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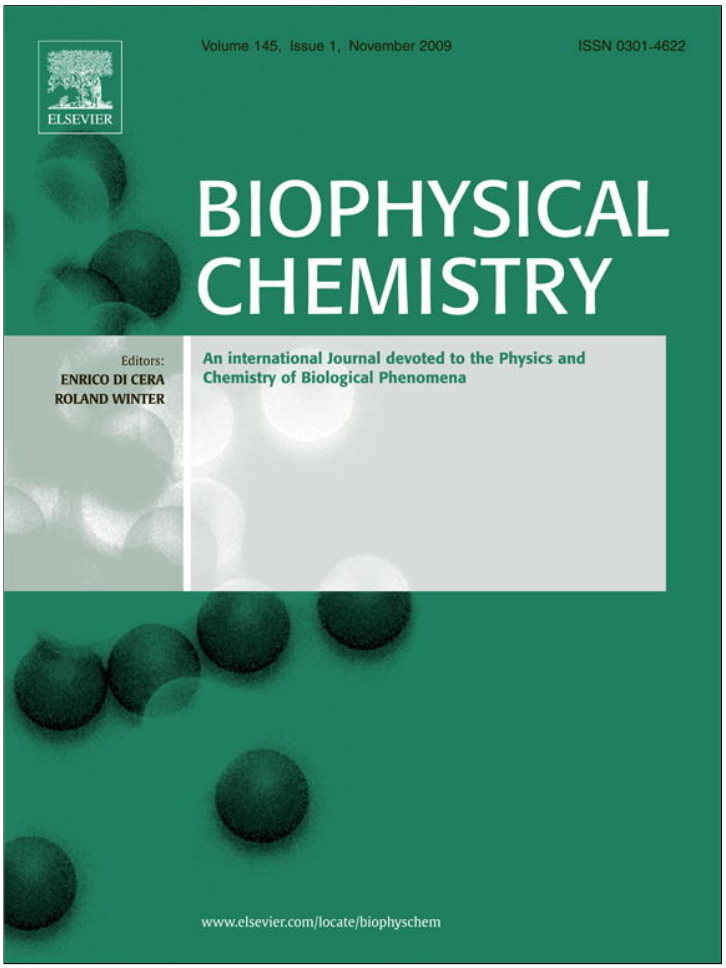

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On photoabsorption of the neutral form of the green fluorescent protein chromophore

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ABSTRACT

We present results of theoretical studies of the photoabsorption band corresponding to the vertical electronic transition S_0–S_1 between first two singlet states of the model chromophore from the green fluorescent protein (GFP) in its neutral form. Predictions of quantum chemical approaches including *ab initio* and semi-empirical approximations are compared for the model systems which mimic the GFP chromophore in different environments. We provide evidences that the protein matrix in GFP accounts for a fairly large shift of about 40 nm in the S_0–S_1 absorption band as compared to the gas phase.

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1. Introduction

In spite of wide applications of the famous green fluorescent protein (GFP) in biology and medicine [1–3] its photophysical properties are still not completely understood. Multiple efforts, both experimental and theoretical, are being undertaken to study absorption and emission processes in the GFP-related model systems ranging from isolated chromophores in the gas phase to numerous wild-type and mutated proteins from the GFP family. The GFP chromophore apparently may appear in the protein and in solution in different protonation forms, two of which, anionic (Fig. 1a) and neutral (Fig. 1b), attract the greatest attention of researchers.

In the wild-type GFP, the neutral form of the chromophore absorbs light at ~400 nm and the deprotonated anionic form absorbs at ~480 nm. Excitation at both wavelengths leads to fluorescence emission at 510 nm. A large shift of emission after 400 nm excitation is explained by the excited state proton transfer along the hydrogen bond chains in the protein connecting the phenolic and imidazolinone sites in the chromophore molecule [4,5].

