Effect of microhydration on the electronic structure of the chromophores of the photoactive yellow and green fluorescent proteins

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Electronic structure calculations of microhydrated model chromophores (in their deprotonated anionic forms) of the photoactive yellow and green fluorescent proteins (PYP and GFP) are reported. Electron-detachment and excitation energies as well as binding energies of mono- and dihydrated isomers are computed and analyzed. Microhydration has different effects on the excited and ionized states. In lower-energy planar isomers, the interaction with one water molecule blueshifts the excitation energies by 0.1–0.2 eV, whereas the detachment energies increase by 0.4–0.8 eV. The important consequence is that microhydration by just one water molecule converts the resonance (autoionizing) excited states of the bare chromophores into bound states. In the lower-energy microhydrated clusters, interactions with water have negligible effect on the chromophore geometry; however, we also identified higher-energy dihydrated clusters of PYP in which two water molecules form hydrogen-bonding network connecting the carboxylate and phenolate moieties and the chromophore is strongly distorted resulting in a significant shift of excitation energies (up to 0.6 eV). © *2011 American Institute of Physics*. [doi:10.1063/1.3660350]

I. INTRODUCTION

Microsolvation is often used to study the effect of individual solvent-solute interactions. For example, the protein environment and bulk water feature multiple hydrogen-bonding interactions, in addition to covalent linkage and electrostatics. These effects can be quantified by considering well-defined model systems, such as microhydrated chromophores.[1,2] Hydrogen-bonding interactions in photoactive proteins can modulate optical properties of the chromophore, as has been demonstrated by theoretical[3,4] and experimental studies.[5–10]

This paper focuses on the chromophores of two important photoactive biomolecules, photoactive yellow (PYP) and green fluorescent (GFP) proteins. PYP, which was found in the *Halorhodospira halophila* bacterium,[11,12] serves as a blue light receptor of its host and is responsible for negative phototaxis.[13] The PYP chromophore is one of the simplest model systems for studying spectral tuning by protein (or solvent) environment, which is relevant, for example, for understanding the properties of fluorescent biomarkers[14] and the mechanism of color vision.[15] GFP, which was isolated from jellyfish *Aequorea victoria*,[16] has a natural function of converting blue to green light. Proteins from the GFP family are widely used as genetically encoded biomarkers for *in vivo* imaging.[17–20]

The isolated PYP and GFP chromophores have attracted attention both from experimentalists and theoreticians. The most commonly used model system representing the GFP chromophore is 4-hydroxybenzylidene-2,3-dimethylimidazolinone (HBDI), whereas for PYP it is para-coumaric acid (pCA). In spite of entirely different biological functions and origins, their electronic structure has several similar features.[21] Anionic forms (deprotonated at the phenol end) play a central role in photophysics of both chromophores and feature a phenolate moiety connected by a methine bridge to either imidazolinone (HBDI) or carboxylate (pCA). The pCA anion exists in two tautomeric forms (carboxylate and phenolate) that have different electronic structure and optical properties.[22,23] Both anionic GFP and PYP (phenolate) chromophores can be represented by the two interacting resonance structures leading to bond-order scrambling and allylic-like molecular orbitals (MOs) spanning the bridge region.[21] The character of the bright state in both chromophores (phenolate PYP isomer) is remarkably similar and can be described as a $\pi \rightarrow \pi^*$ transition between the (allylic-like) HOMO and LUMO.[21] The gas-phase calculations of the model PYP (phenolate and carboxylate isomers)[4,22,24] and GFP (Refs. 25 and 26) chromophores have revealed the resonance (i.e., metastable with respect to electron-detachment) character of the first bright excited state. In both chromophores, the lowest bright state is above electron detachment continuum; however, the type of resonance is different. In the case of $HBDI^-$ (Ref. 25) and the phenolate isomer of pCA^-,[22] the $\pi \rightarrow \pi^*$ state is above the state of the neutral derived by removing the electron from the π orbital (HOMO of the anion)—this type of resonance is called a shape resonance. In the carboxylate isomer of pCA^-,[22] the excited state is a Feshbach resonance, as it is located above the continuum corresponding to the ionization from a lone-pair orbital and is, therefore, uncoupled (in the Koopmans picture) from the continuum (see Ref. 22 for details).

