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Competition between photodetachment and autodetachment of the $2^1\pi\pi*$ state of the green fluorescent protein chromophore anion

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Using a combination of photoelectron spectroscopy measurements and quantum chemistry calculations, we have identified competing electron emission processes that contribute to the 350–315 nm photoelectron spectra of the deprotonated green fluorescent protein chromophore anion, *p*-hydroxybenzylidene-2,3-dimethylimidazolinone. As well as direct electron detachment from S_0, we observe resonant excitation of the $2^1\pi\pi*$ state of the anion followed by autodetachment. The experimental photoelectron spectra are found to be significantly broader than photoelectron spectrum calculated using the Franck-Condon method and we attribute this to rapid (~10 fs) vibrational decoherence, or intramolecular vibrational energy redistribution, within the neutral radical. © *2014 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution 3.0 Unported License.* [http://dx.doi.org/10.1063/1.4874643]

I. INTRODUCTION

Nature has developed a myriad of highly efficient photoactive proteins for gathering energy for chemical processes and initiating specific actions. A particularly well-known example is the green fluorescent protein (GFP) that was first found in the jellyfish *Aequorea victoria*.[1] Not only is GFP used extensively in cell biology and biomedical research as a fluorescent marker but it also undergoes a range of photoconversions.[2–6] An example that is relevant to the work presented in this paper is its ability to act as a light-induced electron donor with the potential to monitor and manipulate redox processes in cells.[7]

The GFP chromophore is formed by intramolecular cyclisation of three residues in the nascent protein, Ser[65]-Tyr[66]-Gly[67], and is localised in the centre of the β-barrel structure of GFP. The chromophore exists in neutral and anionic (deprotonated) forms that both absorb visible light.[8,9] Excitation of either form results in strong fluorescence at 508 nm from the anionic form of the chromophore (the neutral form deprotonates upon photoexcitation to form the fluorescent anionic form). In order to understand the photophysics of GFP, a good starting point is the isolated model chromophore, *p*-hydroxybenzylidene-2,3-dimethylimidazolinone (HBDI) (Fig. 1). Studies of HBDI have revealed that the electronic absorption spectrum of the deprotonated chromophore anion in the gas phase is remarkably similar to that in the protein.[10] However, in the gas phase, the deprotonated chromophore anion does not fluoresce[11,12] and its excited state lifetime[13] has been found to be very similar to that in solution.[14]

A number of recent photoelectron spectroscopy and theoretical studies of gas-phase HBDI have shown that following excitation to the first electronically excited state of the deprotonated anion, $1^1\pi\pi*$, direct and indirect photodetachment processes compete with internal conversion and fragmentation.[15–17] Photoelectron spectra from our group also hinted at the existence of competing electron emission pathways at higher photodetachment energies, however this was not investigated in any detail.[18] Here, we employ a combination of photoelectron spectroscopy and theory to investigate in detail the competition between direct photodetachment and autodetachment of the $2^1\pi\pi*$ state of the anion at these higher photodetachment energies, in the range 350–315 nm, within the $2^1\pi\pi*$–S_0 absorption band (Fig. 2). Such studies of photoinduced electron emission in the GFP chromophore provide valuable information on the intrinsic processes that govern the redox properties of fluorescent proteins following photoexcitation with ultraviolet light.

II. METHODS

A. Experimental

The experimental setup has been described in detail in Ref. 20 although some modifications have been made to improve the signal to noise ratio and resolution. HBDI was synthesised using established methods *via* the azalactone and subsequent aminolysis with methylamine.[21] It was purified by recrystallisation from ethanol. Deprotonated chromophore anions were generated by electrospray-ionisation of HBDI in MeOH with a few drops of NH_3. Deprotonated HBDI anions were mass-selected by a quadrupole, guided into a hexapole trap and focused into the photoionisation region of a collinear velocity-map imaging spectrometer, where they were intersected by nanosecond laser pulses with $\lambda = 350-315$ nm, generated by frequency doubling the output of a Nd:YAG pumped dye laser system. Electrons were accelerated towards a position-sensitive detector where they were imaged using a
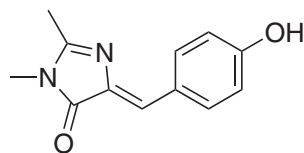
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FIG. 1. The GFP chromophore *p*-hydroxybenzylidene-2,3-dimethyl-imidazolinone (HBDI).

