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Rocha-Rinza, Tomas; Sneskov, Kristian; Christiansen, Ove; Ryde, Ulf; Kongsted, Jacob

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LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

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Unraveling the similarity of the photoabsorption of deprotonated *p*-coumaric acid in the gas phase and within the photoactive yellow protein

Tomás Rocha-Rinza,[a,b] **Kristian Sneskov,**[a] **Ove Christiansen,**[a] **Ulf Ryde**[c] **and Jacob Kongsted**[d]

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Using advanced QM/MM methods, the surprisingly negligible shift of the lowest-lying bright electronic excitation of the deprotonated *p*-coumaric acid (pCA^-) within the photoactive yellow protein (PYP) is shown to stem from a subtle balance between hypsochromic and bathochromic effects. More specifically, it is found that the change in the excitation energy as a consequence of the disruption of the planarity of pCA^- inside PYP is nearly canceled out by the shift induced by the intermolecular interactions of the chromophore and the protein as a whole. These results provide important insights about the primary absorption and the tuning of the chromophore by the protein environment in PYP.

Introduction

The response of a biological cell to radiation is mediated through receptor proteins. For instance, the extremophile bacterium *Halorhodospira halophila*[1] is attracted to red and infrared light which can be used to carry out photosynthesis, whereas it moves away from sources of blue light, which is identified as a potentially harmful wavelength.[2] In *H. halophila,* the photosensory receptor protein associated with the blue-light repellent response is the photoactive yellow protein (PYP).[3] Besides its biological relevance, PYP has become an important model in the study of PAS-domains, as well as for the primary photochemistry and functional folding of biological photoreceptors.[4] The prominence of PYP in photochemistry and photobiology is also due to its advantageous biochemical properties (high water solubility and relatively easy formation of crystals), photochemical stability and the comparatively simple structure of its chromophore, *p*-coumaric acid (pCA), which has only one isomerizable double bond.[2,4] This last issue is especially relevant because the structure of the chromophore, e.g. *cis* or *trans* geometry or protonation state, is of major importance for the function of the photoactive protein. In particular, pCA has a *trans* double bond and is deprotonated (pCA^-) in the electronic ground state of PYP.[5]

At the heart of cellular response to radiation lies the concept of electronic transitions most easily visualized in terms of electronic excitation energies. Essentially, the characteristics of a photoactive protein may be explained by considering the electronic structure of the chromophore; it reflects the frequency interval in which the action of the holoprotein is needed: It is only when the chromophore absorbs light of a suitable wavelength that structural changes in the protein occur and are followed by appropriate action from the host cell.[2] This absorption can be tuned by the protein environment and it is therefore important to consider the interaction between the chromophore and the protein. pCA^- is located – via a thioester bond with Cys69 – in a hydrophobic pocket within the protein, which efficiently isolates the chromophore from the aqueous solvent – see Fig 1. This hydrophobic insulation has been verified by the X-ray structure of PYP[6] and by the observed similarities in absorption properties of pCA^- in gas phase and in different xanthopsins,[7] as opposed to the strong blue shift that occurs in aqueous and alcoholic solutions. Such chromophore insulation is important in view of its strong solvatochromism[7-9] which implies a strong change in the dipole moment after excitation, which is corroborated by RI-CC2 and aug-MCQDPT2 calculations.[8] This indicates that the absorption of pCA and pCA^- (and therefore the biological function of PYP) should be particularly responsive to the different interactions of the chromophore within the protein. In addition, the delocalization of the negative charge of pCA^- is most efficacious when the chromophore is in its planar gas-phase structure. The chromophore geometry may be altered in an amino-acid environment and an absorption shift is thus

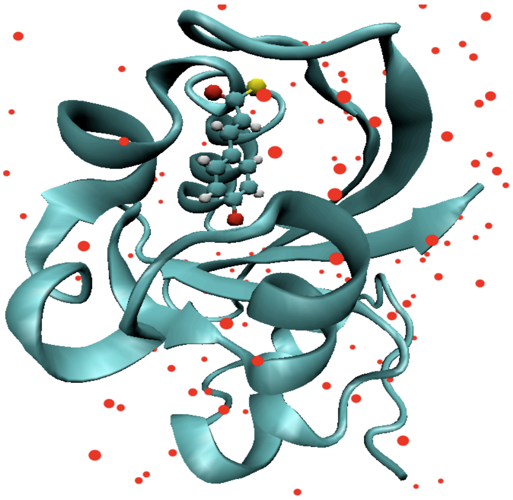

Fig. 1 Deprotonated *p*-coumaric acid buried in a major hydrophobic pocket of the photoactive yellow protein. The amino acids of the core prevent contact between the chromophore and the solvent, displayed by small red circles

expected.

