Chemical and protein shifts in the spectrum of the photoactive yellow protein: A time-dependent density functional theory/molecular mechanics study

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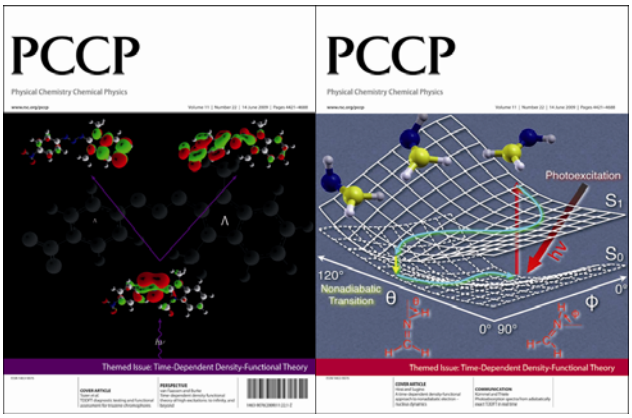

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Chemical and protein shifts in the spectrum of the photoactive yellow protein: a time-dependent density functional theory/molecular mechanics study

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We have studied the light absorption properties of the *p*-coumaric acid chromophore in the photoactive yellow protein (PYP) with a hybrid time-dependent density functional theory/molecular mechanics (TDDFT/MM) method. To critically assess the performance of TDDFT for this specific system, we first evaluated *in vacuo* the excited states of several PYP chromophore models. We then calculated the absorption maximum of the phenolate anion of the thiomethyl-*p*-coumaric acid ($TMpCA^-$) in the protein. Although within the limitations of TDDFT in describing charge-transfer and resonance excited states, we confirm a sizeable red shift in the absorption maximum due to the chemical differences between the free chromophore and that in the protein. The interaction between the chromophore and the protein environment induces a very small spectral shift, in line with experimental evidence. Comparison between the vertical electron detachment energy of the chromophore *in vacuo* and in the protein reveals that the protein stabilizes the choromophore in the excited states by preventing radical formation.

Introduction

The photoactive yellow protein (PYP) is thought to be responsible for the negative phototaxis to blue light of the *Halorhodospira* (*Ectothiorhodospira*) *Halophila* bacteria.[1,2] The protein chromophore, a *para*-hydroxy cinnamic acid covalently bound to a cysteine residue, undergoes a *trans*-to-*cis* isomerization around its unique C=C double bond upon light absorption.[3] This triggers a cascade of structural modifications in the protein that ultimately lead to the generation of a signal, warning the bacteria of the presence of damaging blue light.[4] PYP is an excellent model to study photo-induced signal transduction in photoreceptor proteins because of its small size (125 amino acids), water-solubility, stability and available high resolution crystallographic structures.[3] These reasons have indeed motivated many experimental and theoretical investigations. Although some insights have been obtained on the initial steps of the chromophore upon light absorption (*e.g.* on the isomerization path[5]), there are still many open questions concerning the role of the protein environment during the absorption of light by the chromophore.

The free chromophore of PYP is the *p*-coumaric acid (pCA). In the gas phase, its absorption maximum is in the ultraviolet region of the spectrum[6–8] whereas PYP maximally absorbs in the blue, at 2.78 eV.[9] In PYP the chromophore is found as a phenolate anion, bound to the protein by a thioester linkage to the Cys-69 residue.[10] It forms hydrogen bonds with the Tyr-42 hydroxyl and with the protonated Glu-46. Moreover, the positively charged nearby Arg-52 is believed to compensate, or neutralize, the negative charge of the chromophore, and to stabilize the chromophore in its binding site, shown in Fig. 1.

The total spectral shift (ΔE) of the absorption maximum of the chromophore from its gas phase form to the protein can be divided into two contributions: (i) the effect of the chemical difference between the free chromophore pCA and the chromophore within the protein (ΔE_{chem}), and (ii) the effect of the interaction between the chromophore and the protein environment ($\Delta E_{protein}$). Hence, $\Delta E = \Delta E_{chem} + \Delta E_{protein}$.

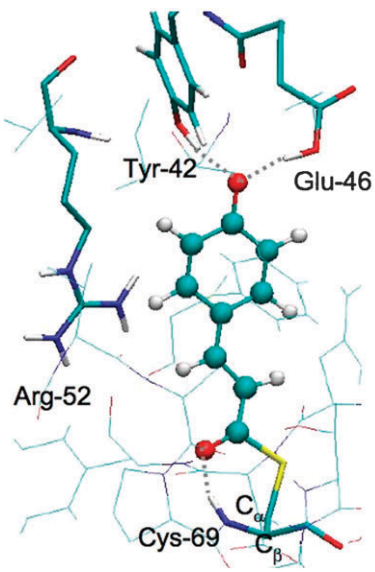

Fig. 1 The binding site of the chromophore (represented in ball and sticks) in the photoactive yellow protein. The main residues interacting with the chromophore are highlighted with thicker sticks.

