

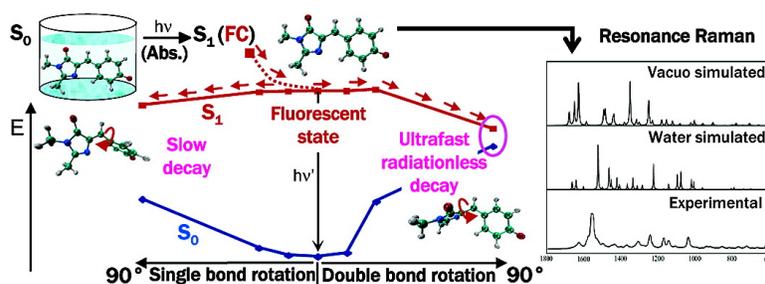
Article

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Solvent Effects on the Vibrational Activity and Photodynamics of the Green Fluorescent Protein Chromophore: A Quantum-Chemical Study

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Abstract: Vibrational activities in the Raman and resonance Raman spectra of the cationic, neutral, and anionic forms of 4'-hydroxybenzylidene-2,3-dimethyl-imidazolinone, a model compound for the green fluorescent protein chromophore, have been obtained from quantum-chemical calculations in vacuo and with the inclusion of solvent effects through the polarizable continuum model. It is found that inclusion of solvent effects improves slightly the agreement with experimental data for the cationic and neutral forms, whose spectra are qualitatively well-described already by calculations in vacuo. In contrast, inclusion of solvent effects is crucial to reproduce correctly the activities of the anionic form. The structural effects of solvation are remarkable both in the ground and in the lowest excited state of the anionic chromophore and influence not only the vibrational activity but also the photodynamics of the lowest excited state. CASPT2//CASSCF photoreaction paths, computed by including solvent effects at the CASSCF level, indicate a facile torsional deformation around both exocyclic CC bonds. Rotation around the exocyclic CC double bond is shown to lead to a favored radiationless decay channel, more efficient than that in gas phase, and which explains the ultrafast fluorescence decay and ground-state recovery observed in solution. Conversely, rotation around the exocyclic CC single bond accounts for the bottleneck observed in the ground-state recovery cycle. It is also speculated that the ultrafast radiationless decay channel would be hampered in protein for unfavorable electrostatic interactions and steric reasons.

Introduction

Several molecular biological and biochemical applications take advantage of the highly fluorescent chromophore generated autocatalytically in the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. The 4-hydroxybenzylidene-imidazolinone fluorophore is formed via post-translational internal cyclization, dehydration, and oxidation of the Ser65-Tyr66-Gly67 tripeptide unit.¹ In wild-type (WT) GFP, the chromophore, protected from the solvent through the β -can structure formed by the protein,² exists in two protonation forms, neutral and anionic. The neutral form is responsible for the short-wavelength absorption, at 398 nm, while the anionic form is associated with the long-wavelength peak, at 471 nm.³ These assignments are supported by the similar absorption maxima observed for model chromophores such as ethyl 4'-hydroxybenzylidene-2-methyl-imidazolinone-3-acetate (HBMIA)⁴ or 4'-hydroxybenzylidene-2,3-dimethyl-imidazolinone (HBDI).⁵ The

above two molecular models have also been employed to compare the vibrational fingerprints of the GFP chromophore in solution and in protein. Raman spectra of various protonation states of HBMIA⁴ and HBDI⁵ in water and resonance Raman (RR) spectra of HBDI⁶ in ethanol have been reported. These spectra can be compared with the Raman spectra of the neutral and anionic forms of the fluorophore in WT-GFP.⁷ Model chromophores have also been employed to investigate the environmental effect (protein versus solvent) on the photodynamics of the fluorophore. These studies have shown a dramatic increase of the radiationless decay in solution at room temperature, which results in complete quenching of the fluorescence.⁸ The ultrafast ground-state recovery dynamics of HBDI, measured in a range of temperatures and viscosities,^{9,10} sets an upper limit on the excited-state lifetime of a few picoseconds, and it was concluded that the dominant radiation-

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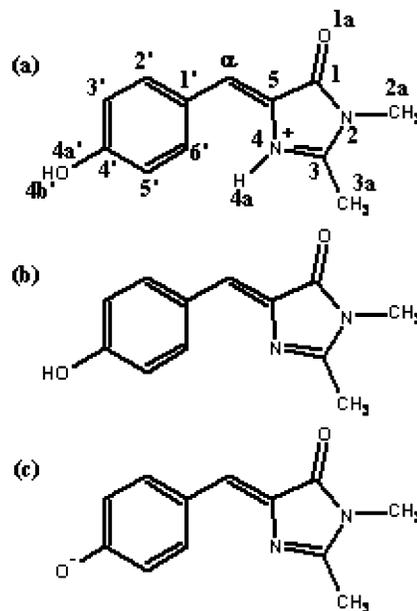
less decay pathway must be internal conversion (IC). More recent femtosecond fluorescence up-conversion measurements^{11–13} revealed a fluorescence decay well-described by a sum of two exponential terms. Owing to the weak viscosity dependence of the IC, it was suggested that the coordinate promoting IC must displace only a small solvent volume, that is, it must be volume-conserving. The concerted twist around the C–C=C bridging bonds (hula twist or two bond flip (TBF)) proposed on the basis of quantum-chemical calculations¹⁴ appeared to be a mechanism respecting the volume-conserving requirement, but a very recent computational study has indicated that the one bond flip or, more precisely, the single bond flip (SBF) around the exocyclic CC single bond would also satisfy the same requirement.¹⁵

More recently, accurate computational studies of the potential energy surfaces and deactivation pathways of the photoexcited state of the GFP chromophore have been reported for the anion in the gas phase¹⁵ and for the neutral chromophore, with inclusion of explicit solvent.^{16,17} The results of these model calculations and of several experimental studies concerning the effect of solvent on the photodynamics of the HBDI chromophore as opposed to the protein environment^{10,12,13} have led to the suggestion that the different behavior between solvent and protein must be driven by some modification of the displacement and curvature of the excited-state potential energy surfaces^{13,15} (PES). In other words, it is becoming apparent that in addition to possible direct effects (steric hindrance, hydrogen bond formation), the protein environment may act also indirectly, by inducing modifications of the structure and/or PES of the chromophore.

In contrast with the excited-state dynamics studies that have shown a dramatic difference between solution and protein environments, the vibrational spectroscopy studies^{4–6} carried out in solution have shown vibrational fingerprints of the chromophore quite similar to those observed in the protein. Indeed, the Raman and infrared studies carried out on several protonation states^{5,6} of the model HBDI chromophore have provided additional support to the assignment of the protonation state (neutral and anionic) of the two chromophoric forms in protein. Several theoretical studies on the vibrational activities of the HBDI chromophore have also been published.^{4,5,18–20} However, to date, all the calculations were carried out in the gas phase,^{5,6,18,19} and they were only partially able to account for the observed intensity distributions. Solvent interactions with a chromophore are prime examples of environmental effects, and they can lead to charge rearrangements and geometry changes in ground and excited states and can consequently affect

Chart 1. Structural Formula of

4'-Hydroxybenzylidene-2,3-dimethyl-imidazolinone (HBDI) in Three Protonation States: (a) Cationic, (b) Neutral, and (c) Anionic.



the vibrational dynamics and excited-state dynamics of the chromophoric units.²¹ Thus, before trying to account for solvent versus protein differences, it is perhaps even more fundamental to clarify the difference between in vacuo and solvent effects on both the spectroscopy and photodynamics of the GFP chromophore. For this reason, the main objective of this work is to investigate computationally the effect of solvation on the vibrational fingerprints and the photodynamics of the HBDI chromophore. It will be shown that the structural modifications induced by the solvent on both ground and excited states of the anionic chromophore affect on one side the vibrational structure and on the other side the deactivation channels of the photo-excited state.

