

## Electronic Excitations of the Chromophore from the Fluorescent Protein asFP595 in Solutions

Alexander V. Nemukhin,<sup>\*,†,‡</sup> Igor A. Topol,<sup>§</sup> and Stanley K. Burt<sup>§</sup>

Department of Chemistry, M. V. Lomonosov Moscow State University,  
Moscow, 119992, Russia, Institute of Biochemical Physics, Russian Academy of  
Sciences, Moscow, 119997, Russia, and Advanced Biomedical Computing Center,  
National Cancer Institute at Frederick, Frederick, Maryland 21702

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**Abstract:** We present the results of modeling spectral properties of the chromophore, 2-acetyl-4-(*p*-hydroxybenzylidene)-1-methyl-5-imidazolone (AHBMI), from the newly discovered fluorescent protein asFP595 in different solvents and compare computational and recent experimental data. The time-dependent density functional theory (TDDFT) method is used to estimate positions of spectral bands with large oscillator strengths for vertical transitions to excited states following geometry optimizations of chromophore coordinates in vacuo and in solutions. The performance of different TDDFT functionals in computing excitations for a simpler chromophore from the green fluorescent protein was tested at the preliminary stage. Properties of various protonation states (neutral, anionic, zwitterionic) for the cis and trans conformations of AHBMI are compared. By using the polarizable continuum model, the following solvents have been considered for AHBMI: water, ethanol, acetonitrile, and dimethyl sulfoxide. It is shown that the bands found experimentally in aqueous solution refer to the cis neutral and cis anionic (or trans zwitterionic) conformations. The computed band positions deviate from experimental ones in water by no more than 35 nm (0.23 eV). In accord with experimental studies, the band shifts in different solvents do not show correlation with the dielectric constant or dipole moment; however, the computed values of the shifts are much smaller than those measured experimentally for the ionic species.

### Introduction

Proteins from the family of the green fluorescent proteins (GFP) are extensively used in molecular and cell biology<sup>1–3</sup> and promise a variety of important biotechnology applications.<sup>4</sup> A newly discovered GFP-like protein from the sea anemone *Anemonia sulcata* asFP595<sup>5</sup> is initially nonfluorescent, but in response to intense green light irradiation at 568 nm, it becomes brightly fluorescent (kindles) with emission at 595 nm. Photoswitching properties of this

kindling fluorescent protein may be useful for information storage in macromolecules or for creating triggerable markers in living cells. The mechanism of kindling is far from clear, and at present, substantial efforts are being undertaken to understand the intriguing properties of asFP595.<sup>6–9</sup>

The model chromophore from the kindling protein, 2-acetyl-4-(*p*-hydroxybenzylidene)-1-methyl-5-imidazolone (AHBMI), was recently elegantly synthesized<sup>9</sup> following crystallographic studies of the chromoprotein asFP595.<sup>6</sup> Its spectral properties in solution and their dependence on the pH and polarity of the solvent were investigated.<sup>9</sup> It was suggested that the bands in aqueous solution at 418 nm (2.97 eV) and 520 nm (2.38 eV) referred to the neutral and anionic states of the model chromophore. These absorption maxima experienced noticeable shifts in ethanol, 2-propanol, and

\* Corresponding author phone: 7-095-939-1096; fax: 7-095-939-0283; e-mail: anem@lcc.chem.msu.ru.

<sup>†</sup> M. V. Lomonosov Moscow State University.

<sup>‡</sup> Russian Academy of Sciences.

<sup>§</sup> National Cancer Institute at Frederick.

dimethylformamide compared to water, although no clear correlation with the dielectric constant of the solvent was seen.

The goal of this work is to model spectral features of the vertical  $S_0$ – $S_1$  transitions of AHBMI in vacuo and in solutions by using the time-dependent density functional theory (TDDFT) method<sup>10,11</sup> in conjunction with the polarizable continuum model (PCM)<sup>12,13</sup> for solvents.

