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Chromophores of the green fluorescent protein studied in the gas phase

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Abstract. Absorption of gas-phase biomolecules has been studied at the heavy-ion storage ring ELISA. Here we discuss the absorption characteristics of the chromophores of the Green Fluorescent Protein (GFP). The gas-phase absorption maximum of the deprotonated chromophore (anion form) is at 479 nm. This is almost identical to one of the two absorption maxima of the protein, being at 477 nm, which is ascribed to a deprotonated chromophore in the protein. The protonated chromophore (cation form) has a maximum at 406 nm in the gas phase. We compare the gas-phase results with absorption profiles of GFP and chromophores in liquids, and argue that the absorption characteristics of GFP are mainly ascribed to intrinsic chemical properties of the chromophore. Evidently, the special β-can structure of GFP provides shielding of the chromophore from the surroundings without significantly changing the electronic structure of the chromophore through interactions with amino acid side chains.

PACS. 33.20.Kf Visible spectra – 87.14.Ee Proteins – 87.64.Ni Optical absorption, magnetic circular dichroism, and fluorescence spectroscopy

1 Introduction

Functional consequences of protein dynamics may conveniently be studied by photoactive proteins because the chromophore, *i.e.* the molecule responsible for light-absorption, acts as a build-in "trigger" that allows synchronization of events on time scales as short as tens of femtoseconds. The absorption properties of such proteins are remarkable. The absorption is intense and the absorption spectra are broad. Moreover, the spectra of emission are significantly red shifted upon absorption which forms the basis for many applications. Most well known is perhaps the Green Fluorescent Protein (GFP) which is found in the jellyfish *Aequorea victoria*. GFP is a relatively small protein whose natural function is to convert blue light to green light [1–4]. This single chain protein, consisting of 238 amino acids, has triggered a revolution in molecular biology since it may be fused into other proteins and by its special absorption and emission characteristics used for continuously monitoring gene expression and developments in living cells [1–4]. Many other photoactive proteins like, *e.g.*, the photoactive yellow protein (PYP) [5], which is found in the purple sulfur bacterium Ectothiorhodospira halophilia, have also been studied. The atomic scale structure of PYP has been determined and the photocycle of PYP has been examined in great details.

To fully understand the photophysics of these proteins, relevant chromophore information must be collected. The electronic and conformational structures of the chromophore molecule in the ground and excited states are responsible for the initial absorption (wave length and intensity). The response of the protein is given by an interplay between the photo excited chromophore and the environment in the protein. Typically, the interaction with the protein determines whether isomerization can take place or not. The protein can also deliver or take away protons from the chromophore and hence change the charge state and electronic structure of the chromophore.

Many studies of the photophysics of such biosystems are performed in solutions. It is, however, clear from observed shifts of the absorption maxima that the environment provided by the protein is not identical to that of aqueous solutions. To shed light on the intrinsic properties of chromophores, we have initiated a series of studies of *gas phase* chromophores. In the present communication we will focus on results obtained with the wild-type GFP chromophore. We used the model chromophore, 4-hydroxybenzylidene-2,3-dimethyl-imidazolinone, shown in Figure 1, which was also used in an experiment by Webber *et al.* [6].

2 Experiment

Our measurements were performed at the new electrostatic ion storage ring in Aarhus, ELISA [7] (see Fig. 2).

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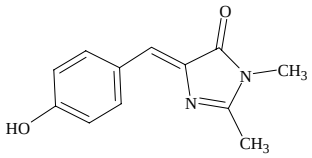

Fig. 1. The structure of the neutral gas-phase GFP-model chromophore used in the present work. Anions are created when the proton is removed from the hydroxyl group and cations are created when a proton is attached to the imidazolidinone ring containing the two nitrogen atoms.

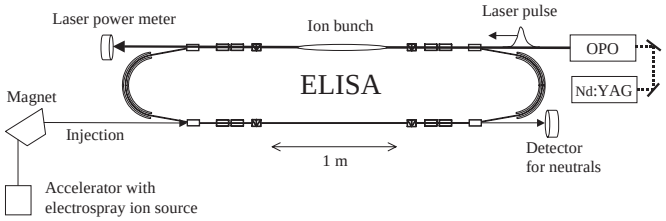

Fig. 2. The electrostatic ion storage ring ELISA equipped with an electrospray ion source, a pulsed laser and a detector for neutral products.

