Photophysics

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UV Excited-State Photoresponse of Biochromophore Negative Ions**

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Abstract: *Members of the green fluorescent protein (GFP) family may undergo irreversible phototransformation upon irradiation with UV light. This provides clear evidence for the importance of the higher-energy photophysics of the chromophore, which remains essentially unexplored. By using time-resolved action and photoelectron spectroscopy together with high-level electronic structure theory, we directly probe and identify higher electronically excited singlet states of the isolated para- and meta-chromophore anions of GFP. These molecular resonances are found to serve as a doorway for very efficient electron detachment in the gas phase. Inside the protein, this band is found to be resonant with the quasicontinuum of a solvated electron, thus enhancing electron transfer from the GFP to the solvent. This suggests a photophysical pathway for photoconversion of the protein, where GFP resonant photooxidation in solution triggers radical redox reactions inside these proteins.*

Electronic resonances are well-known to play an important role in dissociative electron attachment to neutral closed-shell biological molecules, thus producing intermediate radical anion states relevant, for example, to DNA damage.[1,2] A ladder of electronically excited resonance states is also thought to provide fast channels for stabilization of transient negative ion states in the gas phase, as revealed in the case of *para*-benzoquinone.[3] This may have a direct implication on electron-transfer processes in solutions and proteins, where radical anion states, which acquire a bound character, are capable of forming a dense manifold of excited charge-transfer states. These states enhance electron-acceptor dynamics, for example, in photosynthesis.[3]

In contrast, higher electronically excited singlet states S_n ($n>1$) of biochromophore negative ions are much less studied, since focus is usually on their photochemically relevant first excited state, which is important for the primary functioning of proteins containing anionic, deprotonated chromophores. Proteins of the green fluorescent protein (GFP) family,[4] which contain 4-(*para*-hydroxybenzylidene)-5-imidazolinone as a chromophore, have attracted much attention because of their remarkable fluorescent properties, which are widely employed in bioimaging and biotechnology.[5] GFPs may also undergo irreversible phototransformation upon irradiation with UV light.[6] Photoinduced post-translation modification of proteins, which includes decarboxylation (loss of CO_2) of the key glutamate residue Glu222 located in the vicinity of the chromophore,[6,7] results from a largely unknown UV reactivity of the GFP-related chromophores inside the proteins. It has been shown that such photoconversion may also occur with visible light upon consecutive resonant two-photon excitation of the neutral as well as anionic chromophores, presumably populating higher electronically excited states.[8,9]

The phototransformation mechanism remains elusive. van Thor et al. suggested that the oxidative decarboxylation proceeds in GFP according to a so-called Kolbe-type mechanism.[6,10] This mechanism is well-known in chemistry through similar thermal reactions of carboxylic acids, where oxidants such as Mn^{III} are known to act as catalysts.[11] In GFPs, an oxidant state is assumed to be formed upon irradiation with UV or visible light.[6] However, the nature of this state cannot be inferred from the Kolbe reaction, nor can it be directly revealed in the experiment. Moreover, a lack of unambiguous identification of the absorption character in the visible range (one- versus stepwise resonant two-photon absorption) obscures its interpretation. It has been suggested that the electronically excited chromophore (regardless of its protonation state) acts as an electron acceptor, directly oxidizing Glu222 inside the protein.[6,7] However, the reduction of, in particular, an excited anion seems questionable. We propose an alternative photophysical pathway that triggers redox reactions inside the GFP, where the primary step is an excited-state photodetachment from the chromophore anion on irradiation with one-photon UV or two-photon visible light to produce a solvated electron and a radical chromophore. The photooxidized radical chromophore inside the protein, therefore, forms an oxidant state in the decarboxylation of Glu222. The fundamental questions addressed here are the redox properties of the excited GFP chromophore anion and the pattern of excited states.

The higher excited states of the GFP chromophore anion are not amenable to direct characterization in the protein environment due to their low intensity and very fast electronic

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de-excitation. Moreover, the UV absorption of the chromophore anion inside the protein heavily overlaps with the absorption of aromatic amino acid residues, thus making identification of the states even more challenging. These states are electronically bound in the condensed media, whereas they acquire a metastable character in the gas phase, decaying through electron emission. Here, we exploit their resonant nature and directly probe the higher excited states of deprotonated GFP-model chromophores (Figure 1). By using a combination of time-resolved action and photoelectron spectroscopy we show that the photoresponse of the chromophore anions provides compelling evidence for resonant photodetachment from S_n states in the gas phase. The implication of the intrinsic properties of the GFP chromophore anion to those inside the protein is revealed by performing protein excited-state calculations.