The gas-phase experiments investigated the properties of isolated model PYP (Refs. 27–30) and GFP (Refs. 31–34)

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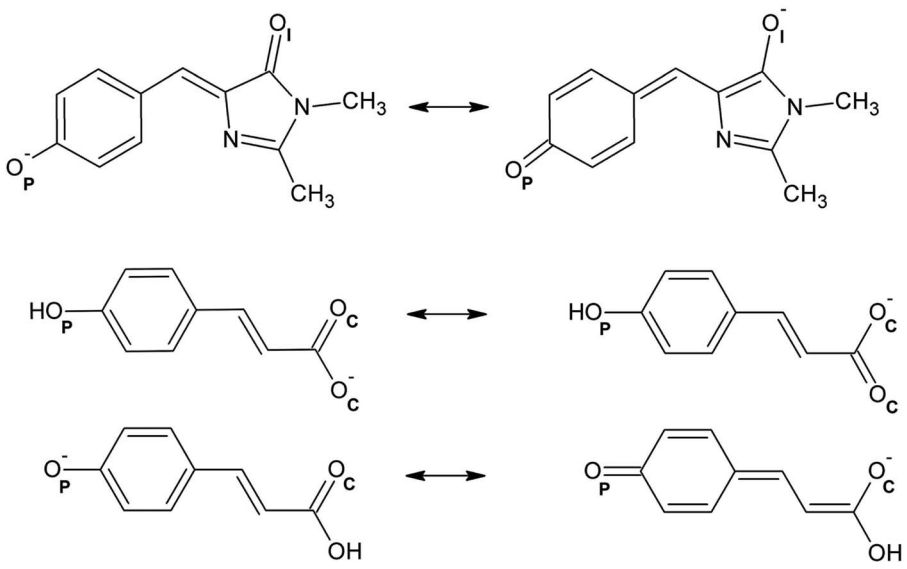

FIG. 1. Model chromophores and hydration sites. From top to bottom: $HBDI^-$, pCA^- carboxylate (PYPa), and pCA^- phenolate (PYPb). Microhydrated structures are labeled according to the hydration centers (P or I for $HBDI^-$, and P or C for pCA^-).

chromophores. The properties of the chromophores in solutions have also been characterized.[35–45] Rocha-Rinza *et al*. have studied photoabsorption of the methylated pCA^- phenolate and carboxylate isomers using gas-phase action spectroscopy. They reported almost identical spectra for both isomers, in contradiction to the theoretical results[22,23,29] that predict higher excitation and detachment energies for the carboxylate species.

In general, protein environment can significantly alter both geometry and electronic structure of the chromophore. In the case of GFP and PYP, however, the protein appears to have a very small net effect on the absorption maximum, as demonstrated by the action spectroscopy studies[27,29,33] and high-level calculations.[46–48] Experimentally, it has been shown[7] (for PYP) that the presence of Glu46 H-bonded to the chromophore blueshifts the absorption maximum by about 0.3 eV. The thioester covalent linkage between the protein and the chromophore, on the other hand, redshifts the absorption maximum, as was demonstrated by mutation studies.[9] The electronic structure calculations tell the same story—the overall small effect on the absorption is due cancellation of (somewhat larger) shifts induced by individual interactions.[48] Thus, it is interesting to investigate how hydrogen-bonding affects the absorption spectrum of the bare chromophore.

The effects of H-bonding on electronic structure of the chromophores were studied by both theory[49] and experiment.[50] Rajput *et al*. have analyzed the influence of H-bonding in microhydrated pCA^- clusters on the spectral tuning of the PYP chromophore[50] employing same action spectroscopy coupled with ion-storage ring and electrospray setup as in their studies of the isolated species.[27–29] They observed that the carboxylate isomer prefers to cluster with one water molecule, whereas the phenolate attaches two water molecules.[50] The photoabsorption maximum of the dihydrated phenolate chromophore showed an unusually large blueshift of 0.71 eV with respect to the bare chromophore. Upon excitation, the system was reported to decay via detachment of the water molecules, in contrast to the relaxation of the bare chromophore via electron detachment or dissociation.[29]

Several theoretical studies investigated the effect of the environment on the chromophores' properties. Gromov *et al*. have thoroughly studied the effects of the protein[4] and hydrogen-bonding interactions.[49] Owing to the anionic nature of the chromophore and partial charge-transfer character of the $\pi \rightarrow \pi^*$ transition, the influence of environment is expected to be pronounced. The key results obtained by Gromov *et al*. are electrostatic stabilization by Arg52 against autodetachment and spectral shifts due to the H-bonding with Tyr42 and Glu46.[4] In contrast to the isolated anion, the first excited state was found to be stable with respect to autoionization. The same result in the hybrid QM/MM calculations of the protein-bound chromophore.[51] Thus, the protein environment converts the exited states into the bound electronic states.[4,51] The stabilizing effect of the protein environment has also been reported for the GFP chromophore whose detachment energy increases in the protein by ~2.2 eV.[47]

In this work, we investigate the effect of microhydration on the relative energy of the excited and ionized states of the pCA^- and $HBDI^-$ chromophores, in order to understand how the solvation and the hydrogen bonds formed in the protein environment affect the spectral properties of the chromophore. The structures of the model chromophores are shown in Fig. 1. We employ high-level electronic structure methods to quantify the effect of adding one and two water molecules to these model systems and analyze the structural and spectral changes induced by microhydration.