The storage ring is based on electrostatic bending and focusing elements, which gives ions of a given energy to charge ratio identical storage conditions regardsless of their mass. Thus, the ring may be used to study ions of light elements as well as heavy molecules, such as biomolecules. In the storage ring, molecular ions may be stored for several seconds and interactions with *e.g.* laser light or electrons can be studied. Since revolution times are of the order of microseconds, each stored ion may typically, at a given position in the ring, be studied about 10^6 times during its lifetime. Moreover, ions that were born hot in the ion source, or made hot by collisions during the acceleration and injection, will have time to cool down by infrared emission before a given measurement starts.

An electrospray ion source was used for production and injection of the biomolecules. The source, which is shown schematically in Figure 3, was constructed and built recently in Aarhus [8]. Briefly, it may be operated in a DC mode with typically 10^5 to 10^6 ions/s or in a pulsed mode with 10^3 to 10^4 ions in a bunch (repetition rate 10 Hz). Anions were formed by electrospraying a chromophore sample dissolved in an ammoniated water/methanol (1:1) solution (pH = 9). For cations we used a solution of water/methanol (1:1) with HCl (pH = 3). The mass spectra of the ion source were remarkably clean. As an example, Figure 4 shows the mass spectrum obtained with GFP-chromophore anions. There is an unambiguous peak at the correct mass of 215 amu (deprotonated ion). With cations of this chromophore the peak is at 217 amu (protonated ion).

In 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. The ions were selected according to their mass to charge ratio by a magnet and injected into the storage ring. About 5 ms after injection,

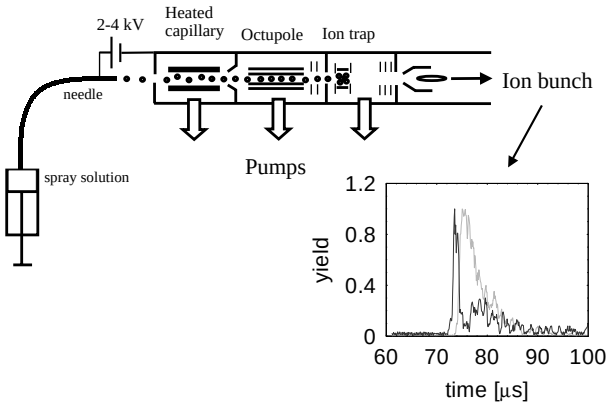

Fig. 3. The principle of the electrospray ion source. The inserted figure shows measured intensities as a function of time. Ion bunches of 2–5 μs were obtained with He as a buffer gas in the ion trap.

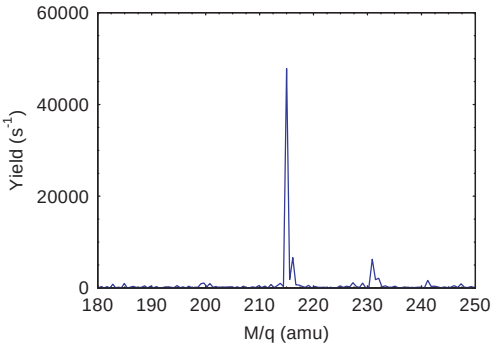

Fig. 4. Mass spectrum of the electrospray ion source containing the GFP chromophore anions at 215 amu.

the ions were irradiated by a laser pulse of 3 ns duration in the straight section opposite the injection side. An OPPO (optical power parametric oscillator; Lambda Physik) pumped with the third harmonic of a Nd:YAG laser (Coherent) was used to create ∼1–2 mJ laser pulses in the region 430–630 nm. Frequency-doubled light from a dye laser (Lambda Physik), pumped by the second harmonic of the Nd:YAG laser, was used for wavelengths shorter than 430 nm. Tuning the laser wave length to an absorption band resulted in the production of neutral particles after electron emission or bond dissociation (photo destruction spectroscopy). Neutrals formed in the straight section opposite the laser-interaction region were counted by a particle detector (see Fig. 2). Because of the low background rate of neutrals and the applied single-particle detection scheme, the technique allows us to monitor weak absorption rates. An example of a decay spectrum (neutrals count rate as a function of time) acquired for the chromophore anion at 490 nm is shown in Figure 5. Collisions between anions and residual gas molecules in the ring (mainly H_2) produce neutrals that account for the small background signal even when the laser is off. It is evident, however, that irradiation at the selected wave length resulted in additional neutrals with an observable decay over a few milliseconds. It would be very useful