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These two contributions have been analyzed experimentally, although chromophores in solution rather than *in vacuo* were mostly taken as references to evaluate the spectral shifts; results are summarized below. Due to the effects of the complex medium, it is therefore difficult to deduce the intrinsic absorption properties of the chromophore.

Kroon *et al.*[11] measured the absorption spectra of PYP hybrids with different chromophore analogues inserted in them; they found that the chemical modification of the chromophore accounts for 80% of the red shift of the absorption spectrum of PYP with respect to the chromophore in water solution at neutral pH. In particular, they attributed a red shift of $\sim$0.58 eV to the deprotonation of the phenolate oxygen of pCA by comparing the absorption of the denatured protein in neutral and alkaline pH; they also attributed a red shift of $\sim$0.74 eV to the formation of the thioester linkage with the protein by comparing the free coumaric acid and the denatured protein at neutral pH: hence $\Delta E_{\mathrm{chem}} \sim -1.32$ eV. A further red shift of $\sim$0.28 eV was ascribed to the chromophore–protein interaction. Changenet-Barret *et al.* measured the absorption spectra in solution of the fully deprotonated *trans* *p*-coumaric acid (pCA^{2-})[12] and of the phenolate anion of the thiophenyl-*p*-coumarate (pCT^{-}).[13] The effect of the presence of the thioester group was to red shift by 0.58 eV the absorption maximum of pCT^{-} to 395 nm (buffer solution at pH 10.6)[13] from the 333 nm of pCA^{2-} (in water).[12] Nielsen *et al.* tried to disentangle the intrinsic absorption properties of the chromophore from the effects of the protein environment by measuring the absorption spectra *in vacuo* of pCT^{-} (which they considered a good model of the chromophore within the protein since the charge does not extend to the phenyl group) and of the carboxylate anion pCA^{-} (which they used as a model of the neutral pCA).[14] By comparing the absorption maximum of pCT^{-} (at 460 nm with full width at half maximum of 51 nm) and of the carboxylate anion pCA^{-} (at 430 nm with full width at half maximum of 25 nm), they estimated a red shift due to the phenol deprotonation and formation of the thioester linkage of 0.19 eV, whereas by comparing the absorption maximum of pCT^{-} *in vacuo* (2.70 eV) and that of PYP (2.78 eV), they estimated a small blue shift of 0.08 eV due to the chromophore-protein interaction. They also measured the absorption spectra in water at neutral and alkaline pH deducing a $\sim$0.6 eV red shift due to deprotonation and a 0.55 eV red shift due to thioester formation, in good agreement with the estimations by Kroon *et al.*[11] The small effects on the absorption maximum due to chromophore–protein interaction were also deduced by mutagenesis experiments of various residues in the chromophore binding-site region. In particular, the positively charged Arg-52 was found not to play an important role in the spectral tuning: when mutated to neutral amino acids, no apparent changes were observed in the spectrum.[15] When Glu-46 and Tyr-42 were mutated with residues that could not form hydrogen bonds with the chromophore, small blue shifts were measured ($\Delta E_{\mathrm{protein}} = 0.18$ eV);[16] this result was corroborated by semi-empirical calculations by He *et al.*[17]

The effects of chemical substitutions, charge status and environment effects on the spectral tuning were also investigated by *ab initio* calculations. Previously reported TDDFT calculations performed by Sergi *et al.* on pCA, its phenolate anion pCA^{-}, and on pCT^{-} *in vacuo* yielded a red shift of 0.53 eV and 0.23 eV due to deprotonation and thioester formation, respectively, hence $\Delta E_{\mathrm{chem}} = -0.76$ eV.[18] They also estimated a very small red shift ($\Delta E_{\mathrm{protein}} = -0.04$ eV) due to the chromophore–protein interaction,[18] when comparing TDDFT excitation energies of the isolated chromophore and of an extended model of the active site including a few residues. MS-CASPT2 excitation energies for the thiomethyl-*p*-coumaric acid ($TMpCA^{-}$) in the crystallographic structure placed its absorption maximum *in vacuo* just 0.20 eV below the absorption maximum of PYP, suggesting $\Delta E_{\mathrm{protein}} \sim 0.20$ eV.[19] Groenhof *et al.* included the effects of the protein by performing a classical molecular dynamics simulation of $TMpCA^{-}$ within the protein with the GROMOS96 force field at constant temperature and pressure.[20] They calculated the TDDFT absorption maximum for the average classical structure of $TMpCA^{-}$ and of few surrounding residues (for a total of 74 atoms) obtaining 2.80 eV and compared this result to that of $TMpCA^{-}$ *in vacuo* (3.10 eV); hence, they found a red shift of 0.30 eV, which includes the effects of the geometrical changes of the chromophore along the trajectory.

