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How far can a single hydrogen bond tune the spectral properties of the GFP chromophore? †

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Photoabsorption of the hydrogen-bonded complex of a neutral and an anionic Green Fluorescent Protein chromophore has been studied using a new dual-detection approach to action-absorption spectroscopy. Following absorption of one photon, dissociation through a single channel ensures that the full absorption spectrum is measured. Our theoretical account of the spectral shape reveals that the anionic 0-0 transition (464 nm) is blue-shifted compared to that of the wild-type protein (478 nm) due to the stronger H-bond in the dimer, and represents an upper bound for that of the isolated anion. At the same time, the apparent effect of the H-bond for the neutral chromophore is as large as 0.5 eV, red-shifting the absorption maximum of the isolated neutral (340 nm) to that measured in the dimer (393 nm) and various proteins (∼395 nm). This shift results from changes in topography of potential-energy surfaces in the Franck-Condon region of the H-bonded systems.

The discovery of the Green Fluorescent Protein (GFP) and its gene has revolutionized biological imaging, with its use as a fluorescent marker. GFP is self-folding and non-toxic, which makes it ideal for tagging into live cells by injecting its gene. The protein chromophore is formed autocatalytically from the residues Ser65, Tyr66 and Glu67 in the central α-helix surrounded by an 11-stranded β-barrel.[1] The chromophore is held in place by an extensive hydrogen-bond network including structural water and amino acid residues.[2] The chromophore of wild type GFP can exist in a neutral state (A) and in an anionic, deprotonated state (B). Fluorescence happens from a deprotonated (I/I*) state, which for the neutral chromophore is reached after excited-state proton transfer,[3,4] where the H-bonding is essential for the functioning of GFP.

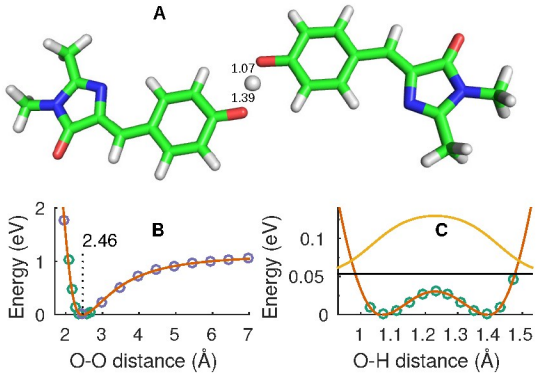

Fig. 1 Two H-bonded model GFP chromophores, one neutral and one deprotonated (anion) forming a singly-charged dimer complex. **A**: The optimized MP2/(aug)-cc-pVTZ dimer structure. **B**: The dimer dissociation curve. **C**: The potential and probability distribution for the ground-state proton transfer. For details see ESI.

The GFP chromophore anion is typically modeled using the deprotonated para-hydroxybenzylidene-2,3-dimethylimidazonilone ($HBDI^-$) molecule, which closely resembles the chromophore in the fluorescent state of GFP.[5] The use of $HBDI^-$ also eases gas-phase measurements, as it is possible to control the spatial position and velocity of the charged molecule. Doing direct absorption-spectroscopy measurements of ions in the gas phase is generally challenging, owing to rather low ion densities. To circumvent this problem, gas-phase measurements have relied on action-absorption spectroscopy, where the response of absorbing one or more photons is observed. Typically, action is fragmentation or electron detachment.

Previous gas-phase action-absorption spectroscopy measurements of the $HBDI^-$ anion show, that *two* photons are required to yield fragmentation[7,8] within the experimental time window

