

Excited State Dynamics of the Isolated Green Fluorescent Protein Chromophore Anion Following UV Excitation

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ABSTRACT

A combined frequency-, angle-, and time-resolved photoelectron spectroscopy study is used to unravel the excited state dynamics following UV excitation of the isolated anionic chromophore of the green fluorescent protein (GFP). The optically-bright S_3 state, which is populated for $h\nu > 3.7$ eV, is shown to decay predominantly by internal conversion to the S_2 state that in turn autodetaches to the neutral ground state. For $h\nu > 4.1$ eV, a new and favourable autodetachment channel from the S_2 state becomes available, which leads to the formation of the neutral in an excited state. The results indicate that the UV excited state dynamics of the GFP chromophore involve a number of strongly coupled excited states.

KEYWORDS

Photoelectron spectroscopy, green fluorescent protein, ultrafast dynamics, electron transfer.

INTRODUCTION

Over the past half-century, molecular biology has been revolutionised by the discovery of naturally occurring fluorescent proteins and their development into a fluorescent probe for *in vivo* biological imaging. The ability to incorporate a fluorescent probe into the genetic encoding of a protein without affecting the biological functionality imparts an unprecedented level of locational specificity that is unachievable with traditional fluorescent probes.¹⁻⁵ Of the fluorescent proteins, the green fluorescent protein (GFP) was the first discovered and remains the most widely utilized. The origins of the optical properties of GFP, as well as many other fluorescent proteins, can be traced to a chromophore enclosed deep within the β -barrel of the body of the protein.^{6,7} In GFP, the chromophore is essentially identical to the deprotonated anion of *para*-hydroxybenzilidene-2,3-dimethylimidazolinone (HBDI⁻, shown inset in Figure 1f) and has been widely employed as a model to investigate the intrinsic photophysics of the chromophore within the protein.⁸⁻¹⁸ In the gas-phase, the S₁ state is well-characterised: the S₁ ← S₀ absorption (action) spectrum is similar to that of the protein and its origin is vertically bound relative to the ground state of the neutral (D₀).^{8,16} The S₁ state decays primarily by internal conversion on a timescale of 1.4 ps;¹⁴ vibrational autodetachment is also an open channel, although this occurs on a 30 ps timescale.¹⁸ Recently, the next optically-accessible excited state of the anion, which is formally the S₃ excited state, has been considered. The action spectrum of HBDI⁻ suggests that the S₃ ← S₀ transition is almost as bright as the S₁ ← S₀ transition and has an onset of ~3.8 eV in the gas-phase.⁹ Bochenkova *et al.* suggest that these states may be important in the photo-oxidation of GFP as the S₃ band of the protein in an aqueous solution is quasi-resonant with the hydrated electron.⁹ In isolation, the S₃ state is a resonance and three photoelectron (PE) spectra recorded with photon energies around the onset of the S₃ state indicate that the PE distribution broadens with increasing photon energy.¹⁵ This spectral broadening was assigned

to vibrational motion on the S_3 excited state surface, followed by autodetachment to a range of co-ordinates of the neutral D_0 state.¹⁵ In order to gain a more comprehensive understanding of the dynamics occurring in the continuum of HBDI^- around the S_3 state, we have performed a frequency-, angle-, and time-resolved PE spectroscopy study that probes these dynamics in detail and show that the primary dominant decay mechanism is internal conversion.

