Vibrationally Resolved Photoabsorption Spectroscopy of Red Fluorescent Protein Chromophore Anions

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Photoabsorption studies of red fluorescent protein chromophore anions have been performed at the ELISA electrostatic heavy-ion storage ring. The broad absorption band due to electronic excitation of the chromophores is tuned to a longer wavelength (redshifted) by extending the electronic conjugation of the molecule. A clear vibrational progression is resolved with $E_{\mathrm{vib}} \sim 380$ and 520 cm^{-1} for two different forms of the chromophore. The vibrational modes correspond to collective motions of the entire molecular structure. It is argued that the excited electronic state has an equilibrium configuration far from that of the electronic ground state, i.e., poor Franck Condon overlap.

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Fluorescent proteins such as the well known green fluorescent protein (GFP)[1] have spectroscopic properties which make them very applicable for biological labeling. There has been an ongoing development towards new proteins with different absorption and emission characteristics, mainly to improve sensitivity and make multicolor labeling possible. The DsRed is a recently cloned, bright red fluorescent protein (RFP) from *Discosoma* sea coral [2–4]. The DsRed protein has an absorption maximum at about 559 nm, whereas the absorption maxima of wild-type GFP are at 395 nm and 475 nm, corresponding to a neutral and a negative chromophore charge state, respectively [1]. Thus, there is a significant redshift of the absorption of DsRed, the origin of which is likely related to the electronic structure of the chromophore molecule. Polar perturbations and hydrogen binding in the protein may of course also have an influence on the absorption.

Here we investigate the absorption properties of bare RFP chromophores, i.e., without external interactions of any kind. In the case of GFP it was demonstrated that the special protective structure of the GFP protein (the so-called β-can) makes the absorption properties of the protein similar to those of the isolated anionic chromophore molecule [5]. We directly demonstrate that the origin of the redshift of DsRed is related to the structure of the chromophore; i.e., it is not a result solely of the protein environment. Moreover, we have identified collective vibrational modes of the excited state, which are detectable only for chromophore ions in the gas phase and not in solution measurements [6,7]. The excited state dynamics is of fundamental importance in photobiology, since photoinduced reactions (e.g., cellular response) depend upon it.

We used model chromophores of the red fluorescent protein, which are shown in Fig. 1. To study the redshifted spectroscopic properties of DsRed, when compared to GFP, different conjugation was inserted in two forms of the DsRed model chromophore. The chromophores have been synthesized and used previously by some of us in solution studies [7], but the photophysics of the chromophores was not studied in the gas phase.

The experiment was performed at the electrostatic ion storage ring in Aarhus, ELISA [5,8–10]. A layout of the experiment at the storage ring is shown in Fig. 2. As a source for the chromophore ions we used an electrospray ion source. Anions were formed by electrospraying a chromophore sample dissolved in an ammoniated

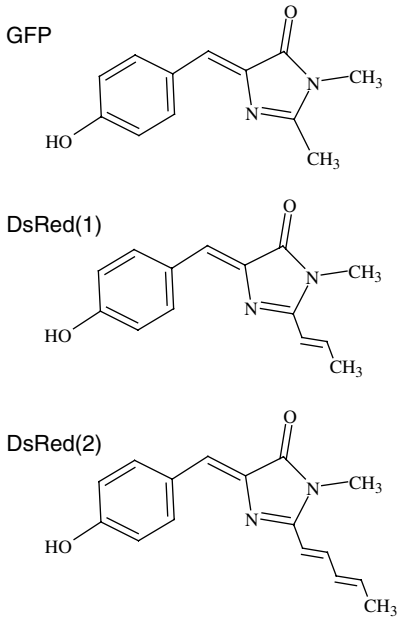

FIG. 1. Structure of model DsRed chromophores in which the GFP chromophore core has been extended by one [DsRed(1)] and two [DsRed(2)] ethylenic bonds.