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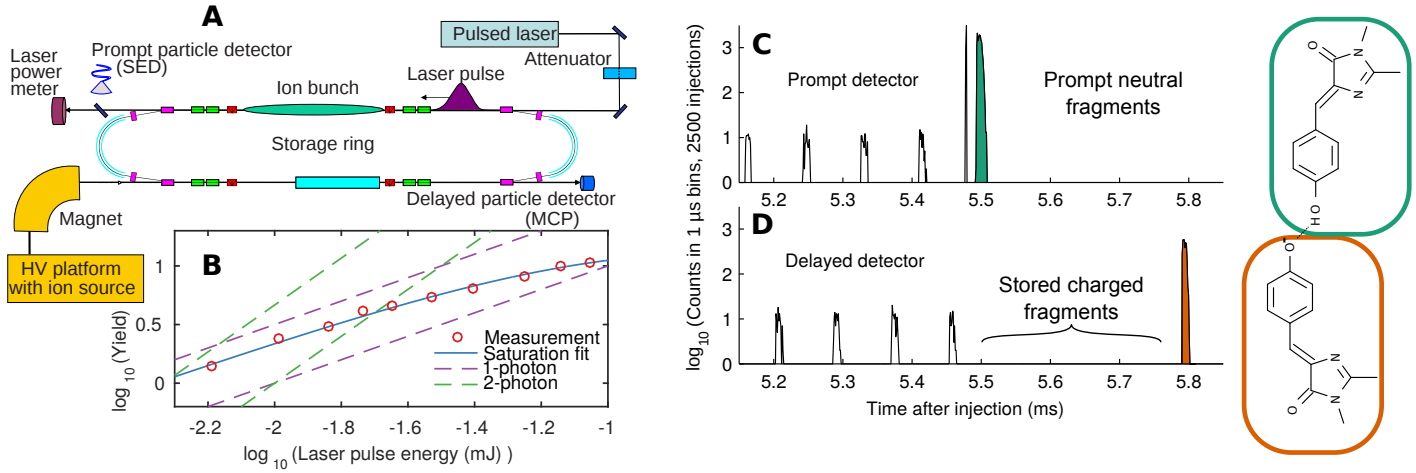

Fig. 2 A: The experimental setup with the electrostatic ion storage ring ELISA.[6] A detailed description is found in ESI. **B**: A power dependence measurement verifies that only a single photon is required to fragment the complex. The axis are logarithmic and the slope of 1 and 2 photon processes are indicated. A 1-photon process model, including saturation (See ESI), is fitted to the data. **C**: The prompt detector measures the neutral laser induced fragments marked with green. **D**: As the laser is fired, the voltages are switched, such that we store the charged laser-induced $HBDI^-$ fragments. Several revolutions later, these stored charged fragments are dumped on the delayed particle detector.

(milliseconds), whereas only *one* photon is needed to detach an electron above the detachment threshold.[8–13] Contributions from both pathways in the same wavelength range notably complicates the interpretation of the spectroscopy measurements for the $HBDI^-$ anion.

Neutral model chromophores with a positive charge located away from the photoactive π-system have previously been used to approximate a neutral chromophore in the gas phase[14,15], and recent spectrocopy measurements of the neutral chromophore have been achieved by Greenwood *et al.*[16] using resonantly enhanced multi-photon ionization.

In this work we have studied the hydrogen-bonded complex of a neutral and a deprotonated HBDI chromophore (Fig. 1). This complex is sufficiently loosely bound, that only a single photon is needed to cause action – the break of the hydrogen bond. At the same time we show, that electron detachement is avoided by the increased electron binding, thereby easing the interpretation of the results significantly. In addition a new technique is employed, in which we measure all action simultaneously with branching into specific fragmentation pathways. With the dimer system, the full *absorption spectrum* is therefore measured as ensured by our new dual-detection technique. We have for the first time been able to directly probe the effect of a single H-bond on the spectral tuning of the GFP chromophore in both protonation states. Thereby we provide important references for the bounds on the adiabatic excitation energies of the isolated chromophores, which are challenging to obtain from either direct experimental measurements or theory. Furthermore, we show that an unexpectedly large shift of 0.5 eV in the absorption maximum of the neutral GFP chromophore inside the protein with respect to that of the isolated molecule is imposed by a single H-bond. We argue that the effect of the H-bonding is in altering the local topography of potential energy surfaces of both the ground and excited states in the Franck-Condon region, rather than in increasing the energy gap between the two electronic states.

The $HBDI \cdot HBDI^-$ dimer complex is studied in the gas phase using the electrostatic ion storage facility ELISA[6]. The chromophore dimers (Fig. 1) are produced in the gas-phase using electrospray ionization with methanol as solvent. The sprayed droplets are evaporated in a heated capillary and charged ions are guided into a radio-frequency ion trap. Ions are accumulated in the trap for nearly 50 ms where they thermalize with a helium buffer gas. The ions are extracted as a bunch, accelerated to 22 keV, mass selected and guided into ELISA (Fig. 2). The dimers are stored in ELISA for 5 ms before they are exposed to light from a tunable Nd:YAG pumped OPO (EKSPLA NT-342B-SH-20) nanosecond laser. The average laser-pulse energy is stabilized at 15 μJ.