In spite of this wealth of data, a balanced evaluation of the spectral shifts where the chromophore is treated at the same level of theory *in vacuo* and within the full protein environment (*e.g.* through a QM/MM scheme) is still missing. Here, we quantify the effects of both the chemical modification and the protein environment within a time-dependent density functional theory/molecular mechanics scheme.[21]

TDDFT[22] is a very convenient method to calculate excited states of large molecules due to its moderate computational cost.[23] Although it yields vertical excitation energies for low-lying valence excited states with an accuracy of $\sim$0.4 eV,[24] with the currently available exchange–correlation functionals and within the adiabatic approximation it exhibits some shortcomings for specific kinds of excitations. In particular it has been shown that TDDFT systematically underestimates the excitation energies of charge-transfer states,[25] and it can also fail to describe high-lying bound states.[26] In the study of the excited state properties of PYP chromophore analogues charge transfer states are indeed involved. Therefore TDDFT results for such systems need to be considered with caution and properly assessed. These problems are common to a wider class of biological chromophores which contain conjugated chains. While, for example, TDDFT seems to predict absorption properties of the green fluorescent protein in good agreement with experiments,[27] it gives inaccurate excitation energies and gradients for other systems such as the chromophore of rhodopsin.[28]

Computational methods

We performed a systematic study of selected singlet excited states of chromophore models of PYP *in vacuo* in order to assess the performance of TDDFT for this specific system. The chromophore models studied are shown in Fig. 2: (a) *p*-vinylphenol (pVP), (b) *p*-coumaric acid (pCA), (c) phenolate anion of the thiomethyl-*p*-coumaric acid ($TMpCA^{-}$) and (d)

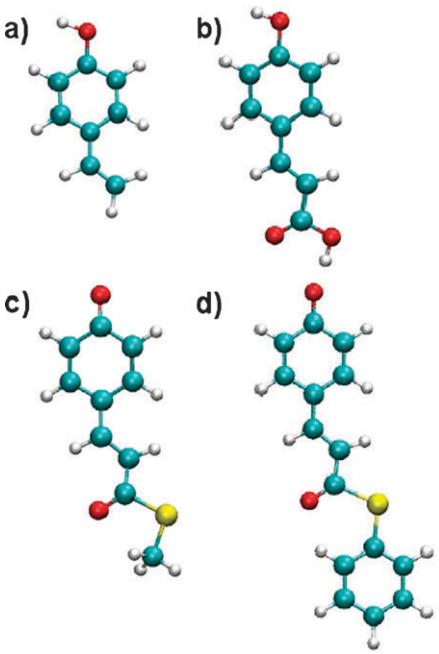

Fig. 2 PYP model chromophores: (a) *p*-vynil-phenol (pVP); (b) *p*-coumaric acid (pCA); (c) thiomethyl-*p*-coumaric acid ($TMpCA^-$) and (d) thiophenyl-*p*-coumarate (pCT^-).

phenolate anion of the thiophenyl-*p*-coumarate (pCT^-). Reference values provided by experiments or high-quality *ab initio* calculations were used for comparison. The effect of the protein environment was taken into account by considering the excited states of $TMpCA^-$ in PYP with a quantum mechanics/molecular mechanics (QM/MM) scheme,[29,30] specifically TDDFT/MM, where the environment is treated classically at a molecular mechanics level of theory, while the chromophore is treated quantum mechanically within TDDFT.[21]

Chromophores *in vacuo*

Firstly we calculated the ground state structures of the chromophore models (*trans* conformers) *in vacuo* by optimizing the geometries at a density functional theory (DFT) level of theory. We performed the DFT calculations with the CPMD[31] and GAUSSIAN[32] codes so to test different basis sets and approximations. For the geometry optimization with the CPMD code we used Troullier-Martins pseudo potentials for the core-electrons,[33] a plane wave basis set with a kinetic energy cutoff of 70 Rydberg, the PBE exchange–correlation functional,[34] orthorhombic simulation cells with edges at 3.5 Å from each side of the molecules, and the Martyna-Tuckerman method[35] for neglecting the electrostatic interaction between neighbouring cells. With the GAUSSIAN code, we treated the core-electrons explicitly and we employed the cc-pVTZ basis set[36] and the B3LYP exchange–correlation functional.[37]

