

Spectral Tuning of the Photoactive Yellow Protein Chromophore by H-Bonding

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ABSTRACT Spectral tuning in the photoactive yellow protein (PYP) is investigated by performing gas-phase absorption measurements on a PYP-model chromophore with two water molecules hydrogen-bonded to it. The photoabsorption maximum shows an unusually large blue shift of 0.71 eV in going from the bare to the hydrogen-bonded chromophore. It is concluded that several interactions within the PYP protein are mutually canceling each other, yielding an absorption maximum that is close to the absorption maximum of the bare chromophore. The system breaks apart upon photoexcitation in the gas phase by releasing the two water molecules, leaving the chromophore itself intact. The hydrogen-bonding interactions thus play an important role in stabilizing the gas phase chromophore against photofragmentation. The relaxation dynamics for the breakup process was also studied, and the timescale of relaxation via fragmentation was found to be <25 ns.

INTRODUCTION

Hydrogen-bonding plays an essential role in the structure and functional properties of proteins (1,2). In photoactive proteins, H-bond interactions may also cause spectral shifts of the light-absorbing molecular group (chromophore) within these proteins, as has been proved by considerable theoretical (3–7) and experimental efforts—especially those based on mutation studies (8–13). Apart from H-bonding effects, the linkage of the chromophore to the apo-protein can also have an effect on the spectral tuning of the protein (12).

Hydration of biomolecules is one of the simple ways to introduce H-bond(s) to a molecular system and several studies on hydrated biomolecules have been reported (14–21). Most of these studies, however, have been concerned with structural investigations conducted by infrared spectroscopy. The excited-state dynamics of the chromophore has also been a theme of several investigations (8,22,23). Here we report on gas-phase absorption studies to directly quantify the spectral shift of the electronic transition by hydrogen-bonding of two water molecules to the chromophore *trans-p*-coumaric acid (pCA) found within the photoactive yellow protein (PYP). This water-soluble protein with 125 amino acids from the purple halophilic bacterium *Halorhodospira halophila* (24–26) is believed to act as a blue-light sensor for the bacterium, and to be responsible for phototaxis—i.e., the movement of an organism as a consequence of its exposure to light. The PYP chromophore is one of the simplest model systems for studying spectral tuning by protein environments, an issue of great importance in detecting fluorescent markers (27) in biology and in studying the mechanics of color vision (28). Importantly, the H-bonding network in the immediate neighborhood of the chromophore may play

a decisive role in the photocycle of the PYP-protein, as suggested by ultrafast infrared spectroscopy (29).

Gas-phase absorption of the isolated PYP chromophore has already been reported (30), which reveals an absorption maximum of the biologically relevant pCA anion at ~430 nm, close to the absorption maximum of PYP at 446 nm (31). Previous gas-phase photoabsorption studies have shown that, for many cases, the gas-phase photoabsorption maximum of a model chromophore agrees rather well with the photoabsorption maximum within the protein (32). This implies that either the interactions within the protein have negligible effects on the absorption of the chromophore or that effects, even though significant, are mutually canceling, rendering the absorption maximum basically unchanged. In this article, we are able to show, directly, that in PYP there are some very significant counteracting tuning contributions.

The chromophore with two water molecules attached to the bare pCA-anion is shown in Fig. 1. The water molecules introduce two H-bonds to the phenolic end of the chromophore, which mimic the H-bonds present within the protein at the same end of the ion but with two amino-acid residues Glu⁴⁶ and Tyr⁴² instead of water.

These gas-phase studies provide information about three different aspects of the photophysics of the PYP chromophore: 1), the absorption profile of the solvated chromophore; 2), daughter masses yielding information on how the chromophore is stabilized upon photoexcitation; and 3), the timescale of the molecular relaxation.

EXPERIMENT

The photoabsorption spectra were measured at the electrostatic ion storage ring ELISA (34) (Fig. 2). The solvated system of the anionic chromophore was generated from a solution of the chromophore sample in ethanol using an electrospray-ion source. After the electrospray, the ions

Submitted September 7, 2009, and accepted for publication October 19, 2009.