Ions stored in ELISA are confined by electrostatic fields only and thus the required voltages are proportional to their kinetic energy. Therefore, by switching the voltages in the ring promptly after the laser pulse, we are able to store charged photofragments which have a fraction of kinetic energy according to the fragment mass.[17] In this experiment we store charged, laser induced $HBDI^-$ fragment chromophores (monomers) for four and a half revolutions in ELISA after which they are dumped on a detector. Because of the low binding energy of the hydrogen bond, all action is prompt in the sense that it happens much faster than the revolution time ($\sim 80\mu$s) in ELISA. Using this technique, we are able to monitor a given fragmentation channel, by storing only the associated photofragments, while the prompt detector (Fig. 2) monitors the full action absorption yield (all *neutral* photofragments). In Fig. 2 C and D the accumulated counts on the detectors are shown ($\lambda = 450$ nm). The observed laser induced signal is either neutrals (C) or charged fragments (D), as indicated by the colored regions. The experimental action absorption cross section is obtained by analyzing the change in laser induced counts as a function of wavelength (for further details, see ESI).

We also provide a theoretical account of the experimental

spectral shape. The MP2/(aug)-cc-pVTZ ground-state and XM-CQDPT2[18]/(aug)-cc-pVTZ excited-state calculations, as well as the spectral shape analysis are described in the ESI. Firefly version 8.0[19] is used for all electronic structure calculations.

The optimized structure of the dimer system (Fig. 1A) reveals a double well potential where the proton is located closer to one of the chromophores at the equilibrium configuration in one of two equivalent minima (1.07 Å and 1.39 Å). The barrier for proton transfer is 30 meV, as calculated through the O-H bond length relaxed geometry scan at the fixed equilibrium O-O distance (Fig. 1C). The ground-state probability distribution shows, that the proton is not confined by this barrier. The dimer zero-point energy corrected binding is 1.2 eV (Fig. 1B) and while this is a remarkably strong hydrogen bond it is still the weakest bond in the dimer. Following photoabsorption we therefore expect only one fragmentation channel:

$$\mathrm{Dimer}^- + h\nu \rightarrow \{\mathrm{Dimer}^-\}^* \rightarrow \mathrm{HBDI} + \mathrm{HBDI}^-, \qquad (1)$$

which happens either in the hot ground state following internal conversion or directly from the excited state.

In principle, electron detachment is also possible, however, the vertical detachment thresholds are calculated to be 3.38 eV for the anionic part and 4.29 eV for the neutral chromophore which is beyond the energy range in this study. In the experiment, the full action-absorption spectrum is measured on the prompt detector, since both channels (fragmentation and potential electron detachment) lead to fast action producing one neutral particle. The specific fragmentation channel into two separate chromophores is measured on the delayed detector (Fig. 2D). It is verified that only a single photon is needed to cause the signals (Fig. 2B), and that no spectral difference between the two channels (prompt and delayed) are observed (Fig. 3B). We therefore conclude that the full 1-photon absorption cross section is measured, with the only available channel being the breaking of the hydrogen bond.

In Fig. 3A we show the measured absorption cross section with the ion source trap cooled with liquid nitrogen. We deduce that the $S_0 \rightarrow S_1$ transition in the anion has the highest oscillator strength and peaks approximately at 2.72 eV (455 nm). Another peak arises at 3.15 eV (393 nm) in the spectrum which we assign to absorption of the neutral chromophore in the dimer system. Two sticks represent the calculated vertical excitation energies of the bright transitions: At 2.72 eV with an oscillator strength 1.44 for the anion, and at 3.04 eV with an oscillator strength 0.27 for the neutral. The predicted intensities correspond very well to the observed peak heights, when an almost flat 'anionic' background under the neutral absorption peak is subtracted (illustrated in grey in Fig. 3A).

Fig. 3C illustrates the calculated Franck-Condon spectra at the optimized geometry (details in ESI). We note that the experimental spectrum is inhomogenously broadened due to the ground state proton dynamics, and for the anion, the most redshifted transition should correspond to that of the equilibrium configuration of one of the two equivalent minima. The prominent bond length alternating (BLA) modes are visible in the theoretical spectra of the protein environment, Fig. 3D, as well as the dimer,