1. Computational Methods

To model Raman and resonance Raman spectra, we obtained equilibrium structures in the ground state and in the optically allowed excited state, along with vibrational frequencies and normal coordinates in the ground state. The model chromophore investigated in this study is HBDI (see Chart 1). It includes the complete conjugate system of the real GFP chromophore, and it is the chromophore investigated in solution in several vibrational studies.^{5,6,19} The exact correspondence between the chromophore investigated theoretically and experimentally is particularly important when observed and computed vibrational frequencies and activities are discussed, since these can be strongly affected by the presence or absence of substituents. The vibrational spectra were simulated in vacuo and by including the solvent effect through the polarizable continuum model (PCM).^{22–24} In the PCM method the solvent is treated as an infinite continuum dielectric and the solvent response to the presence of the electric field generated by the solute charge distribution (assumed to be contained in a volume of known form and dimension, the molecular cavity) is represented in

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terms of an apparent charge spread on the cavity surface. The solvent chosen in these calculations is water, since this was used to measure the experimental Raman spectra,⁵ and the solvent reaction field was equilibrated to the solute electronic density also in the calculation of excited states. The inclusion of this latter effect may lead to an overestimate of the stabilization for the excited states. Several levels of theory (HF, CIS, and CASSCF) and basis sets (6-31G* and 6-31+G*) were considered, and all the calculations were carried out with the Gaussian 98 and Gaussian 03 suite of programs.^{25,26} Vibrational activities were evaluated for three different protonation states (anionic, neutral, cationic) of the HBDI model chromophore. Raman intensities were obtained by the standard algorithms available in Gaussian^{25,26} while RR intensities were simulated on the basis of computed molecular parameters along the lines described in refs 27 and 28. Vibrational frequencies were uniformly scaled by 0.9 to improve the agreement between computed and observed frequencies, and a Lorentzian line width of 5 cm⁻¹ was superimposed to each computed vibronic activity to facilitate the comparison with the observed spectra. For resonance with a strongly dipole-allowed transition, the fundamental quantity that governs RR intensities is the displacement parameter B_k , which represents the projection of the geometry change upon excitation, onto normal vibrations of the ground state. In this sense, RR intensities provide information on ground-state structure through the active frequencies, but also on the primary relaxation of the excited state, through the selection of few among the usually large number of active modes.

The effect of solvent on the photoexcited reaction path was investigated only for the anionic form of the chromophore. The path was computed at CASSCF(12,11)/6-31G* level, with the inclusion of solvent effects through the PCM method, and energies were corrected via single-point CASPT2 computations. Since the complete π -system of HBDI (16 electrons in 14 orbitals) is too large for CASSCF calculations, we selected 12 electrons in 11 orbitals as the best compromise between computer speed and accuracy. One of the three excluded orbitals is localized on the amidic nitrogen, while the remaining two correspond to the highest and lowest energy π -orbitals of the benzene ring. In addition, the excited-state reaction path was calculated by using an S_0/S_1 state average approach to prevent convergence problems in energy calculations. Vibrational frequencies at the CASSCF(12,11)-optimized geometry of S_0 were computed with a reduced active space (10,8). Because of energy convergence problems, CASPT2 calculations were carried out without the inclusion of PCM

solvent effects. Thus, the PT2 correction was extracted from the CASPT2 calculations in vacuo and summed to the CASSCF (PCM-corrected) energies. The CASPT2 calculations were carried out with MOLCAS-5.²⁹

The CASSCF(12,11) relaxed structures of the ground and excited states were also employed to simulate RR spectra for the anionic form. Notice, however, that because of the incomplete π -active space, the CASSCF-simulated spectra are not expected to be superior to the spectra simulated on the basis of CIS excited-state calculations. Indeed, it should be noted that, although the neglect of two benzenic orbitals in the active space does not influence the quality and the details of the reaction path, the same does not hold for the prediction of vibrational frequencies and activities, since the benzenic region of the chromophore is described, at CASSCF level, in a somewhat unbalanced way and vibrational modes partially localized in this molecular region are expected to be predicted less accurately. Thus, although the CASSCF level is generally superior to the combination of HF and CIS, in this case the vibrational predictions will have to be evaluated critically by taking into account the above considerations.

2. Vibrational Activities in Vacuo and Solvent

In this section we discuss the Raman and resonance Raman spectra simulated for the three protonation forms of HBDI shown in Chart 1. The ground-state relaxed structures of the three forms were evaluated at HF/6-31+G* level of theory. Excited-state relaxed structures are also required to simulate the RR spectra, and these were evaluated at CIS/6-31+G* level of theory. Our previous experience^{30,31} indicates that this level of theory ensures a reliable description of the vibronic structures in electronic spectra of conjugated molecules. Furthermore, the CIS method can be deemed reliable for the description of the lowest excited state since this is dominated by single excitations. In addition, we also employed the CASSCF relaxed structures of the ground and excited states to simulate RR spectra for the anionic form, since the photoreaction path was calculated at this level of theory. Detailed simulations of RR intensities have never been reported, except for the results from nonthermally equilibrated molecular dynamics simulations.^{18,20} Since RR intensities are directly related to the displacement of the excited-state potential energy surface (PES) with respect to the ground state, the comparison between observed and simulated intensities provides strong indications on the reliability of the predicted relaxed structures in S_0 and S_1 .

In Figure 1, we present the Raman spectra simulated for HBDI anion, neutral, and cation (HF/6-31+G* level of theory) in vacuo and by including the solvent effect (water) through the PCM method. Raman spectra computed in vacuo at B3LYP/6-31G* level were reported in previous studies,^{6,19} and intensity distributions were discussed in some detail for the neutral form of the chromophore but not for other protonation states.¹⁹ A cursory inspection of Figure 1 shows immediately that, while for the neutral and the cationic forms of the chromophore the Raman spectra simulated in vacuo agree well with the observed spectra, the Raman spectrum predicted in vacuo for the anionic form is significantly different from the observed spectrum. Indeed, the stronger activity is computed, for the anionic form, at 1350 cm⁻¹, a region where, experimentally, only weaker

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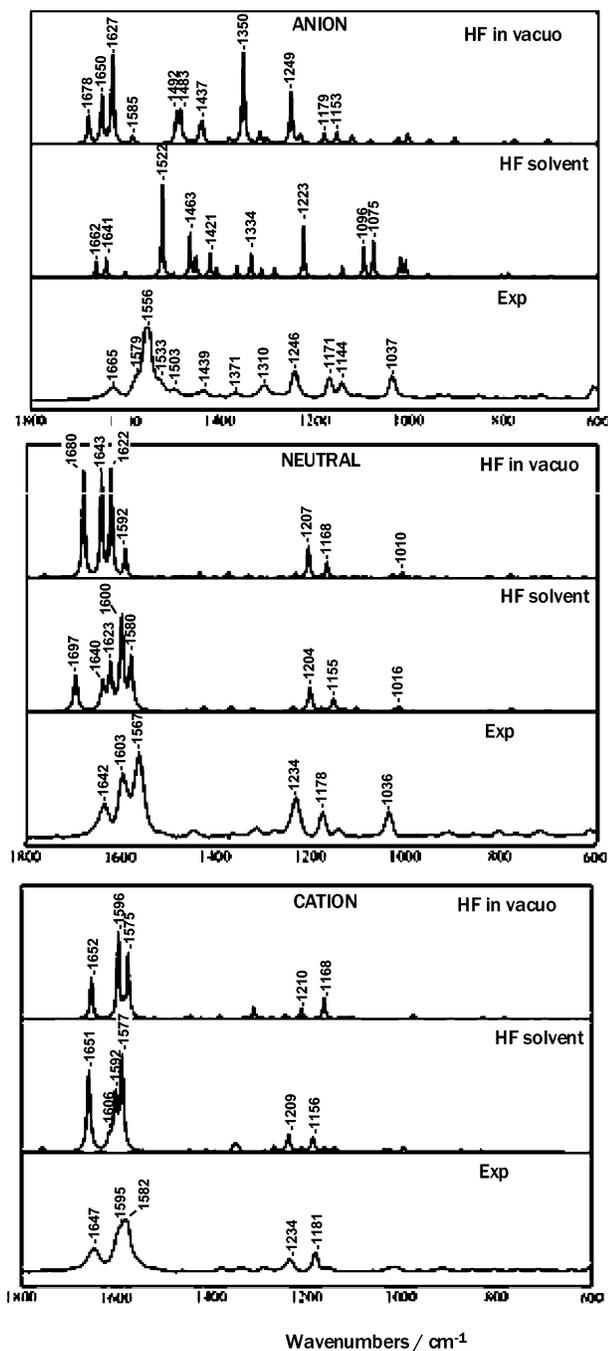


Figure 1. Raman spectra (from HF/6-31+G* calculations) simulated in vacuo and in water (with PCM) and comparison with the corresponding observed experimental spectra from ref 5. From top to bottom: anionic, neutral, and cationic forms of the HBDI chromophore.