The purpose of this paper is to dissect all these potential contributions to the spectral tunning of pCA$^-$ in the ground state of PYP. We do so by calculating the vertical excitation energy $S_0 \Rightarrow S_1$ of different models of the pCA$^-$ chromophore within the protein using full quantum mechanics calculations, as well as a coupling between time-dependent density functional theory and molecular mechanics. As opposed to earlier works,[10-12] this paper is not focused on the specific influence of close-lying amino acids, but in considering the whole protein using a very advanced force-field, which includes not only a higher-order multipole description (truncated at the quadrupole level) of the permanent charge distribution, but also a direct inclusion of protein polarization effects via anisotropic polarizabilities. Overall, this study reveals that the spectral shift of the chromophore is small only because of a subtle balance between bathochromic and hypsochromic effects. Thereby, we elucidate the underlying photochemistry intrinsically hidden when two numbers appear identical, thus providing novel insight into the absorption properties and potential color-tuning mechanisms of PYP.

Computational details

Based on full quantum mechanics (QM) calculations, as well as on a coupling between time-dependent density functional theory (TDDFT) and molecular mechanics (MM), we calculate the vertical excitation energy $S_0 \Rightarrow S_1$ of pCA$^-$ in different environments that mimic the surroundings of the chromophore within PYP. We performed a partitioning of the PYP molecule into QM and MM subsystems in a similar fashion to other investigations.[13-16] However, in variance to previous QM/MM studies, which have used only a fixed point-charge model of the MM surroundings, we describe the environment of the chromophore in a much more detailed way, by considering atom-centered charges, dipoles, and quadrupoles, as well as induced dipoles determined through anisotropic polarizabilities. Both sets of localized properties were calculated for all residues in the protein, using the LoProp approach.[17] The force field was calculated according to the method outlined in Ref. 18 at the DFT(B3LYP)/6-31+G* level. We also calculated the force field using the larger and more flexible aug-cc-pVDZ basis set, but that led to negligible changes in the final prediction of the excitation energies and are not presented here. All calculations of the force field were performed with the Molcas 7.4 quantum chemistry program.[19]

This treatment is a significant improvement with respect to earlier theoretical investigations in which only a few of the residues comprising PYP were investigated[10-12] and QM/MM studies describing the MM region with point charges and the QM system semiempirically, where the explicit polarization between the QM and MM system was not taken into account.[13-16] In fact, it has been shown previously that different point-charge models (e.g. those of the Amber 94 and 03 force field) may give excitation energies that differ by up to 0.6 eV.[18] We note that related QM/MM or polarizable dielectric continuum model (DC) schemes have been presented before[20-22] all differing primarily in their treatment of the coupling between the QM and MM or DC subsystems. The reported excitation energies are obtained at the CAM-B3LYP/6-31+G* level of theory. This functional has previously been shown to give accurate excitation energies[23] and a good basis set convergence is already observed with the 6-31+G* basis set. However, for benchmark purposes, we also calculated vertical excitation energies using the RI-CC2[24,25] linear-response module of Turbomole[26,27] (without any multipoles and polarizabilities). The QM/MM calculations were performed using a locally modified version of Dalton.[28] In this model, the effective Hamiltonian includes the contributions from the MM multipoles, as well as a mutual account of chromophore–protein polarization. The latter is iterated to self-consistency using either the ground-state or the perturbed electronic density of the chromophore. For details on this method as well as the implementation we refer to Ref. 29. In the QM/MM calculations, all multipoles and anisotropic polarizabilities were zeroed for MM sites within 1.2 Å of any QM atom, i.e. for the Cα and link hydrogen atom of Cys69 (see below). All vacuum geometries were optimized with the B3LYP/cc-pVTZ approximation. The geometries of both the vacuum and in-protein structure of the chromophore are available as supporting information.

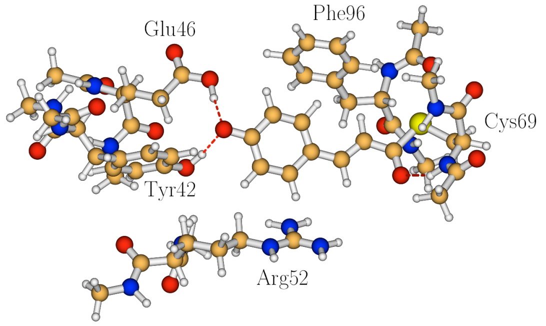

Fig. 2 Deprotonated *p*-coumaric acid and its closest residues within the photoactive yellow protein.