II. COMPUTATIONAL DETAILS

The model systems used to represent the PYP and GFP chromophores with two sites of hydration are shown in Fig. 1. The geometry of $HBDI^-$ was optimized with density functional theory (DFT) using the ωB97X-D long-range and

dispersion-corrected hybrid density functional[52] that includes long-range Hartree–Fock exchange mitigating the notorious self-interaction error. The 6-31+G(d,p) basis set has been used for these calculations. For $HBDI^-$, the average deviation of the computed bond lengths from the structure[26] optimized using a larger basis set (cc-pVTZ) is around 0.005 Å. Both isomers of pCA^- were optimized with resolution of identity Møller-Plesset perturbation theory (RIMP2) (Refs. 53–56) with the aug-cc-pVDZ basis set.[57] The cyclic pCA^- isomers (Fig. 6) were optimized with ωB97X-D/aug-cc-pVDZ. The following convergence thresholds were used in the optimization procedure: 1×10^{-6} hartree for the energy, 1×10^{-4} hartree/Å for the energy gradient, and 1.2×10^{-3} Å for displacements. All microhydrated pCA^- structures have C_s symmetry except PYPa-W_C, PYPa-$W_C W_P$, and the cyclic isomers. All $HBDI^-$ structures are non-planar and have C_1 symmetry.

Binding energies (D_e) of different microsolvated isomers were computed with ωB97X-D/6-311++G(2df,2pd) (Ref. 58) and with RIMP2/aug-cc-pVDZ for the GFP and PYP chromophores, respectively, as the differences between the ground-state energies of the microsolvated molecule and the dissociation products. The grid used in all DFT calculations contained 75 points in the Lebedev[59] radial grid and 302 points in the Euler-Maclaurin[60] angular grid.

Vertical excitation energies for pCA^- were computed using equation-of-motion coupled-cluster method with singles and doubles (EOM-CCSD) for excitation energies (EOM-EE-CCSD) (Refs. 61–64) with the 6-31+G(d,p) basis set; for the lower symmetry cyclic isomers we used 6-31+G(d). Previous benchmark calculations[22] for the bare chromophore have shown relatively minor basis set dependence of excitation energies, e.g., increasing the basis beyond a polarized double zeta basis affects the transition energies of the lowest excited states by 0.1–0.2 eV. Moreover, these errors are systematic, and are expected to cancel out when solvent-induced shifts are computed.[65]

Vertical detachment energies were computed by EOM-CCSD for ionization potentials (EOM-IP-CCSD) (Refs. 66–68) with the 6-311+G(df,pd) basis; for the cyclic isomers we used 6-31+G(d,p). Calculations of the ionization energies of uracil[69] by EOM-IP-CCSD with various bases show that typical error bars in IEs computed with these basis sets are about 0.2 eV.

EOM-CC methods[64] provide accurate and balanced treatment of dynamical and static correlation energy, accurately reproducing excited and ionized states properties. In these calculations, we employ well-behaved closed-shell reference states; thus, the target excited and ionized states are not affected by spin-contamination. The anticipated error bars for these methods are 0.1–0.3 eV.[22,69–71]

Excitation energies for all microsolvated $HBDI^-$ have been calculated with SOS-CIS(D),[72] scaled opposite spin configuration interaction singles with the second-order perturbative doubles correction, and the cc-pVTZ basis set. This method has been shown to give accurate results for the excitation energies of the isolated and protein-bound anionic GFP chromophore.[25,47] Detachment energies were calculated with ωB97X-D/6-311++G(2df,2pd). These detachment energies were corrected by using energy additivity scheme based on the EOM-IP-CCSD/6-311(2+,+)G(2df,2pd) extrapolated value for the bare chromophore from Ref. 25:

$$DE_{\text{hydrated,corrected}} = DE_{\text{bare,corrected}} + DE_{\text{hydrated,DFT}} - DE_{\text{bare,DFT}}, \quad (1)$$

where $DE_{\text{bare,corrected}}$ is the best estimate of the detachment energy of bare $HBDI^-$ calculated by ωB97X-D/6-311++G(2df,2pd) and corrected by the DFT/EOM-IP-CCSD difference computed in a smaller basis set.[25]

We also performed benchmark calculations (for $HBDI^-$) to quantify the dependence of excitation energies on the general and auxiliary basis used in the SOS-CIS(D) (Ref. 72) calculations. The results are given in supplementary materials.[73] All calculations were performed with Q-Chem.[74]

III. RESULTS AND DISCUSSION

A. Optimized structures and binding energies of the mono- and dihydrated chromophores

The equilibrium structures of the bare[22,25] and monohydrated chromophores are shown in Fig. 2. The effect of microsolvation on the structure is relatively small, e.g., the magnitude of the changes in bond lengths is about 0.01 Å. The effect is larger for $HBDI^-$. This is because it is more symmetric and the two resonance configurations[26] contribute almost equally to the chromophore structure leading to more allylic character of the bridge relative to pCA^- (Ref. 22) (Fig. 1). The water molecules stabilize one resonance structure more than another, which changes the bond lengths patterns and reduces the allylic character. Similarly, the phenolate form of pCA^- has more allylic character[22] than the carboxylate, which results in a more pronounced geometry changes due to microhydration.