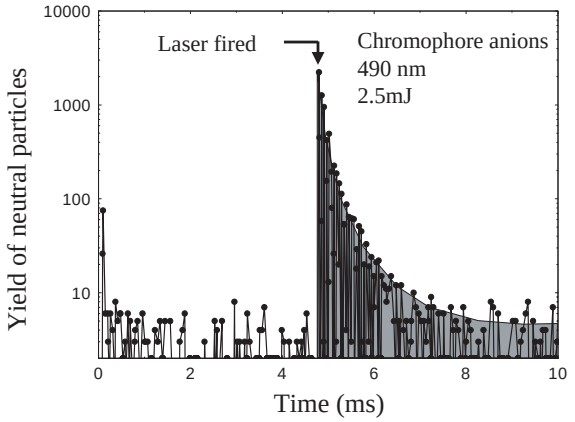

Fig. 5. Yield of neutrals as a function of time after the injection of GFP-model chromophore anions into ELISA. One revolution of the ion bunch takes about 50 μs. The ions were irradiated after 4.75 ms with one 2.5 mJ laser pulse at 490 nm photons. The grey area represent signal due to photon absorption.

if a mass spectrum of the fragment neutrals arriving at the detector could be obtained. At the moment this is not possible and we do not know the exact decay mechanism after photo absorption. In a separate experiment we measured the collisional induced fragment spectrum of 100 keV chromophore ions colliding with N_2. For cations as well as anions of the chromophore, positively charged fragments were found primarily at mass 56 amu.

Yield of neutrals (integrated over time) were recorded as a function of the wave length and produced the absorption spectrum (Fig. 6). The neutral yield was found to be almost proportional to the laser power squared, indicating that we mainly deal with a signal of a two-photon absorption process (depletion of the ion bunch caused deviations from a pure quadratic power dependence at high laser power) [9]. The yield of neutrals may be expressed as:

$$N_{\text{neutrals}} = N_{\text{ions}}(\sigma\Phi)^2, \tag{1}$$

where N_{neutrals} is the number of neutrals, N_{ions} the number of ions in the ion bunch, σ the cross-section for photon absorption, and Φ the photon flux. The absorption at a specific wave length was calculated as the square root of the ratio between the total number of counts due to laser excitation and the laser power squared.

3 Results and discussion

The measured absorption bands are shown in Figure 6, where we compare the absorption of the chromophore in three different media: the protein (GFP) [1], *in vacuo* [9,10], and in aqueous solutions of different pH [9,10]. The protein absorption spectrum shows two absorption bands that are ascribed to a neutral chromophore in the protein (absorption maximum at 395 nm) and a deprotonated chromophore (absorption maximum at 477 nm).

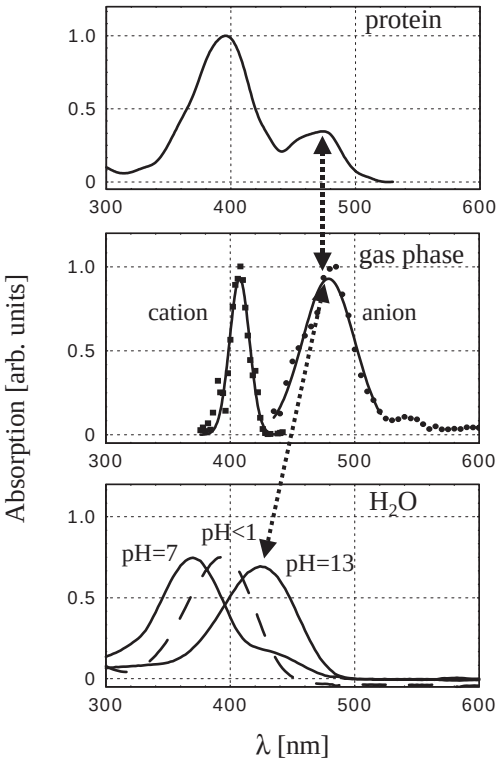

Fig. 6. Top: absorption spectrum of the wild-type *Aequorea victoria* GFP. The two peaks are ascribed to a neutral and a negative charge state of the chromophore in the protein [1], respectively. Middle: absorption spectra of the GFP-model chromophore *in vacuo* (the curves are Gaussian fits). Bottom: absorption spectra obtained with the GFP-model chromophore in aqueous solutions of different pH values.