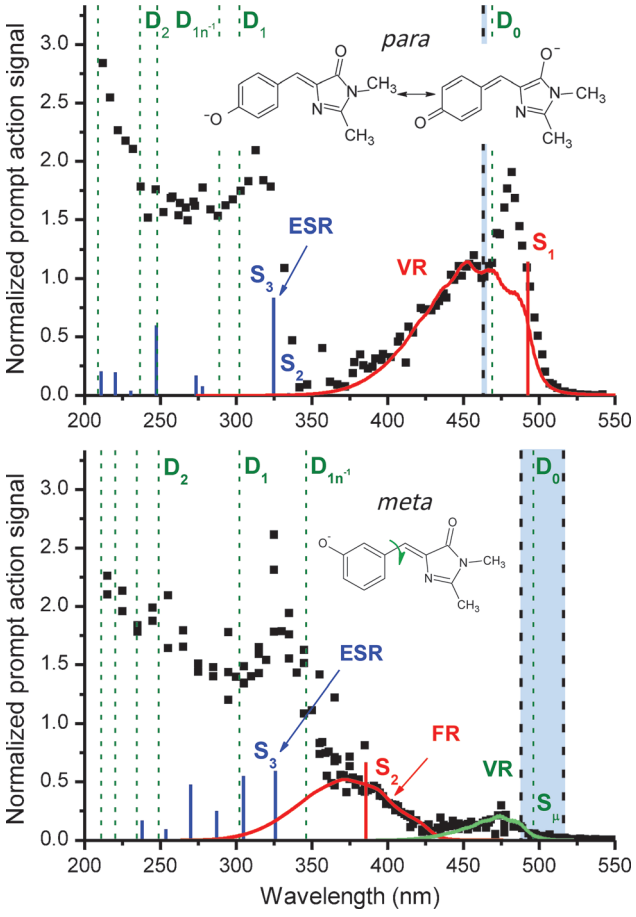

Figure 1. Prompt action spectra of the GFP *para*- and *meta*-chromophore anions (black dots) together with calculated one-photon spectral profiles (colored curves) and vertical excitation energies (colored vertical lines). The experimental spectra are normalized with respect to the calculated oscillator strengths of the brightest transitions. The relative intensities of the higher excited states in the UV region (blue lines) are multiplied by 10. The positions of the calculated π^{-1} (D_n) and n^{-1} (D_{nn}^{-1}) neutral radical states with respect to the planar ground-state equilibrium geometry of the chromophores are shown as green dashed lines. The experimental vertical detachment thresholds are depicted with black dashed lines. Note the significant spread of vertical detachment energies as a result of inhomogeneous broadening in the *meta* case highlighted in blue. Different laser settings were used above and below 420 nm, where the spectra from the two regions were merged. VR, FR, ESR stand for vibrational, Feshbach, and excited-shape resonances, respectively.

The action spectroscopy experiment is conducted at the electrostatic ion-storage ring ELISA.[12] The number of neutral photofragments is used as a measure of the absorption cross-section after normalization to the number of photons and stored ions.[13] The details of the experimental and theoretical methods are described in the Supporting Information.

We have previously shown that the prompt action spectrum of the native GFP *para*-chromophore anion within the visible range at low excitation energies is completely dominated by an intense bound-bound $S_0 \rightarrow S_1$ transition, and electron autodetachment here proceeds from the $\pi^1\pi^{*1}$ S_1 state through vibrational resonances (VRs).[14] The high density of states of Franck–Condon-active vibrational modes is recognized to cause a relatively large blue-shift of the absorption cross-section away from the most intense $0 \rightarrow 0$ transition at 492 nm (red curve, upper panel in Figure 1). Furthermore, there is a significant contribution from a sequential multiphoton $S_0 \rightarrow S_1$ absorption to the first peak at 482 nm below the detachment threshold. In this spectral region, there is a strong and wavelength-dependent competition between prompt photodetachment and delayed action in the hot ground state after fast internal conversion.[14–17] Internal conversion is a predominant channel at 482 nm, which also enables an efficient sequential absorption of more than one photon within the same laser pulse. Prompt electron emission after one-photon absorption dominates through the entire spectrum below 460 nm. The multiphoton contribution at low excitation energies results in an enhanced signal in the action spectrum above the calculated absorption profile.[18]

We focus here on the higher electronically excited states of the GFP chromophore anion in the previously unexplored UV region down to 210 nm, as well as address the UV/Vis spectroscopic properties of the *meta* analogue of the GFP chromophore to probe the electronic continuum of the molecular anion with an essentially different electronic structure. In all cases, only a prompt response is observed, which is associated with resonant photodetachment (Figure 1). Remarkably, an enhanced action signal of the *para* anion only appears below 325 nm (3.8 eV), which is well above the anion detachment threshold previously found at 2.68 ± 0.1 eV.[19–22] This intense and broad feature is ascribed to excitation to the $\pi^1\pi^{*0}\pi^{*1}$ S_3 anion state, which is the first excited shape resonance (ESR) that decays very fast to π^1 D_0 (Figure 2).