For the ground state geometries obtained for each chromophore model, we then calculated, with both codes, the lowest-lying singlet vertical excited states. In addition to the PBE and B3LYP functionals, we also used the PBE0[38] and BLYP[39] exchange–correlation functionals, with CPMD and GAUSSIAN, respectively. For the CPMD calculations, the Tamm-Dancoff approximation was adopted; this approximation in TDDFT usually does not significantly alter the quality of the excitation energies.[40] With the plane-wave basis set implementation, simulation cells larger than for the ground state geometry optimization calculations (5 Å at each side of the molecule) were chosen so to ensure the convergence of the virtual Kohn–Sham (KS) orbitals.[41] Further details about convergence tests with respect to the cell dimensions and the basis set can be found in ref. 42.

QM/MM calculations

The protein environment was taken into account with the QM/MM scheme implemented in CPMD.[30] The QM/MM boundary was set at the C_α–C_β bond of Cys-69 (see Fig. 1) and the unsaturated bond was capped with a hydrogen atom. The $TMpCA^-$ chromophore (QM subsystem) was described by DFT, whereas the environment (protein + solvent, MM subsystem) was described with the GROMOS96 force field,[43] which has already been satisfactorily applied in PYP simulations.[5,20,44] The chromophore was considered in an orthorhombic QM simulation cell of volume $18 \times 14 \times 14$ Å^3 embedded in a MM cubic box of volume of $69 \times 69 \times 69$ Å^3, which included the protein immersed in a water solution with a saline concentration of 0.15 M. Starting from the crystallographic structure of PYP at 0.82 Å of resolution[45] (entry 1NWZ in the PDB data base[46]), the whole system was first equilibrated at a MM level of theory. Then a QM/MM simulated annealing was performed in order to obtain a representative configuration of the chromophore within the protein at zero temperature. Finally the vertical excited states of $TMpCA^-$ at this configuration were calculated taking into account the protein environment with the TDDFT/MM scheme.[21] To further investigate the effect of the adopted QM/MM scheme on the electronic excitations, we performed additional calculations of the $TMpCA^-$ chromophore immersed in the electric field generated by the point charges of neighbouring residues using a localized basis set.[32]

Results and discussion

PYP chromophore models *in vacuo*: assessment of TDDFT

To assess the performance of TDDFT for describing the light absorption of PYP, we carried out a systematic study of the excited states of the chromophore models shown in Fig. 2, comparing our results with reference experimental or high-level *ab initio* calculations.

Different conformers are possible depending on the orientation of the *trans* isomerizable bond with respect to the sulfur group; the hydrogen attached to the phenolic oxygen in the neutral chromophore can also have two different orientations. Since our final goal is to understand the properties of the chromophore inside the protein, we chose conformers compatible with the crystallographic structure.

The gas phase fluorescence excitation and emission spectra, originally attributed to the free pCA by Ryan *et al.*,[47] turned out to correspond to the smaller pVP molecule,[48] because decarboxylation of pCA occurred during the experiment. For

pVP, the experimental value of the origin of the S_1–S_0 electronic transition (33 200 cm^{-1}, *i.e.* 4.12 eV) was determined.[47,49] The emission and excitation spectra for pVP were also calculated with the EOM-CCSD/LVC (Linear Vibronic Coupling) and TDDFT/Franck–Condon scheme; even if the EOM-CCSD spectra had to be red-shifted by $\sim$0.4 eV to fit the experiments, the main features were correctly reproduced.[49] Neglecting vibrational contributions, we will compare the experimental data for the origin of the S_1–S_0 electronic transition with the first singlet excited state.

For pCA, MS-CASPT2 and EOM-CCSD results by Ko *et al.*[6] and EOM-CCSD results.by de Groot *et al.*[7] and by Gromov *et al.*[8] are available. Although, some disagreements exist between these two sets of data concerning the ordering and character of the two lowest excited states, the energies of the absorption maximum are fairly similar. Recently experiments on methyl 4-hydrocinnamate, a neutral model chromophore with similar properties (and excitation energies) to pCA, have been performed in the gas phase.[7]

Molina *et al.*[19] calculated the MS-CASPT2 excitation energies of $TMpCA^-$ at the crystallographic structure (0.85 Å of resolution),[3] which differs from the relaxed gas phase structure in the bond length alternation.