Protein preparation and force field derivation

All calculations in this investigation are based on the X-ray structure of PYP[6] (PDB accession code 1nwz). First the chromophore was removed from the protein by cutting the Cys69 link through the Cα–Cβ bond and filling the valences by a hydrogen atom both in the chromophore and in the protein. This effectively changed Cys69 into a glycine residue. The chromophore was assumed to be negatively charged (see Figure 2). The protein structure was prepared using the protein preparation wizard[30] in the Schrödinger software graphical interface Maestro.[31] Hydrogen atoms were added to the structure in accordance with a physiological pH (7.0). Thereby, all Asp and Glu residues, except Glu64, were assumed to be negatively charged. Glu64 was assumed protonated and thus neutral[32]. From a detailed study[33] of the hydrogen-bond structure and the solvent exposure, it was decided that the two His residues in the protein are protonated on the Nε2 atom. All Arg and Lys residues were assumed to be positively charged, while the remaining amino acids were considered to be neutral. The total charge of the apo- and holo-protein were –5 and –6 respectively. The latter comprises

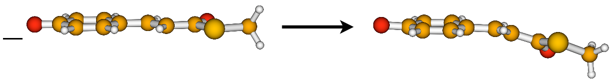

Fig. 3 Change in geometry of the chromophore when going from vacuum to within the photoactive yellow protein.

the negative charge of the chromophore. Most calculations were performed with such charged proteins. However, some calculations were performed in which the charge of the holoprotein was neutralized by adding six Na$^+$ counter ions. The ions were added by the Amber9 tleap module, which places the ions at points with the largest electrostatic potential around the protein[34]. Three distinct sets of positions for the counter ions were tested. In addition, the protein preparation wizard checked the flip states of the imidazole rings of the Hie residues, together with the side chain amides of Asn and Gln residues. This resulted in flips of the side chain amides of Asn13, Gln22 and Gln99. X-ray water molecules within 5 Å of the chromophore were retained in the calculations (molecules 1005 and 1036). In the last step of protein preparation, hydrogen positions were optimized while keeping heavy atoms fixed.

Results and discussion

First, we considered the geometrical constraints imposed on the chromophore by the protein environment. These structural effects were evaluated by a geometry optimization of the free (isolated) form of deprotonated methyl *p*-thiocoumarate (pCTM$^-$) in vacuo. In Figure 3, the vacuum structure is compared with the in-protein geometry. It is clearly seen that the chromophore is distorted from a planar vacuum structure to a non-planar geometry inside PYP. This distortion of the chromophore causes a change in an excitation energy from 3.33 to 3.24 eV, i.e. the protein change of the chromophore structure conveys a bathochromic shift of approximately –0.09 eV.

Second, we consider the electrostatic and polarization effects of the protein environment on the pCA $S_0 \Rightarrow S_1$ excitation energy by using the in-protein structure and introducing various MM perturbations in the form of close-lying residues capped with CH_3CO– and –$NHCH_3$ groups (see Figure 2). We compare these QM/MM excitation energies with full QM results on the chromophore–residue systems in Table 1, in order to assess our QM/MM calculations.

It can be seen that the errors introduced by the QM/MM partitioning are essentially negligible. It is also noteworthy that for the pCTM$^-$+Cys69 system, the QM/MM and QM calculations give identical results, although the Cα–Cβ bond is cut only in the QM/MM approach.

The oscillator strengths (length gauge) of the transition for the considered model systems are also reported in Table 1. It can be seen that the Tyr42, Glu46 and Cys69 residues lead to an increase in the intensity of the investigated lowest bright state, while the residues Phe96, Thr50 and Arg52 generally serve to decrease the intensity. Comparing the oscillator strength calculated for the isolated chromophore with the full QM/MM predictions, we find that the net effect of the protein environment is to increase the intensity of the lowest bright state. This increase in the intensity may be relevant for the biological action of PYP in virtue of its low abundance in the cytoplasm: *H. halophila* has only around 500 PYP molecules per cell.[35] Generally, we find that the shifts caused by the individual protein residues are in good agreement with a similar analysis previously presented by Gromov et al. using CC2 and EOM-CCSD[10].

Table 1 $S_0 \Rightarrow S_1$ vertical excitation energies (in eV) and oscillator strengths of the considered systems capped with CH_3CO– and –$NHCH_3$ groups.