EXPERIMENTAL METHODS

The experiment has been described in detail elsewhere.¹⁹⁻²¹ Briefly, HBDI^- anions were produced electrospray ionisation of a ~ 1 mM solution of HBDI in methanol that was regulated to pH 10 by addition of NH_3 . The free HBDI^- ions are trapped in a radio frequency ring-electrode ion trap operating at ambient temperature (~ 300 K). The ion packet was injected into a time-of-flight mass spectrometer and mass-selected HBDI^- was irradiated at the centre of a penetrating-field velocity-map-imaging assembly. In the frequency-resolved PE imaging experiments, laser pulses of ~ 6 ns duration with photon energies ranging between 2.38 eV (520 nm) to 4.39 eV (282 nm) were generated using an optical parametric oscillator pumped by a Nd:YAG laser. In the time-resolved experiments, femtosecond pulses at 4.13 eV (300 nm) were used as the pump and 1.55 eV (800 nm) were used as the probe pulse. All time-resolved laser pulses were derived from a commercial femtosecond system. The 4.13 eV pump was generated by frequency doubling the 1.55 eV fundamental in a β -barium borate (BBO) crystal and combining this in a second BBO crystal with 1.03 eV (1200 nm) pulses produced by an optical parameter amplifier. PE spectra were extracted from the raw velocity-map images using a polar onion peeling algorithm,²² and were calibrated using the known spectrum of Γ^- . The experimental resolution is around 5% and the cross-correlation between pump and probe pulses was 75 fs, offering a temporal resolution of ~ 40 fs.

RESULTS AND ANALYSIS

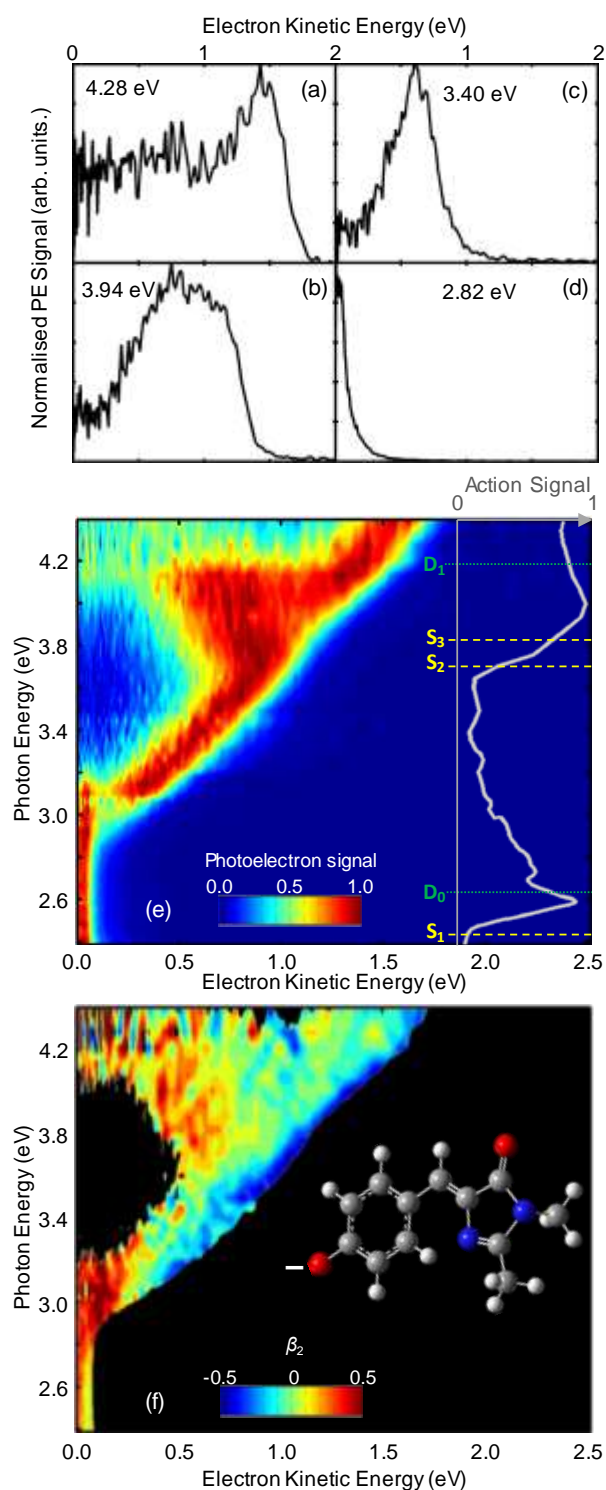


Figure 1: Frequency- and angle-resolved photoelectron (PE) spectra of HBDI. Representative PE spectra are shown in (a) – (d). The complete set of peak-normalised PE spectra is shown in (e), together with the action spectrum and calculated energies, taken from reference (9). The PE anisotropy parameters are shown in (f) with the structure of HBDI inset.