CCD (charge-coupled device) camera. Anion-only and laser-only images were subtracted from images recorded following interaction of the laser light with the anions, to eliminate background counts from anions and scattered laser light. The resulting photoelectron images were processed using the pBasex method.[22] The velocity-map imaging spectrometer was calibrated using the photoelectron spectrum of I^- and has a resolution of ∼5%.

B. Computational

All calculations were performed using the Gaussian09 suite of programmes.[23] The geometric structures of the deprotonated HBDI chromophore anion and its corresponding neutral radical were optimised using the B3LYP hybrid functional[24–27] and the 6-311++G(3df,3pd) basis set.[28] Frequency calculations were performed to confirm that a minimum in the potential energy surface was reached.

The vertical detachment energies (VDEs) were obtained using the electron propagator theory (EPT) method with the outer valence Green's function (OVGF) propagator[29,30] and a

6-311++G(3df,3pd) basis set. The suitability of this method was determined by comparing photodetachment energies calculated using different basis sets with experimental measurements of the VDE[31] and benchmarking against the high-resolution photoelectron spectrum of the phenoxide anion. The EPT/6-311++G(3df,3pd) method gives a VDE of 2.280 eV for the phenoxide anion, which is within 1% of the experimental value of 2.253(6) eV reported by Gunion *et al.*[32]

The vertical excitation energies (VEEs) of the singlet excited electronic states of the deprotonated HBDI chromophore anion were calculated using the CAM-B3LYP/6-311++G(3df,3pd) method. The long range corrected version of B3LYP using the Coulomb Attenuating Method (CAM) was chosen for its potential to describe excited states with charge-transfer character.[33]

The photodetachment spectrum was calculated using the Franck-Condon (FC) method implemented in Gaussian09.[34] This type of calculation requires the harmonic frequencies and normal modes of the deprotonated HBDI chromophore and corresponding neutral radical as input. These were obtained using the B3LYP/6-311++G(3df,3pd) method. The FC calculation included the first 500 vibrational states of the anion that were composed of up to 3 normal modes and whose Boltzmann populations were ≥10% of the ground vibrational state. The vibrational temperature of the anions was assumed to be 300 K.

III. RESULTS AND DISCUSSION

Fig. 3 shows photoelectron spectra recorded at 350 nm (3.54 eV), 328 nm (3.78 eV), and 315 nm (3.94 eV), plotted as a function of electron binding energy (eBE), where eBE = $h\nu$ − eKE. Each spectrum is dominated by a broad asymmetric feature. The vertical axes of the spectra have been scaled so that the low eBE edges are superimposed on one another and it is clear that the threshold for photodetachment is
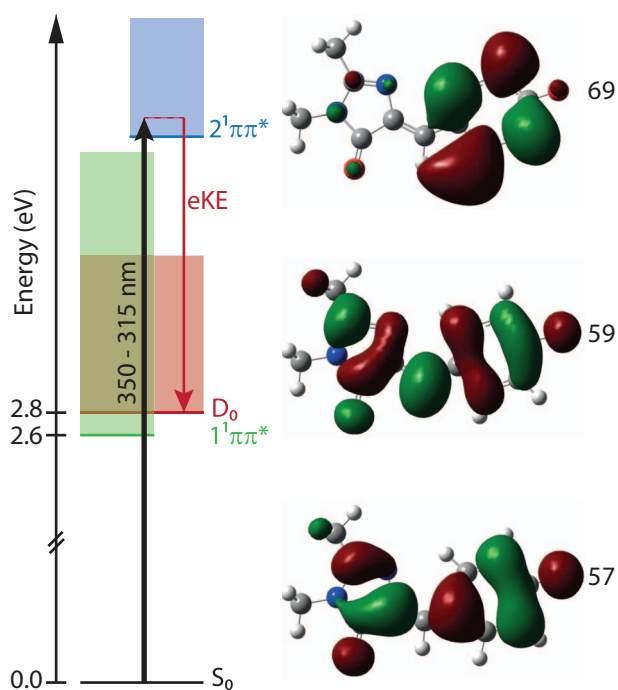