Estimates of the absorption spectra of biological chromophores in the gas phase provide important information on the role of protein environment in tuning chromophore's spectral properties. Following experimental studies performed at the heavy-ion storage ring, Andersen and co-authors reported the absorption maxima at 479 nm for the anionic GFP chromophore [6,7]. Also an absorption maxima at 406 nm was attributed for the cationic species in which (compared to the neutral form) the proton was attached to the imidazolinone nitrogen. Recently, the same group reported a maximum absorption at 415 nm for the synthetic chromophore called "neutral$^+$" [8] shown in Fig. 2. This charged molecule which carries a positive charge in the $-NH_3$ group, presumably well separated from the "neutral" fragment of the chromophore, was supposed to mimic spectral properties of the gas phase GFP chromophore in its neutral form. Upon correcting the measured value of 415 nm for the presence of the charged group by using the results of quantum chemistry calculations (TDDFT with the B3LYP/6-311++G(d) approximation at the MP3 optimized geometry configuration) for the "neutral$^+$" and the true neutral species the authors finally reported the absorption maximum for the gas phase neutral GFP chromophore at 399 nm [8]. Since both gas phase values (399 nm for neutral and 479 nm for anionic) were found to coincide with those known for the chromophore inside the protein matrix (400 nm and 480 nm, respectively) the conclusion was that "the absorption properties of the green fluorescent protein to a high degree are determined by the intrinsic chromophore properties" [8]. Therefore, the role of the protein environment was suggested to be negligible.

The latter conclusion may be challenged following the results of careful quantum chemical calculations for a series of model systems which include the anionic and neutral forms of the GFP chromophore. It should be noted that accurate *ab initio* treatment of the vertical S_0–S_1 excitation in the GFP-like chromophores is still a hard task for quantum chemistry, and it is difficult to expect theoretical errors less than 15 nm in this range of 400–500 nm [9]. We do not concentrate in this paper on the anionic species which is a subject of many thorough theoretical studies [9–17], but mention that the agreement between

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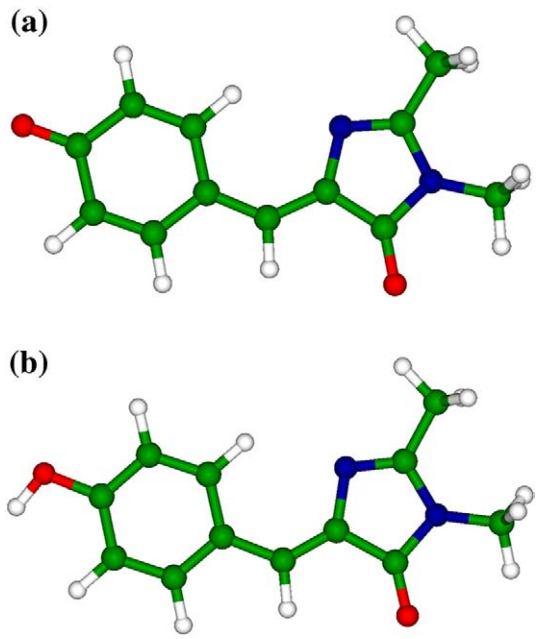

Fig. 1. Anionic (a) and neutral (b) forms of the GFP chromophore in the cis-conformation. Here and in other figures, carbon atoms are shown in green, oxygen in red, nitrogen in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the best theoretical estimates for the gas phase GFP chromophore and the experimental estimate of 479 nm [7,8] are consistent within the claimed error bars of ±15 nm. However, this is not the case for the neutral chromophore. In this case we compare the previously and newly obtained theoretical values for the several model systems which mimic the neutral GFP chromophore and provide evidences that the protein matrix in GFP accounts for a fairly large shift in the S_0–S_1 absorption band compared to the gas phase.

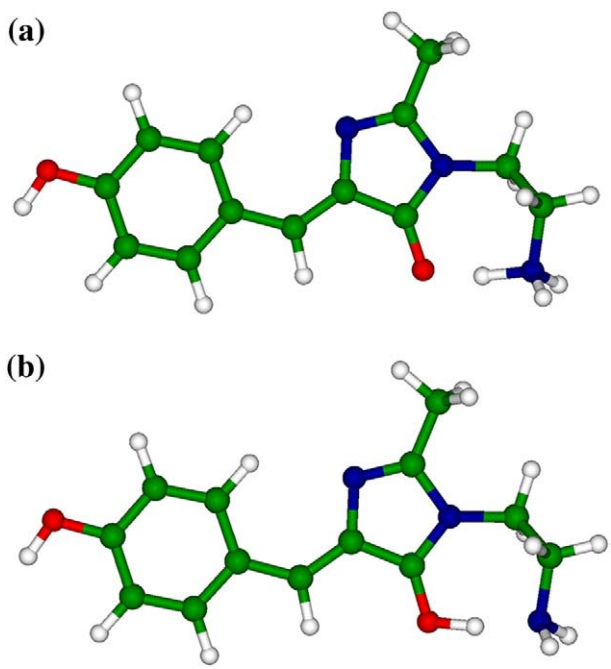

Fig. 2. Structures of the "neutral$^+$" chromophore. Panel (a) — the structure assumed in Ref. [8], panel (b) — the lower energy structure with the proton re-located from the NH_3^+ group to the carbonyl oxygen.