Representative examples of PE spectra are shown in Figures 1a-d, while Figure 1e summarises all frequency-resolved PE spectra of HBDI for photon energies between 2.39 eV and 4.39 eV (48 individual PE spectra in Supporting Information). The PE spectra have been

normalized to have a maximum intensity of unity, which accentuates the spectral changes as a function of photon energy. The absolute PE yield, however, is strongly modulated across this range because of the variations in photo-induced detachment cross-sections. To highlight this variation, the action (absorption) spectra leading to “prompt” electron loss as reported in the recent study of Bochenkova *et al.* is also shown, together with their calculated energetics of relevant electronic states.⁹

Despite $\text{HBD}\Gamma$ having an observed adiabatic detachment energy of 2.7 eV,^{10,12,23} PE signal was observed between $2.39 \text{ eV} < h\nu < 2.70 \text{ eV}$, although signal levels rapidly became very weak at the lower photon energies. This below-threshold contribution is facilitated by the $\sim 300 \text{ K}$ temperature of the anions in our experiment, which corresponds to an internal energy of $\sim 310 \text{ meV}$. The PE spectra in this range are all essentially identical (independent of photon energy) and consist of a peak centred at $eKE \sim 0.03 \text{ eV}$ and a feature decaying exponentially from $eKE = 0 \text{ eV}$. These features remain identifiable up to $h\nu = 3.1 \text{ eV}$. However, from $h\nu = 2.8 \text{ eV}$, a new PE peak is observed at higher eKE that increases linearly with photon energy as might be expected for a direct (vertical) detachment process. For $h\nu > 3.7 \text{ eV}$, however, the eKE distribution broadens with increasing photon energy, reaching a spectral width of $eKE \sim 1 \text{ eV}$ at $h\nu \sim 4.1 \text{ eV}$. The high energy edge of this PE feature remains linear with photon energy but the average eKE of the spectrum shifts to increasingly lower energy to yield a bimodal distribution. In fact, the low energy side of this bimodal distribution remains approximately constant in eKE with increasing $h\nu$, as can be seen more clearly from the individual PE spectra shown in the Supporting Information. The maximum of the low energy peak is around $eKE \sim 0.6 \text{ eV}$. For $h\nu > 4.1 \text{ eV}$, the progressively broadening feature abruptly collapses to a PE spectrum that has a feature consistent with direct detachment as well as a very broad and featureless distribution of electrons ($0 < eKE < 1.3 \text{ eV}$). The relative intensity of this broad distribution is lower than the low energy peak

observed for $3.7 < h\nu < 4.1$ eV, however, their integrated PE yields are approximately the same. The broadened feature contributes about 60% of the total PE yield, with the other 40% arising from the constant binding energy PE feature at high eKE (see Supporting Information).

In addition to the frequency-resolved PE spectra, the photoejection anisotropy parameter, β_2 , which quantifies the PE angular distribution,²⁴ are plotted in Figure 1f. These show that there is a small but consistent difference in $\beta_2(eKE)$ for the peak correlated with a direct detachment process and the feature at lower eKE in the $3.7 < h\nu < 4.1$ eV range. The sole PE peak between $3.2 < h\nu < 3.6$ eV has $\beta_2 \sim 0$ over the width of this constant binding energy peak remains this value for $h\nu > 3.6$ eV. Around $h\nu \sim 3$ eV and between $0.1 < eV < eKE < 0.2$ eV, the PE anisotropy is $\beta_2 \sim 0.4$.

The spectral broadening of the PE spectra over the $3.7 \text{ eV} < h\nu < 4.1 \text{ eV}$ range suggests that excited state dynamics are occurring in competition with autodetachment. This broadening was previously assigned to vibrational motion on the S_3 state.¹⁵ However, the spectral broadening could also arise from internal conversion of the S_3 state to the lower-lying S_2 state followed by autodetachment. The S_2 state has a calculated energy that lies within 0.1 eV of the S_3 state⁹ suggesting that pathways of coupling these two states may be present.