bands are observed. Notice that considerable intensity is predicted also at 1627 cm^{-1} , but this frequency region is considerably higher than the frequency of the dominant experimental band,⁵ at 1556 cm^{-1} . Raman intensity calculations carried out for the anionic form with a larger basis set (6-311++G**) did not improve the results. The same figure shows that inclusion of solvent effects generally improves the agreement between computed and observed intensities. The change in intensity distribution is, however, dramatic for the anion: inspection of Figure 1 shows that the agreement between the Raman spectrum computed in water and the observed spectrum is remarkably improved, although we notice an underestimate for some of the

Table 1. Computed Bond Length Changes upon Inclusion of Solvent Effects through the PCM Method ($S_0^{\text{PCM}} - S_0^{\text{vacuo}}$ and $S_1^{\text{PCM}} - S_1^{\text{vacuo}}$) ($\text{\AA} \times 10^3$) of the Exocyclic CC Bonds of the Neutral, Cationic, and Anionic Forms of the HBDI Chromophore

exocyclic bond	anion		neutral		cation	
	HF/6-31+G* (S_0) and CIS/6-31+G* (S_1)					
	$S_0 - S_0$	$S_1 - S_1$	$S_0 - S_0$	$S_1 - S_1$	$S_0 - S_0$	$S_1 - S_1$
C1'C α	31	-11	-1	-3	6	-4
C α C5	-22	11	3	9	-2	12

CASSCF(12,11)/6-31G*						
$S_0 - S_0$	$S_1 - S_1$					
C1'C α	35	-12				
C α C5	-20	9				

predicted frequencies. The simulated spectrum is now sufficiently close to the observed spectrum to allow for a one-to-one correspondence between observed and computed bands to be made for almost all the prominent bands in the spectrum. Thus, the computed 1522, 1463, 1421, 1334, 1223, 1096, and 1075 cm^{-1} bands correlate with the bands observed⁵ at 1556, 1503, 1439, 1310, 1246, 1171, and 1144 cm^{-1} , respectively. Since only ground-state calculations are involved in the evaluation of the above-discussed Raman intensities, it can be concluded that the dramatic intensity change between simulations in vacuo and in water is strongly influenced by changes in the ground-state structure. Indeed, inspection of the computed S_0 equilibrium geometries with or without solvent effects shows that the largest modifications are predicted for the anionic chromophore, the species showing the largest changes in Raman intensities. The more substantial geometry changes are localized on the exocyclic CC bonds, as summarized in Table 1. It is seen that the effect of solvent is to elongate the single CC bond and to shorten the double CC bond. In other words, the effect of solvent is similar to that of a positive counterion placed close to the phenolic oxygen, which stabilizes the resonance structure in which the negative charge is located on the phenolic oxygen. Notice that the solvent modifies only slightly the ground-state geometry of neutral and cationic HBDI, a trend reproducing the minor changes computed for Raman intensities.

The improvement, on both Raman intensities and normal mode description, obtained upon inclusion of solvent effects can be better appreciated by considering the shifts of the dominant Raman bands upon isotopic substitution. In Table 2, we compare the isotopic shifts computed in vacuo with those computed in water and with the experimental values.⁵ It is interesting to note that, although the overall appearance of the Raman spectrum predicted in vacuo for the neutral form agrees well with the observed spectrum (see Figure 1), a more thorough inspection of the isotopic shifts of the two most intense predicted Raman bands shows a remarkable discrepancy with the experimental shifts, especially for the band predicted at 1643 cm^{-1} and assigned, on the basis of its intensity, to the 1603 cm^{-1} observed band (compare the isotopic shifts in columns two and six of Table 2). The discrepancies are eliminated upon inclusion of solvent effects (see column four in Table 2), an indication that, in addition to a better numerical agreement between computed and observed frequencies, the shape of the associated normal coordinates also improves by including the PCM correction. Inspection of Table 2 shows that the cation is apparently the only species weakly affected by solvent effects. For the anionic form, finally, the situation is similar to the

Table 2. Neutral, Cationic, and Anionic Forms of HBDI: Summary of Isotopic Shifts of the Most Intense Bands in Raman, in the Region 1500–1700 cm^{-1} , and Comparison between Computed (in Vacuo and PCM) and Observed⁵ Values

isotopomer	in vacuo		PCM		expt	
	neutral					
C α	-8	0	-2	-2	0	-8
C1	-2	-1	-5	-1	-3	-2
C5	-9	-1	-1	-2	-1	-5
N4	-16	-1	0	0	+3	-6
C3	-35	0	0	0	0	0
HBDI	1643	1622	1600	1580	1603	1567
	cation					
C α	-2	-9	-2	-6	+1	+0
C1	0	0	0	-1	+5	+2
C5	-1	-5	-1	-5	0	0
N4	-1	+4	+5	0	+5	-2
C3	-1	+5	+3	-7	+5	+4
HBDI	1596	1575	1592	1577	1595	1582
	anion					
C α	-3		-5		-7	
C1	-1		-5		-3	
C5	-2		-1		-2	
N4	-5		0		-2	
C3	-21		0		+1	
HBDI	1627		1522		1556	

neutral form: the isotopic shifts predicted in vacuo are substantially different from the experimental values, and the latter are in very good agreement with those predicted upon inclusion of solvent effects.

The above discussion on computed equilibrium structures and static (off-resonance) Raman intensities shows that the ground state of the anionic form of HBDI is remarkably affected by solvent effects.

It is now interesting to consider the RR spectra since intensities, in this case, are connected with the S_0 – S_1 geometry change.^{27,28} This implies that, if the solvent affects in a similar way the PES of the two states, the S_0 – S_1 geometry change will be similar in vacuo and solvent, and one may expect a reasonable prediction of RR intensities also from calculations in vacuo. In contrast, the RR spectra simulated in vacuo and by inclusion of solvent effects, compared in Figure 2 with the experimental spectra,⁶ show a trend similar to the off-resonance Raman spectra just discussed. The RR spectra of the cationic and neutral forms computed in vacuo are in good agreement with the observed spectra, and inclusion of solvent effects improves moderately the agreement. The RR spectra predicted in vacuo for the anionic form are similar to the corresponding Raman spectra, and they disagree remarkably with the observed spectra. Inclusion of solvent effects improves significantly the agreement with experimental data. Notice that this improvement implies that the solvent and in vacuo predicted S_0 – S_1 geometry changes must be rather different for the anionic chromophore. Indeed, as with the ground state, the excited-state geometry is also affected by solvent effects, and dominant changes occur in the exocyclic CC bonds (see Table 1). More important, the solvent modifies (with respect to gas phase) the ground-state and excited-state exocyclic bonds in an *opposite* way. Inspection of column 3 of Table 1 shows that in solvated S_1 the C α C5 bond (double bond in the ground state) is elongated with respect to gas phase S_1 , while the C1'C α bond (single bond in the ground state) is shortened in the solvent with respect to the gas