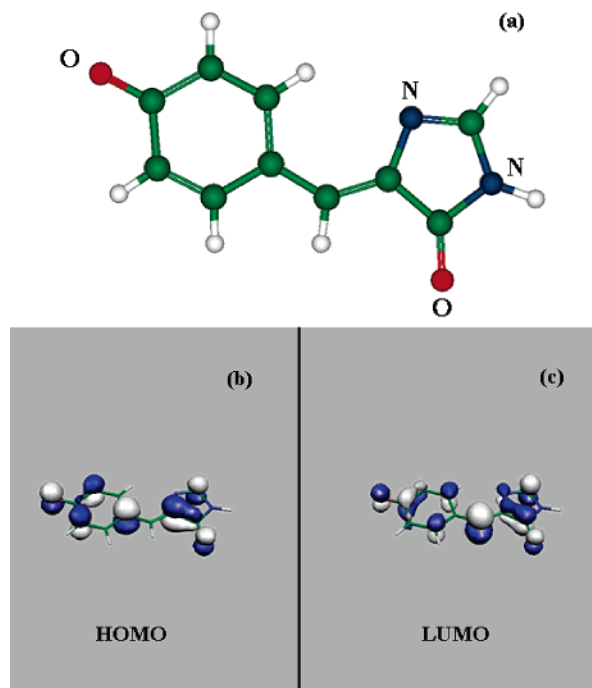
Since the pioneering estimates of the  $S_0$ – $S_1$  excitation energies in the GFP chromophore at the semiempirical INDO/S level by Voityuk and coauthors,<sup>14</sup> numerous attempts to calculate optical spectra of the GFP-like chromophores have been described in the literature. The reviews of Helms<sup>15</sup> and recent publications of Das et al.,<sup>16</sup> Marques et al.,<sup>17</sup> Laino et al.,<sup>18</sup> Toniolo et al.,<sup>19</sup> Martin et al.,<sup>20</sup> Vendrell et al.,<sup>21</sup> Altoe et al.,<sup>22</sup> Sinicropi et al.,<sup>23</sup> and Lopez et al.<sup>24</sup> present achievements of the theory in this field. Presently, it is believed that either the CASPT2//CASSCF<sup>20–23</sup> or TDDFT<sup>17,21,24</sup> method may provide reasonable excitation energies, although accurate prediction of the spectral band positions is still a problem. The state-of-the-art CASPT2//CASSCF approach was found to reproduce the absorption wavelength with a less than 40 nm error.<sup>23</sup>

The vast majority of theoretical simulations have been carried out for the simplest model GFP chromophores, for example, 4-hydroxybenzylidene-1,2-dimethylimidazolinone (HBDI).<sup>15–24</sup> The larger chromophore from asFP595, AHBMI, closely resembles the chromophores from the red fluorescent proteins DsRed and HcRed, whose spectral properties have been modeled in vacuo by using the TDDFT method in the B3LYP/6-31++G(d,p)//B3LYP/6-31+G(d,p) approximation.<sup>25,26</sup> The TDDFT technique, also at the same level, was applied by Xie and Zeng<sup>27</sup> for the characterization of another closely related chromophore, 4'-hydroxybenzylidene-2-methyl-imidazolin-5-one-3-acetate (HBMIA), in various protonation states of cis and trans isomers in vacuo followed by estimates of solvent effects in aqueous solution. For all these simulations, there are some quantitative discrepancies between computed and experimental absorption bands either in chromoproteins<sup>25,26</sup> or in solutions.<sup>27</sup>

In the following sections, we describe, first, the calculations of excitation energies for the anionic form of the simplest GFP model chromophore by exploring different TDDFT functionals and, second, the TDDFT calculations for the asFP595 chromophore in the B3LYP/6-311++G(2df,p)//B3LYP/6-31+G(d,p) approximation. In the latter case, optimization of the geometry parameters of all species and estimates of the spectral properties have been performed for gas-phase and solvent environments (water, ethanol, acetonitrile, and dimethyl sulfoxide) within the polarizable solvation model. These data are compared to the measurements of the absorption bands for this chromophore in water, ethanol, and dimethylformamide.<sup>9</sup>

## Results for the GFP Chromophore

Different estimates of the possible errors of the TDDFT approximation for the GFP-like chromophores may be found in the literature. For vertical excitation energies of the  $S_0$ –



**Figure 1.** Structure of the GFP chromophore (a) and views of the HOMO (b) and LUMO (c) orbitals computed in the B3LYP approximation.

$S_1$  transitions, these range from 0.44<sup>21</sup> to 0.1 eV.<sup>24</sup> Therefore, we performed preliminary calculations for the simplest model system relying on the available gas-phase experimental results for the excitation energy which refers to the GFP anionic state: 2.59 eV (479 nm).<sup>28</sup> Calculations have been carried out with conventional options of Gaussian 03.<sup>29,10</sup> Figure 1 illustrates equilibrium geometry configuration of the cis anionic form of the GFP chromophore. The coordinates of the molecule have been optimized by using the B3LYP/6-31++G(d,p) method.