Molecular System	QM	QM/MM	Error(QM/MM-QM)	Osc. strength
pCTM$^-_{\text{in-protein strcuture}}$	3.24	-	-	1.036
pCTM$^-$+Cys69	3.14	3.14	0.00	1.131
pCTM$^-$+Tyr42	3.36	3.38	0.02	1.222
pCTM$^-$+Glu46	3.37	3.39	0.02	1.134
pCTM$^-$+Arg52	3.34	3.31	-0.03	0.533
pCTM$^-$+Thr50	3.30	3.30	0.00	1.020
pCTM$^-$+Phe96	3.17	3.19	0.02	0.870
pCTM$^-$+5 residues[a]	3.32	3.28	-0.04	1.062
pCTM$^-$ in PYP	-	3.34	-	1.101

[a] The 5 residues considered simultaneously are Cys69, Tyr42, Glu46, Arg52 and Phe96.

Encouraged by these observations, the total electrostatic and polarization effects of the protein environment were deduced by performing QM/MM calculations on the full PYP structure. We then obtain an excitation energy of 3.34 eV, i.e. a hypsochromic shift of 0.10 eV as compared to the excitation energy of the isolated chromophore with the in-protein structure in Figure 1. We thus observe that two oppositely directed effects ultimately give rise to a very small residual shift of 0.01 eV. We stress that the total shift is due to these opposite effects and not simply because there is no significant intermolecular interaction between the chromophore and the protein when the former is embedded in the hydrophobic pocket. This important result should be compared to that of Coto et al.,[13] where a cruder force-field description (e.g. neglect of explicit polarization) led to the conclusion that the protein environment only serve as a fine-tuning of the excitation energy. Finally, it is clear that while the investigated shift of the excitation energy is of very high quality, the absolute value is not so: The calculated excitation energy is 3.34 eV as compared to the experimental result 2.78 eV. Indeed, it is from a theoretical point-of-view very challenging to reproduce the latter value, requiring the inclusion of many different considerations, such as dynamic effects, solvation, larger basis sets, and more accurate methods. However, these effects are assumed to cancel upon consideration of the shift and is thus of less importace for the present analysis.

The deprotonated phenyl *p*-thiocoumarate (pCTP$^-$) has been conjectured to be a good model chromophore and its vacuum absorption spectrum has been measured.[7] In order to

Table 2 Shift of the $S_0 \Rightarrow S_1$ excitation energies (eV) relative to the isolated form of the chromophore used in experiment (pCTP$^-$) and that used in this study (pCTM$^-$).

Chromophore	E_{exci}	ΔE
pCTP$^-_{\text{vac}}$	3.22	-
pCTM$^-_{\text{vac}}$	3.33	+0.11
pCTM$^-_{\text{in-protein structure}}$	3.24	-0.09
pCTM$^-_{\text{PYP}}$	3.34	+0.10
Total shift relative to pCTP$^-_{\text{vac}}$		+0.12
Exp. shift relative to pCTP$^-_{\text{vac}}$		+0.08
Total shift relative to pCTM$^-_{\text{vac}}$		+0.01

scrutinize these experimental findings, we investigated the effects of replacing the terminal phenyl group of pCTP$^-$ with a methyl group on the calculated excitation energies. The transition energy of the optimized structure of pCTP$^-$ is 3.22 eV. This can be compared with the previously reported pCTM$^-$ $S_0 \Rightarrow S_1$ excitation energy of 3.33 eV (Table 2). Regarding the change in excitation energy induced by the protein, we get a total shift of 0.12 eV for the excitation energy of PYP in comparison with that of pCTP$^-$ in vacuum. This is in reasonable agreement with the corresponding experimental value of 0.08 eV.

In the calculations collected in Table 2, the total charge of PYP is -6. To investigate if this net charge affects the results of the calculation of the PYP electronic excitation energy, we constructed three neutral structures by adding six counterions at different positions. On average, the result from these calculations reproduce the corresponding result without any counterions added, i.e. giving an excitation energy of 3.34 eV.