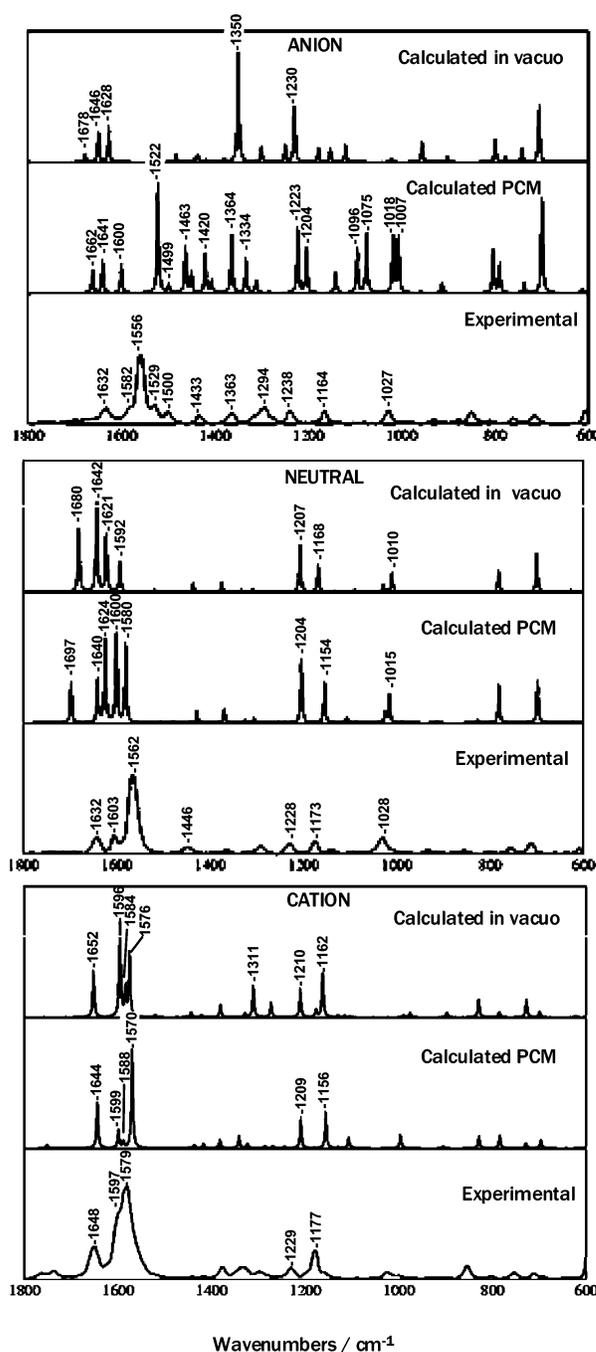
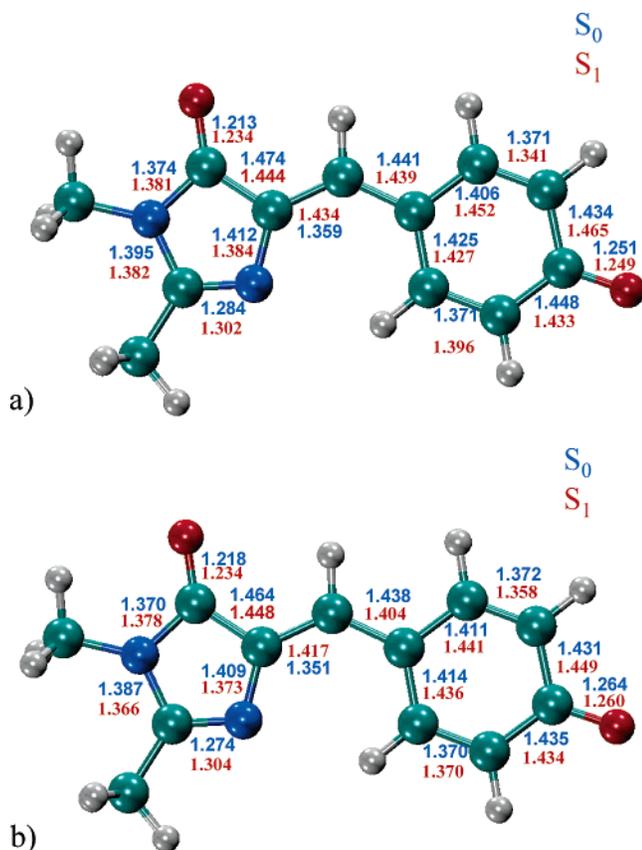


Figure 2. RR spectra (from HF and CIS/6-31+G* calculations) simulated in vacuo and in water (with PCM) and comparison with the corresponding observed experimental spectra from ref 6. From top to bottom: anionic, neutral, and cationic forms of the HBDI chromophore.

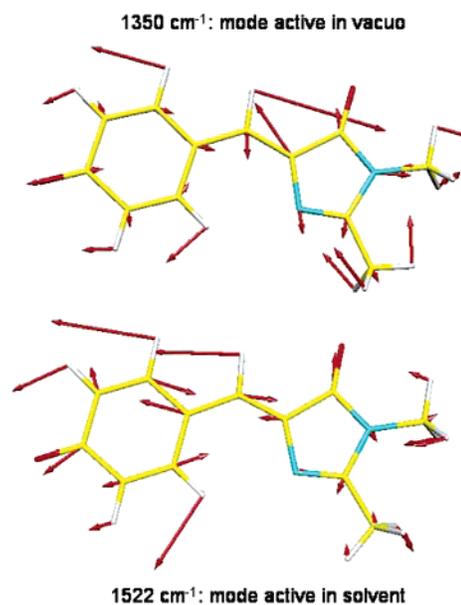
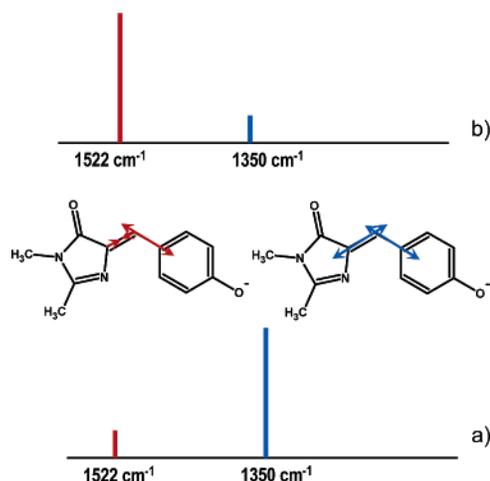
phase. Column 2 of Table 1 shows that bond length changes go in the opposite direction in the ground state. As a result, inclusion of solvent effects induces an S_1 – S_0 geometry change in which the two exocyclic CC bond lengths are modified in opposite ways: the CC single bond shortens upon excitation while the CC double bond lengthens upon excitation. The PCM-corrected ground- and excited-state bond lengths computed at CASSCF(12,11)/6-31G* and HF(S_0) or CIS(S_1)/6-31+G* levels of theory are collected in Figure 3. The exocyclic CC bond length variations (S_1 – S_0) are summarized in Table 3, where they are compared with those predicted from gas phase calculations. Table 3 shows that the exocyclic bond length

Table 3. Computed Bond Length Changes (S_1-S_0) ($\text{\AA} \times 10^3$) of the Exocyclic CC Bonds, upon Excitation to S_1 , for the Neutral, Cationic, and Anionic Forms of the HBDI Chromophore

exocyclic bond	anion				neutral			cation		
	CAS	CAS	HF-CIS	HF-CIS	CAS	HF-CIS	HF-CIS	CAS	HF-CIS	HF-CIS
	in vacuo	PCM	in vacuo	PCM	in vacuo	in vacuo	PCM	in vacuo	in vacuo	PCM
	631G*	631G*	631+G*	631+G*	631G*	631+G*	631+G*	631G*	631+G* planar	631+G* planar
C1'Ca	44	-3	8	-34	-78	-63	-65	-16	-44	-58
CαC5	45	74	33	66	99	81	83	30	61	76

**Figure 3.** Relaxed structures of the ground (S_0) (FC structure) and lowest excited state (S_1) (FS structure) of the anionic form of HBDI. (a) From CASSCF(12,11)/6-31G* calculations and (b) from HF/6-31+G* (S_0) and CIS/6-31+G* (S_1) calculations.

changes upon excitation are opposite in solvent (*out-of-phase* geometry change) and in vacuo (*in-phase* geometry change) only for the anionic chromophore. For neutral and cationic HBDI the direction of bond length changes is not altered by the solvent, and this explains why the predicted RR spectra do not change dramatically in gas phase and in solvent. For the anion, the *out-of-phase* (solvent) versus *in-phase* (gas phase) CC bond length variation implies remarkably different RR intensities, since among the Raman active modes, intensity will be enhanced for those modes that mimic the S_1-S_0 geometry change. Specifically, the 1522 cm^{-1} frequency normal mode (Figure 4, bottom), which shows the largest RR intensity in the simulations with solvent (see Figure 2), is indeed characterized by an *out-of-phase* motion of the two exocyclic CC bonds. The same vibrational mode cannot be active in the simulations in vacuo where activity is enhanced, on the contrary, for the mode with frequency 1350 cm^{-1} , (see Figure 4, top) characterized by an *in-phase* motion of the two exocyclic CC bonds. A schematic representation of the selective enhancement of intensities by the RR mechanism is depicted in Figure 5, where the shape of

**Figure 4.** Vectorial representation of the most active mode in the Raman and RR spectra of the anionic form of HBDI. (Top) Most active mode in vacuo. (Bottom) Most active mode in solvent.**Figure 5.** Schematic representation of the selective enhancement of active modes for the anionic form of HBDI through the RR mechanism. (a) The S_1-S_0 geometry change is dominated by the in-phase variation of the exocyclic CC bonds, and the band at 1350 cm^{-1} is selectively enhanced in the RR spectrum schematically shown at the bottom. (b) The S_1-S_0 geometry change is dominated by the out-of-phase variation of the exocyclic CC bonds, and the band at 1522 cm^{-1} is selectively enhanced in the RR spectrum schematically shown at the top.

the normal modes selectively enhanced in the top or bottom part of Figure 5 is intentionally simplified as localized on the exocyclic CC bonds.