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Editor: David D. Thomas.

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0006-3495/10/02/0488/5 \$2.00

doi: 10.1016/j.bpj.2009.10.025

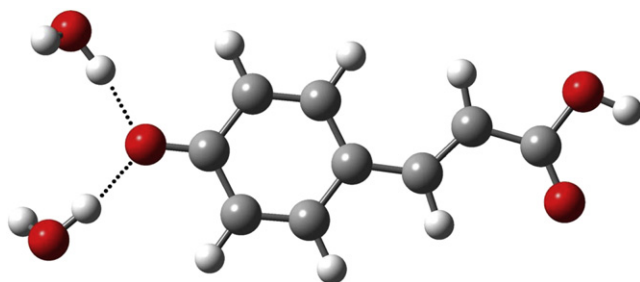


FIGURE 1 Optimized structure of the chromophore-water complex, provided by DFT(B3LYP/6-311++G(d,p)) level calculations using the Gaussian package (33). The minimum-energy structure was found to be a phenolate with the two water molecules being H-bonded with the oxygen atom on the phenolate. The two water molecules do not form mutual H-bonds. The other possible structure, in which one water molecule is attached to the phenolic oxygen and the other is attached to the carboxyl group, was found to be slightly higher in energy by 0.08 eV (1.85 kcal/mol).

were collected in a cylindrical ion trap with helium as a buffer gas. The trap potential was lowered every 100 ms for a duration of 30 μ s to extract the ions as a bunch. This bunch of ions was then mass-analyzed by a 90° bending magnet and injected into the ring for storage.

After 40 ms of storage in ELISA, the ion bunch was photoexcited with a laser pulse. An EXSPLA NT320 laser system with a tunable wavelength range from 210 nm to 2100 nm and a pulse duration of 3 ns was used as a photon source. The photoabsorption spectra were recorded by measuring the count of neutral photofragments generated after laser excitation as a function of wavelength (i.e., action spectroscopy (35)). More details on the experiment and data analysis can be found elsewhere (30). In brief, the absorption cross-section σ at a specific wavelength may be related to measurable quantities by

$$N_{\text{neutrals}}(\lambda) = N_{\text{ions}} \times \sigma(\lambda) \times \int \Phi dt \quad (1)$$

or

$$\sigma(\lambda) \propto \frac{N_{\text{neutrals}}(\lambda)}{N_{\text{ions}}} \times \frac{1}{E_{\text{laser}} \times \lambda}, \quad (2)$$

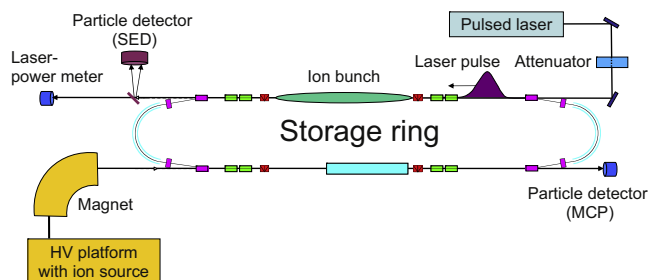


FIGURE 2 ELISA storage ring equipped with a multichannel plate (MCP) detector for delayed events and a secondary emission detector (SED) for prompt and delayed detection of photofragments.

where N_{neutrals} is the number of laser-induced neutrals (integrated over time), N_{ions} the number of ions in the ion bunch (proportional to the collision-induced neutrals arriving at the detector before firing the laser), Φ the photon flux, E_{laser} the laser-pulse energy, and λ the wavelength. Because we do not have a direct measure of the number of ions in the storage ring, we only obtained relative absorption cross sections.

We investigated the microsolvation of the *trans* *p*-coumaric acid as well as two methyl-substituted derivatives of *trans* *p*-coumaric acid in their deprotonated form, the methoxy derivative *trans* *p*-CH₃OC₆H₄CHCHCOO[−], which is a carboxylate and the methyl ester derivative *trans* *p*-OC₆H₄CHCHCOOCH₃, which is a phenolate. It was found that the carboxylate species prefer to cluster with one water molecule whereas the phenolate species prefer to attach two water molecules. This gives support to the assumption that the structure of pCA[−](H₂O)₂ is one in which both water molecules are attached to the phenolate end of pCA[−] (see Fig. 1).