FIG. 2. Schematic energy level diagram illustrating the excitation scheme in the deprotonated HBDI anion. The vertical axis marks the vertical excitation energy of the $1^1\pi\pi*$ state of the anion[19] and the vertical detachment energy (Table I). Also shown are the main ground-state CAM-B3LYP/6-311++G(3df,3pd) orbitals involved in the transitions to the $1^1\pi\pi*$ and $2^1\pi\pi*$ states (Table II).
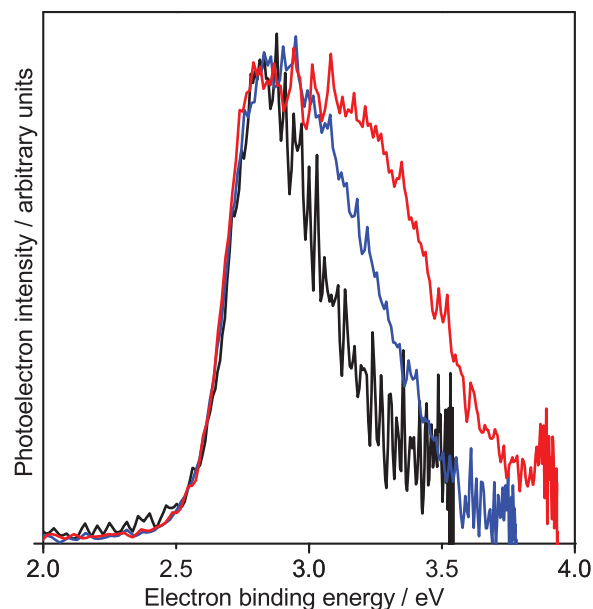

FIG. 3. Photoelectron spectra recorded at 350 nm (black), 328 nm (blue), and 315 nm (red), plotted as a function of electron binding energy.

TABLE I. Calculated VDE and D_0-S_0 ADE (0-0 transition) together with experimental photoelectron spectrum (PES) measurements. A description of the experimental measurements is provided in the text.

Method	eBE (eV)
EPT/6-311++G(3df,3pd)	2.777[a] (0.874)[b]
B3LYP/6-311++G(3df,3pd)	2.775[c]
PES (350 nm)	2.82 ± 0.10[d]
PES (355 nm)	2.68 ± 0.1[e]
PES (330 nm and 269 nm)	2.85 ± 0.10[f]
PES (355 nm, 268.1 nm, and 201.5 nm)	2.8 ± 0.1[g]

[a]VDE.
[b]Pole strength.
[c]0-0 transition.
[d]This work (see text).
[e]Reference 15.
[f]Reference 18.
[g]Reference 16.

independent of laser wavelength. From these measurements, our visual estimation of the threshold for electron detachment is 2.5 ± 0.1 eV. Interestingly, the peaks broaden on the high eBE side as the laser wavelength is decreased. Measured values for the full-width at half-maximum (FWHM) are 0.32 ± 0.03 eV, 0.59 ± 0.01 eV, and 0.79 ± 0.01 eV at 350 nm, 328 nm, and 315 nm, respectively. More importantly, at 315 nm the spectral profile is clearly not that of a simple Franck-Condon envelope. There is a new feature centered around 3.2 eV eBE and there is also some evidence of vibrational structure in the range 2.9–3.1 eV. This suggests that autoionisation of a higher lying excited state of the anion is contributing to the photoelectron spectrum.