2. Computational methods

We considered either the isolated chromophore species as shown in Figs. 1 and 2, or the molecular clusters constructed on the base of the coordinates of heavy atoms from the crystal structure PDBID:1EMG [18]. To prepare the cluster systems (as one illustrated in Fig. 3) we surrounded the chromophore molecule by the nearest amino acid residues and water molecules, and kept the coordinates of their C_α atoms frozen as in the crystal structure upon optimization of all other coordinates.

In majority of calculations, the equilibrium geometry parameters of the model systems in the ground electronic state S_0 were obtained in the density functional theory (DFT) approach. For the isolated chromophore molecules (Fig. 1) we used either the B3LYP/6-31 + G (d,p) or PBE0/cc-pVDZ approximation. When considering the "neutral$^+$" structure (Fig. 2) the MP2/6-311 + G(d,p) approximation was also applied. Ground state geometry parameters of the molecular clusters were optimized by using the B3LYP/6-31 + G(d,p) method.

To compute vertical excitation energies from the respective minima on the S_0 potential surfaces of the model systems we considered several quantum chemical approaches. Vast amount of the results presented below was obtained with the semi-empirical ZINDO method [19]. As shown here and elsewhere [16,20] a strategy to estimate the S_0–S_1 excitation energies at the DFT-optimized equilibrium geometry parameters performs perfectly for these model systems. Another inexpensive approach is the time-dependent DFT (TDDFT) approximation [21]. From the *ab initio* quantum chemical side, it is feasible to perform calculations of the vertical $\pi\pi^*$ transition energies for the isolated chromophore molecule by using a very expensive and highly correlated approach, the state-averaged CASSCF wave functions augmented by perturbative corrections: multireference second-order Møller–Plesset perturbation theory (MRMP2) [22] and versions of the multiconfigurational quasidegenerate perturbation theory (MCQDPT2) [23–25]. These techniques, however, are computationally demanding, and their execution requires advanced skills and extreme care, as the application of the method involves: (i) a careful selection of a large number of active space orbitals in fairly large basis sets; (ii) converging the state- veraged CASSCF solutions corresponding to the $\pi\pi^*$ transition, especially in realistic basis sets; (iii) a careful and often ambiguous treatment of perturbative corrections to the reference CASSCF solutions.

Here, the B3LYP, MP2, TDDFT, ZINDO calculations have been carried out with the Gaussian03 program [26]. Calculations in the PBE0, MRMP2 and various versions of MCQDPT2 have been performed with the PC GAMESS program [27].

3. Results and discussion

(i) Ground state geometry configurations.

Computed ground state equilibrium geometry parameters of the anionic and neutral forms of the GFP chromophore (Fig. 1a,b) are consistent with the results of previous theoretical studies [14–17,28]. In the original paper of Lammich et al. [8] describing the gas phase "neutral$^+$" species (Fig. 2a), the authors reported the results of MP3 and B3LYP/6-31 + G(d) calculations of its structural parameters showing, in particular, a strong hydrogen bonding between the carbonyl oxygen of the imidasole ring and one of the ammonium hydrogens. More accurate calculations performed in this work apparently indicate that another structure with the proton attached to oxygen, but not to ammonium nitrogen (Fig. 2b) in fact refers to the lowest energy potential energy minimum. According to the MP2/6-311 + G(d,p) results the energy of the latter (Fig. 2b) is 0.5 kcal/mol lower than that of the initially suggested structure "neutral$^+$" (Fig. 2a). Apparently, the excitation energy of the structure shown