When the relaxation process after photoexcitation is a fast one, the absorption signal appears only at the secondary electron-emission detector located directly after the laser-interaction region (see Fig. 2), and the decay time for the process cannot be resolved by the storage-ring technique. This was the case for solvated ions under study here, and hence, a linear time-of-flight spectrometer with submicrosecond time resolution (36,37) was used to study the fast relaxation dynamics in a crossed-beam experiment using 355-nm laser light.

After photoexcitation, the chromophore complex breaks apart into neutral and charged fragments. The mass of the charged fragment was determined by switching the voltages of the storage ring to settings for a given fragment mass, and subsequently dumping the stored particles, if any, onto the multichannel plate detector (38) to register the amount of a specific fragment.

RESULTS AND DISCUSSION

Fig. 3 shows the absorption profile for the microsolvated and the bare pCA[−] chromophore. A large blue shift of 0.71 eV (85 nm) is observed upon solvation of pCA[−] by just two water molecules.

The only observed photofragment of pCA[−](H₂O)₂ was pCA[−]. Thus, photoexcitation leads to the emission of the two water molecules from the cluster, which leaves the parent chromophore intact. The excited state (*S*₁) has less electron density on the phenolic oxygen (calculated, e.g., in (39)), and as a consequence, it has a weaker H-bonding, which in turn may lead to release of the two water molecules in the excited state. Upon photoexcitation using 400 nm light, the bare pCA[−] relaxes via photodetachment as well as fragmentation (39,36). The presence of the two H-bonds prevents the chromophore from fragmenting after photoexcitation, even though the photon energy (3.59 eV) is much

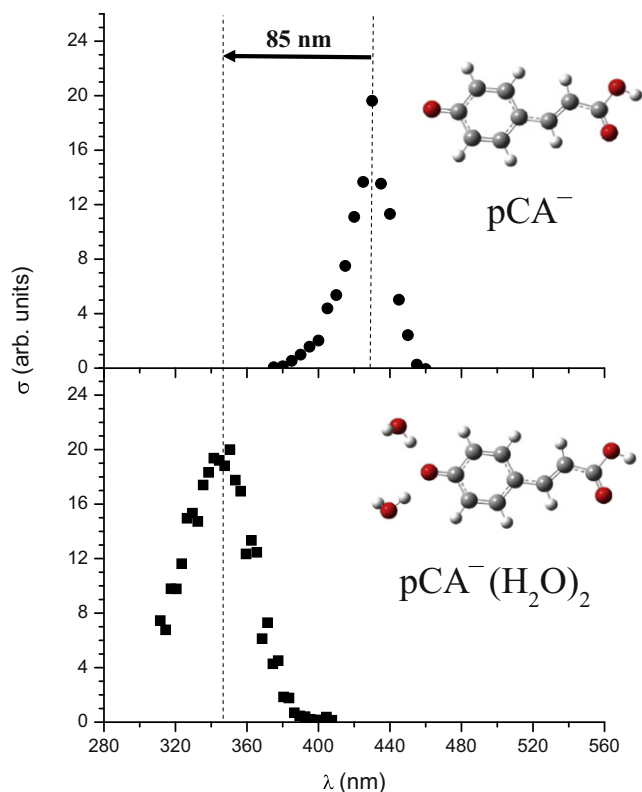


FIGURE 3 Absorption spectra of deprotonated *p*-coumaric acid (pCA^-) in bare and hydrogen-bonded forms. Multiple data sets were collected for the anion cluster $\text{pCA}^-(\text{H}_2\text{O})_2$ and were subsequently binned with a bin width of 3 nm. The maximum of absorption is at 430 ± 5 nm for pCA^- and 345 ± 5 nm for $\text{pCA}^-(\text{H}_2\text{O})_2$.

higher than needed to fragment the bare chromophore (30). A similar situation appears for aniline $^+(\text{H}_2\text{O})_n$ ($n = 4\text{--}20$) clusters (19), where photofragmentation with photons of 1.65–4.66 eV energy shows that most of the photon energy flows to the hydrogen-bonding networks. This photon energy is then used-up for liberation of water molecules, with the aniline cation surviving the irradiation.