In Table 1, we collect computed properties of the vertical excitations of the GFP anion calculated in different approximations. Excitation energies, corresponding wavelengths, and oscillator strengths are presented. The first part of Table 1 shows the dependence of computed parameters on the type of exchange-correlation functional in TDDFT while retaining the same basis set 6-31++G(d,p). The second part of Table 1 illustrates the basis set dependence for B3LYP as a choice of the particular functional in TDDFT. The dominant contribution to this  $\pi \rightarrow \pi^*$  type excitation refers to the HOMO  $\rightarrow$  LUMO transition; however, some mixtures from other orbitals appear for nonhybrid functionals. The sequence number (first, second, or third) of the needed excitation in every case is easily recognized by the value of the oscillator strength; other states (even with lower excitation energies) are characterized by  $f$  values close to zero. All the states with low oscillator strengths below the bright state are of the same  $\pi \rightarrow \pi^*$  origin.

As follows from these simulations, all local-density approximation (LDA) and gradient-corrected DFT functionals give very similar results. Hybrid functionals also provide values of the same quality; however, the more Hartree–Fock (HF) exchange is included in the functional, the greater is

**Table 1.** Characteristics of the Vertical Excitations of the GFP Anion Calculated in Different Approximations<sup>a</sup>

variables	excitation number	energy of HOMO (au)	$\Delta E$ (eV)	$\lambda$ (nm)	oscillator strength, $f$
Different Functionals (FUN) in FUN/6-31++G(d,p)//B3LYP/6-31++G(d,p) Calculations					
RHF	1	-0.114	3.50	353.8	1.206
SVWN <sup>b</sup>	2	-0.060	2.94	421.1	0.788
BVWN <sup>c</sup>	3	-0.060	2.94	421.9	0.798
BP86 <sup>d</sup>	3	-0.037	2.94	421.1	0.795
VXSC <sup>e</sup>	3	-0.033	3.02	410.1	0.834
MPW1PW91 <sup>f</sup>	2	-0.061	3.11	399.1	0.933
B972 <sup>g</sup>	2	-0.053	3.09	400.9	0.919
B1LYP <sup>h</sup>	2	-0.054	3.08	402.7	0.924
B3LYP <sup>i</sup>	2	-0.055	3.05	406.0	0.902
BHandHLYP <sup>j</sup>	1	-0.082	3.23	384.4	1.038
Different Basis Sets (BS) in B3LYP/BS//B3LYP/6-31++G(d,p) Calculations					
6-31G	2	-0.036	3.25	381.2	0.930
6-31G(d)	2	-0.032	3.22	384.5	0.899
6-31+G(d)	1	-0.054	3.06	404.8	0.906
6-31++G(d,p)	2	-0.055	3.05	406.0	0.902
6-31++G(2df,p)	2	-0.056	3.03	408.8	0.880

<sup>a</sup> In all cases, geometry coordinates have been computed in the B3LYP/6-31++G(d,p) approximation. The first part of the table shows the dependence of computed parameters on the type of functional in TDDFT while retaining the same basis set 6-31++G(d,p). The notation of functionals is given according to the Gaussian 03 system.<sup>29</sup> The second part of the table illustrates basis set dependence for B3LYP as a choice of the exchange-correlation functional in TDDFT.

<sup>b</sup> SVWN: (S) Slater exchange (Slater, J. C. *Quantum Theory of Molecules and Solids*; McGraw-Hill: New York, 1974; Vol. 4), (VWN) Vosko, Wilk, and Nusair correlation (Vosko, S. H.; Wilk, L.; Nusair, M. *Can. J. Phys.* **1980**, *58*, 1200). <sup>c</sup> BVWN: (B) Becke's 1988 exchange functional (Becke, A. D. *Phys. Rev. A* **1988**, *38*, 3098) and VWN correlation functional. <sup>d</sup> BP86: Becke's 1988 exchange, (P86) Perdew correlation functional (Perdew, J. P. *Phys. Rev. B* **1986**, *33*, 8822). <sup>e</sup> VXSC: van Voorhis and Scuseria's gradient corrected functional (Van Voorhis, T.; Scuseria, G. E. *J. Chem. Phys.* **1998**, *109*, 400). <sup>f</sup> MPW1PW91: Modified Perdew-Wang exchange and Perdew-Wang 91 correlation functionals (Adamo, C.; Barone, V. *J. Chem. Phys.* **1998**, *108*, 664). <sup>g</sup> B972: Wilson, Bradley, and Tozer's modification to the initial B971 functional (Orig.: Hamprecht, F. A.; Cohen, A. S. J.; Tozer, D. J.; Handy, N. C. *J. Chem. Phys.* **1998**, *109*, 6264. Modif.: Wilson, P. J.; Bradley, T. J.; Tozer, D. J. *J. Chem. Phys.* **2001**, *115*, 9233). <sup>h</sup> B1LYP: (B1) Becke's one-parameter hybrid exchange functional (Becke, A. D. *J. Chem. Phys.* **1996**, *104*, 1040); (LYP) Lee, Yang, and Parr correlation functional (Lee, C.; Yang, W.; Parr, R. G. *Phys. Rev. B* **1988**, *37*, 785). <sup>i</sup> B3LYP: (B3) Becke's three-parameter hybrid exchange functional (Becke, A. D. *J. Chem. Phys.* **1993**, *98*, 5648); LYP correlation. <sup>j</sup> BHandHLYP: (BHandH) Half-and-half hybrid exchange functional [ $0.5E_x(\text{HF}) + 0.5E_x(\text{LSDA}) + 0.5x\Delta E_x(\text{Becke88})$ ]; LYP correlation.

the energy gap  $\Delta E$  and the deviations from experiment are larger. The basis set dependence is saturated fairly fast.