The difference in the electronic structures of the *para* and *meta* anions[24] is evident in their action absorption spectra in the photochemically relevant low-energy part. The S_1 spectral region of the *meta* anion is theoretically expected to be at the very red edge of the visible region around 700 nm, with a very low oscillator strength because this charge-transfer state is optically dark. Therefore, the impact of the S_1 state is eliminated from the otherwise low action absorption cross-section at 500 nm close to the opening of the electronic continuum, which is here assigned to rotational/vibrational autodetachment out of a dipole-bound state (S_μ). The

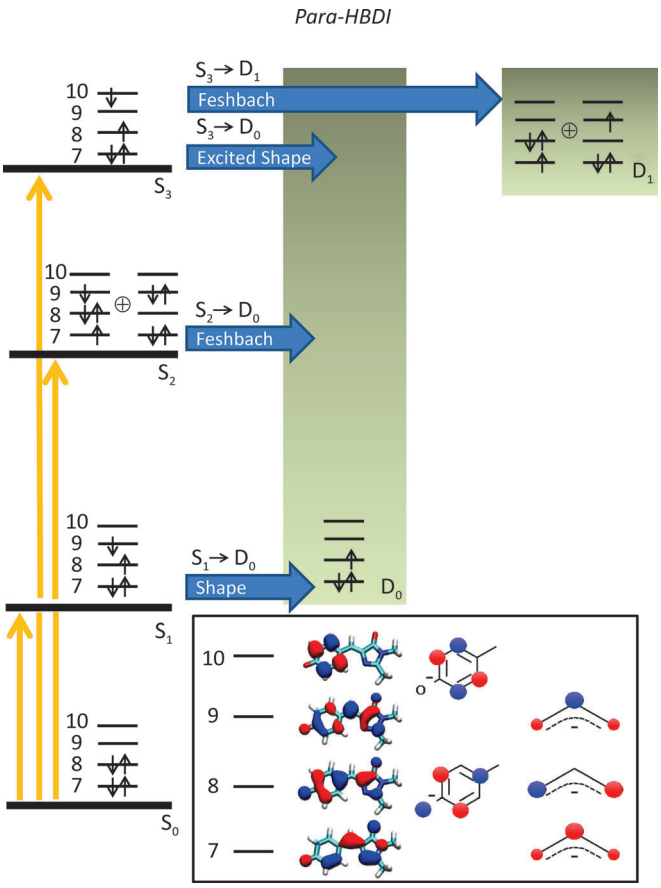

Figure 2. Schematic energy level diagram for the GFP *para*-chromophore anion. Shown are the π and π* XMCQDPT2[23] natural orbitals primarily involved in the excitations as well as their simplified Hückel representation that emphasizes the nature of the transitions. The autoionizing decays are denoted as shape or Feshbach type according to their nature.