The absorption spectrum of the gas phase chromophore anion reveals that the absorption band has a maximum at 479 nm and a full width at half maximum of 45 nm. There is, thus, almost no difference between the absorption bands obtained with the protein and the chromophore *in vacuo*. It is seen that the absorption maximum for the anion form of the chromophore is shifted to 426 nm when recorded in an alkaline aqueous solution. This large shift may be explained by hydrogen bond interactions which localize the active electrons of the chromophore in the anion form. There is a significant amount of electron-delocalization for the anion form [9], which makes the chromophore sensitive to perturbations from the environment and is, in general the reason for the red-shifted absorption.

The similarity between the absorption band of the gaseous anion and the second absorption band of the protein is striking. In the protein, the chromophore is covalently attached to an α-helix that runs up the axis of a hollow cylinder formed by eleven β-strands [1,11, 12]. The cylindrical shape of the rigid β-can protects the buried chromophore from solvent and dioxygen quenching of excited states [12,13]. At the same time, the rigidity of the protein may prevent radiationless stabilization of the chromophore through *cis-trans* photo-isomerization.

Our results indicate that the actual environment of the chromophore inside the protein cavity is much closer to vacuum than to bulk solution, assuming that the second band is indeed due to the anion as suggested by previous studies [14–18] and our measurements [10]. In other words, the electron delocalization in the *Aequorea victoria* GFP chromophore matches the one of the gaseous anion. This does not preclude, however, that the protein environment does affect the displayed photophysics. Indeed, the charge state of the chromophore prior to excitation is directly determined by its local environment of proton donating and proton accepting groups, which explains why the photophysics of a single chromophore can differ significantly from protein to protein and between mutants [1–4].

The chromophore cation in the gas phase exhibits an absorption maximum at 406 nm and a width of about 20 nm. It is not possible to make direct comparisons with protein data as this chromophore charge state is not present in GFP. However, we may compare with the measurement performed in solutions. Here the absorption in the acidic solution (pH < 1) has a maximum at 396 nm, *i.e.* a blue shift of only 10 nm with respect to the gas-phase case. It may be argued that the shift is significantly smaller for cations than for anions because of less electron delocalization for GFP-chromophore cations [9].

It is our hope that the newly obtained gas-phase data may provide a basis for theoretical developments in this area, and we end the discussion by briefly considering the status of calculations. The chromophore in GFP is not terminated by the two methyl groups that were used in our model system (Fig. 1), and different theoretical approaches have used different groups R and R′ to terminate the chromophore and replace the two links to the remaining protein in GFP. The difficulty in calculating the absorption maximum for the deprotonated chromophore (anion form) *in vacuo* is reflected in the various reported values, which are 284 nm (substituents R, R′ = CH_3, CH_3) [19], 402 nm (adiabatic excitation; R, R′ = CH_3, CH_3) [19], 448 nm (R, R′ = CH_3, CH_2CH_2OH) [20], 444 nm (R, R′ = CH_3, CH_2CH_2OH) [16], and 479 nm (R, R′ = H, H) [16]. The measured absorption spectrum of the gaseous anion provides the most direct test of such calculations. A perfect agreement is found with the semi-empirical calculation by Weber *et al.* [16] (479 nm), whereas *ab initio* based models significantly overestimate the gas-phase excitation energy [19]. In the case of cations, the calculation by Voityuk *et al.* gives an absorption maximum at 401 nm (R, R′ = CH_3, CH_2CH_2OH) [21] and at 404 nm (R, R′ = CH_3, $CH_2COOCH_2CH_3$) [22], both in good agreement with our gas-phase measurements.

To summarize, a sensitive absorption technique for studies of gas-phase bio-molecules has been used by coupling an electrospray ion source with the ELISA storage ring. With this technique we studied the absorption characteristics of the GFP chromophore anion and cation. We present evidence that the exact location of the absorption band of the anionic form of the GFP chromophore is ascribed almost purely to the intrinsic chemical properties of the chromophore rather than to a result of interactions with amino acid side chains in its vicinity. Absorption measurements with model chromophores of mutated GFP proteins are currently being performed in our laboratory to investigate to what extend our findings apply to other systems.

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