A somehow discouraging result is that, in all approximations, the deviations from the experimental parameters (2.59 eV, 479 nm)<sup>28</sup> are considerable, giving rise to at least a 0.35 eV error. The best performance of the B3LYP method is characterized by the 0.44 eV error. These error bars seem to be typical for TDDFT applications to such complex systems.

## Results for the Gas-Phase and Aqueous Structures of the Chromophore from asFP595

The panels of Figure 2 show equilibrium geometry configu-

rations of cis and trans isomers of the neutral, anionic, and zwitterionic forms of the chromophore AHBMI. We do not overcrowd the pictures with the computed geometry parameters, which have been reported in many papers describing the GFP-like chromophores.<sup>2</sup> Instead, we collect the Cartesian coordinates of the corresponding structures optimized for the gas-phase conditions in the B3LYP/6-31+G(d,p) approximation in the Supporting Information. We should mention that the only nonplanar structure among those considered here refers to the cis zwitterionic species. This is due to the repulsion of the nearby hydrogen atoms from the five- and six-member rings, occurring in this particular arrangement. Reoptimization of the geometry parameters in the dielectric continuum corresponding to the aqueous solution ( $\epsilon = 80$ ) leads to small changes by no more than 0.02 Å in bond lengths and 1° in angles.

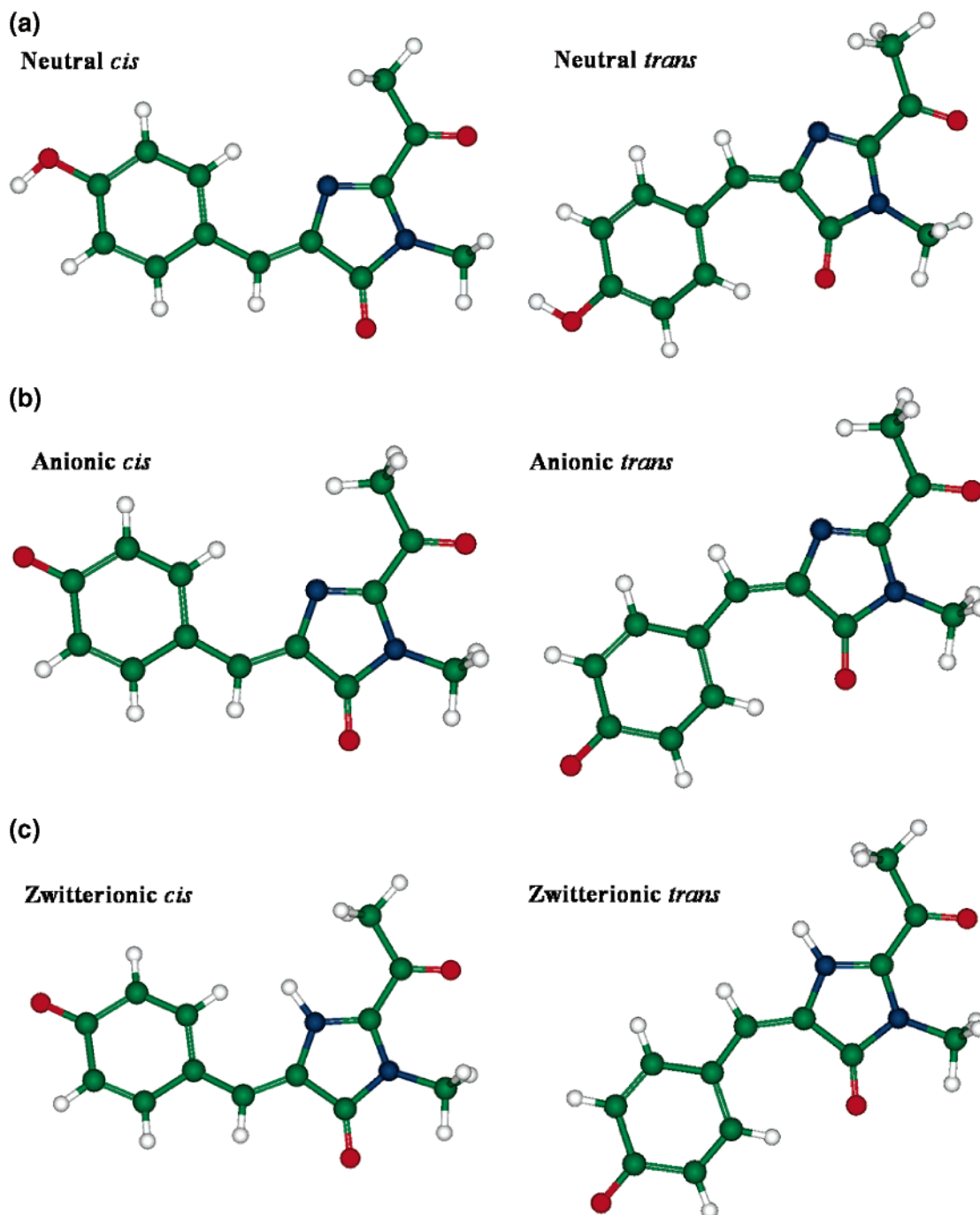
In Table 2, we present the total energies in vacuo and free energies in aqueous solution and the relative (trans vs cis) energies for these structures calculated in the B3LYP/6-31+G(d,p) approximation. According to these data, cis conformations are preferable for all species except the zwitterionic structure in aqueous solution.