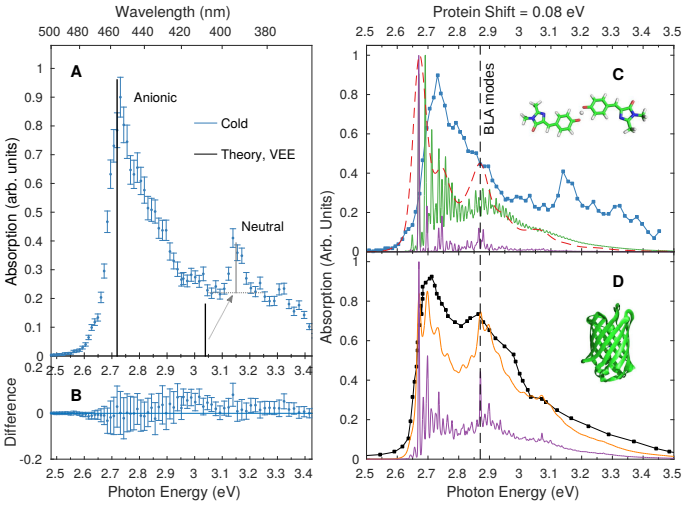

Fig. 3 A: Prompt absorption spectrum measured with cooled ion trap. The sticks show the vertical excitation energies and relative oscillator strengths of our theoretical model. For details see Table S1 in ESI. **B**: Difference between the prompt channel and the stored HBDI^- fragment channel. **C**: The dimer spectrum as presented in A is shown in blue. Our theoretical spectra are shown as lines. The purple (100 K, 2 meV HWHM) and red (300 K, 21 meV HWHM) lines include only modes isolated on the anion in the dimer, whereas the green (100 K, 2 meV HWHM) curve includes all vibrational modes (ESI Fig. S1). **D**: The low temperature fluorescence excitation spectrum by Lossau *et al.*[3] and the calculated spectra of S65T GFP containing the anionic chromophore. (Purple: 100 K, 2 meV HWHM. Orange: 150 K, 7 meV HWHM) The protein calculations are described in Ref. 20.

Fig. 3C, with a similar frequency (ESI Table S2). The shoulder caused by these modes are clearly visible in the experimental protein spectra, but are blurred due to the ground state proton dynamics in the dimer. The BLA modes account for a strong fingerprint of the anionic chromophore and are used to align the dimer spectrum to that of the protein, resulting in a slight shift of only 0.08 eV between the 0-0 transition of the dimer (464 nm) and that of the wild-type protein (478 nm). The strength of the hydrogen bond in the dimer (H-bond length of 1.4 Å) is stronger than that in the protein (H-bond length of 1.8 Å) thus the excitation energy is correspondingly larger. It is noteworthy, that different GFPs also have slightly different anionic absorption maximum due to different local H-bonding environments around the chromophore (e.g. wtGFP: 470-475 nm[1] and EGFP: 487-489 nm[21,22]). At the same time a stronger interaction can blue-shift the absorption much further, like in teal FPs where the maximum is shifted down to about 453 nm, caused by electrostatic interactions with a nearby positively charged residue.[23]

When an isolated deprotonated chromophore is excited from the singlet ground state S_0 into the first excited singlet state S_1, electron density moves from the rings to the middle carbon atom of the bridge moiety.[11,12,20] In the dimer complex, excitation of the anion thus causes electron density to move away from the hydrogen bond which makes it weaker. The hydrogen-bond interaction therefore lowers the excited-state energy *less* than it lowers the ground-state energy. This results in a blue shift of the $S_0 \rightarrow S_1$ dimer transition compared to that of the deproto-

nated monomer. The obtained 2.67 eV (464 nm) 0-0 transition in the dimer is therefore an *upper bound* on the adiabatic excitation energy of the monomer chromophore anion, consistent with the value (2.52 eV (492 nm)) reported in Ref. 20. We note that the 0-0 transition has the largest Franck-Condon strength for $HBDI^-$.

The two available excited-state decay channels, electron detachment and internal conversion, yield different action absorption profiles for $HBDI^-$.[20] Electron emission due to one-photon absorption becomes the predominant action only at the blue part of the absorption spectrum, which correlates with the opening of the electron continuum at 2.73 eV (454 nm).[9,13] Action (fragmentation) following internal conversion, visible after sequential two-photon absorption, however, only probes the red part of the absorption spectrum and peaks at 2.57 eV (482 nm),[7,24] close to the calculated XMCQDPT2/(aug)-cc-pVDZ vertical excitation energy 2.52 eV (492 nm) of the monomer[20]. This is consistent with the upper bound of 2.67 eV (464 nm) found here.