In order to glean further insight into the decay mechanisms, the dynamics of the S_3 state were probed using time-resolved PE spectroscopy. In these experiments, the S_3 excited state was populated using femtosecond photoexcitation at 4.1 eV (300 nm), which was subsequently probed at 1.55 eV (800 nm). The excitation energy was chosen to energetically coincide with the spectral broadening observed in the $3.7 < h\nu < 4.1$ eV region of the frequency-resolved PE spectra as well as the local maximum in PE yield from the action spectra.

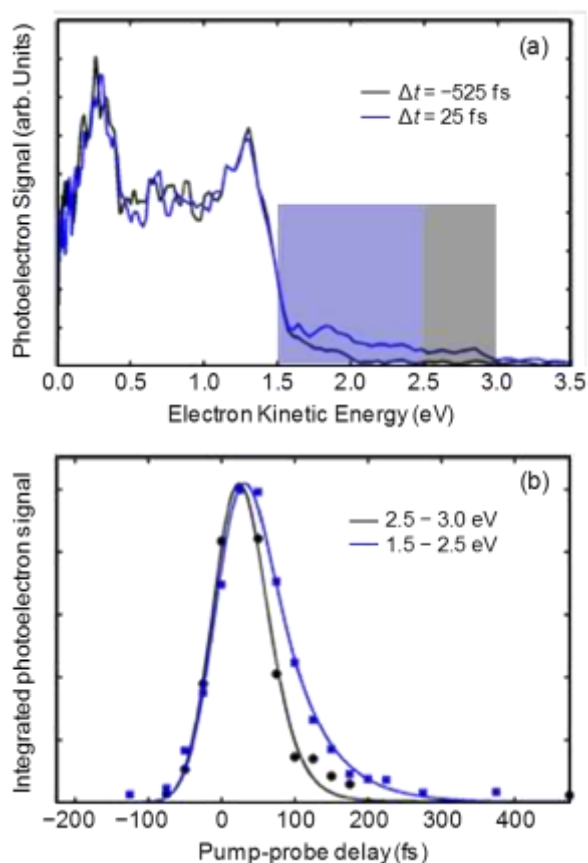


Figure 2: (a) Time-resolved photoelectron (PE) spectra of HBDI Γ excited at 4.1 eV. (b) Decay dynamics of PE signal taken over the spectral windows shown in (a).

Figure 2a shows two representative pump-probe PE spectra recorded with the probe arriving before pump pulses ($\Delta t = -525$ fs) and near the temporal overlap of the two pulses ($\Delta t = 25$ fs). Both spectra show two peaks centred at ~ 0.25 eV and ~ 1.28 eV. The appearance of the PE spectrum between $0.5 < eKE < 1.5$ eV is consistent with the 4.1 eV (300 nm) PE spectrum obtained in the frequency-resolved experiments. The peak at $eKE \sim 0.25$ eV arises from a non-resonant two-photon detachment processes from the 1.55 eV probe; the PE spectrum at $eKE < 0.5$ eV is consistent with the PE spectrum recorded at 3.1 eV (400 nm). At $\Delta t \sim 0$, an additional PE feature between $1.5 \text{ eV} < eKE < 3.0 \text{ eV}$ is observed. This can be assigned to photodetachment from the excited state population; the PE spectrum of the time-dependent feature extends approximately an additional 1.55 eV beyond that of the pump-only PE spectrum. The breadth of the time-dependent PE feature suggests that it contains

contributions from both excited state population leading to both prompt autodetachment (high eKE electrons) and delayed autodetachment (lower eKE).

