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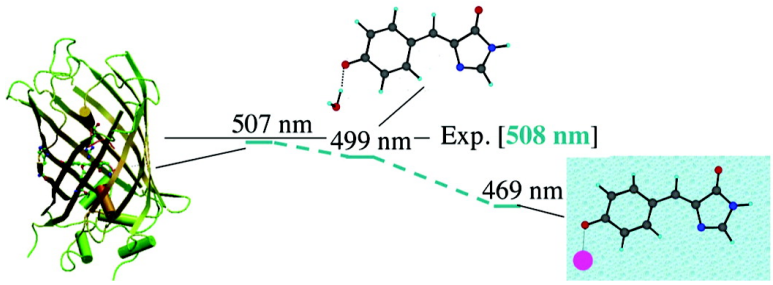
J. Am. Chem. Soc., **2005**, 127 (33), 11534-11535• DOI: 10.1021/ja045269n • Publication Date (Web): 30 July 2005



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Published on Web 07/30/2005

Properties of the Emitting State of the Green Fluorescent Protein Resolved at the CASPT2//CASSCF/CHARMM Level

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Green fluorescent proteins (GFPs) have been found in numerous bioluminescent organisms, such as the jellyfish *Aequorea victoria*[1] and the sea pansy *Renilla reniformis*.[2] Wild-type GFP (wt-GFP), features a *p*-hydroxybenzilideneimidazolinone chromophore (see **1** in Figure 1) located at the center of a barrel-like protein backbone.[3,4] The two peaks seen in the absorption spectra are attributed to distinct chemical states of the chromophore. The A state is assigned to the neutral form and the B state to a deprotonated (anionic) form. In contrast, the fluorescence spectrum shows only one peak attributed, within the three-state model,[4−8] to the I state (see Figure 1) featuring the anionic chromophore of B.

Presently, it is still not clear how the protein environment of wt-GFP tunes the spectral properties of the hydroxybenzilideneimidazolinone anion. Indeed, in water solution the same anion displays fluorescence and absorption peaks (λ_{max}) 18 and 70 nm blue-shifted with respect to the corresponding I state values.[4,9] Here, to determine the nature of the wt-GFP emitting domain, we employ "brute-force" ab initio QM/MM computations to model the ground (S_0) and singlet excited (S_1) states of I and of its chromophore in water. The results indicate that the emitter corresponds to a slightly perturbed H-bonded chromophore- - -H_2O pair. The rest of the protein seems to be designed in such a way to mimic an environment that is more similar to the gas-phase than to the solution.

Only few quantum chemical calculations have been performed for wt-GFP. The chromophore in vacuo has been investigated by Voityuk et al.[10−12] at the NDDO-G, INDO/S-CI, and CISD level, by Helms et al.[13] using post-SCF ab initio methods (CIS, MCSCF//MCQDPT), and by Das et al.[14] (SAC/SAC-CI). Attempts have been reported to study the chromophore in the protein[15,16] and in solution.[17−19] However, these calculations have not addressed the problem of the quantitative evaluation of absorption and fluorescence λ_{max}. In the past we reported[20] a quantum chemical study of the S_1 properties of the gas-phase wt-GFP chromophore. The results suggest that the theory required to correctly model its structural and spectroscopic parameters must include the treatment of dynamic electron correlation. In particular, the use of an ab initio CASPT2//CASSCF strategy (i.e. geometry optimization at the CASSCF level and energy evaluation at the CASPT2 level) yields an absorption λ_{max} only 15 nm off the observed value.[21] The same strategy has been recently employed within a QM/MM scheme to investigate the visual pigment rhodopsin.[22] The results indicate that the method is able to reproduce the absorption λ_{max} of the protein with a less than 40 nm error (less than 5 kcal mol^{-1}). Here the same computational scheme is used to investigate the wt-GFP chromophore in water and in the protein environment.

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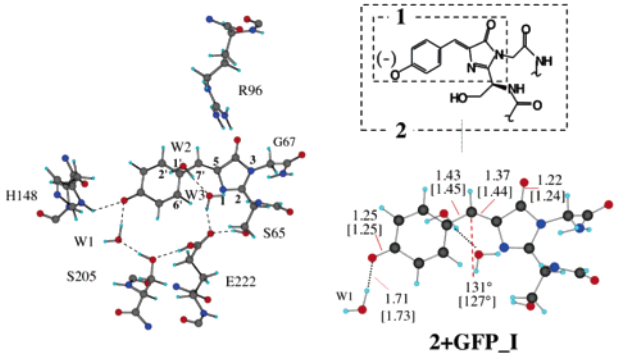

Figure 1. (Left) Cavity of the I state model. (Right) S_0 and S_1 (square brackets) CASSCF/CHARMM equilibrium structure of the I state chromophore. Parameters in Å and degrees.