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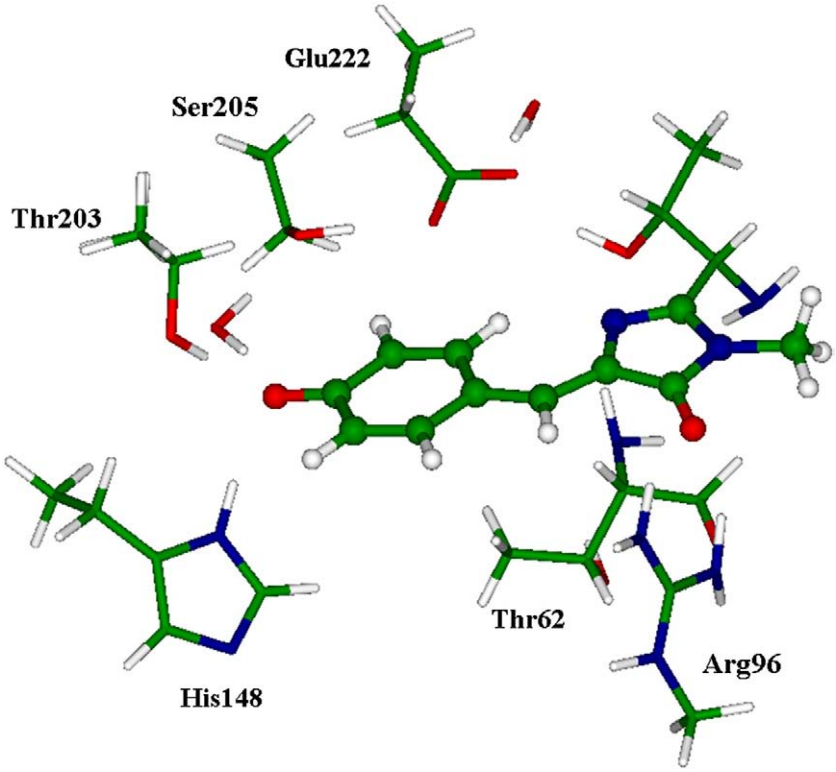

Fig. 3. The molecular cluster based on the PDBID: 1EMG structure for simulating properties of the anionic chromophore inside the protein.

in Fig. 2b may not coincide with that of the true neutral form of the GFP chromophore (Fig. 1b).

(ii) Anionic form of the isolated chromophore.

The computed vertical excitation energy for the anionic form of the gas phase GFP chromophore was reported in several previous works. There is no principle disagreement in all of them, and the experimental value of the wavelength of 479 nm [7,8] is basically reproduced in reliable calculations with the errors of about 15 nm. We make a reference to the most recent theoretical paper [9] in which the problems of using advanced quantum chemical approaches are discussed in details. Here we report the results of calculations for the anionic form only to justify the theoretical approaches utilized for the neutral form. Namely, we obtained the precise experimental value of 479 nm by using the ZINDO approach and the B3LYP/6-31 + G(d,p) optimized geometry parameters, and the value of 491 nm by using the MRMP2/cc-pVDZ approach with the state-averaged CASSCF(14/12) electron density and the PBE0/cc-pVDZ geometry parameters.

(iii) Absorption of the neutral form of the isolated chromophore.

The S_0–S_1 absorption band maximum transition for the neutral GFP chromophore in the gas phase (Fig. 1b) was calculated in several previous projects. Lammich et al. [8] reported the value 359 nm computed in the TDDFT approximation (B3LYP/6-311 + + G(d) using the MP3 optimized ground state geometry configuration). Close values, 362 nm [16] and 358 nm [29], were also obtained with the TDDFT approach. Toniolo et al. [28] performed a re-parameterization of the semi-empirical AM1 approach what allowed them to estimate the absorption maximum at 343 nm. Highly correlated *ab initio* treatment at the SAC–CI (symmetry-adapted cluster–configuration interaction) level carried out by Das et al. [13] resulted in the value 372 nm. In the later calculations with the improved SAC–CI algorithms Hasegawa et al. reported the absorption wavelength at 383 nm [30]. Bravaya et al. [17] optimized the equilibrium geometry parameters in the PBE0/cc-pVDZ approach and estimated electronic excitations by using three versions of the perturbation corrections (MRMP2 [22], MCQDPT2 [23] and aug-MCQDPT2 [24]) with the electronic density averaged over four first states in the CASSCF(16/14)/cc-pVDZ approximation. Such obtained values for the absorption maxima were as follows: 343 nm (MRMP2), 334 nm (MCQDPT2), 399 nm (aug-MCQDPT2). It was pointed out that treatment of the neutral form of the GFP chromophore in this formalism was complicated since the CASSCF method resulted in the wrong ordering of the electronic states even with the almost full π-electron active space. As noticed later by Granovsky [http://classic.chem.msu.su/gran/gamess/index.html/mcqdpt2.pdf], an application of the MCQDPT2 method in certain cases might not result in accurate estimates for the excitation energy. Therefore, the reported values [17] for the MCQDPT2 and aug-MCQDPT2 approximations should not be considered as fairly reliable.

Calculations performed in this work also specified difficulties in applications of electronic structure methods to describe the neutral form of the GFP chromophore. Table 1 includes the data obtained with the TDDFT approach by using either B3LYP, or BP86 functional, showing energy gaps, wavelengths and oscillator strengths for the first two bands, S_0–S_1 and S_0–S_2. Apparently, the TDDFT methodology exhibits a mixing of electronic configurations when considering

Table 1
The results of calculations performed in this work for the neutral GFP chromophore (Fig. 1b).