It has been reported[10] that all excited states of pCTM$^-$ are auto-ionizing. In such a case, these states lie in the ionization-continuum and should strictly be treated with definite methods, such as complex absorbing potentials[36] or the all-electron mixed-basis approach[37-39] which involves both plane waves and atomic orbitals as basis functions. Nonetheless, the agreement between the absorption found in experiment and predicted using different theoretical methods, including linear response theory (in both CC and DFT forms) together with multi-referential methodologies (CASPT2, MRMP2 and aug-MCQDPT2) regarding the $S_0 \Rightarrow S_1$ excitation transition of pCTM$^-$ in vacuum is noteworthy. Therefore, these approximate excitation energies have been used as references to determine the effect of the neighboring amino acids on pCTM$^-$ within PYP. For example, CC2 and EOM-CCSD excitation energies of the isolated pCTM$^-$ were used in Ref. 10 to show that the effect of Arg52 on the energetics of the $S_0 \Rightarrow S_1$ transition is negligible, although it provides a stabilization with respect to autoionization. Another check of the validity of the reported excitation energies and oscillator strengths is through the use of damped response theory.[40] Here the absorption is determined directly. This allows us to calculate an absorption spectrum in a direct way, circumventing the troublesome metastable state. It is evident from the spectrum in Fig. 4 that a strong absorption is indeed located at the frequencies corresponding to the calculated excitation energies. There is no sign of spurious absorption due to artificially discretized states lying in the continuum.

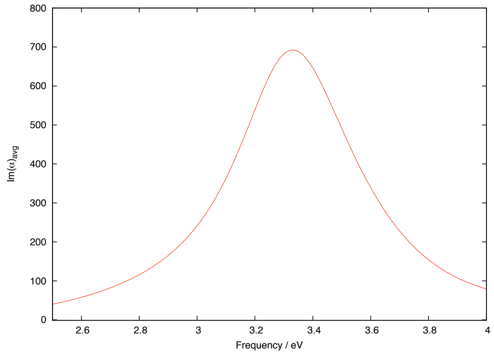

Fig. 4 Absorption spectrum of the vacuum pCA- structure obtained using damped linear response theory at the CAM-B3LYP level.

Table 3 Comparison of shift of the $S_0 \Rightarrow S_1$ excitation energy (eV), obtained with CAM-B3LYP/6-31+G* and RI-CC2/aug-cc-pVTZ. The shift is relative to the isolated chromophore, using in-protein geometries for all groups, i.e. $\Delta E^{\text{model}} = E^{\text{model}}(\text{pCTM}^-\text{+X}) - E^{\text{model}}(\text{pCTM}^-)$.

X	TDDFT	ΔE^{TDDFT}	RI-CC2	$\Delta E^{\text{RI-CC2}}$
Cys69	3.14	-0.10	2.66	-0.11
Tyr42	3.36	+0.12	2.92	+0.15
Glu46	3.37	+0.13	2.93	+0.16
Arg52	3.34	+0.10	2.84	+0.07
Thr50	3.30	+0.06	2.84	+0.07
Phe96	3.17	-0.07	2.70	-0.07

It is well known that the standard exchange–correlation functionals used in DFT lack the inclusion of certain non-covalent interactions, most notably dispersion effects. Consequently, an additional test of the accuracy of the presented analysis was performed by doing a numerical comparison with second-order approximate coupled cluster singles and doubles (CC2) and TDDFT on the model systems. In Table 3, we report the spectral shift obtained by TDDFT(CAM-B3LYP)/6-31+G* and RI-CC2/aug-cc-pVTZ levels of theory. It can be seen that the two descriptions provide almost identical shifts for the considered model systems, thus serving as another validation of the use of DFT in the analysis presented in this work.

Conclusions

In this paper, we have studied how the surrounding protein tunes the lowest-lying electronic excitation energy of the bound chromophore in PYP using unusually detailed QM/MM studies. We show that the surprisingly small shift between the protein and gas phase chromophore models stems from a subtle balance between effects due to change of structure of the chromophore inside the protein and perturbations from the protein environment on the excitation energy.

Our combined QM/MM approach provides valuable insights into the primary absorption of the chromophore within PYP and it could be further extended to study other relevant intermediates in the PYP photocycle, which could lead to the ultimate understanding of the biological function of this photoreceptor.

Notes and references

[a] *The Lundbeck Foundation Center for Theoretical Chemistry and the Center for Oxygen Microscopy (COMI), Department of Chemistry, University of Aarhus, DK-8000 Aarhus C, Denmark*

[b] *Present address: Instituto de Qumica, UNAM, Circuito Exterior Ciudad Universitaria, Delegación Coyoacán C.P. 04510 México, D.F. México*

[c] *Department of Theoretical Chemistry, Lund University, Chemical Center, P.O. Box 123, SE-22100 Lund, Sweden*

[d] *Department of Physics and Chemistry, University of Southern Denmark, DK-5230 Odense M, Denmark. Fax: (+)45 6615 8760; Tel: (+)45 6550 2304; E-mail: kongsted@ifk.sdu.dk*

† Electronic Supplementary Information (ESI) available: XYZ files of the vacuum and in-protein chromophore structures. See DOI: 10.1039/b000000x/

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