Our wt-GFP model is derived from monomer A deposited in the PDB archive as file 1GFL. The I state has been set up by deprotonation of the chromophore *p*-OH and protonation of E222 yielding a neutral cavity. The orientation of the residues S205 and E222 are then relaxed to generate the accepted I state structure[6,8] featuring the H-bonding network −O(−)- - -W1- - -S205- - -E222- - -S65− (see Figure 1). Within our QM/MM scheme (see the Supporting Information, SI) the MM protein is kept frozen during the optimization while the QM moiety **2** (see Figure 1), comprising the chromophore and the two S65 and G67 linkages − and the position/orientation of three TIP3P water (W1, W2, and W3) are relaxed. The computations in solution have been carried out embedding the QM model **1** (derived from the substructure **1** of Figure 1) in a box of 701 TIP3P waters using periodic boundary conditions (the Na^+ counterion is treated at the MM level). The λ_{max} of a gas-phase binary complex (**1- - -H_2O**), of the chromophore in solution **(1+water)**, of the protein I state **(2+GFP_I)**, of the protein B state (**2+GFP_B**) and of a I state model with a disrupted W1- - -chromophore H-bond (**ub-2+GFP**) are evaluated as vertical differences in S_1 and S_0 energies. The quality of the computed S_1 and S_0 equilibrium structures for the solution and protein models have been assessed via simulation of the resonance Raman (RR) spectra of **1+water** and computation of λ_{max} of a model of the wt-GFP B state (**2+GFP_B**), respectively.[23]

As show in Table 1, for all models the computed λ_{max}'s compare well (error lower than 5 kcal mol^{-1}) with the observed quantities. Comparison of the gas-phase model **1**[20] with **1- - -H_2O** in Figure 2 reveals that hydrogen bonding of the chromophore to a *single* H_2O molecule leads to a dramatic change in S_0 structure. More specifically, this becomes remarkably similar to that found in **2+GFP_I**. Such change is explained by the increased anionic character of the oxygen due to stabilization of the negative charge

10.1021/ja045269n CCC: $30.25

Table 1. CASPT2//CASSCF/CHARMM Absorption and Emission λ_{max} and Change in Dipole Moment $\Delta\mu$

structure	exc.	λ_{max} (nm)[a]		$\Delta\mu$ (D)
1	$S_0 \rightarrow S_1$	465[b]	[480][c]	1.0
	$S_1 \rightarrow S_0$	507[b]	–	1.0
1- - -H_2O	$S_0 \rightarrow S_1$	453	–	4.0
	$S_1 \rightarrow S_0$	499	–	1.7
1+water	$S_0 \rightarrow S_1$	434	[425][d]	6.6
	$S_1 \rightarrow S_0$	469	[490][d,e]	5.4
2+GFP_I	$S_0 \rightarrow S_1$	468	[495][f]	4.3
	$S_1 \rightarrow S_0$	507	[508][g]	2.3
2+GFP_B[h,i]	$S_0 \rightarrow S_1$	442	[476][i]	5.4 [6.8][j]
	$S_1 \rightarrow S_0$	492	[503][i]	3.3
ub-2+GFP	$S_0 \rightarrow S_1$	471	–	0.8
	$S_1 \rightarrow S_0$	521	–	1.2

[a] Experimental values in square brackets. [b] Data for the 2,3-dimethyl derivative of **1** (HBDI). See ref 20. [c] Data from ref 21. [d] Data for HBDI. See ref 24. [e] While the calculated λ_{max} for **1+Water** cannot be directly compared with the experiment (the fluorescence in water has a quantum yield below 0.0001), the chromophore in a C_2H_5OH glass is highly fluorescent (λ_{max} ca. 490 nm). [f] See refs 5 and 25. [g] See refs 5, 6, and 8. [h] The B state model (see also SI) is derived from **2+GFP_I** by relaxing the T203 in a conformation allowing formation of an additional hydrogen bond with the phenate oxygen. [i] See ref 8. [j] See ref 7.

through hydrogen bonding. As reflected by the $\Delta\mu$ change reported in Table 1, (see charge analysis in the SI) upon $S_0 \rightarrow S_1$ excitation the oxygen charge migrates toward the imidazolinone moiety. This change in electronic structure is followed by a stretching relaxation that leads to the emitting states (i.e. to the S_1 equilibrium structure).