To gain quantitative insight, the time-dependent PE feature can be divided into two spectral windows as shown in Figure 2a. The $2.5 < eKE < 3.0$ eV window probes population of the initially excited state and the $1.5 < eKE < 2.5$ eV window probes population of the intermediate state to which the initial state has decayed. The time-varying integrated PE signals for each of these spectral windows are given in Figure 2b and were fitted to an exponential decay function, $f(t) = A\exp(-(t-t_0)/\tau)$, convoluted with the instrument response function (FWHM = 75 fs). The amplitude (A), lifetime (τ), and time-zero (t_0), were allowed to vary. The t_0 for both spectral windows were less than 5 fs apart and can be assumed to be essentially identical. The lifetime of the initially excited state was found to be 25 fs, which is less than our temporal resolution and so we may only quote this as <40 fs. The lifetime of the intermediate state was found to be 55 fs, which is still extremely fast, although visibly slower than that of the initially excited state.

DISCUSSION

The PE peak at $eKE = 0.03$ eV in the range $2.6 < hv < 3.1$ eV has previously been assigned to vibrational autodetachment (VAD) from the S_1 excited state.⁸ The exponential tail decaying from $eKE = 0$ eV has been correlated with statistical (thermionic) electron emission.¹² These features remain identifiable up to $hv \sim 3.1$ eV. However, from $hv > 2.8$ eV, a new PE peak is observed at higher eKE . This new feature corresponds to direct photodetachment into the continuum. Between $2.8 < hv < 3.1$ eV the two processes are in competition and VAD is dominant because the S_1 state is predominantly excited. In the higher energy window, $3.2 < hv < 3.7$ eV, the cross-section for excitation to the S_1 sharply decreases and the PE signal is probably dominated by direct detachment. The photoejection

anisotropy is significantly more positive around $h\nu \sim 3.1$ eV as shown in Figure 1f. This might be a consequence of the interference between autodetachment and direct detachment into the continuum leading to a different β_2 value than for exclusively direct detachment, which is dominant for $3.2 < h\nu < 3.7$ eV.

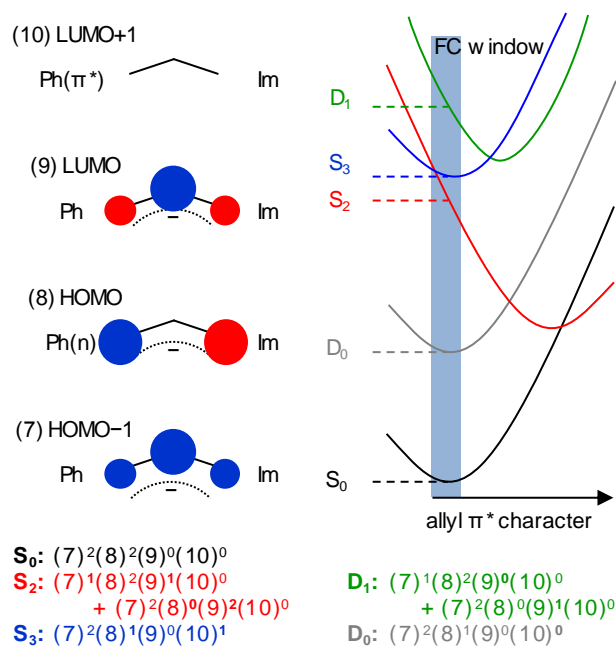


Figure 3: Left: Molecular orbitals of HBDI⁻ with contributions to central allyl bridge that connects the phenol (Ph) with the imidazole (Im) shown schematically. Right: Schematic of potential energy curves of the various anionic and neutral excited states considered as a function of allyl antibonding character. Below: Electronic configurations of the various electronic states, where the bold numbers indicate where electrons have been moved.

The frequency-resolved PE spectra combined with the prompt action-spectra from the Andersen group⁹ show that for $h\nu > 3.7$ eV higher-lying excited states are accessed. Using high-level calculations, Bochenkova *et al.* showed that the S_2 and S_3 are very close in energy at 3.7 eV and 3.8 eV, respectively, in the vertical Franck-Condon (FC) region (see Figure 1e). The increase in the action spectrum shows that the PE peak that is increasing linearly with photon energy for $h\nu > 3.7$ eV is predominantly arising from an indirect detachment process. In Figure 3, we show the molecular orbital (MO) configurations of the relevant electronic states of HBDI⁻ and its neutral as calculated by Bochenkova *et al.*⁹ To a first approximation,