A widely cited contribution to the question of the cis-trans isomerization of the GFP-like chromophore in the ground electronic state was the study of He et al.<sup>30</sup> These authors used NMR spectroscopy to characterize conformations of HBDI in water for the neutral, cationic, and anionic states. They found that, for the model chromophore, the cis isomers must be lower in energy by 0.8, 2.1, and 2.3 kcal/mol for the cationic, neutral, and anionic states, respectively. The corresponding activation barriers for cis-trans isomerization were estimated as 11.7, 13.1, and 13.1 kcal/mol for the cationic, neutral, and anionic forms, respectively.<sup>30</sup>

Recent quantum chemical calculations also provide support to a somewhat greater stability of cis isomers. In the semiempirical quantum mechanical/molecular mechanical calculations of Toniolo et al.<sup>31</sup> for the GFP chromophore in vacuo and inside the shell of explicit water molecules, it was observed that the cis conformer was more stable than trans as a consequence of "the larger dipole moment in the cis conformer". Wilmann et al.<sup>26</sup> presented, along with other findings for the HcRed fluorescent protein, the results of quantum chemical modeling of the cis and trans conformations of the HcRed chromophore in vacuo. According to the B3LYP/6-31++G(d,p)//B3LYP/6-31+G(d,p) calculations, the coplanar cis conformer is 1.7 kcal/mol lower in energy than the coplanar trans isomer.<sup>26</sup> The most recent work of Xie and Zeng<sup>27</sup> reported lower energies of the cis conformations for all protonation states (neutral, anionic, and zwitterionic) of the HBMI chromophore, as computed in the B3LYP/6-31++G\*\*//B3LYP/6-31+G\*\* approximation.

Therefore, our results are in line with previous findings showing a slightly lower energy of the cis forms for neutral and anionic species.

In Table 3, we present the results of calculations of vertical excitation energies, corresponding wavelengths, and oscillator strengths computed in the B3LYP/6-31++G(2df,p)//B3LYP/6-31+G(d,p) approximation both in vacuo and in aqueous solution. For the solution, geometry parameters have been



**Figure 2.** Structures of the AHBMI chromophore: (a) neutral, (b) anionic, (c) zwitterionic forms.

**Table 2.** Energies in Vacuo and Free Energies in Aqueous Solution (au) of the Forms of AHBMI (Figure 2)<sup>a</sup>

species	neutral	anionic	zwitterionic
Gas Phase			
cis	-837.843 79	-837.327 00	-837.812 65
trans	-837.841 14	-837.324 06	-837.811 78
energy of trans vs cis	1.66	1.84	0.54
Aqueous Solution			
cis	-837.849 76	-837.383 38	-837.826 37
trans	-837.847 15	-837.381 36	-837.832 82
energy of trans vs cis	1.64	1.27	-4.04

<sup>a</sup> Relative energies are given in kcal/mol.

optimized by using the PCM method. These data show noticeable solvent-induced shifts in band positions: -0.15 eV (+23 nm) for cis neutral, -0.09 eV (+18 nm) for cis anion, and -0.07 eV (+14 nm) for cis zwitterion. In accord

with experimental findings,<sup>9</sup> the intensities (oscillator strengths) of bands assigned to the neutral chromophore are lower than those of the ionic species.