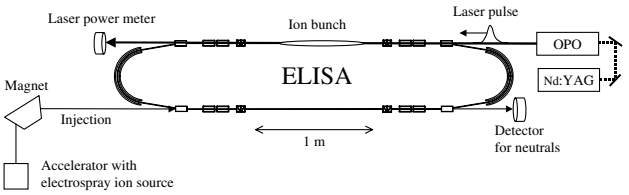

FIG. 2. The electrostatic ion storage ring ELISA equipped with an electrospray ion source, a pulsed laser, and a detector for neutral products.

water/methanol (1:1) solution (pH = 11). Just after the ion source, a cylindrical ion trap accumulated ions for 0.1 s before they were accelerated as an ion bunch to a kinetic energy of 22 keV. Anions, i.e., deprotonated molecules [masses 241 amu and 267 amu for DsRed(1) and DsRed(2), respectively], were selected by a magnet and injected into the storage ring. About 11 ms after injection the ions were irradiated by a single laser pulse of ~3 ns duration in the straight section opposite the injection side. A storage time of several milliseconds before irradiation ensured that ions collisionally excited during production and injection had decayed. An OPO (optical parametric oscillator) pumped with the third harmonic of a Nd:YAG laser was used to create ~1 mJ laser pulses in the spectral region 430–650 nm. Tuning the laser wavelength to an absorption band resulted in the production of neutral particles (after electron emission or bond dissociation). Neutrals formed in the straight section opposite the laser-interaction region were counted by a particle detector (channel-electron multiplier). We estimate to have only 10^3–10^4 ions in the ring at the time, but the technique is very sensitive as we count individual photofragments (neutral molecular fragments) with high efficiency. Our technique resembles that of fluorescence excitation methods with supersonic jets, except that we address charged (protonated/deprotonated) chromophores, which are present in many biological systems [11]. A detailed description and analysis of the decay will be given elsewhere [12].

We recorded the yield of neutral fragments as a function of the wavelength and time after photoabsorption, which makes up the 3D absorption spectrum (Fig. 3). A measure for the absorption cross section at a specific wavelength was obtained from

$$N_{\text{neutrals}} = N_{\text{ions}} \sigma \int \Phi dt \tag{1}$$

or

$$\sigma \propto \frac{N_{\text{neutrals}}}{N_{\text{ions}}} \frac{1}{E\lambda}, \tag{2}$$

where N_{neutrals} is the number of laser-induced neutrals (integrated over time), N_{ions} is the number of ions in the ion bunch (proportional to the neutrals arriving at the detector prior to firing the laser), σ is the cross section for photon absorption, Φ is the photon flux, E is the laser-pulse energy, and λ is the wavelength. As is seen in Fig. 3, the decay of the excited chromophore ions happens over typically a few hundred μs or less. The absorption of a single photon raises the temperature of the chromophore ion to about 900 K (slightly wavelength dependent in the absorption region). The decay rate is related to the activation energy of the dissociation, as will be discussed in a more comprehensive report [12]. Here we continue the discussion of the time-integrated absorption signal, which carries information about the excitation dynamics.

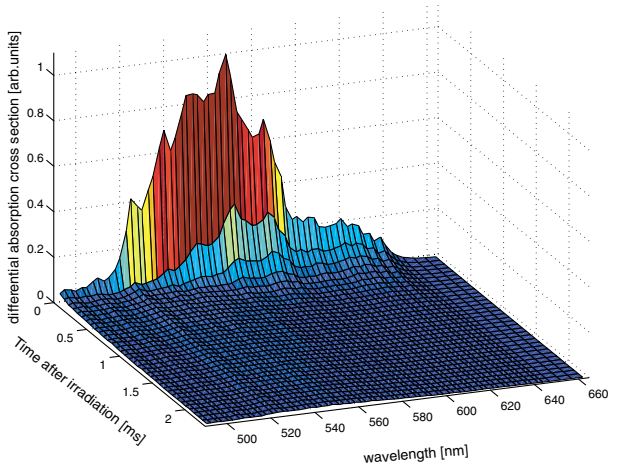

FIG. 3 (color online). Yield of neutrals detected as a function of time and laser wavelength for DsRed(2) anions.

The normalized, integrated, wavelength-dependent absorption signal of the two DsRed chromophore anions is shown in Fig. 4. Both forms show very broad absorption profiles with clear superimposed structures (easily seen also in Fig. 3). We first note, that the absorption of the long form [DsRed(2)] is shifted considerably to the red compared to the short form [DsRed(1)]. The absorption maxima are at $\lambda = 521$ nm and 549 nm for DsRed(1) and DsRed(2), respectively (see Fig. 4). Both are significantly redshifted with respect to the GFP model chromophore anion with the smaller conjugation (Fig. 1; maximum at 479 nm [5,13]). This directly shows the importance of the electron conjugation in the isolated chromophore without perturbations of surroundings in liquids and proteins. Perturbations in water are significant as seen in Table I; DsRed(1) anions have the absorption maximum at 460 nm in water and DsRed(2) anions at 482 nm [7], both significantly below the gas-phase maxima. We attribute the shifts caused by the polar water medium to a charge localization effect diminishing the π conjugation. The energy shift of the absorptions is about the same for the three chromophore anions.