the MOs can be roughly classified into different types of localised MOs that are useful as a guide to further discussions. MO(7) is primarily of π bonding character on the allyl moiety that couples the phenol to the imidazole rings. MO(8), which is the highest occupied MO (HOMO), is essentially non-bonding on the allyl bridge, while MO(9) has π^* character there. The $S_2 \leftarrow S_0$ transition is one in which π bonding orbitals are replaced for antibonding orbitals over the allyl. Hence, population of the S_2 state in the FC region is likely to involve rapid wavepacket motion leading to a stretching of the allyl bridge. The $S_1 \leftarrow S_0$ transition is similar in that it has π^* character on the allyl bridge and initial wavepacket dynamics of the allyl stretching mode on the S_1 state was predicted to be on the order of 10 fs.^{25,26} For the S_2 state, the antibonding character is increased and one might expect the S_2 state to be more repulsive along the stretching coordinate of the allyl bridge (at the FC) with initial nuclear wavepacket motion that is similar to or faster than on the S_1 state. In contrast, the $S_3 \leftarrow S_0$ transition is one in which MO(8) is excited to MO(10), which is localised on the phenol-ring. The main structural differences are thus expected to be on the phenol and one might anticipate only modest structural changes along the allyl coordinate. Finally, we consider the neutral states, D_0 and D_1 . The ground D_0 state corresponds to loss of an electron from MO(8). As MO(8) is essentially non-bonding on the allyl bridge, the D_0 state will have a similar minimum energy geometry to the S_0 state along the allyl stretching coordinate. Indeed the PE feature for direct detachment is relatively sharp given the molecular size of HBDI.^{12,23} The S_3 excited state is correlated with D_0 in a one electron Koopmans picture; S_3 is a shape resonance, which typically have very short autodetachment lifetimes (10s fs). In contrast, the S_2 state does not correlate with the D_0 state (*i.e.* S_2 is a Feshbach resonance), but it is correlated with the D_1 excited state of the neutral. The above picture has been schematically summarised as potential energy curves along the allyl stretch (π^* character) in Figure 3 and

we will use this as a guide to understanding the dynamics that are evident in the frequency-resolved PE spectra.

The spectral broadening in the $3.7 < h\nu < 4.1$ eV range suggests that excited state dynamics are occurring before electron emission. The $S_3 \leftarrow S_0$ excitation is significantly brighter than the $S_2 \leftarrow S_0$ excitation, hence, we may assume that the S_3 state is initially populated in this range, and direct detachment is a very minor channel that can essentially be ignored. The observed broadening in the PE spectra could arise from vibrational dynamics on the S_3 state that precedes autodetachment, as suggested by Mooney *et al.*¹⁵ Alternatively, because the S_2 state is in very close proximity to the S_3 state in the FC region, it is conceivable that there exists a strong coupling or conical intersection between these two states that can transfer population from S_3 to S_2 on a timescale faster than autodetachment. Such dynamics have indeed been observed in some quinone anions.^{21,27,28}

The latter of the two scenarios above is more consistent with the experimental results. The PE angular distribution across the bimodal peak is not constant. The anisotropy over the high energy component is slightly negative ($\beta_2 \sim -0.3$), while that across the lower eKE portion is positive ($\beta_2 \sim +0.2$). Because the PE angular distribution depends sensitively on the MO from which the electron is detached, the difference in the anisotropy across the bimodal distribution reflects a change in the electronic character.²¹ Additionally, the S_3 state minimum energy geometry is not expected to be very different to that of the D_0 state, so it would be difficult to reconcile the large spectral red-shift of (~ 0.8 eV) assuming vibrational motion occurring on a timescale of <40 fs. In contrast, the S_2 state is expected to have its minimum energy geometry far from the FC region because of the strong π^* character on the allyl bridge. As a result, one might envisage that the S_2 and D_0 potential energy surfaces will be far from parallel along the allyl stretching coordinate, as shown schematically captured in Figure 3. Furthermore, the dynamics of along the allyl stretch are known to be very fast (10 fs).^{25,26}

Of course, these dynamics occurring on the S_2 state would be in competition with autodetachment. However, as the S_2 state is not correlated with the D_0 state, the $S_2 \rightarrow D_0$ autodetachment will be slower allowing for the vibrational wavepacket to sample a larger area of the S_2 potential energy surface. This would in turn lead to a large spectral width in the PE emission as observed.