The effects discussed above are confirmed also at CASSCF-(12,11)/6-31G* level of theory: the simulated RR spectra are collected in Figure 6, which shows discrepancies between

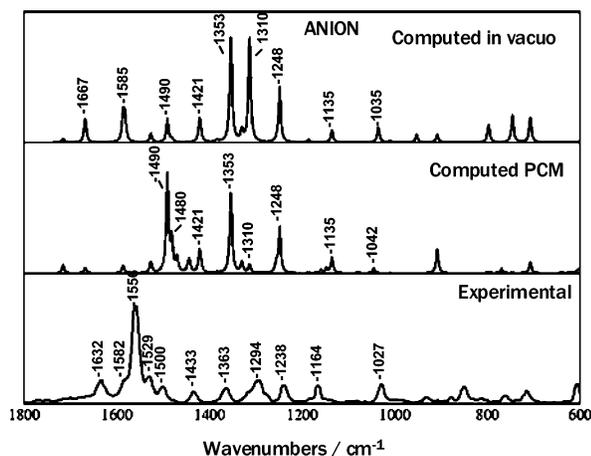


Figure 6. RR spectra (from CASSCF/6-31G* calculations) simulated in vacuo and in water (with PCM) and comparison with the corresponding observed experimental spectra from ref 6 for the anionic form of the HBDI chromophore.

observed and gas phase computed spectra comparable to the HF/CIS results. Inclusion of solvent effects improves the simulated RR spectrum, although the quality of the final simulation is less satisfactory compared to the HF/CIS results. As discussed in the previous section, the lower accuracy of CASSCF spectral simulations can be attributed to the incomplete π -orbital active space selected in the calculations. This leads to an unbalanced description of the normal coordinates and, more specifically, of the out-of-phase exocyclic stretching motion whose frequency (1490 cm^{-1}) is remarkably underestimated. Nevertheless, the major solvent effect, namely the out-of-phase geometry change upon excitation and the consequent RR enhancement of different normal coordinates in gas phase and in solution, is confirmed also at this level of theory. We will show in the next section that this remarkable geometrical effect, which is responsible for the dramatic difference between Raman spectra simulations in vacuo and in solvent, also has implications on the photodynamics of the anionic HBDI chromophore, since the relaxation of the S_1 state from the vertical Franck–Condon (FC) structure (ground-state equilibrium geometry) to the optimized or fluorescent state (FS) structure, here discussed in connection with RR activities, represents the first step of the two-state/two-mode deactivation path of the excited chromophore. The different FS structure in solvent, as compared with the gas phase, will also influence the preferential channel of ultrafast radiationless deactivation, characterizing the second step of the photoreaction. In other words, the discrepancy discussed here, between in vacuo simulated and experimental Raman spectra of anionic HBDI in solution,^{5,6} may also imply that the deactivation path predicted in vacuo¹⁵ does not represent the preferential deactivation channel in solution.

3. Solvent Effects on the Photoexcitation Reaction Path

In the previous section, we discussed the solvent effect on the first relaxation step from the FC to the FS region of the S_1 state of HBDI anion and we anticipated that changes (compared to the gas phase) induced by solvent on this first step may drive the subsequent evolution on the excited PES, leading, ultimately, to a relaxation channel that may be different from that

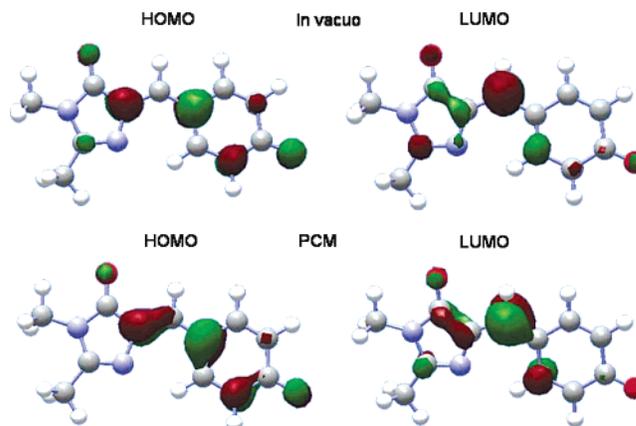


Figure 7. Molecular orbitals of the anionic form of HBDI involved in the single excitation that dominates the S_1 state. (Top) MOs from CASSCF calculations in vacuo. (Bottom) MOs from CASSCF + PCM contribution.

preferentially followed in gas phase. In solvent, the initial FC \rightarrow FS relaxation is dominated by an *out-of-phase* change of the exocyclic CC bond lengths, in contrast with the *in-phase* change computed in the gas phase. As discussed in the previous section, the PCM correction modifies the FC (i.e., ground state) structure with respect to gas phase calculations. In this regard, it is interesting to compare the CASPT2 computed vertical excitation at this FC structure with the experimental data. Calculations predict a vertical excitation of 461 nm, as compared with the 425 nm⁵ or 432 nm⁶ observed in water. Notice that the CASPT2 calculations include the solvent effects only on the CASSCF part, and their contribution is to increase the vertical excitation energy from 493 to 461 nm. It can also be expected that the PT2 contribution goes in the same direction, and this would lead to an even better agreement with the experimental data.

Inspection of the excited-state wave function computed in vacuo and with the PCM contribution indicates minor changes, since the state is dominated in both cases by the HOMO \rightarrow LUMO excitation and the HOMO and LUMO orbitals computed in vacuo and after PCM correction are very similar (see Figure 7). Nevertheless, small changes in the atomic charge distribution of both ground and excited states lead to a significant change in the computed dipole moment of the S_1 state. As shown in Figure 8b, the change in dipole moment, computed with the PCM-corrected wave functions, is 8.6 D, in close agreement with the value and the direction of the observed change of 6.8 D.³² This remarkable change (the corresponding value in vacuo is only 2 D) can be accounted for by inspection of Figure 8 (bottom) where atomic charge variations upon excitation are collected from both gas phase and PCM-corrected calculations. It is seen that the largest differences between gas phase and solvent are generally located at the two opposite ends of the chromophore, thus leading to more substantial dipole moment change upon excitation in the case of the PCM-corrected calculations. As a result, the net charge transfer upon photoexcitation is of the order of 0.3 e from the phenolic to the imidazolinone ring in solvent, while it is only of the order of 0.05 e in gas phase (see Figure 8).

We have already discussed the evolution of the excited state from the FC to the FS structure (Figure 3): this relaxation

(32) Bublitz, G.; King, B. A.; Boxer, S. G. *J. Am. Chem. Soc.* **1998**, *120*, 9370–9371.