To assist with the interpretation of the photoelectron spectra and to understand the role of the different electronic states, we have calculated the VDE, using the EPT method, and the adiabatic detachment energy (ADE), as the difference between the ground vibrational level of the anion and the ground vibrational level of the neutral radical (Table I). These values are compared with the electron binding energy corresponding to the maximum intensity in the experimental photoelectron spectrum, determined by fitting the calculated photoelectron spectrum convoluted with a Gaussian to the experimental spectrum recorded at 350 nm (see Fig. 4), and other experimental values reported in the literature.[15, 16, 18]

The calculated VDE is 2.78 eV, which is lower than the maximum intensity in the experimental photoelectron spectrum (2.82 ± 0.1 eV). It is within the experimental errors of all previously reported experimental measurements. Toker *et al.* reported a value of 2.68 ± 0.1 eV, which they determined by fitting a Franck-Condon envelope to an experimental photoelectron spectrum recorded at 355 nm.[15] Their Franck-Condon envelope was calculated by taking a Fourier transform of Lax's autocorrelation function using PBE0/aug-cc-pVDZ optimised geometries and frequencies. Horke and Verlet reported a value of 2.8 ± 0.1 eV, based on measuring the maxima in photoelectron spectra recorded at 355 nm, 268.1 nm, and 201.5 nm.[16] We reported a value of 2.85 ± 0.1 eV, based on fitting a Franck-Condon envelope calculated using ezSpectrum[35] to experimental photoelectron spectra recorded at 330 nm and 269 nm.[18] Our calculated VDE
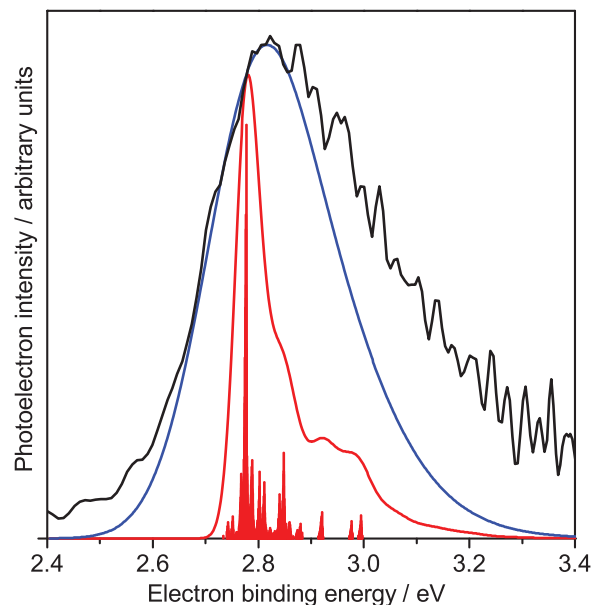

FIG. 4. Experimental photoelectron spectrum recorded at 350 nm (black) together with a calculated D_0–S_0 photoelectron stick spectrum at 300 K (red) and the calculated stick spectrum convoluted with Gaussians with HWHM = 200 cm^{-1} (red) and 900 cm^{-1} (blue).

is very close to the value reported recently by Bravaya and Krylov which was calculated using the ωB97X-D/aug-cc-pVTZ method with ωB97X-D/cc-pVTZ optimised geometries (2.76 eV).[36]

To identify the electronic state responsible for the broadening on the high eBE side of the photoelectron spectra, we have calculated the VEEs of the singlet excited states of the anion.[31] Table II lists the VEEs and main configurations of the first two singlet excited states of the deprotonated HBDI anion with oscillator strengths $f > 0.002$. The CAM-B3LYP/6-311++G(3pd,3df) molecular orbitals (MOs) involved in these transitions are presented in Fig. 2. The Highest Occupied Molecular Orbital (MO 57) is delocalised across the whole molecule. It has two out-of-phase π bonds across the central C-C-C bridge, with a node at the central C-atom, and a pair of out-of-phase delocalised π orbitals on the phenoxide ring. The first singlet excited state is dominated by a transition from the HOMO to MO 59 and involves a $\pi^* \leftarrow \pi$ transition on the central C-C-C bridge. The calculated VEE (3.1 eV) is in line with values determined at similar levels of theory,[37] although it is higher than those reported from action spectroscopy measurements[10, 11] and higher levels of theory.[19, 38, 39]

The second singlet excited state with appreciable oscillator strength is dominated by a transition from the HOMO (MO 57) to MO 69. As can be seen in Fig. 2, MO 57 is

TABLE II. Main configurations, VEEs and oscillator strengths of the first two $^1\pi\pi^*$ excited states of the deprotonated HBDI anion.