The microhydrated clusters and corresponding binding energies are shown in Figs. 3–6. The lowest energy monohydrate of PYPa is PYPa-W_C in which water forms two hydrogen bonds with the carboxy oxygens making this structure more stable. The phenolate isomer, PYPb-W_P, has two energetically equivalent structures that have slightly higher binding energy than PYPa-W_C. We found that PYPb-$W_P W_{P2}$ (Fig. 4) isomer is slightly lower in energy (by 0.03 eV) than PYPb-$W_P W_{P1}$, which was suggested to be the most stable structure in Ref. 50. In the former case, the two water molecules form a hydrogen bond with each other, which results in additional stabilization. We also found several cyclic dihydrated isomers (Fig. 6). PYPa-cyclic is slightly higher in energy than the lowest PYPa dihydrate, PYPa-$W_C W_C$ (by 0.03–0.04 eV), whereas PYPb-cyclic is significantly less stable than PYPb-$W_P W_{P2}$ (by about 0.5 eV). The cyclic isomers might play an important role in the pCA^- isomerization by proton transfer via a water chain. Since PYPa is more stable in solution whereas PYPb is more stable in the gas phase, such a pathway may be responsible for the isomerization upon introducing pCA^- into gas phase by electrospray.

We observe that binding energies are nearly additive for all microhydrated systems, except cyclic isomers. The energy required to detach all water molecules is approximately

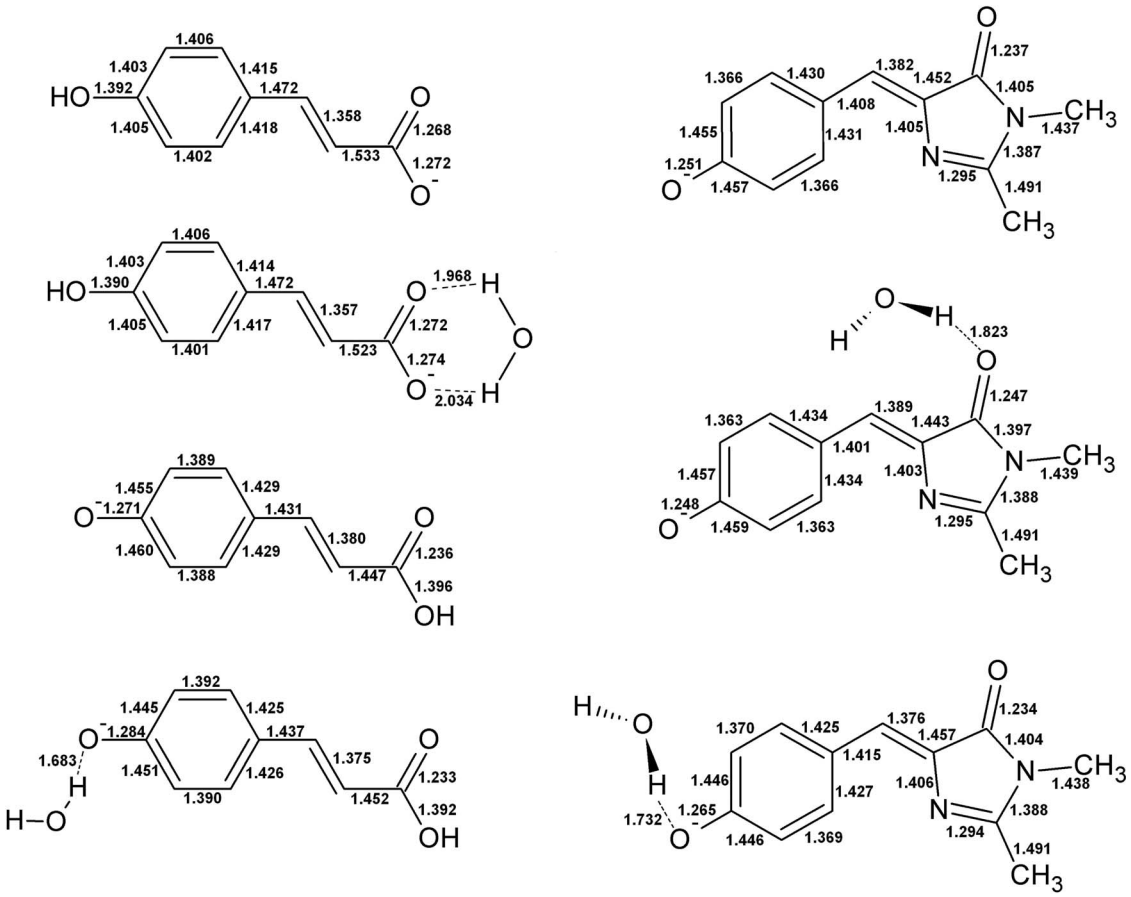

FIG. 2. Equilibrium structures of pCA^- (left column), $HBDI^-$ (right column), and their monohydrates.