Within the protein, in addition to H-bonds, there are other interactions as well (a covalent linkage, steric hindrance, counterion) that may lead to a very different way of dissipation of the absorbed energy. The time-of-flight measurements showed relaxation dynamics with a dissociation time faster than 25 ns for the solvated anion cluster and a kinetic energy release of 3 meV for the neutral fragments (36,37). The low kinetic-energy release indicates that hot water molecules may be released (bearing in mind that the chromophore stays intact).

Fig. 4 shows the PYP chromophore with its nearest amino-acid residues within the protein environment. The phenolic oxygen of the chromophore is connected to the amino-acid residues Glu⁴⁶ and Tyr⁴² by H-bonds, and the chromophore is covalently linked to the protein via a thioester linkage to cysteine residue 69. In addition, the H-atom of the amino group of the cysteine residue forms

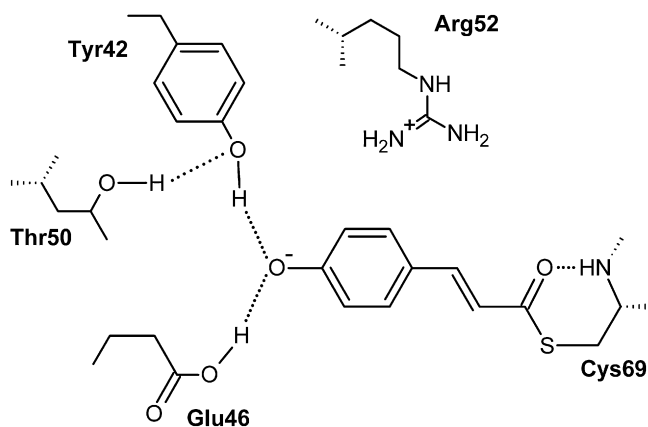


FIGURE 4 PYP chromophore and the residues surrounding it within the protein.

an H-bond to the carbonyl oxygen of the chromophore. The absorption in gas phase of the PYP chromophore agrees fairly well with the absorption within the protein (30), despite the presence of these H-bonds which individually affect the absorption maximum, as is evident from this work.

The role of the active site residue Glu⁴⁶ in PYP, which is hydrogen-bonded to the pCA^- anion at the phenolic end, was studied by substitution at this site (10). It was concluded that substitution by residues with similar volumes but no hydrogen-bonding capability results in a red shift when compared to absorption of the wild-type PYP. This implies that the presence of H-bonding interactions at the phenolic end of the chromophore tends to blue-shift the absorption maximum. The system, $\text{pCA}^-(\text{H}_2\text{O})_2$, can be thought of as simulating these two H-bonding interactions (Glu⁴⁶ and Tyr⁴²), and indeed we see a significant blue shift.

The importance of covalent linkage of the chromophore to the protein was investigated by mutation studies. They show that the absence of the covalent link blue-shifts the absorption maxima from 446 nm in wild-type PYP to 435 nm in the C69G mutant (12). Thus, the thioester linkage between the protein and the chromophore red-shifts the absorption and contributes to nullifying the blue shift introduced by the presence of the two H-bonds at the phenolic end of the deprotonated chromophore. Nielsen et al. (30) have measured the absorption spectrum, in vacuum, of the deprotonated *trans-p*-coumaric acid and deprotonated *trans*-thiophenyl-*p*-coumarate and found them to be 430 nm and 460 nm, respectively, with a 30-nm red shift caused by replacement of oxygen by sulfur. An additional factor that may contribute to the red shift is the H-bond between the carbonyl oxygen and amino group of the cysteine residue. As this bond is expected to pull the electron density from the phenolate end of the chromophore, which will result in increased charge delocalization, a red shift is expected. The presence of a H-bond at the carboxylate end of the chromophore may also cause a weakening of the H-bonds at the phenolic end, and as a consequence, reduce their blue-shift.