Inspection of Table 1 indicates that, in contrast to the solvated chromophore, **1- - -H_2O** "mimics" closely the properties of the I state with differences in the absorption and emission λ_{max} less than 15 and 10 nm, respectively. Furthermore, the $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_0$ $\Delta\mu$ agree within 0.6 D. This value reflects the similar magnitude of the instantaneous increase in negative charge of the imidazolinone moiety (e.g., for $S_0 \rightarrow S_1$, +0.23 e^- in **1- - -H_2O** and +0.26 e^- in **2+GFP_I**). In contrast, and consistently with its ca. 40 nm blue-shifted absorption, **1+water** display a larger (+0.36 e^-) negative charge increase that leads to a larger $\Delta\mu$ change and shorter S_1 C1′−C7′ bond. The spectral difference between **1+water** and **1- - -H_2O** could be primarily attributed to the more effective Na^+ stabilization of S_0 (featuring the negative charge on the oxygen) with respect to S_1 (where part of the negative charge has moved away from the oxygen and toward the imidazolinone).

The data reported in Figure 2 and Table 1 indicate that **2**- - -W1 (i.e. the "internal" **1- - -H_2O** complex) represents the emitting domain of wt-GFP. This is confirmed by the values of the absorption (470 nm) and emission (502 nm) λ_{max} of the **2**- - -W1 moiety taken with its **2+GFP_I** geometry (see SI for details). To demonstrate the importance of the bonding to W1, we report the S_0 and S_1 equilibrium structures of a (ca. 20 kcal mol^{-1}) higher-energy I-state configuration (**ub-2+GFP**) where such interaction has been disrupted and W1 is now linked to S205. In Figure 2 we show that in **ub-2+GFP** the S_0 and S_1 equilibrium structure of the chromophore becomes close to the gas-phase structure **1**. As shown in Table 1 this also displays $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_0$ $\Delta\mu$ closer to the corresponding gas-phase quantities.

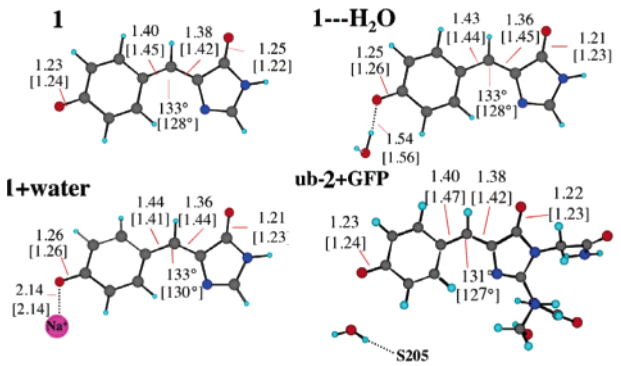

Figure 2. S_0 and S_1 (square brackets) CASSCF/CHARMM optimized structures of the models discussed in the text. Parameters in Å and degrees.

In conclusion, CASPT2//CASSCF/6-31G*/CHARMM computations can be used to study the structure and spectroscopy of GFPs. Remarkably, our results imply that, in the protein environment, the positively charged R96 counterion does not perturb much the electronic and molecular structure of the **2**- - -W1 chromophore.[26] This can only be explained by admitting that the charges distributed in the various parts of the protein cavity counterbalance each other in both the S_0 and S_1 states. A similar counterion quenching effect has been documented for rhodopsin[22] despite its completely different protein and chromophore structure.

Acknowledgment. We are grateful to Dr. S. R. Meech for helpful discussion. Funds are provided by Università di Siena (PAR 02/04), HFSP (RG 0229/2000-M), and CINECA. N.F. and T.A. are grateful for the EC fellowships HPMF-CT-2001-01189 and HPMF-CT-2002-01769.

Supporting Information Available: Methodological details; coordinates of all optimized structures; tables of energies, charge distribution, μ and oscillator strength f; simulated RR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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(26) An isolated R96- - -**2**- - -W1 moiety taken with its 2+GFP_I geometry displays an absorption λ_{max} 20 nm red-shifted with respect to **2**- - -W1 (see also SI). Thus, the rest of the protein seems to be able to quench this R96 effect.

JA045269N