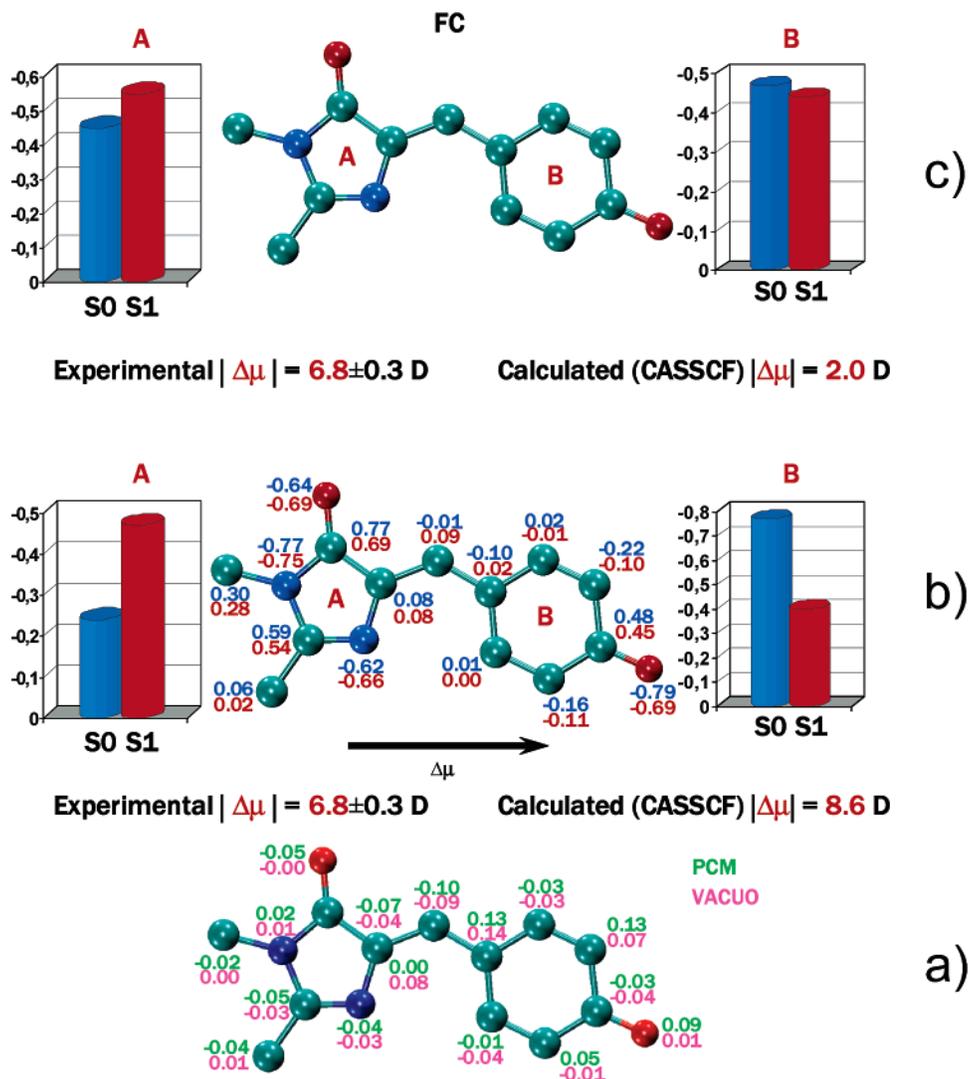


Figure 8. (a) Mulliken atomic charge variation upon excitation (S_1-S_0) from gas phase (magenta) and solvent-corrected (green) calculations and (b and c) charge relocation (from the phenolic (B) to the imidazolone ring (A)) upon excitation to the S_1 state, according to CASSCF(12,11) calculations. The atomic charge of carbon C_α is not included in the sum. (b) Charge relocation and Mulliken atomic charges for the ground and excited states of the anionic form of the HBDI chromophore computed from PCM-corrected CASSCF(12,11)/6-31G* calculations. (c) Charge relocation computed from in vacuo CASSCF(12,11)/6-31G* calculations.

involves in-plane coordinates, mainly stretching modes, among which those remarkably active in Raman and RR spectra. The above-discussed charge distribution and wave function of the S_1 state hardly change along the $FC \rightarrow FS$ relaxation path. As shown in Figure 3, the *out-of-phase* change of the exocyclic CC bonds upon excitation drives the exocyclic chromophore structure from a marked alternation (well-defined single and double bonds (FC)) to a more delocalized situation in which both CC bond lengths are comparable (FS) and close to 1.43 Å (at CASSCF level). This behavior is remarkably different from that computed in the gas phase,¹⁵ where the FC structure shows a more delocalized structure and the FS structure shows a notable difference (0.03 Å) between the two exocyclic CC bond lengths. The PES, in the FS region, is remarkably flat. From the FS region, the system can evolve in several different ways: (i) it can deactivate radiatively or it can rotate around the two exocyclic CC bonds to produce (ii) a twisted intermediate that we will call TW2 if rotation occurs around the CC bond, which is markedly double in the ground state, leading to the double bond flip (DBF) mechanism, (iii) a twisted intermediate TW1

for rotation around the other CC bond, mainly single in the ground state, leading to the already mentioned SBF mechanism and (iv) a hula-twist intermediate HT if simultaneous rotation around both exocyclic bonds occurs.

The emission from the FS is computed at 504 nm, a value underestimated with respect to the experimental value of ca. 490 nm.^{10,13} As with the ground-state absorption, the PCM correction on the CASSCF portion of the CASPT2 calculations increases the excitation energy from 518 to 504 nm. The still underestimated value is most likely attributable to the lack of PCM correction in the PT2 part of the CASPT2 computed energies.

Our calculations on the HT intermediate, obtained by simultaneous rotation of 90° of the two exocyclic bonds, show an S_0-S_1 surface touching.³³ Nevertheless, the CASPT2 energy of the HT structure is 11 kcal/mol above the FS structure and about 8 kcal/mol above the FC structure. Owing to its high

(33) The lowest energy conical intersection (CI) structure of the HT conformation was not determined because of problems in the CI algorithm in the presence of PCM corrections.

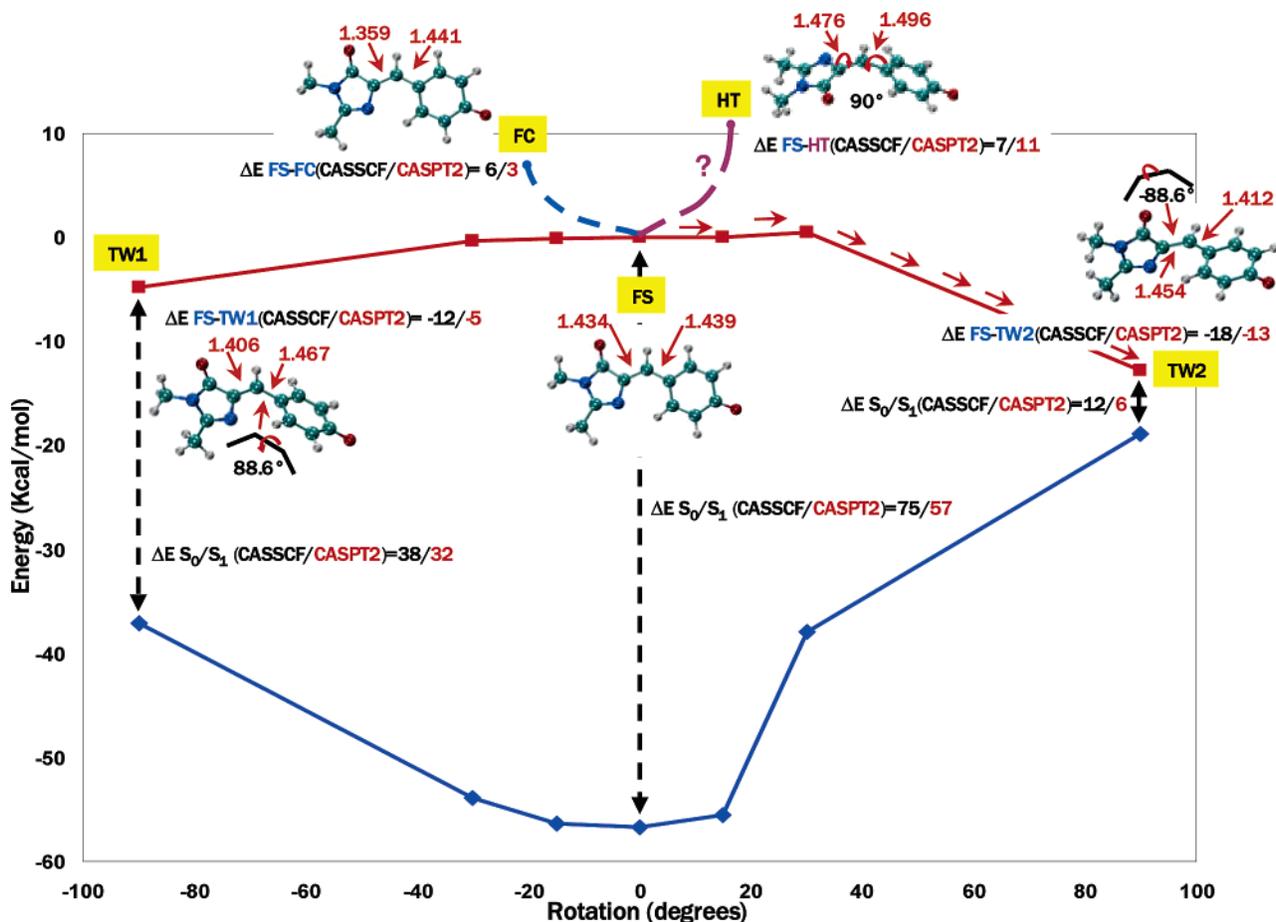


Figure 9. CASPT2 energy profiles for the electronic S_1 and S_0 states of the anionic form of HBDI, along the computed FS \rightarrow TW1 and FS \rightarrow TW2 relaxation coordinates. CASPT2 and CASSCF relative energies are in kilocalories per mole. The stream of arrows indicates the reaction path responsible for ultrafast radiationless decay.

energy, we deem highly unlikely that the HT plays a role in the deactivation of the excited state in solution.