The absorption spectra of pCT^- and of the carboxylate anion of pCA *in vacuo* were experimentally measured by Nielsen *et al.* with absorption maxima at 2.70 eV and 2.89 eV, respectively.[14]

TDDFT excited state calculations for most of these chromophore models have been already performed;[7,18,48,50] however a systematic assessment of the performance of TDDFT for these systems has not been previously carried out. DFT yields accurate ground state structures of these chromophores *in vacuo*[42] in very good agreement with available CCSD results.[8,49]

We evaluated the lowest-lying singlet vertical excited states of the selected chromophores with TDDFT and different exchange–correlation functionals (as discussed in the previous section). The kind of electronic transitions and the magnitude of oscillation strengths of the excited states given by TDDFT are generally comparable to the reference MS-CASPT2 results. The excitation energies of selected excited states of the various chromophore models are shown in Table 1, including the lowest-lying excited state of pVP (for which experimental information is available) and the excited states with the highest oscillation strength (corresponding to the first absorption maximum) of pCA, $TMpCA^-$ and pCT^-. These excitations correspond to $\pi \rightarrow \pi^*$ (mainly HOMO $\rightarrow$ LUMO) transitions.

Table 1 TDDFT excitation energies with PBE, PBE0, BLYP and B3LYP exchange–correlation functionals of the lowest-lying singlet excited state of pVP and of the absorption maximum of pCA, $TMpCA^-$ and pCT^-. The HOMO–LUMO PBE gaps are shown in square brackets for comparison. The PBE TDDFT calculations for $TMpCA^-$ in the crystallographic structure is shown in parentheses

	Excitation energy/eV				
Molecule	PBE	PBE0	BLYP	B3LYP	Ref.
pVP	4.20 [3.38]	4.68	4.17	4.57	4.12[a]
pCA	4.01 [2.89]	4.46	3.80	4.20	4.93[b], 4.95[c]
$TMpCA^-$	3.40 [1.91] (3.24[f])	3.56	3.11	3.32	2.58[d]
pCT^-	3.28 [1.88]	3.34	3.09	3.05	2.70[e]

[a] Experiments.[47] [b] CASPT2.[6] [c] EOM-CCSD.[7] [d] CASPT2.[19] [e] Experiments.[14] [f] Calculation for the crystallographic structure.

TDDFT with the PBE and BLYP functionals yield excitation energies for the lowest-lying excited state of pVP in very good agreement with experiments, while the hybrid PBE0 and B3LYP functionals yield slightly higher excitation energies. This excitation involves insignificant charge redistribution and corresponds to a covalent excited state, consistently with previous TDDFT results for covalent excited states of aromatic hydrocarbons.[51]

For the larger neutral pCA, TDDFT underestimates the excitation energy of the state with highest oscillation strength in comparison to CASPT2 and EOM-CCSD. The TDDFT excitation energies with pure xc-functionals (PBE and BLYP) are about 1 eV lower than the MS-CASPT2 and EOM-CCSD results. PBE0 and B3LYP yield higher excitation energies; the are still, respectively 0.47 eV and 0.73 eV below the CASPT2 reference value, but interestingly fairly close to the experimental measurement *in vacuo* for methyl 4-hydrocinnamate (4.36 eV), which should have similar excitation properties to pCA.[7] This excitation involves some degree of charge transfer. For charge transfer excitations the TDDFT corrections usually lead to the bare Kohn–Sham excitation energy, underestimating the excitation energy.[25,52] With pure xc-functionals this effect is more severe, since the KS gaps are generally smaller than for hybrid functionals. In pCA the optically active $\pi \rightarrow \pi^*$ excitation is not purely charge-transfer since the electron-donor and electron-acceptor orbitals do overlap to a certain degree, hence the TDDFT excitation energy is not equal to the KS excitation energy, as can be seen from Table 1; however charge transfer effects are present to some extent and mostly affect the PBE and BLYP results.

For the $\pi \rightarrow \pi^*$ excitations of the anionic chromophore models $TMpCA^-$ and pCT^- a peculiar behavior is found: although there is some charge transfer between the phenyl and the vinyl groups upon excitation, the TDDFT excitation energies are not underestimated, but, on the contrary, they are larger than reference values. For $TMpCA^-$, all functionals overestimate the excitation energy, with the overestimation larger for the hybrid functionals with respect to the corresponding pure ones. The MS-CASPT2 result[19] is for the chromophore with the protein crystallographic structure, while our TDDFT results have been evaluated for the ground state geometry *in vacuo*. Hence we calculated the TDDFT(PBE) excitation energy of $TMpCA^-$ at the crystallographic structure for further comparison, obtaining 3.24 eV, *i.e.* 0.16 eV below the value for the relaxed geometry *in vacuo* (3.40 eV). The TDDFT excitation energy in the crystallographic structure is indeed closer to the MS-CASPT2 value, but the small structural difference does not totally account for the discrepancy in excitation energy. In the case of pCT^-, TDDFT also overestimates the excitation energies with the different xc-functionals by more than 0.30 eV. Although this is a reasonable result, the overestimation seems to persist with all functionals for the anionic systems. We also note that the excitation energies of pCT^- and $TMpCA^-$ energies are fairly