The neutral chromophore is notoriously harder to handle in the gas phase because it is difficult to manipulate by electric fields in traps or rings, and magnetic mass selection is not applicable. Nevertheless, a recent experiment by Greenwood *et al.*[16] has achieved an absorption profile by using resonantly enhanced multi-photon ionization on an evaporated neutral sample. They have revealed an absorption maximum at 340 ± 5 nm (3.65 eV). The absorption of the free neutral chromophore is thus significantly blue shifted compared to the absorption band in the protein as well as in the dimer system. It is noteworthy that the absorption maxima of GFP related proteins containing a neutral chromophore are remarkably insensitive to the local chromophore environment.[25–27] Only a very strong hydrogen bond S65T/H148D GFP (O-O distance of 2.32 Å, 0.14 Å shorter than that in the dimer) can, at most, shift the absorption maximum to 410 nm.[27] With regards to the neutral absorption, the dimer and various proteins are therefore very much alike. The absorption of the isolated neutral chromophore thus represents a special case. To account for this, it is noted that the chromophore is a strong photoacid, where the O-H distance is strongly affected upon photoexcitation. In the absence of a viable proton acceptor, the electron-density redistribution in the isolated chromophore should therefore lead to a dramatic increase of the O-H distance. As a consequence, photoexcitation is expected to be associated with excitation of the Franck-Condon active O-H stretching mode. The effect of having a hydrogen bond in the dimer, as well as in proteins, is that the O-H distance in the ground state is also increased and therefore favors absorption closer to the 0-0 transition (Fig. 4). In other words, the surfaces are shifted in the OH-bond direction and not in energy.

The vibrational analysis of the isolated neutral chromophore yields an O-H stretching frequency of 3821 cm^{-1}. Using the experimental maximum of 340 nm[16] as a reference and the typical scaling factor of 0.98 for the MP2 harmonic frequencies, we find the 0-0 transition of the free HBDI chromophore to be located around 390 ± 6 nm (3.18 eV), which is in very close agreement with our dimer data as well as with the protein data (Fig. 4).

In conclusion, we have demonstrated that it is possible to generate and store hetero chromophore complexes bound by a single

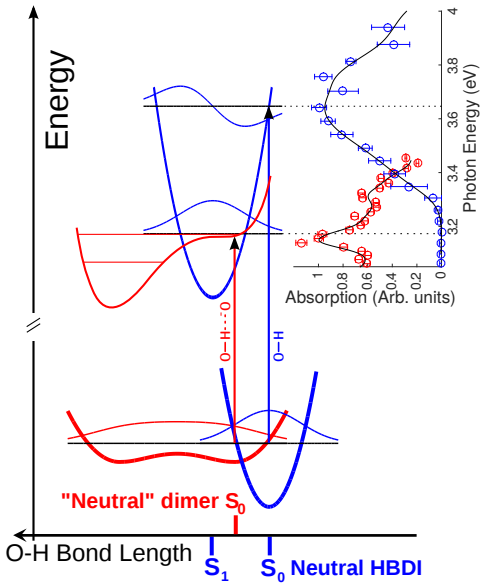

Fig. 4 The calculated ground-state potential energy surfaces as presented in Fig. 1 (red) and the OH stretching mode harmonic potential (blue). The conceptual excited state potentials are also shown. The difference in the absorption maxima (the inset) is well correlated with the 0-1 excitation of the O-H stretch mode.

hydrogen bond in the gas phase. The low bond strength provides fast fragmentation (action) after one-photon absorption. Consistent with theory, and similar to the protein environment, there is no sign of electron detachment in the measured region, even though electrons are clearly seen from the anion monomer at these wavelengths. With our new dual-detection technique it is ensured that the full absorption spectrum of the dimer is measured.

Based on the spectral analysis, the 0-0 transition energy in the anionic part of the dimer is identified to be 2.67 eV (464 nm), blue shifted by only 0.08 eV compared to that of the wild-type protein, due to a stronger hydrogen bond in the dimer. Importantly. this adiabatic excitation energy represents an upper bound on that of the isolated monomer anion. As a neutral HBDI chromophore is a part of the complex, it is also accessible for measurements in the gas phase, which has only recently been achieved for the isolated chromophore by Greenwood *et al.*[16]. The apparent shift of almost 0.5 eV between the free neutral chromphore and the dimer as well as proteins is assigned to the excitation of the Franck-Condon active O-H stretch mode (3821 cm^{-1}) in the free HBDI monomer.

In the dimer complex the absorption spectrum of the two monomers overlap which is further broadened by the ground state proton dynamics. Excitation of the complex might therefore lead to electronic excitations which oscillate between the two chromophores. One might be able to see such effects using femtosecond pump-probe techniques while monitoring UV-generated photoelectrons.

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