Conversely, the rotation around either one of the exocyclic CC bonds leads to the intermediates TW1 and TW2, both characterized by an energy lower than the FS structure (see Figure 9). The calculations of the photoreaction path at CASSCF(12,11)/6-31G* or CASPT2 level do not indicate a preferential initial channel of deactivation, in contrast with gas phase calculations¹⁵ since rotation around the two CC exocyclic bonds is almost barrierless for both channels (see Figure 9). The energy profile along the reaction coordinate remains almost flat up to a twisting of about 30°. After this region, the energy starts lowering significantly. The TW1 intermediate is computed to be about 5 kcal/mol (12 kcal/mol) lower in energy than the FS structure at CASPT2 (or CASSCF) level, while the TW2 intermediate is even more stable: 13 kcal/mol (18 kcal/mol). More important, the energy gap between S_0 and S_1 is significant for the TW1 structure: 32 kcal/mol at CASPT2 level (38 kcal/mol at CASSCF level), as can be expected since the ground-state energy does not increase dramatically when the CC single bond is twisted. The computed energy separation is larger than that of the corresponding channel in gas phase¹⁵ (21 kcal/mol at CASPT2 level). This suggests that the TW1 channel, in solution, is unlikely to be responsible for the fast IC experimentally documented. Conversely, the S_0 – S_1 gap is remarkably reduced at the TW2 structure: 6 kcal/mol at CASPT2 level (12 kcal/mol at CASSCF), owing to the considerable energy increase

of the ground state when rotating around the exocyclic CC double bond. Such a small energy gap (6 kcal/mol) is compatible with a real crossing in the surrounding region and, consequently, with an efficient radiationless decay.³⁴ For this reason, we identify the TW2 channel as the dominant radiationless decay route of the photoexcited anionic chromophore in solution. Notice that the remarkable approaching of the two states S_0 and S_1 is strictly related with the presence of solvent. Indeed, in gas phase, the corresponding deactivation channel leads to a much larger S_0 – S_1 energy separation of ca. 13 kcal/mol¹⁵ at CASPT2 level, owing to the less dramatic energy increase of the ground state whose CaC5 bond is considerably longer in the gas phase.

In addition to the S_0 – S_1 energy gap, the charge redistribution, represented in Figure 10, is also relevant to differentiate the TW1 and TW2 intermediates. As shown in Figure 10, the negative charge in the excited state is almost completely localized on the imidazolinone ring for the TW1 structure and on the phenolic ring for the TW2 structure. The charge localization is completely reversed in the ground state.

In summary, the second step (namely evolution from the FS structure) of photoexcited reaction path in solution is characterized by a more stable TW2 intermediate, which corresponds to a reduced S_0 – S_1 energy gap (compared with gas phase), and by the less stable twisted intermediate TW1, which corresponds

(34) The conical intersection structure could not be determined for the same reason as that indicated in ref 33.

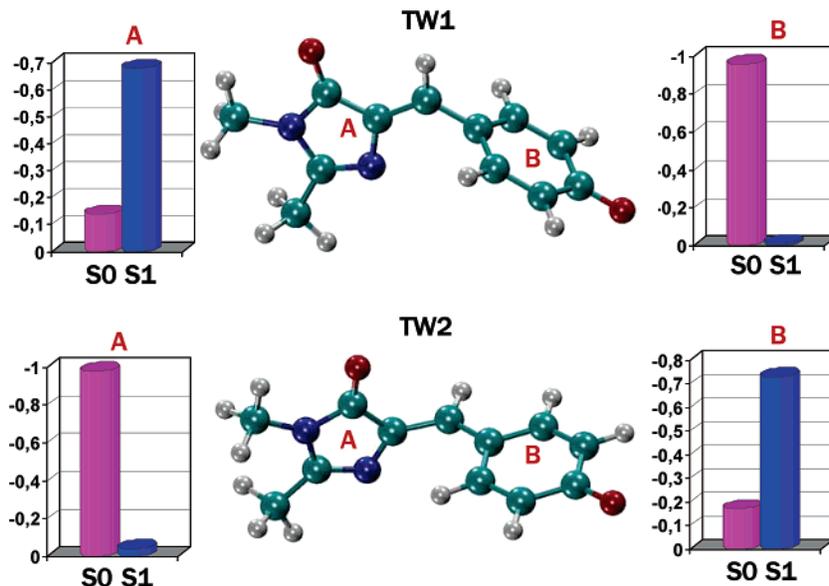


Figure 10. Charge relocation (from the phenolic (B) to the imidazolinone ring (A)) upon excitation to the S_1 state, according to CASSCF(12,11) + PCM calculations. The atomic charge of carbon C_α is not included in the sum. (Top) Charge relocation for the TW1 structure of the HBDI chromophore. (Bottom) Charge relocation computed for the TW2 structure.

to an increased (compared to gas phase) S_0 – S_1 gap. Furthermore, the lower energy of the TW2 intermediate implies a deeper energy profile and, consequently, a faster acceleration in the direction of the S_0 – S_1 surface touching.

4. Discussion

The computed CASPT2 photoexcitation reaction path in solution shows that the channel involving rotation around the $C\alpha C5$ bond leads to the TW2 structure whose energy separation from the ground state is considerably reduced as compared with the other low energy intermediate TW1, obtained upon rotation around the $C1'C\alpha$ bond. For this reason, the TW2 channel is identified as the primary radiationless deactivation channel in solution. One requirement of the radiationless decay mechanism in solution is that it should be volume-conserving to account for the weak viscosity dependence experimentally observed.^{11–13} Although the HT structure strictly satisfies this requirement, we have shown that its high energy precludes its contribution to radiationless deactivation in solution. Nevertheless, the TW2 structure (similar to the TW1 structure responsible for the major decay in gas phase¹⁵) does not imply substantial displacement of solvent molecules and can be considered as acceptable also in the light of volume conservation.

Experimentally, the fluorescence intensity of HBDI increases by about 3 orders of magnitude by lowering the temperature to 77 K.^{10,12} According to the mechanism outlined in the previous section, this behavior can occur only if a small barrier characterizes the reaction path along the DBF coordinate or it may reflect the influence of glassy dynamics.¹² The CASPT2 reaction path is almost barrierless, but a barrier of the order of 1–2 kcal/mol can be considered compatible with the accuracy of the method. Fluorescence decay measurements have shown a biexponential character with the shortest time constant in the range of 400–500 fs and the slower time constant of the order of 2 ps.¹¹ These two decay times have been interpreted as due to an intrinsically nonexponential character of the intramolecular mechanism leading to IC. More specifically, to the relaxation from the planar along the twisted coordinate (<500 fs compo-

nent) followed by the $S_1 \rightarrow S_0$, IC occurs on a time scale of the order of 1–3 ps.¹¹ This explanation is plausible, also in the light of our computed energy path in solution.

Thus far we have mainly focused our attention on the branch of the computed photoexcited reaction path corresponding to the formation of the TW2 intermediate, since this is the channel that accounts for the ultrafast radiationless decay. However, according to the calculated reaction path, we cannot rule out population of the alternative channel, leading to the TW1 intermediate (see Figure 9). This alternative deactivation route will bring the system to a region where radiationless deactivation will be much slower because of the large computed energy gap (32 kcal/mol) and because infrared fluorescence can compete. Experimentally, ground-state recovery studies⁹ have shown that ca. 95% of the initial population of the excited state is recovered within 5 ps, but the remaining fraction does not recover to the ground state on a time scale of at least several hundred picoseconds, indicating the presence of a bottleneck in the ground-state recovery cycle. We attribute this bottleneck to the TW1 deactivation channel, whose S_0 – S_1 energy gap and TW1 \rightarrow TW2 equilibration (as indicated by simple TW1 \rightarrow TW2 rate constant calculations) are compatible with a lifetime in the range of nanoseconds.

