1 (v/v) methanol−water solutions (solid blue lines). All spectra are normalized on the absorption maximum in the range 310−420 nm. Calculated vertical excitation energies are marked as vertical dashed lines.

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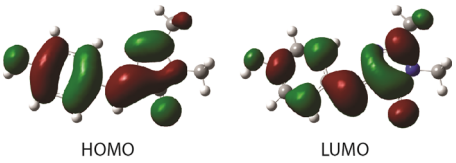

Figure 3. CAM-B3LYP/6-311++G(d,p) orbitals for *p*-HBDI.

at the ortho positions, where there is already increased electron density arising from the resonance effect of the lone-pair of the phenolic oxygen atom donating into the aromatic ring. Conversely, substituting electron-withdrawing fluorine atoms at the ortho positions to form DFHBDI results in a slight blue shift compared with *p*-HBDI, both in the gas phase and in solution.

Our results show that the absorption maximum of *p*-HBDI in vacuo is blue-shifted substantially by 0.5 eV (55 nm) compared with the protein. This is considerably more than that estimated by the indirect action spectroscopy measurements of Rajput et al.[32] using *p*-HBDI "neutral+" analogs. In fact, Filippi et al.[34] show that these analogs may actually provide an environment closer to the protein than vacuum and, using the CASPT2/cc-pVDZ method, they determine a value of 3.58 eV for the absorption maximum of the isolated chromophore, which is close to our experimental value of 3.65 ± 0.05 eV. Polyakov et al.[35] have reported an SOS-CIS(D) excitation energy of 3.83 eV, which is also in reasonable agreement with our experimental observation. Our calculated value for the VEE is 3.71 eV, which is in good agreement with our experimental value and the calculated values of Filippi et al.[34] and Polyakov et al.[35]

In aqueous solution, the absorption maxima of the isolated neutral chromophores are all red-shifted compared with the absorption maxima of the isolated neutral chromophores in vacuo by ∼0.3 eV (30 nm) (Table 1).

This sensitivity of the absorption spectrum of the chromophore to its environment is strikingly dependent on the oxidation state. Whereas the absorption maximum of the neutral chromophore in vacuo is blue-shifted by 0.3 eV compared with that in aqueous solution and by 0.5 eV compared with that of the protein in its neutral form, the absorption maximum of the deprotonated chromophore anion in vacuo is red-shifted by 0.32 eV compared with that of the anion in aqueous solution and is remarkably similar to that of the protein in its anionic form (Figure 4). In both the anionic and neutral forms of the chromophore, the first excited singlet state is dominated by a transition from the HOMO to the LUMO. For the anionic form this involves a $\pi^* \leftarrow \pi$ transition on the central allyl bridge, with negligible change in dipole moment,[17] whereas for the neutral chromophore it involves an ethylene-like $\pi^* \leftarrow \pi$ transition (Figure 3), with a more significant change in dipole moment.[47] These differences, together with the differences in hydrogen bonding and other interactions between the chromophore and the protein, are

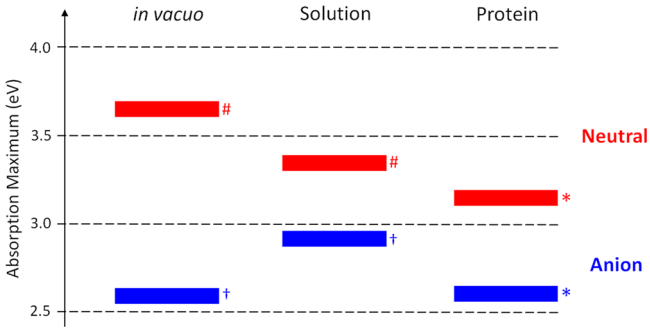

Figure 4. Plot summarizing the measured absorption maxima for the neutral and deprotonated anionic forms of the GFP chromophore in vacuo, in solution (neutral−1/1 (v/v) methanol−water pH = 7; anion−NaOH pH = 13) and in the protein (#, our results; †, Nielsen et al.,[5] ∗, Chalfie et al.[4]).

clearly responsible for the different effects of the environment on the absorption spectra of the anionic and neutral forms of the chromophore.

In summary, we have reported the first action spectrum of the GFP chromophore using its neutral form in vacuo. We find the absorption maximum to be 3.65 ± 0.05 eV (340 ± 5 nm), which is blue-shifted by 0.5 eV (55 nm) compared with that of the protein with its chromophore in a neutral form. We conclude that the direct hydrogen bonding and other interactions between the neutral GFP chromophore and its environment have a significant influence on the electronic structure of the chromophore and that the similarity between the absorption spectra of the isolated deprotonated chromophore anion in vacuo and the anionic form of the protein[5] is likely to be a coincidence. We show that the absorption maximum can be tuned by substituting electron-withdrawing or electron-donating groups at the ortho positions on the phenol moiety. These results provide an important benchmark for theory that will allow the influence of the protein on the chromophore to be quantified in more detail. Determining the time scales for electronic relaxation following photoexcitation of the isolated neutral GFP chromophore in vacuo will be important for the development of new chromophores for future synthetic photochemical systems. As well as new developments for cell biology methodologies, such as RNA fluorophores which mimic GFP,[48] there is huge potential for designer chromophores to be exploited in molecular electronics[49] and for purely photonic devices based on the nonlinear molecular response.[50]

■ ASSOCIATED CONTENT

Supporting Information

Further computation results including optimized geometries of the three molecules, their VEEs, comparison of the vertical excitation energy of *p*-HBDI with previous theoretical calculations, and calculations of VEEs of higher excited states. The chemical synthesis and experimental methods are also

Table 1. Vertical Excitation Energies (eV) with Corresponding Wavelengths (nm) in Parentheses, and Oscillator Strengths of the S_1−S_0 Absorption Maxima of Neutral Chromophores in 1/1 (v/v) Methanol−Water Solution, in Vacuo and in Protein

chromophore	protein	solution	isolated in vacuo (expt)	isolated (theory)	*f*
p-HBDI	3.13 (395)	3.35 ± 0.01 (370 ± 1)	3.65 ± 0.05 (340 ± 5)	3.71 (334)	0.765
DMHBDI		3.20 ± 0.01 (388 ± 1)	3.44 ± 0.05 (360 ± 5)	3.55 (349)	0.794
DFHBDI		3.39 ± 0.01 (366 ± 1)	3.70 ± 0.06 (335 ± 5)	3.70 (335)	0.723

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described in more detail, and the two-color REMPI spectra are compared with one-color REMPI spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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