### Comparison with Experimental Results for Excitations in Solution for the AHBMI Chromophore

The calculation results shown in Tables 2 and 3 for the aqueous solution allow us to confirm the experimental assignment that the band at a lower wavelength (418 nm) refers to the neutral chromophore and to suggest the cis neutral form (453 nm) as a primary candidate. Although the computed value for the trans isomer (437 nm) is closer to the experimental measurements, it is difficult to ignore the



**Table 3.** Excitation Energies, Corresponding Wavelengths, and Oscillator Strengths Computed in the B3LYP/6-311++G(2df,p)//B3LYP/6-31+G(d,p) Approximation

structure	$\Delta E$ , eV	$\lambda$ , nm	oscillator strength $f$
Gas Phase (Geometry Optimized in Vacuo)			
cis neutral	2.89	430	0.53
cis anionic	2.56	484	0.80
cis zwitterionic	2.38	521	0.68
Aqueous Solution (Geometry Optimized in Solution)			
cis neutral	2.74	453	0.72
cis anionic	2.47	502	0.94
cis zwitterionic	2.31	535	0.85
trans neutral	2.84	437	0.82
trans anionic	2.61	474	1.18
trans zwitterionic	2.48	500	1.10

higher energy of the trans structure (1.6 kcal/mol) and a possible fairly large rotational barrier for cis–trans isomerization in solution.<sup>30</sup> Even for the cis form, the discrepancy between calculated and experimental values (35 nm or 0.23 eV) falls within accepted error bars of the TDDFT model, as discussed above.

A comparison of computational and experimental results for anion species appears even more encouraging. The experimental band in water (520 nm) may be assigned to either the cis anionic (502 nm) or trans zwitterionic (500 nm) forms of the chromophore. In both cases, the deviations from experimental band positions are fairly small (18 nm or 0.09 eV and 20 nm or 0.10 eV, respectively). The cis anionic structure seems more preferable, considering that this band appears experimentally at basic pH.<sup>9</sup>

For different solvents, we reoptimized geometry configurations of the chromophore by using the B3LYP(6-31+G(d,p) approximation and the PCM model. There is no option in Gaussian 03 to treat dimethylformamide (DMF). Therefore, we considered acetonitrile and dimethylsulfoxide (DMSO), whose dielectric constants  $\epsilon$  and dipole moments  $\mu$  bracketed those of DMF. Then, excitation energies, wavelengths, and oscillator strengths were computed in the B3LYP/6-311++G(2df,p) approximation. The results are presented in Table 4.

**Table 4.** Comparison of Calculated (B3LYP/6-311++G(2df,p)//B3LYP(6-31+G(d,p)) Excitation Energies and the Corresponding Wavelengths for All Considered Solvents<sup>a</sup>

solvent	neutral		anionic		zwitterionic	
	$\Delta E$ , eV	$\lambda$ , nm	$\Delta E$ , eV	$\lambda$ , nm	$\Delta E$ , eV	$\lambda$ , nm
Cis Isomers						
gas phase ( $\epsilon = 1$ )	2.89	430	2.56	484	2.38	521
ethanol ( $\epsilon = 24.3$ )	2.74	453 ( <b>425</b> )	2.46	504 ( <b>542</b> )	2.30	538
acetonitrile ( $\epsilon = 36.3$ )	2.74	453 ( <b>422*</b> )	2.47	502 ( <b>572*</b> )	2.31	537
DMSO ( $\epsilon = 47.2$ )	2.71	458 ( <b>422*</b> )	2.43	511 ( <b>572*</b> )	2.27	545
water ( $\epsilon = 80$ )	2.74	453 ( <b>418</b> )	2.47	502 ( <b>520</b> )	2.32	537
Trans Isomers						
ethanol ( $\epsilon = 24.3$ )	2.83	438 ( <b>425</b> )	2.60	476 ( <b>542</b> )	2.46	504
water ( $\epsilon = 80$ )	2.84	437 ( <b>418</b> )	2.61	474 ( <b>520</b> )	2.30	538

<sup>a</sup> Shown in bold are the experimental results. By asterisk, we distinguish the wavelengths measured in DMF ( $\epsilon = 38.3$ ).

For the band associated with the neutral form in water (418 nm), an agreement between our theoretical estimates and experimental results<sup>9</sup> is reasonable: in both cases, the band position is predicted to be slightly sensitive to the solvent. For the band assigned to the anionic form, the distinctions are much larger, and the observed shifts of up to 50 nm when moving from water to DMF are not reproduced computationally.