Based on the time-resolved PE spectra, the dynamics of the $S_3 \rightarrow S_2$ internal conversion is extremely fast (sub-40 fs). However, the internal conversion is also in competition with autodetachment from the S_3 state. This is evidenced in the frequency-resolved by the PE feature that increases linearly with eKE as the photon energy is increased. This PE feature is predominantly due to the $S_3 \rightarrow D_0$ autodetachment channel because direct detachment into the continuum is a minor channel in this energy range⁹ (see action spectrum in Figure 1e). From the frequency-resolved spectra, the PE yield due to $S_3 \rightarrow D_0$ autodetachment is ~40% of the total PE yield, with the remaining 60% arising from S_2 autodetachment (see Supporting Information). Based on competing kinetics internal conversion occurs on a <65 fs timescale while $S_3 \rightarrow D_0$ autodetachment is slower. However, we note that the true lifetime will be significantly faster given that the $S_3 \rightarrow D_0$ autodetachment is a very favourable process, but it is limited by our time resolution. The spectral broadening observed over the $3.7 < h\nu < 4.1$ eV range can thus be assigned to vibrational dynamics on the S_2 state that leads to a broad PE spectrum in this range. The overall decay of this S_2 state feature occurs on a timescale of 55 fs, and can be correlated to the $S_2 \rightarrow D_0$ autodetachment.

Perhaps the most striking feature of the frequency-resolved PE spectra is that at $h\nu > 4.1$ eV, the PE spectra abruptly change from the bimodal distribution (between $3.7 < h\nu < 4.1$ eV) to a very broad distribution (see Figure 1a and b). This change is suggestive of a new decay pathway becoming available at this energy, and happens this to coincide with the

calculated location of the D_1 excited state of the neutral in the FC region.⁹ The D_1 state is not correlated with the S_3 state, but it is correlated with the S_2 state in a one electron Koopmans' interpretation (see Figure 3). Hence, we propose that, as the photon energy increases and becomes higher than the D_1 energy, autodetachment from the S_2 state becomes much faster by virtue of the favourable $S_2 \rightarrow D_1$ autodetachment channel. Indeed, it can be seen in the frequency-resolved PE spectra, and more clearly in the PE spectra at $h\nu = 4.28$ and $h\nu = 3.94$ eV (Figure 1a and b), that the peak around $eKE \sim 0.6$ eV, that can be assigned to $S_2 \rightarrow D_0$ autodetachment, relative to the peak arising from $S_3 \rightarrow D_0$ autodetachment is decreasing. Concomitantly, a new feature emerges at lower eKE .

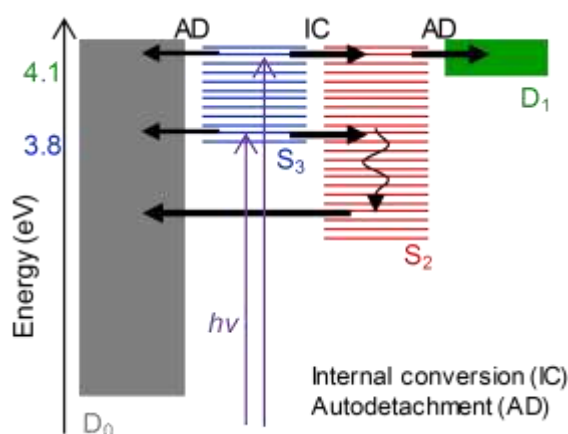


Figure 4: Summary of the dynamics occurring in HBDI^- following excitation below and above the onset of the D_1 excited state. The black horizontal arrows indicate the competing processes and their thickness reflects their respective rates.