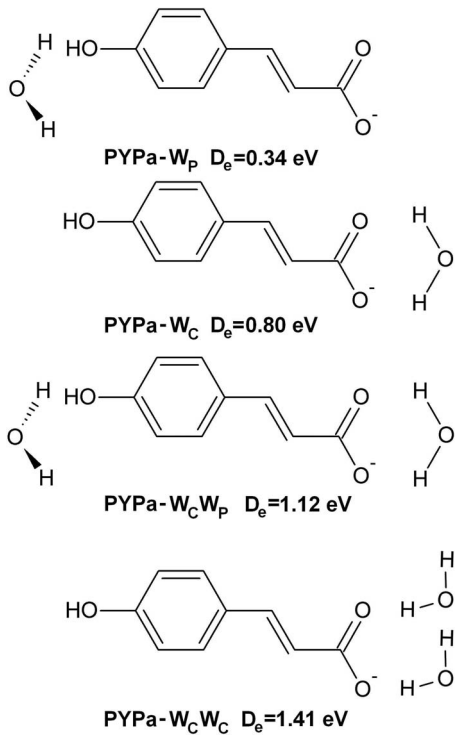

FIG. 3. Structures and binding energies (D_e, eV) of microsolvated model systems of the pCA^- (PYPa, carboxylate) chromophore.

equal to the sum of the energies required to detach each water molecule (the deviations are very small), as illustrated in Fig. 7. This suggests that the interaction energy between water molecules is negligible in comparison to the interaction energy between the chromophore and water. However, we expect higher non-additive contributions to interaction energy in larger systems, in which the polarization effects giving rise to three-body interactions are more pronounced (see, for example, Ref. 65).

B. Electronically excited and ionized states of microhydrated pCA^-

Previous excitation and detachment energy calculations for the bare PYP and GFP chromophores[4,22,24–26,75] show that the excited states are above the detachment continuum. Such resonance states are common in molecular anions.[26,76] In the protein environment, the excited state is stable with respect to autoionization, thus the environment plays an important role in stabilizing the anionic species.[47,75]

Vertical excitation and detachment energy calculations for microhydrated pCA^- are given in Table I. The calculations show that in most cases microhydration has a minor effect on the excitation energies (about 0.1–0.3 eV), whereas the detachment energies change dramatically (up to 1.2 eV). The water molecules stabilize both ground and excited states

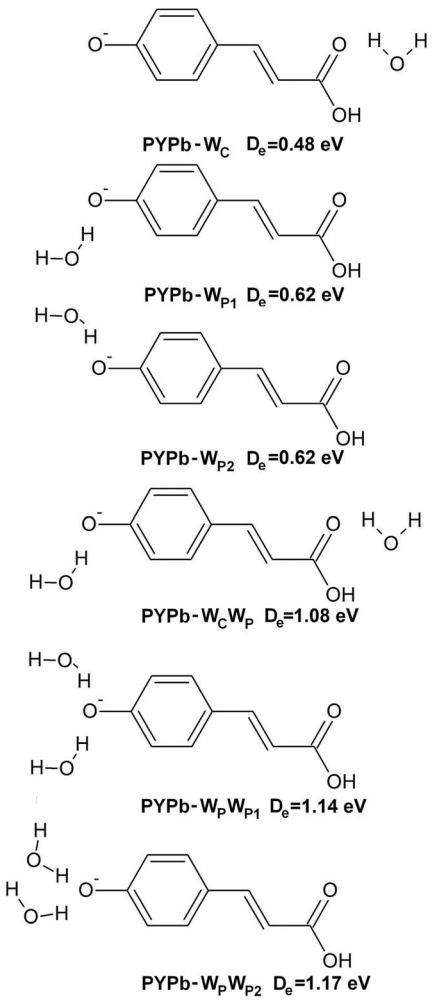

FIG. 4. Structures and binding energies (D_e, eV) of microsolvated model systems of the pCA$^-$ (PYPb, phenolate) chromophore.

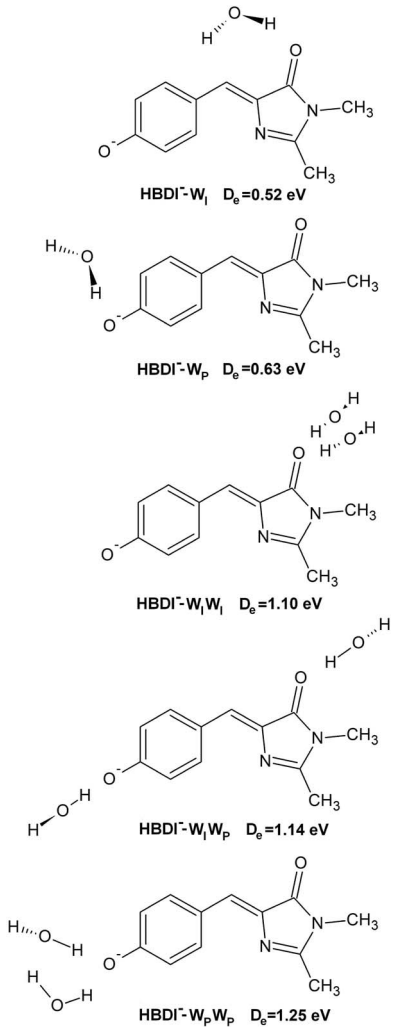

FIG. 5. Structures and binding energies (D_e, eV) of microsolvated model systems of the HBDI$^-$ chromophore.