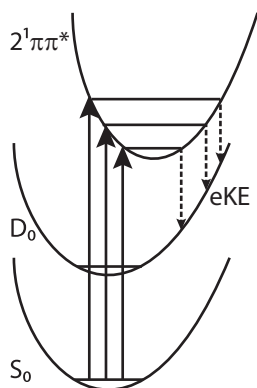| State	Main configuration	VEE (eV)	f
$1^1\pi\pi^*$	0.69(57–59)	3.128	1.050
$2^1\pi\pi^*$	0.62(57–69)	4.391	0.058

FIG. 5. Cartoon illustrating how resonant excitation of the $2^1\pi\pi^*$ state results in broadening on the low eKE (high eBE) side of the photoelectron spectrum.

delocalised across the whole molecule and has two out-of-phase π-bonds on the phenoxide unit, whereas MO 69 has two π^*-bonds on the phenoxide ring and has virtually no electron density on the imidazolinone unit. This indicates that the transition involves a $\pi^* \leftarrow \pi$ transition on the phenoxide ring and charge-transfer from the imidazolinone unit to the phenoxide ring. The calculated VEE is 4.4 eV, which is reasonably close to the maximum in the action absorption spectrum of deprotonated HBDI (310 nm or 4.0 eV) recorded at the ELISA storage ring in Aarhus.[40] Thus, we believe the most likely explanation for the broadening on the high eBE side of the photoelectron spectrum is resonant excitation of the $2^1\pi\pi^*$ state followed by vibrational motion within the $2^1\pi\pi^*$ state and autodetachment (Fig. 5). In this case, the profile of the experimental photoelectron spectrum will reflect the Franck-Condon overlap between the ground electronic state of the anion (S_0), the $2^1\pi\pi^*$ state of the anion and the ground electronic state of the neutral radical (D_0), in addition to a contribution from direct electron detachment (the Franck-Condon overlap between S_0 and D_0). Following resonant excitation of the $2^1\pi\pi^*$ state, ultrafast dynamics through conical intersections between the $2^1\pi\pi^*$ state and lower electronic excited states of the anion may compete with autodetachment from the $2^1\pi\pi^*$ state. Such processes will also influence the shape of the photoelectron spectrum.

In order to determine if other processes are also contributing to the photoelectron spectra, we have calculated the photoelectron spectrum corresponding to direct photodetachment from the ground electronic state of the anion to D_0. Fig. 4 shows the calculated photoelectron stick spectrum. The most intense line in the spectrum is the 0-0 transition at 22387 cm^{-1} (2.78 eV). The experimental instrument resolution is ~5% and corresponds to a half width at half maximum (HWHM) of 0.025 eV at 1 eV eKE (2.5 eV eBE) and 0.01 eV at 0.5 eV eKE (3 eV eBE). To compare the low eBE edges of the calculated spectrum and the experimental spectrum recorded at 350 nm, the calculated spectrum is convoluted with Gaussians with HWHM of 200 cm^{-1} (0.025 eV).