Theoretical studies on the electronic structure of the PYP chromophore, considering the effects of nearby amino acids on the ordering and location of states, have been carried out by Gromov et al. (4). Their studies show that the presence of a nearby Arg⁵² counterion results in the stabilization of the chromophore against autoionization, but it has only minor spectral importance. In addition, the Tyr⁴² and Glu⁴⁶ amino acids, both of which form a hydrogen bond to the phenolic oxygen, have a major effect on increasing the vertical excitation energy to S₁ state by up to 0.69 eV. Their calculations also show that the linkage with the Cys⁶⁹ amino-acid residue reduces this energy by as much as 0.2 eV. However, the model chromophore in their case is the deprotonated *p*-coumaric thio-methyl ester (with a sulfur atom) and hence, a direct comparison with our experimental results is not straightforward. They have also calculated the effect of adding two water molecules to a bare PYP chromophore model, and the first excitation energy is shown to increase by <0.1 eV (5). The model chromophore for this calculation is the deprotonated form of *p*-coumaric methyl ester, which is a better candidate for comparison with our experimental results. Apparently, the theoretical and experimental values for the blue shifts obtained upon solvation with two water molecules do not agree.

The 85-nm blue shift associated with the H-bonds at the phenolate group of the pCA[−] chromophore may be related to the change in dipole moment ($|\Delta\vec{\mu}| = 5$ D) associated with the S₀-S₁ excitation (39). This change is caused by an electron-density transfer from the phenoxy ring to the β -carbon, and the blue shift is a consequence of the electron pull-off effect of the two hydrogen bonds.

We can expect the strength of the H-bonds to dictate the extent of spectral tuning as it directly relates to the extent of localization of the negative charge on the chromophore anion. The presence of the hydrogen bonds cause a differential shift in the ground, and in our case, the first excited state leads to an increased energy gap. The blue shift will hence depend on the species forming the H-bond, and it may be different when amino-acid residues replace water molecules inside the protein.

We cannot compare our results with those recorded in solution, because the model does not exist as a phenolate in solution. A suitable model for comparison in solution phase is the deprotonated form of *p*-coumaric methyl ester ([−]OC₆H₄CHCHCOOCH₃), which shows an absorption maximum at 356 nm in methanol (39). This value is not far from the absorption of pCA[−](H₂O)₂ at 345 nm. In solution phase, apart from H-bonds at the phenolic end of the chromophore, the carbonyl oxygen is also free to form H-bonds. In addition, there will be polar and counterion interactions. Together they may result in red-shifting the solution phase maximum, with respect to that of the micro-solvated anion.

The implications of these results are not limited to the PYP chromophore. In nature, protein pockets holding chromo-

phores often form one or more H-bonds with the chromophore, which almost always participates in the photocycle of the protein. That such interactions can also play a significant role in the spectral tuning of the photoabsorption maxima is clearly evident from this work.

CONCLUSION

We have measured the gas-phase photoabsorption spectra of the deprotonated form of *trans-p*-coumaric acid solvated with two water molecules. An unusually large blue shift of 0.71 eV is observed in going from the bare, pCA[−] ion, to the solvated ion, pCA[−](H₂O)₂. The two water molecules, which attach to the phenolic end of the bare ion, mimic two similar H-bonds present within the protein environment. We thus prove that the interactions within the PYP protein individually influence the photoabsorption maximum of the chromophore it encloses, but the interactions are countercanceling so that the gas-phase photoabsorption maximum agrees well with that observed in the native protein. This study also shows that, in gas phase, the presence of H-bonds stabilizes the chromophore against autoionization and fragmentation. The presented measurements highlight the fact that photoabsorption studies in gas phase can be useful to shed light on the issue of spectral tuning within photoactive proteins.

This work was supported by the Lundbeck, Carlsberg, and Willum Kann Rasmussen Foundations, and the Danish Research Agency (contract No. 272-06-0427).

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