The major difference with the reaction path computed in the gas phase¹⁵ is that in solvent the TW2 channel becomes the preferential radiationless deactivation route. It should be noted that rotation around the exocyclic double bond leads to cis–trans photoisomerization with a consequent variation of the ground-state cis and trans populations. Photoisomerization upon irradiation has indeed been reported³⁵ along with ground-state thermal recovery, which provides further support to the plausibility of the TW2 deactivation channel in solution.

In contrast with the synthetic model chromophores in solution, the protein environment suppresses dramatically the radiationless decay. In light of the predominant decay mechanism established in solution, we can speculate on what can be the effect of protein

(35) He, X.; Bell, A. F.; Tonge, P. J. *FEBS Lett.* **2003**, *549*, 35–38.

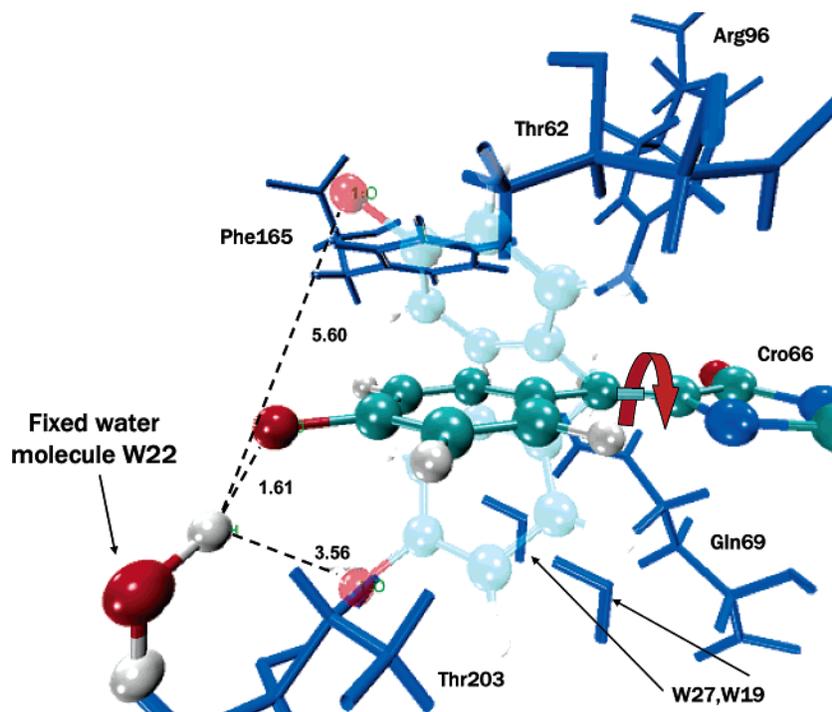


Figure 11. Schematic representation of the hydrogen bond breaking and disfavored steric interactions associated with an hypothetical rotation of the chromophore around the exocyclic CC double bond (DBF mechanism) inside the protein.

on it. We can envisage at least three reasons for suppression of this decay channel. First, the exocyclic CC double bond torsion will be strongly hindered for steric reasons, as can be better appreciated by inspection of Figure 11, which shows the chromophore surrounded by the closest residues from the protein environment,³⁶ along with (shaded) two chromophore structures corresponding to two arbitrary snapshots taken during the hypothetical attempt to rotate around the exocyclic CC double bond. It is seen that the chromophore would collide with several residues in the attempt to rotate among THR62, PHE165, and THR203. A second reason for suppression of this channel is also shown in Figure 11, by the breaking of the hydrogen bond between water W22 and the phenolic oxygen of the chromophore. Both these factors would induce a relevant barrier along the torsional reaction coordinate. There is a third, more subtle factor, due to electrostatic interactions, that would contribute to suppress the efficiency of the TW2 decay path, namely, the charge localization on the twisted intermediate. As shown in Figure 10, the negative charge is localized, for the TW2 intermediate, on the phenolic ring. In contrast, a positive charge (ARG96) is located, in the protein, close to the amidic oxygen of the chromophore. Thus, one can expect that the reaction path involving translocation of the negative charge from the imidazolone to the phenolic ring (namely the TW2 reaction path) will be strongly disfavored in protein. Furthermore, as can be seen in Figure 10, the negative charge in the ground state, at the TW2 structure, is indeed located on the imidazolone ring. Thus, the ground state will be stabilized by the electrostatic interaction with the protein, while the excited state will be destabilized with a consequent increase of the S_0 – S_1 gap and decrease of the radiationless decay efficiency. Notice that the opposite would happen at the TW1 intermediate

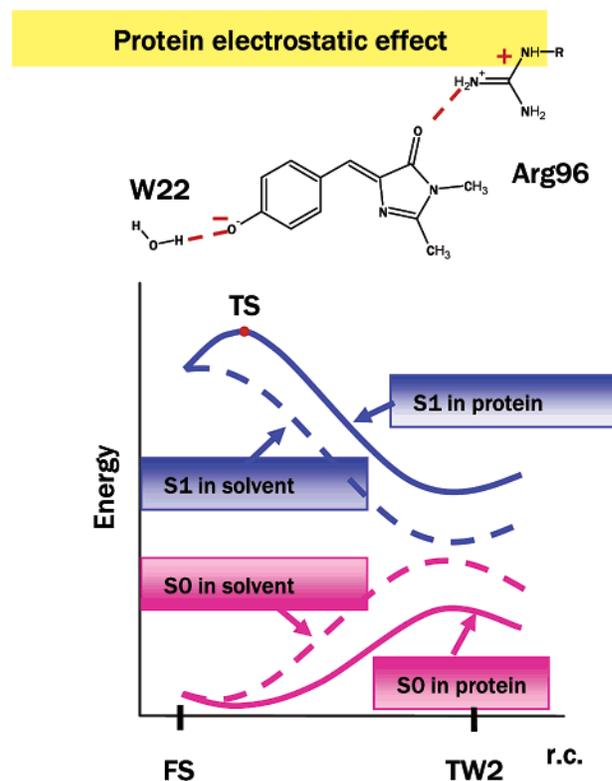


Figure 12. Schematic representation of the electrostatic effects induced by the protein residues on the S_1 and S_0 energy profiles of the anionic form of the HBDI chromophore.

structure. The above-discussed effects on the energy profiles of the DBF coordinate are schematically represented in Figure 12. In summary, although the protein might somewhat modify the electronic structure of the chromophore with respect to the solution, on the basis of the results of the present solution study, we suggest one plausible reason for the suppression of fluo-

(36) Brejc, K.; Sixma, T. K.; Kitts, P. A.; Kain, S. R.; Tsien, R. Y.; Ormo, M.; Remington, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2306–2311.

rescence in protein, namely the reduced efficiency of the TW2 radiationless decay channel which is strongly favored in solution and would be strongly hampered in the protein.

5. Conclusions

We have presented a computational study on the vibrational spectra and photoexcitation decay path of HBDI in solution, a model compound for the GFP fluorophore. The calculations indicate that the solvent modifies substantially the relaxed structures of the ground and excited states of the various protonation forms of the HBDI chromophore. The solvent-induced geometrical modifications are reflected in a closer agreement of the computed intensity distributions in Raman and RR spectra with the corresponding experimental data. The effect is dramatic in the case of the anionic form of the chromophore and is mainly attributed to an opposite S_1-S_0 geometry change in gas phase with respect to the solvent, which results in the enhancement of different vibrational modes in gas phase as compared with the solution. At the same time, solvent-induced changes on the relaxed ground- and excited-state structures affect also the topology of the deactivation path of the photoexcited chromophore. As with the gas phase,¹⁵ the radiationless deactivation in solution is well-represented by a two-state/two-mode mechanism, but in contrast with the favored SBF mechanism in gas phase, the preferential radiationless deactivation channel occurs, in solution, upon rotation around the exocyclic CC double bond. The same mechanism is shown to be highly hampered in protein, owing to a combination of steric and electrostatic factors, and its inefficiency accounts for the

experimentally documented increase of the fluorescence quantum yield. While we cannot rule out that other radiationless deactivation mechanisms are or become active in protein, the present study demonstrates a remarkable difference between solvent and protein environments, which may justify the dramatic quenching of radiationless decay in the latter.

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Supporting Information Available: One table containing CASPT2 energies of the structures discussed in the text and Cartesian coordinates of all the structures discussed in the text, computed at HF/6-31+G*, CIS/6-31+G*, and CASSCF-(12,11)/6-31G* levels of theory, with or without PCM corrections included. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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