We notice that the computed wavelength for the AHBMI chromophore in DMSO in the cis neutral form (458 nm) agrees perfectly with the measurements for the related chromophore HBMIA in DMSO (460 nm).<sup>32</sup>

## Discussion and Conclusion

The application of the TDDFT method for estimates of excited state parameters for fairly large molecules including the fluorescent protein chromophores is becoming very popular. The results of simulations described in this paper contribute to this growing field of computational chemistry. To some extent, the data collected in Table 1 confirm the observation formulated in ref 24 that the use of LDA-based functionals could lead to somewhat better agreement with experimental results; however, we cannot achieve such small errors in excitation energies of 0.1 eV as reported in ref 24 for the chromophore of the blue fluorescent protein. The 40 nm deviations in band positions for the GFP chromophore (or 5 kcal/mol as reported in ref 23, which equals 0.22 eV), illustrate the efficiency of the CASPT2//CASSCF approach. The wavelengths at 402 nm reported recently by Xie and Zeng<sup>27</sup> for the HBMIA chromophore (following their results of TDDFT calculations and estimates of aqueous shifts) show noticeable deviations from the experimental band position of this chromophore in DMSO at 460 nm. Most likely, the errors 0.2–0.4 eV may be expected when computing excited-state energies of GFP-like chromophores.

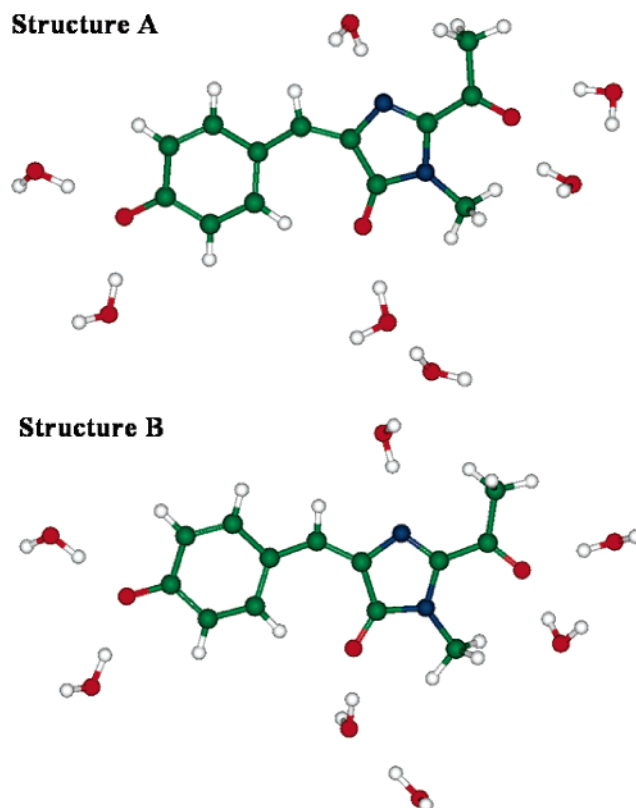
Within these error bars, our TDDFT simulations of spectral bands of the asFP595 chromophore in water are consistent with experimental findings.<sup>9</sup> In simulations, we can expand the experimental knowledge and assign particular conformations to the molecule in different protonation states. From energy considerations, the neutral form should correspond to the cis isomer (consistent with the results of NMR studies<sup>30</sup> of the GFP chromophore), and its absorption band is computed to be at 453 nm (2.74 eV). The deviation from

the experimental band position is 35 nm (0.23 eV). The anionic form of the chromophore observed at neutral and slightly basic pH values absorbs at 520 nm (2.38 eV) in experiments. Theoretically, this band could be assigned to either the cis anion (501 nm or 2.47 eV) or the trans zwitterion (500 nm or 2.48 eV) conformations, giving rise to about 0.1 eV errors. Interestingly, while all TDDFT calculations for molecules in vacuo overestimate excitation energies compared to experimental data, our simulations for the neutral form in aqueous solution result in a value which is underestimated by 0.23 eV. In accord with experimental results, the computed intensities of the neutral form are lower than those of the ionic form. Our calculations also show that the absorption bands in aqueous solution are red-shifted compared to the gas-phase positions by 14–23 nm, depending on the protonation state of the chromophore.

An agreement between our theoretical estimates by using TDDFT and PCM models and experimental results<sup>9</sup> for shifts of the band assigned to the neutral form in various solvents is reasonable. In both studies, the band position is predicted to be slightly sensitive to the solvent. However, an obvious discrepancy is noticed for other solvents, since in calculations the largest shift compared to the value in water ( $\epsilon = 80$ ,  $\mu = 1.85$  D) occurs for DMSO ( $\epsilon = 47.2$ ,  $\mu = 3.96$  D), but in experiments, it occurs for ethanol ( $\epsilon = 24.3$ ,  $\mu = 1.69$  D). For the band associated with the anionic form, the agreement is much worse. Experimentally, the large shifts are observed when moving from 520 nm in water ( $\epsilon = 80$ ) to 542 nm in ethanol and to 572 nm in DMF ( $\epsilon = 38.3$ ,  $\mu = 3.82$  D). Our simulations result in the band position lying within 10 nm at most for all considered solvents: water, ethanol, acetonitrile ( $\epsilon = 36.6$ ,  $\mu = 3.92$  D), and DMSO ( $\epsilon = 47.2$ ,  $\mu = 3.96$  D). The only common observation is that both in theory and in experiment no correlation with either dielectric constant or dipole moment of the solvent molecules is seen.