similar in agreement with the idea of Nielsen *et al*. that pCT^- can be considered a good model of the chromophore in the protein.[14] These overestimated results might be due to the low-lying continuum threshold for these anionic molecules. The ionization potentials (or electron detachment energies) of the isolated pCA^- and $pCTA^-$ (where pCTA stands for *p*-coumaric thio acid) were found to lie below their excitation energies with semi-empirical[17] and EOM-IP/6-31G* calculations.[8] We calculated the vertical electron detachment energy (VDE) of $TMpCA^-$ and pCT^- with spin-unrestricted DFT. For $TMpCA^-$ the VDE calculated with B3LYP is 3.04 eV, lower than the excitation energy of its first singlet excited state (3.25 eV); with BLYP the VDE is 2.85 eV, larger than the energy of the first excited state (2.37 eV) but lower than the excitation energy of the absorption maximum (3.11 eV); similarly with PBE the VDE is 3.02, the excitation energy of the first excited state is 2.24 eV, but the excitation energy of the first absorption maximum is 3.40 eV. For pCT^- the B3LYP VDE (3.16 eV) and the absorption maximum (3.05 eV) are very similar; the same holds for the BLYP with a VDE of 3.02 eV and absorption maximum of 3.09 eV. Also in the experiments, Nielsen *et al*. inferred the presence of resonance excited states.[14] Therefore, resonance states seem relevant for the spectra of anionic chromophores models of PYP *in vacuo*.

The derivative discontinuity of the exchange–correlation functional with respect to the number of electrons[53] was found to play an important role in describing ionization processes with TDDFT;[54] this could also be important for describing resonance states. Maitra *et al*. found that, besides the derivative discontinuity of ground state DFT, a strong frequency dependence in the TDDFT xc-kernel is necessary for a correct description of the excitation of a molecule near the dissociation limit (*e.g*. a molecule composed of two different open-shell fragments at large separation).[55,56] Therefore, with the present functionals, which do not describe the derivative discontinuity, and within the adiabatic approximation in TDDFT, which considers a frequency independent xc-kernel, resonance states are not accurately described with TDDFT.

We also tested for the TDDFT(PBE) calculations of $TMpCA^-$ the asymptotically corrected SAOP potential.[57] The first excited state with a significant oscillation strength has an excitation energy of 3.55 eV, slightly higher than without the asymptotic correction. Hence no significant improvement was obtained for the excitation energy of the $\pi \rightarrow \pi^*$ excitation and the incorrect asymptotic behaviour of the pure and hybrid functionals used in this work does not seem to be the cause of the discrepancy of our TDDFT excitation energies for the anionic chromophore models with the reference values.

Because of the different behaviours of the TDDFT results for the different kinds of excited states involved in each of the chromophore models, no easy correction such as a rigid shift of the excitation energies can be applied, as for example for the green fluorescent protein family.[58]

$TMpCA^-$ within the photoactive yellow protein

$TMpCA^-$ is a good model of the light absorbing chromophore in PYP since its phenolate oxygen is deprotonated and it contains a $-S-CH_3$ group, in a similar way to the natural chromophore linked to PYP through Cys-69 (see Fig. 1 and 2c). Therefore, the excitation energies of $TMpCA^-$ within the protein environment should closely correspond to the light absorption energies of PYP. The TDDFT/MM excited states of $TMpCA^-$ within the protein were calculated on the relaxed QM/MM structure using different QM/MM schemes and exchange–correlation functionals (see Methods for further details). The TDDFT excitation energies of the state with highest oscillation strength (it corresponds to a $\pi \rightarrow \pi^*$ predominantly HOMO–LUMO transition) are 3.45 eV with PBE and 3.51 eV with PBE0; these values overestimate the experimental excitation energy in the protein (2.78 eV[9]) by about 0.70 eV and are very similar to the values *in vacuo*. Calculations with localised basis sets performed with GAUSSIAN also give excitation energies close to their values *in vacuo*, namely 3.06 with BLYP and 3.21 with B3LYP.

The difficulty of our TDDFT/MM results to accurately reproduce the experiments can be due to several reasons: (i) unrepresentative structure of the chromophore and protein environment (as obtained by the QM/MM simulated annealing procedure); (ii) poor description of the chromophore-protein interaction by the adopted QM/MM scheme (although we tested two different schemes obtaining similar trends), (iii) performance of TDDFT in correctly describing the excited state of interest (as also pointed out for other systems *e.g*. in ref. 28).