Although our calculated spectrum does not include the high density of low intensity transitions on the high eBE tail of the spectrum,[15] the overall profile is very similar to those obtained by Toker *et al.*[15] and by Bravaya and Krylov.[36] How-

ever, the calculated spectrum convoluted with an instrument resolution of 200 cm^{-1} HWHM is considerably narrower than the experimental photoelectron spectrum recorded at 350 nm (FWHM =0. 32 $\pm$ 0.03 eV). It is also worth noting that this calculated spectrum is narrower than the experimental photoelectron spectrum recorded at 355 nm by Toker *et al.* (FWHM ~0.25 eV).[10] We find that a Gaussian function of 900 cm^{-1} (0.11 eV) HWHM is required to fit the low eBE edge of the 350 nm spectrum. This is consistent with the instrument function of 0.09 eV (730 cm^{-1}) HWHM that Bravaya and Krylov found necessary to reproduce the 355 nm photoelectron spectrum of Toker *et al.*[36] Even with this unrealistically broad instrument function, the experimental spectrum recorded at 350 nm is broader than the calculated spectrum on the high eBE side, indicating that resonant excitation of the $2^1\pi\pi^*$ state followed by vibrational motion and autodetachment is competing with direct photodetachment at 350 nm (Fig. 5).

The broadening of the spectrum on the low eBE (high eKE) side of the spectrum does not change with wavelength in the range 350–315 nm. This suggests that it is not controlled by Franck-Condon overlap between the $2^1\pi\pi^*$ state of the anion and the D_0 state of the radical, i.e., autodetachment followed by resonant excitation of the $2^1\pi\pi^*$ state. We believe the most likely explanation is vibrational decoherence, or intramolecular vibrational energy redistribution, within the high density of vibrational energy levels in the D_0 state of the radical. The calculated photoelectron stick spectrum (Fig. 4) assumes that the vibrational states have infinite lifetime. If there were rapid decoherence or IVR in D_0, the D_0 lines would have a spectral width corresponding to their decoherence lifetime. Modelling the D_0-S_0 Franck-Condon spectrum and convoluting with Gaussians of HWHM 900 cm^{-1} is equivalent to an instrument function with HWHM 200 cm^{-1} and a lifetime broadening of HWHM 700 cm^{-1} (corresponding to a lifetime of around 10 fs). This 10 fs timescale is also remarkably similar to the effective vibrational decoherence timescale deduced from analysis of the decay of the autocorrelation function used to calculate the $1^1\pi\pi^*$-S_0 absorption spectrum above ~2.75 eV where the electron emission active modes are excited directly.[15,41]

Finally, the small peak visible in the 315 nm spectrum around 3.9 eV eBE (~50 meV eKE) merits some discussion (Fig. 3). Similar low eKE features have been observed in spectra recorded around 500 nm and have been attributed to vibrational autodetachment from the resonantly excited $1^1\pi\pi^*$ state[15,17] or thermionic emission following internal conversion back to S_0.[16] In order to investigate the origin of this feature, we have calculated the VEE of the first excited electronic state of the neutral radical at the optimised geometry of the anion. We find that this state arises predominantly from ionisation out of MO 55 and has a VEE of 1.562 eV, which translates into a VDE of 4.339 eV. This is very close to the VEE of the $2^1\pi\pi^*$ state of the anion and also to the VDE to D_1 determined by measuring peak maxima in our earlier photoelectron spectra,[18] and thus we conclude that the small peak visible around 3.9 eV is likely to arise from direct detachment to D_1 or from vibrational autodetachment from the $2^1\pi\pi^*$ state of the anion to D_1.

IV. SUMMARY

Using a combination of photoelectron spectroscopy measurements and quantum chemistry calculations, we have identified several processes that contribute to the 350–315 nm photoelectron spectra of a model chromophore of the green fluorescent protein. At all photon wavelengths, direct detachment from S_0 to D_0 is an important electron emission channel but resonant excitation of the $2^1\pi\pi*$ state, followed by autodetachment to D_0, is also significant. At 315 nm, the D_1 detachment channel is accessible and we see evidence for either direct or indirect electron detachment to this channel. We attribute the overall broadening of the photoelectron spectra to rapid ($\sim$10 fs) vibrational decoherence, or ultrafast intramolecular vibrational energy redistribution, within the high density of vibrational energy levels of the D_0 state of the neutral radical. Studies such as this, aimed at improving our understanding of competing light-induced electron emission processes in the isolated chromophore anion, are important for understanding, and ultimately exploiting, the redox properties of GFP and for benchmarking theoretical calculations.

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