of the anionic chromophore by a similar value, which results in the mutual cancellation and an overall small change in the excitation energy. Interestingly, the cyclic forms of pCA$^-$ show more pronounced effect of microhydration on the excitation energies (≈0.3–0.6 eV), whereas detachment energy changes less. Large shifts of excitation energy in this case are due to distortion of the planar π-conjugated system of the chromophore. For almost all the structures (except PYPb-W$_C$ and all cyclic forms of pCA$^-$, Table I), the microhydration converts the first excited state to a bound state (see Fig. 8). The same effect was reported for the deprotonated para-coumaric methyl thioester (pCTMe$^-$)—upon hydration of this species by two water molecules, detachment energy increases by 0.7 eV, which is sufficient to stabilize the resonance.[75] Gromov *et al.* emphasizes[4] that in the case of the native PYP chromophore hydrogen bonding plays a similar role, however, the dominant stabilization effects are due to the positively charged Arg52 residue that acts as a counterion leading to the detachment energy increase by several eV.[4] Similar conclusions have been derived from a recent computational study of GFP.[47] The effects of hydrogen bonding in PYP were reported[4] to be most prominent for the states that are less affected by the Arg52 residue. Our results for excitation energies show blueshifts of 0.1–0.2 eV, which is consistent with the results of Gromov *et al.*,[4] but is in contradiction with the experimentally reported shifts of 0.71 eV for a dihydrated pCA$^-$. Interestingly, PYPb-cyclic shows the shift in excitation energy of 0.6 eV, which is close to the experimental observation.

The HBDI$^-$ excitation and detachment energies are collected in Table II. Similar to pCA$^-$, bare HBDI$^-$ features the resonance excited state.[25] The addition of water molecules raises the detachment energy more than excitation energy, thus converting the resonance into a bound state.

In sum, our results show a moderate change in excitation energies for both the GFP and PYP chromophores (around

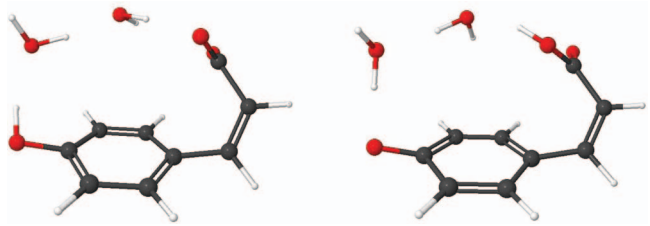

FIG. 6. Structures of the microhydrated pCA$^-$ cyclic isomers. (Left) Cyclic isomer of PYPa (D_e = 1.38 eV). (Right) Cyclic isomer of PYPb (D_e = 0.68 eV).