As far as the first point is concerned, the TDDFT(PBE) excitation energies of $TMpCA^-$ in the calculated ground state structure in gas phase and in the protein crystallographic structure differ by an amount (0.16 eV) which does not account for the large difference between our TDDFT/MM results and the experimental value. The vibrational motion of the chromophore and the protein are also unlikely to be responsible for such difference. Groenhof *et al*. performed classical MD simulations, and calculated the TDDFT excitation energies of the chromophore with a few adjacent residues at an averaged structure along the MD trajectory,[20] they obtained an absorption maximum at 2.80 eV very close to experiments, however, the structures of chromophores or non-standard residues, obtained in a classical MD might not be particularly reliable hence it is difficult to assess the accuracy of such a result.

Reason (ii) refers to the approximate description of the interaction between the chromophore and its environment by the electrostatic potential between the charge distribution of the chromophore (QM) and the static point charges in the (MM) environment. With the non-polarizable GROMOS force field, the polarization of the environment upon excitation of the chromophore cannot be described; in reality the excitation of the chromophore can polarize the environment which can in turn affect the charge distribution of the chromophore in the excited state. These effects could be taken into account by enlarging the QM subsystem so to include part of the chromophore binding site; however TDDFT excitation energies of a PYP chromophore model in isolation and with some residues of the binding site were calculated, yielding very similar results.[18]

We believe reason (iii), *i.e.* the shortcomings of TDDFT excitation energies to be the source of our inaccurate TDDFT/MM excitation energy of PYP. TDDFT and TDDFT/MM overestimate indeed the excitation energies of $TMpCA^-$ *in vacuo* and within the protein by a similar amount with respect to our chosen reference data: *e.g.* with PBE 0.66 eV *in vacuo* (for the crystallographic structure) and 0.67 eV within the protein.

Spectral shifts

From the TDDFT excitation energies of the selected chromophore models and the TDDFT/MM excitation energies of $TMpCA^-$ in the protein, we evaluated the spectral shifts due to the chemical modification and the chromophore–protein interaction as follows: $\Delta E_{chem} = E_{exc}(TMpCA^-) - E_{exc}(pCA)$ and $\Delta E_{protein} = E_{exc}(PYP) - E_{exc}(TMpCA^-)$, where $E_{exc}(X)$ is the maximum absorption energy of the isolated X molecule and $E_{exc}(PYP)$ is the maximum absorption energy of $TMpCA^-$ within the protein. The results are shown in Table 2 together with a summary of relevant experimental and theoretical spectral shifts reviewed in the introduction.

The spectral shift due to the chemical modification can be divided into the effects of the deprotonation of the phenolate oxygen (evaluated by comparing the excitation energies of pCA and pCA^-) and the formation of the thioester linkage (evaluated by comparing the excitation energies of pCA^- and $TMpCA^-$); our results are comparable to previous studies addressing these effects.[18] We predict a red shift due to the chemical modification of the chromophore of about 0.65 eV with the pure xc-functionals and of about 0.90 eV with hybrid xc-functionals. For the chromophore-protein interaction a very small shift (0.05 eV) is predicted, in the blue direction with PBE and in the red direction with PBE0; estimations of the protein shifts with BLYP and B3LYP are also small. Since the magnitude of the shift is smaller than the accuracy expected for TDDFT, these results confirm that the protein shift is small, but cannot reliably define its direction.

Keeping in mind the performance of TDDFT for describing the excited states of the studied chromophore models, discussed in the previous sections, we can assess the reliability of the values obtained for the spectral shifts, although a precise comparison with experiments is difficult due to the different reference systems used to evaluate the shifts as discussed in the introduction. Since TDDFT underestimates the excitation energy of pCA and overestimates that of $TMpCA^-$, ΔE_{chem} is underestimated by TDDFT. Hence, a red shift due to the chemical modification possibly larger than 0.90 eV (the largest value obtained with TDDFT) can be expected. In comparison to the small red shift estimated by Nielsen *et al.* for the effects of the chemical modification,[14] TDDFT yields a larger red shift, which would be even larger if we take into account the expected shift introduced by the intrinsic biases of TDDFT on the involved transitions. However, this apparent discrepancy is due to the different chromophore models used as reference in the experiments and in our calculations for deriving the effect of the chemical difference: the neutral free chromophore of PYP, pCA, in our calculations and the carboxylate anion pCA^- in the experiments, which is different from the neutral molecule. In fact, taking as experimental reference for the free chromophore the value for methyl 4-hydrocinnamate[7] and comparing it with pCT^-[14] gives $\Delta E_{chem} = -1.66$ eV, consistent with our predictions.