The authors of ref 9 suggested that the solvent protonic acidity rather than the solvent polarity accounts for observed shifts in band positions in different solvents. In particular, they assumed that “hydrogen bonds between the negatively charged phenolic oxygen and its surrounding solvent shells substantially increase the energy required for excitation”.<sup>9</sup> We verified this hypothesis by performing calculations for an extended molecular model, illustrated in Figure 3. We added seven water molecules in order to saturate hydrogen bonds of electronegative atoms and optimized (in solution) geometry parameters of the entire system in the B3LYP/6-31+G(d,p) approximation.

The obtained structures A and B differ by arrangements of the solvent molecules near the imidazole part of the chromophore. Structure A possesses negligibly lower total free energy in solution by 0.02 kcal/mol. In both structures, the phenolic oxygen is involved in the hydrogen bond network with the neighboring solvent molecules; however, the TDDFT [B3LYP/6-311++G(2df,p)] calculations in aqueous solution do not show noticeable changes in excitation energy. Compared to the case of implicit solvation within the continuum model (Table 3:  $\Delta E = 2.614$  eV,  $\lambda = 474$  nm), a new model results in the following values:  $\Delta E = 2.608$  eV and  $\lambda = 475$  nm for structure A and  $\Delta E = 2.58$



**Figure 3.** Equilibrium geometry configurations of the trans anion of AHBMI with seven explicit water molecules.

eV and  $\lambda = 480$  nm for structure B. Therefore, although the inclusion of explicit solvent molecules in the continuum model may account for slight changes in band position ( $\sim 5$  nm), hydrogen bonding of the chromophore with solvent species can hardly cause a substantial increase of excitation energy as suggested in ref 9.

In previous paragraphs, we cited the computed band positions for only cis isomers since they possess lower energies (except for the zwitterionic form). Theoretical calculations of  $pK_a$  values of the GFP chromophore<sup>32,33</sup> confirm that all protonation states may occur in solutions. The data obtained in NMR studies of the GFP chromophore in aqueous solution<sup>30</sup> show the barriers for cis–trans transitions for neutral and anionic forms as high as 10 kcal/mol or larger; however, such cis–trans isomerization cannot be excluded as discussed, for instance, in the paper of Xie and Zeng.<sup>27</sup> Although we investigated, in this work, the trans isomers as well, we could not find better agreement with the results of measurements than that presented above for the cis isomers.

We refer the last comment in our discussion to the statement in the paper of Yampolsky et al.<sup>9</sup> about an assignment of the observed weak red fluorescence of the model chromophore in DMF at 603 nm to the same process of absorption–emission as in the native protein asFP595. From simulations described in this paper and elsewhere,<sup>35</sup> this is hard to justify. In protein, the chromophore apparently resides in the trans conformation<sup>6–8</sup> in contrast to the solvent (the observed band at 520 nm in water is uniformly shifted by varying the solvent,<sup>9</sup> and therefore, there are no reasons to assume that in DMSO the chromophore is not in the cis

form). On the other hand, all calculations for gas-phase solutions and protein<sup>35</sup> predict greater wavelengths for cis structures compared to those of the trans species. From both theory<sup>35</sup> and experiment,<sup>8</sup> it follows that the chromophore in asFP595 is excited in the trans conformation and emits in the cis form. Therefore, the observed weak fluorescence in DMF<sup>9</sup> most likely should be assigned to another species.

In conclusion, we report calculations of the spectral properties of the chromophore, AHBMI, from the kindling fluorescent protein asFP595 in vacuo, water, ethanol, acetonitrile, and dimethylsulfoxide in various protonation states in cis and trans conformations by using the TDDFT [B3LYP/6-311++G(2df,p)] and PCM models for geometry parameters optimized for each environment in the B3LYP/6-31+G(d,p) approximation. Despite the simple treatment of the solvent shifts by the dielectric continuum model,<sup>36</sup> the calculation results agree with the majority of conclusions formulated in the experimental studies of this chromophore in water, ethanol, and dimethylformamide.<sup>9</sup> However, some discrepancies with experimental results are underlined with respect to the solvent shifts of the ionic form of the chromophore, as well as with the interpretation of the weak fluorescence in DMF.

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**Supporting Information Available:** Cartesian coordinates of the computed structures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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