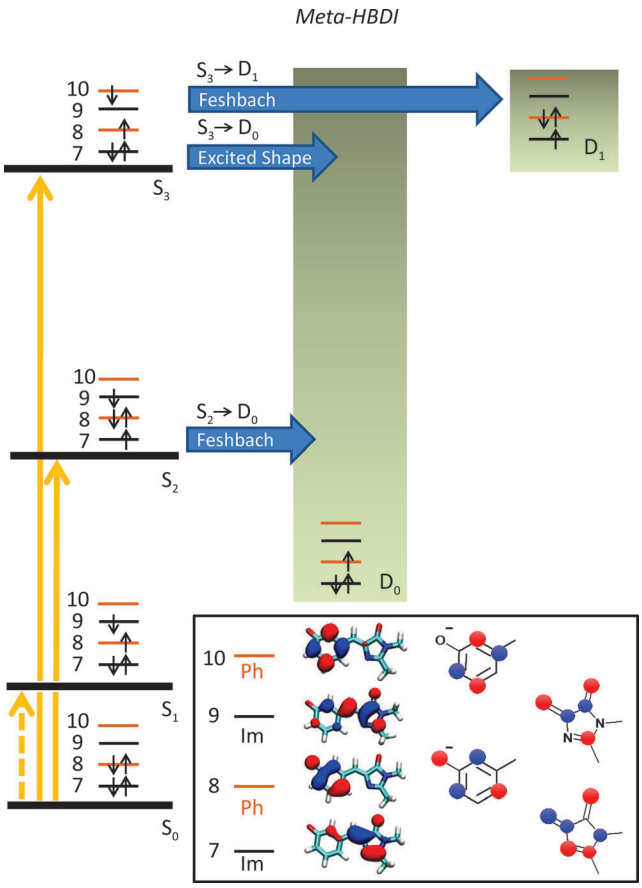

Figure 3. Schematic energy level diagram for the GFP *meta*-chromophore anion. Shown are the π and π* XMCQDPT2[23] natural orbitals primarily involved in the excitations as well as their simplified Hückel representation that emphasizes the nature of the transitions. The autoionizing decays are denoted as shape or Feshbach type according to their nature. Im = methylidene–imidazolinone, Ph = phenolate.

calculated dipole moment of the neutral radical core at a planar geometry (7.4 D) and the spectral profile computed based on the S_0 and D_0 equilibrium structures support this (green curve, lower panel in Figure 1).

The vertical detachment energy of the *meta* anion was determined from our separate photoelectron measurements[19] (see the Supporting Information). Its detachment threshold is strongly influenced by inhomogeneous broadening caused by internal rotation of the anion along the single C–C bridging bond in S_0, thus red-shifting the detachment energy. From the photoelectron spectra obtained at various wavelengths, the vertical detachment energy is found to be in the range of 2.40–2.54 ± 0.1 eV, where the latter value refers to the planar structure. These values are somewhat lower than that of the *para* anion (2.68 ± 0.1 eV),[19] where the negative charge is stabilized through a resonance between the two mesomeric structures (see Figure 1).

An additional shoulder below 425 nm is observed in the case of the *meta* anion, as seen in Figure 1. It is ascribed to the brightest $S_0 \rightarrow S_2$ transition localized at the heterocyclic ring (Figure 3). This resonance state is of Feshbach type (FR) with respect to the open D_0 continuum, which results in a long lifetime and a weak interaction with the electronic continuum. The bound-bound calculations of the spectral profile reproduce well its experimental low-energy part that is not hidden under the overlapping broad ESR resonance (red curve, lower panel in Figure 1).

Despite a clear difference in the visible region of the spectrum, both chromophores show a remarkable similarity at about 3.8 eV (325 nm), which is ascribed to the appearance of the first ESR, as well as above 3.8 eV, where a rather dense manifold of the electronic states of both the anion (S_n) and the neutral radical core (D_n) smears the signatures of the chromophores chemical structure. The electronic ladder of resonances efficiently induces electron detachment, thus opening a pathway to the radical chemistry of the GFP chromophores.

In the GFP protein, the higher excited states of the *para* anion appear to be only slightly blue-shifted to above 4.1 eV (300 nm, Figure 4). Each state is individually affected by the protein, and the order of states is different from that in the gas phase. However, despite the relatively low intensities of the corresponding $S_0 \rightarrow S_n$ transitions, the density of S_n states is high, similar to that of the isolated anion. This high density of states forms a quasiband inside the protein that is well-