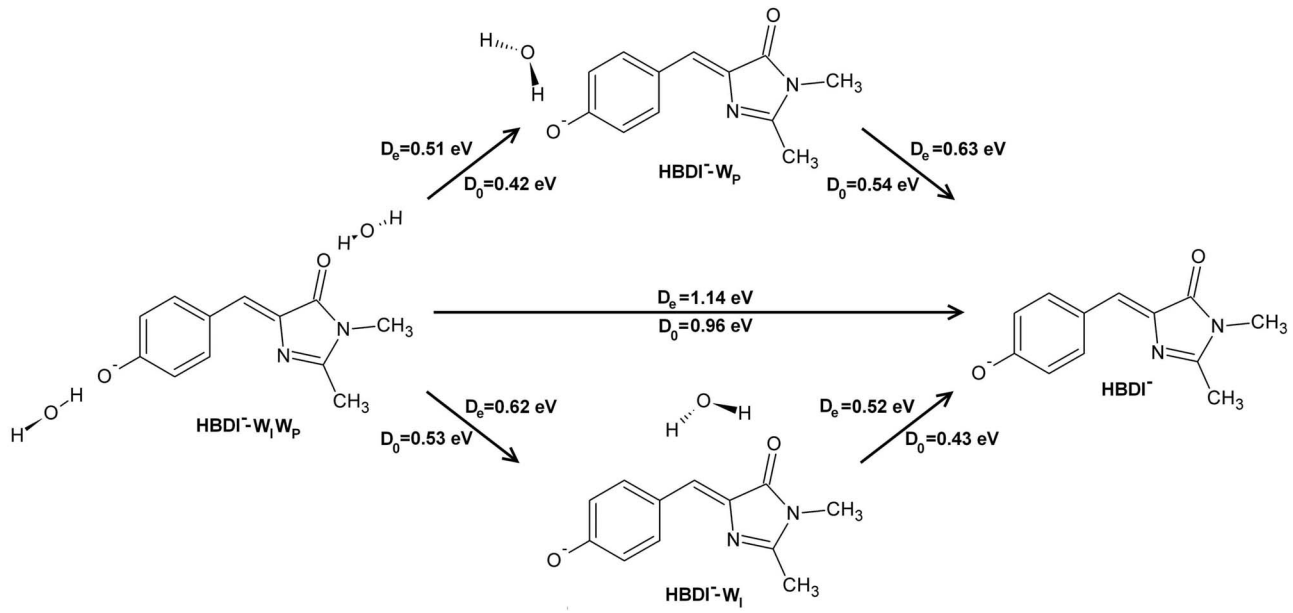

FIG. 7. Binding energies (D_e, eV) of the $HBDI^-$-water complexes.

TABLE I. Vertical excitation energies (eV), oscillator strengths (f_l, in parenthesis), and detachment energies (eV) of microhydrated pCA^-. Excitation energies and transition dipole moments were computed by EOM-EE-CCSD/6-31+G(d,p) and EOM-EE-CCSD/6-31+G(d), respectively; ionization energies—by EOM-IP-CCSD/6-311+G(df,pd).

	Excitation energy		
System	$\pi \rightarrow \pi_1^*$	$\pi \rightarrow \pi_2^*$	Detachment energy
PYPa (Ref. 22)	4.50 (0.03)	4.89 (0.019)	3.91
PYPa-W_C	4.47 (0.03)	5.13 (0.26)	4.72
PYPa-$W_C W_C$	4.49 (0.03)	5.21 (0.58)	5.10
PYPa-cyclic[a]	4.79	5.58	4.29
PYPb (Ref. 22)	3.19 (1.06)	4.23(0.10)	2.92
PYPb-W_C	3.12 (1.11)	4.27 (0.09)	3.06
PYPb-W_{P1}	3.25 (1.01)	4.32 (0.08)	3.29
PYPb-$W_C W_P$	3.20 (1.07)	4.34 (0.07)	3.37
PYPb-$W_P W_{P1}$	3.38 (0.98)	4.41 (0.06)	3.63
PYPb-$W_P W_{P2}$	3.37 (1.01)	4.34 (0.07)	3.72
PYPb-cyclic[a]	3.78 (0.26)	3.99 (0.13)	2.97

[a]Excitation and detachment energies for the cyclic isomers were calculated by EOM-EE-CCSD/6-31+G(d) and EOM-IP-CCSD/6-31+G(d,p).

TABLE II. Vertical excitation and detachment energies (eV) of the microhydrated deprotonated HBDI. Excitation energies were computed by SOS-CIS(D)/cc-pVTZ, detachment energies—by ωB97X-D/6-311++G(2df,2pd).

	Excitation energy		Detachment energy	
System	$\pi \rightarrow \pi_1^*$	$\pi \rightarrow \pi_2^*$	DFT	corrected
$HBDI^-$	2.61	4.38	2.66	2.45
$HBDI^-$-W_I	2.59	4.50	3.07	2.86
$HBDI^-$-W_P	2.64	4.34	3.13	2.92
$HBDI^-$-$W_I W_P$	2.59	4.40	3.34	3.13
$HBDI^-$-$W_I W_I$	2.62	4.59	3.26	3.05
$HBDI^-$-$W_P W_P$	2.71	4.31	3.45	3.24

0.1 eV). However, microsolvation does not represent the solution environment; several solvation shells are required to correctly simulate bulk solvent.[65] Interestingly, in most cases, the microhydration does not affect the transition dipole moments, which change by less than 10%. However, the second bright transition in PYPa shows larger sensitivity—monohydration changes it by more than an order of magnitude (see Table I). This can be explained by the solvation-induced changes in the wavefunction. The second transition in PYPa has a mixed character dominated by excitations from HOMO-1 (which is localized on the carboxylate moiety) and the HOMO (delocalized over the entire π-conjugated system). Addition of a water molecule on the carboxylate side stabilizes HOMO-1 more than the HOMO thus increasing the HOMO contribution to the second bright transition. Since the HOMO has a larger overlap with the target virtual orbital, this leads to the increase of the oscillator strength (see supplementary materials[73] for more details).

C. Theory versus experiment: Microhydrated clusters of the PYP chromophore

Recent experimental work of Rajput *et al.*[50] on photoabsorption of microhydrated pCA^- reported interesting results on the energetics of H-bonding and spectral tuning. It was observed that the phenolate primarily binds two water molecules, whereas the carboxylate only appears as

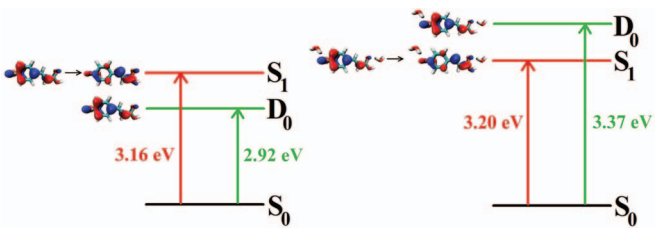

FIG. 8. Electronically excited and ionized states in PYPb and PYPb-$W_C W_P$.