As for the shift due to the chromophore-protein interaction, if we assume that the overestimations of the TDDFT excitation energies for $TMpCA^-$ *in vacuo* and within the protein are comparable, the value obtained for $\Delta E_{protein}$ could be reliable. Indeed the small spectral shift due to the interaction between the chromophore and the protein obtained with TDDFT is in agreement with the experiments by Nielsen *et al.*,[14] to reach their conclusions Nielsen *et al.* measured the absorption spectrum of the pCT^- chromophore model. This is a good model of the chromophore of PYP since the charge does not extend beyond the phenyl group in pCT^- and its maximum absorption energy is not too dissimilar from that of $TMpCA^-$ (*e.g.* 3.26 eV *vs.* 3.40 eV with PBE). In previous theoretical works, small effects of the protein environment on the absorption of the chromophore were also deduced.[18,19]

The electrostatic field created by the protein environment around the chromophore does not seem to substantially alter the electronic distribution in $TMpCA^-$. In fact, the HOMO and LUMO Kohn–Sham orbitals, which are involved in the studies excitation, are similar in shape *in vacuo* and within the protein.[42] However, even if the electrostatic field of the protein does not seem to have a crucial role in modulating the absorption maximum, it might have a stabilizing effect on the excited state and prevent the formation of radical chromophore species within the protein. We therefore calculated the VDE for $TMpCA^-$ as the energy difference between $TMpCA^-$ and the radical $TMpCA^\bullet$ within the QM/MM scheme. With PBE the VDE turned out to be 6.22 eV, which is substantially higher than 3.45 eV, the energy

Table 2 TDDFT spectral shifts due to chemical modifications (ΔE_{chem}), chromophore-protein interaction ($\Delta E_{protein}$) and total ($\Delta E = \Delta E_{chem} + \Delta E_{protein}$)

	ΔE_{chem}/eV	$+\Delta E_{protein}$/eV	ΔE/eV
PBE	−0.61	0.05	−0.56
PBE0	−0.90	−0.05	−0.95
BLYP	−0.69	−0.05	−0.74
B3LYP	−0.88	−0.11	−0.99
Experiments	−1.32,[11] −0.19[14a] −1.66,[7,14b] −1.15[14c]	−0.28,[11] 0.08[14]	−1.60,[11] −0.11,[14a] −1.07[14b]
Other theories	−0.76[18d]	−0.04[18d], −0.30[20d], 0.20[19e]	−0.80[18d]

[a] *In vacuo* calculated using as reference pCT^- and the carboxylate anion pCA^-. [b] *In vacuo* calculated using pCT^- and methyl 4-hydrocinnamate. [c] In aqueous solution. [d] TDDFT. [e] MS-CASPT2.

of the absorption maximum within the protein. This large energy difference demonstrates that the lowest-lying excited states of TMpCA correspond to non-ionizing bound states, unlike in the case of the chromophore *in vacuo*. This result agrees with experimental evidence showing that the protein environment prevents radical formation of the chromophore.[59] Of course the protein environment has also other roles, which are not apparent in the calculation of vertical excitation, but are very important for the isomerisation process such as lowering of the isomerisation barrier with respect to the gas phase and steric constraints determining the isomerisation path.[5]

Conclusions

We have assessed the reliability of TDDFT for describing the excited states of different chromophore models of PYP. Some of the optically active excitations of the chromophore models studied are characterized by excitations involving charge transfer. For pCA this translates into a TDDFT underestimation of the excitation energy with respect to CASPT2 and EOM-CCSD results especially with pure xc-functional such as PBE and BLYP.[25,52] However, for the anionic chromophore models the scenario is more complex since auto-ionizing resonance states are reached upon excitation due to the proximity of the continuum threshold from the ground state. TDDFT within the adiabatic approximation does not accurately describe such states[56] and overestimates the excitation energies of the anionic chromophore models studied in this work, *i.e.* $TMpCA^-$ and pCT^-; similarly the TDDFT/MM excitation energy of $TMpCA^-$ within the PYP environment is overestimated.

Keeping in mind the performance of TDDFT for the system under investigation, we can draw the following conclusions on the spectral tuning for PYP: (i) there is a sizeable red shift due to the chemical modification of the chromophore upon binding to the protein, likely to be larger than 0.9 eV if measured with respect to the neutral pCA; experimental measurements of the absorption spectrum of the free pCA chromophore would be useful to clarify this issue. (ii) The spectral shift due to the chromophore-protein interaction is very small as predicted by Nielsen *et al.*[14] and the protein stabilizes the chromophore in the excited states preventing radical formation.

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