We observe vibrational progressions, which are of rather low energy. High energy modes, of which there are plenty [7], probably overlap each other and make up

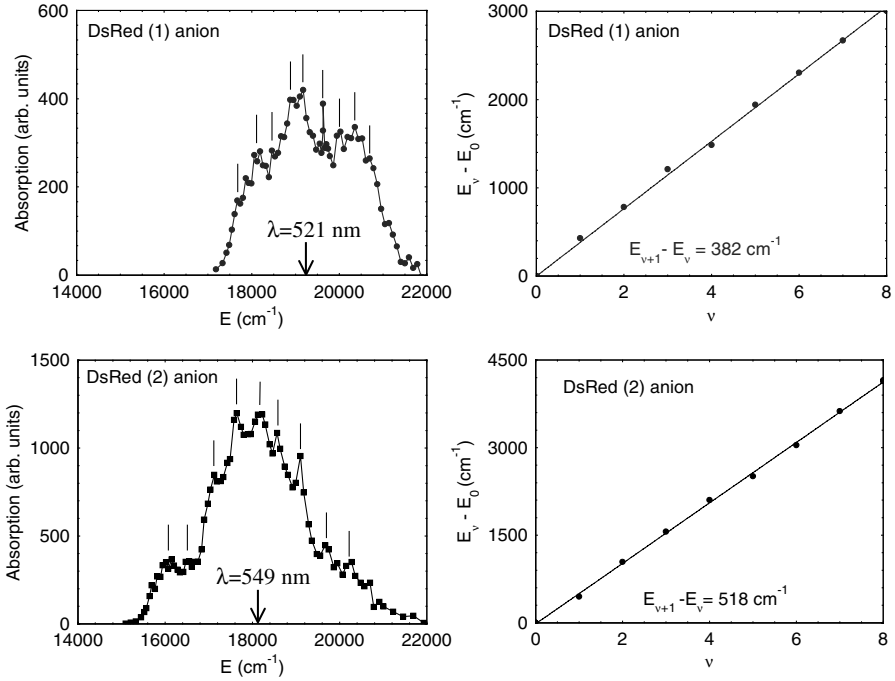

FIG. 4. The time-integrated absorption spectra of DsRed(1) and DsRed(2) anions. Shown by bars are vibrational structures, the energies of which are shown to the right. The vibrational spacing is 382 cm^{-1} for DsRed(1) and 518 cm^{-1} for DsRed(2) with an estimated uncertainty of 10 cm^{-1}.

the underlying broad continuous absorption band. We performed resonance Raman spectroscopy of the chromophore anions (alkaline solution) by 407-nm excitation [12]. Indeed, also many low-energy modes around 500 cm^{-1} were observed — apart from the high energy modes reported in Ref. [7]. To identify these modes, *ab initio* calculations in the spectral region around 500 cm^{-1} were carried out. Only collective modes spread out over the entire structure were seen. The fact that many levels are populated across the absorption band implies that the structure of the electronically excited state is far from that of the ground state. Indeed the photoabsorption instantaneously creates a molecule far from equilibrium, which is a good starting point for recognition of the absorption of radiation in biological systems.

To summarize, we studied the absorption characteristics of two red fluorescent protein chromophore ions. We demonstrate that the redshift of DsRed compared to GFP may be a direct consequence of the extended conjugated system in the DsRed chromophore. Our present storage ring technique reveals vibrational progressions of the gas-phase chromophore ions. In the particular case of DsRed(1) and DsRed(2) anions, a low frequency mode of 382 cm^{-1} and 518 cm^{-1}, respectively, is ascribed to a deformation of the entire molecule.

TABLE I.

	Gas phase	Solution	Shift (nm)	Shift (eV)
GFP	479	426	53	0.32
DsRed (1)	521	460	61	0.32
DsRed (2)	549	482	67	0.31

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