monohydrate. However, the apparent mass distribution was affected by the experimental conditions.[77] Our theoretical results lend no support to preferential binding, i.e., the analysis of binding energies does not reveal any "magic numbers." Binding energies of single water molecule by the carboxylate (PYPa-W_C, Fig. 3) and the phenolate (PYPb-W_{P1} and PYPb-W_{P2}, Fig. 4) are comparable (difference is about 0.18 eV), therefore, the formation of a stable hydrogen bond is anticipated in both cases. The binding energy of the dihydrated carboxylate (PYPa-W_CW_C) is even higher than for the phenolate (PYPb-W_PW_{P2}), therefore, one would except to observe the formation of monohydrates and dihydrates in both phenolate and carboxylate species. A possible explanation might be due to the potential role of cyclic dihydrates in the carboxylate-phenolate isomerization. In the cyclic isomers the carboxylate and phenolate moieties are connected via a chain of H-bonds forming a perfect path for a low-barrier proton migration, and, therefore, the carboxylate-to-phenolate isomerization upon ion extraction from solution (carboxylate is the lowest energy isomer in solution, whereas in the gas phase, phenolate is more stable) may proceed through the formation of these isomers, leading to preferential formation of phenolate dihydrates. However, one can expect similar structures being formed with three and more water molecules. Thus, the selectivity of binding of one (carboxylate) and two (phenolate) water molecules is likely to be due to the effects of the trap conditions on the resulting mass distribution rather than intrinsic energetics of microsolvated chromophores.

The reported[50] photoabsorption maximum of the dihydrated chromophore had an unusually large blueshift of 0.71 eV with respect to the bare chromophore. Our results show much smaller shift (0.1–0.2 eV) for the proposed PYPb-W_PW_{P1} isomer, in agreement with Gromov *et al*.[4] The PYPb-cyclic isomer shows large blueshift (0.6 eV), which is close to the experimental one. The absolute values of calculated excitation energies are systematically higher (by about 0.3 eV (Ref. 22)); however, we expect much better accuracy for the excitation energy shifts due to error cancellation. Therefore, the experimental absorption maximum could be explained by assuming a strongly non-Boltzmann population of different isomers in the electrospray-generated sample. However, even under non-equilibrium conditions it is not clear why the signal from the lowest-energy PYPb-W_PW_{P1} isomer that should appear 0.2 eV blueshifted with respect to the bare chromophore is missing in the action spectrum.

Upon excitation, the dihydrate was reported[50] to decay via the detachment of the water molecules, in contrast to the bare chromophore, which undergoes electron detachment or dissociation.[29] Our calculations confirm that, as expected, the energy of water binding is smaller than the energy for breaking covalent bonds in the molecule or electron detachment, and that this energy provides sufficient relaxation for the energetic chromophore to drop below the fragmentation/detachment onset. This holds for all isomers (see Figs. 3–6 for water binding energies, and Ref. 22 for the dissociation and detachment energies of pCA^-). A possible explanation for missing electron-detachment channel in microhydrated pCA^- is stabilization of the resonance excited state with respect to the continuum that shuts down autoionization and relatively low cross sections from direct detachment relative to electron-excitation transitions.

Despite the detailed characterization of electronic structure of excited and electron-detached state of the microhydrated PYP chromophores, the experimental data by Rajput *et al*.[50] cannot be fully understood on the basis of the present theoretical results. More theoretical and isomer-specific experimental studies are necessary to explain the observed mass distribution of microhydrates and the origin of the 0.7 eV shift in the observed absorption maximum of dihydrated PYPb, as well as the absence of the direct detachment channel, which is energetically allowed for most of the pCA^- phenolate microhydrated clusters.

IV. CONCLUSIONS

We performed comprehensive calculations of mono- and dihydrated clusters of the model GFP and PYP chromophores in their anionic states (deprotonated HBDI and pCA). We do not observe significant three-body effects in the binding energies of the dihydrated species. The lowest-energy isomers of the microhydrated species feature nearly unperturbed chromophores hydrated at the obvious CO sites that host excess negative charge (phenolate, imidazolinone, carboxylate). In these isomers, the microhydration has small effect on the excitation energies (blueshifts of 0.1–0.2 eV), however, it increases VDE by 0.2–1.2 eV, which reverses the relative order of the excited and ionized states. Thus, these isomers cannot explain experimentally observed large blueshifts in excitation energies.

We have identified several unusual isomers of dihydrated pCA^- in which the chromophore is highly distorted and water molecules form a bridge between the carboxylate and phenolate moiety. We expect that these isomers may play a role in the carboxylate-phenolate isomerization of pCA^-. These isomers feature small changes in the electron detachment energies, but large (0.6 eV) blueshifts in the excitation energies, which is close to the experimentally observed shift of 0.7 eV.[50]

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