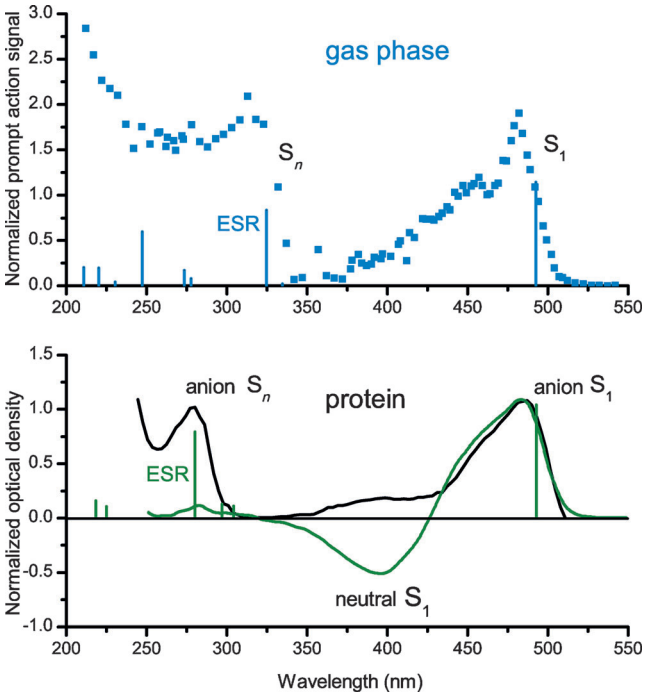

Figure 4. UV/Vis action absorption spectrum of the native GFP chromophore anion in the gas phase (top) and its absorption spectra inside various GFP proteins (bottom). The absorption spectrum of the entire protein EGFP, primarily containing the anionic chromophore,[25] is shown in black. The differential absorption spectrum of the 254 nm photoconverted (primarily containing the anionic chromophore) and pre-illuminated wild-type GFP (primarily containing the neutral chromophore) (adapted from Ref. [7]) is shown in green, where positive values refer only to the absorption of the anionic chromophore inside the protein, while absorption of Tyr and Trp residues are eliminated at 280 nm. The vertical bars represent the calculated vertical transitions, with intensities multiplied by 10 in the UV region.

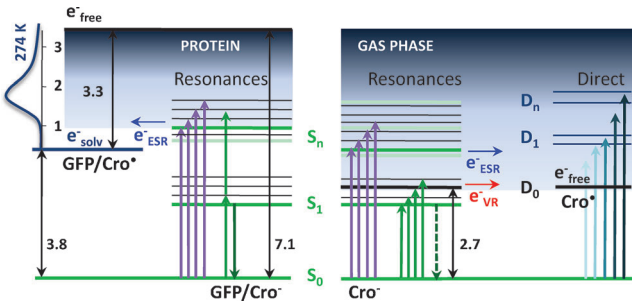

Figure 5. Schematic energy level diagram (in eV) of the GFP chromophore (Cro) anion with respect to the formation of free and solvated electrons in the gas phase and inside the protein, respectively. The absorption spectrum of the hydrated electron at 274 K is shown to the left (adapted from Ref. [28]). Note that one-photon absorption in the UV region as well as two-photon excitation with visible light populate higher excited states of the anion, which have a true (quasi)resonant nature with respect to the formation of a free (solvated) electron and a radical.

separated from the photochemically active S_1 state, hence creating a band gap.

These higher excited states might play a major role in the UV photophysics of the GFP as intermediate anion states, from which charge transfer to the solvent occurs, thereby resulting in formation of a solvated electron and a radical chromophore inside the protein. Our theoretical estimate leads to a threshold value of 7.1 eV for the vertical ionization of S65T GFP. Free electrons in bulk aqueous solution are experimentally found to be bound by 3.3 eV.[26] Therefore, the threshold for the formation of a solvated electron is located at about 3.8 eV, just below the quasiband of the higher excited states of the chromophore anion inside the protein (Figure 5). This makes GFP resonant photooxidation possible over a wide UV range.

The efficiency of the GFP resonant photooxidation in solution should, however, depend on the excitation wavelength, since a coupling between the transient S_n states and the final states of the solvated electron (hence, the tunneling rates) vary with energy. The most efficient photoconversion should occur at higher energies, where the solvated electron exhibits a quasicontinuum with a very dense manifold of excited states. By taking into account the onset of the quasicontinuum of the solvated electron at approximately 1 eV, which refers to excitation from its ground *s* state to excited *p* states (see Figure 5),[27] a threshold of about 4.8 eV (258 nm) is obtained. This is consistent with the experimental findings, where the rate of GFP photoconversion is significantly higher at 254 nm than at 280 nm.[7] In this energy range, the bright ESR state plays a major role (see Figure 4).

Once the electron is transferred to the solvent, it is plausible that secondary radical reactions are initiated between the chromophore and the nearby glutamate residue, where the radical chromophore might serve as an acceptor in electron transfer from Glu222, thereby initiating its decarboxylation. This reveals new insight into the mechanisms underlying the GFP phototransformation with UV or multiphoton visible light, and suggests GFP resonant photooxidation in solution to be a trigger to the radical chemistry of GFPs.

In summary, we have experimentally and theoretically identified higher excited states of the isolated GFP *para*- and *meta*-chromophore anions in the previously unexplored UV region down to 210 nm, thereby revealing a high density of electronic states and their major role in the photoresponse of these chromophores. The molecular resonances in UV serve as a doorway for efficient electron detachment in the gas phase, appearing well above the detachment thresholds of the anions. By performing calculations on the GFP protein, we have shown the existence of a rather dense manifold of such states also inside the protein, which is a direct consequence of the intrinsic properties of the anionic chromophore. Importantly, they are found to be resonant with the quasicontinuum of a solvated electron, thus suggesting a new photophysical pathway for the photoconversion of GFP in UV region.

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