For completeness, we consider the alternative scenario in which the S_2 state plays no role in the dynamics. In this case, all dynamics observed between $3.7 < h\nu < 4.1$ eV would be occurring on the S_3 state. The change in PE spectra at $h\nu = 4.1$ eV could then be assigned to either a conical intersection with the S_2 state that becomes available at this energy, or the opening of the $S_3 \rightarrow D_1$ autodetachment channel. The problem with the latter is that the S_3 state is not correlated with the D_1 state. Additionally, in either case, one would expect that the PE yield in the $S_3 \rightarrow D_0$ autodetachment channel (peak at highest eKE) would transfer to a

lower eKE and this is not experimentally observed. Instead, the contribution of the $S_3 \rightarrow D_0$ autodetachment channel to the total PE yield does not change between $h\nu = 3.9$ and 4.3 eV (see Supporting Information) and the feature at $eKE \sim 0.6$ eV loses PE signal to become the feature at lower eKE . Therefore, only population that has first undergone internal conversion from the S_3 excited state is involved in detachment through the new channel. Finally, one could interpret the bimodal feature as being composed of $S_3 \rightarrow D_0$ (peak at $eKE \sim 0.6$ eV) and direct $S_0 \rightarrow D_0$ detachment (peak increasing linearly in eKE with $h\nu$), as done by Mooney *et al.*. However, based on the integrated electron yields in these channels, this would suggest that the cross section for $S_3 \leftarrow S_0$ excitation relative to direct $S_0 \rightarrow D_0$ detachment is $\sim 3:2$. This is completely inconsistent with the absorption spectrum shown in Figure 1e, which clearly shows that the absorption cross section is ~ 10 times greater than direct detachment. Thus, the experimental data, when viewed holistically, strongly points to a process in which the S_3 population rapidly converts to the S_2 state.

SUMMARY

In summary, using frequency-, angle- and time-resolved photoelectron spectroscopy, we have assigned the dynamics following UV excitation of the chromophore anion of the green fluorescent protein and this is summarised in Figure 4. Excitation with $h\nu > 3.7$ eV leads to the population of the bright S_3 excited state that decays through a combination of autodetachment to the D_0 ground neutral state and internal conversion to the nearby S_2 excited state, with the latter having a higher yield. The subsequent dynamics on the S_2 state involve rapid vibrational motion that is in competition with autodetachment from the S_2 state to the D_0 neutral ground state. When $h\nu \geq 4.1$ eV, the $S_2 \rightarrow D_1$ autodetachment channel opens up and this is favoured over the $S_2 \rightarrow D_0$ channel based on a Koopmans' interpretation. In GFP, the excited states cannot autodetach as the vacuum is replaced by a protein that is

surrounded by water.²⁹ Bochenkova *et al.* showed that the UV excited states are quasi-resonant with the hydrated electron in aqueous GFP such that autodetachment could be replaced by charge-transfer, which could lead to photo-oxidation of the chromophore.⁹ In the limit that the isolated HBDI⁻ has a similar electronic structure in GFP, our results suggest that this pathway will not be very efficient because the optically bright S₃ state rapidly converts to the S₂ state. Although the dynamics on S₂ lead to a broad spectrum that will enhance overlap with the hydrated electron state(s), one would also expect a very weak coupling between the donor and acceptor states. This is because the S₂ state is correlated with the D₁ excited neutral state and charge-transfer would require a two-electron transition. Nevertheless, the lifetime of the S₂ state in the protein may be sufficiently long to enable charge-transfer. We stress that these arguments are based on the assumption that the S_n excited states of gas-phase HBDI⁻ are similar in the protein, which requires further investigation; however, UV excitation in the protein is experimentally complicated by the fact that several protein residues also absorb in this range. Hence, gas-phase studies provide an important portal to explore the UV excited photo-physics of GFP.

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SUPPORTING INFORMATION AVAILABLE: Discussion of photoelectron (PE) yields in the different autodetachment channels; individual PE spectra that make up Figure 1e. This information is available free of charge via the internet <http://pubs.acs.org>.

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