Method		ΔE, eV	λ, nm	f
B3LYP//B3LYP/6-31G*	S_0–S_1	3.54	350	0.7
	S_0–S_2	4.25	292	0.1
B3LYP//B3LYP/6-31 + G**	S_0–S_1	3.46	359	0.7
	S_0–S_2	4.18	296	0.1
BP86//B3LYP/6-31G*	S_0–S_1	3.26	381	0.5
	S_0–S_2	3.69	336	0.2
BP86//B3LYP/6-31 + G**	S_0–S_1	3.19	389	0.6
	S_0–S_2	3.65	340	0.2
MRMP2(CASSCF(14/12)/cc-pVDZ //PBE0/cc-pVDZ	S_0–S_1	3.40	364	0.9
ZINDO//B3LYP/6-31 + G**	S_0–S_1	3.45	360	1.0

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electronic excitations in the neutral form of the chromophore. The same problem is encountered in the CASSCF-based calculations. The CASSCF(14/12)/cc-pVDZ method predicts almost degenerate energy values for the first and second excited states. Application of the MRMP2 theory above the averaged over 3 states CASSCF electron density allowed us to estimate the absorption maximum for the S_0–S_1 transition at 364 nm. The ZINDO//B3LYP/6-31 + G** method, which produces a precise experimental value for the anionic form of the GFP chromophore, describes this transition with the oscillator strength close to 1.0 and gives 360 nm for the wavelength.

From the previous and newly obtained results we may conclude that the reliable electronic structure methods predict the absorption maximum for the neutral GFP chromophore between 360 and 380 nm.

(iv) Absorption of the "neutral$^+$" species.

Experimental estimate for the wavelength of absorption maximum in the "neutral$^+$" species was 415 ± 5 nm [8], and the authors assumed that this value referred to the structure shown in Fig. 2a. According to our calculations, another structure (Fig. 2b) with somewhat lower energy may be formed in the gas phase as well.

The computed here vertical excitation energies converted to the corresponding wavelengths are as follows: the TDDFT approach with the B3LYP functional gives 360 nm for both forms, while the use of the BP86 functional results in 399 nm for both forms (the difference between two conformations of "neutral+" accounts for less than 1 nm).

The results obtained with the ZINDO//MP2/6-311 + G(d,p) and MRMP2//PBE0/cc-pVDZ approaches are summarized in Table 2.

Unlike TDDFT, both these approaches predict a noticeable difference in absorption maxima between the true neutral GFP chromophore and the "neutral$^+$" species in the gas phase.

We may speculate that the experimentally studied molecule "neutral$^+$" [8] better describes a perturbed cationic form of the GFP chromophore rather than the true neutral form. Two structures (Fig. 2a and b) of the "neutral+" species in the gas phase most likely coexist since their energies differ by about 0.5 kcal/mol. The results of ZINDO calculations for both forms (the second column of Table 2) are consistent with the experimental estimate at 415 ± 5 nm [8].

(v) Calculations with molecular clusters.

The primary goal of present calculations with molecular clusters was to obtain computationally the shifts in absorption maximum wavelengths when comparing the isolated chromophore and the chromophore inside the protein.

Our cluster model (Fig. 3) accounts for the effect of the amino acid side chains nearest to the chromophore moiety. With this model we mimicked computationally two protonated forms (anionic and neutral) of the chromophore inside the protein matrix. An initial model based on the PDBID: 1EMG structure (Fig. 3) included the chromophore in the anionic form, the side chains of Thr62, Arg96, His148, Thr203, Ser205, Glu222 and two water molecules. The optimized geometry parameters of the cluster in the B3LYP/6-31 + G(d,p) approximation corresponded to the anionic form of the chromophore in the protein environment. The computed absorption wavelength in the ZINDO//B3LYP approximation results in the value 502 nm which is about 20 nm above the experimental value for GFP containing the anionic chromophore.

Then we added a proton to the model system which principally may be shared by the chromophore and the His148 side chain. Optimization of geometry parameters showed that the added proton was located at the phenolic oxygen accounting for the neutral form of the chromophore inside the protein (Fig. 4). The computed in the ZINDO//B3LYP/6-31 + G(d,p) approximation the absorption wavelength maximum is at 411 nm which is about 10 nm above the experimental value for GFP containing the neutral chromophore. We note that for both model clusters (Figs. 3 and 4), the deviations of computed wavelengths from the corresponding experimental data are small enough (+20 and +10 nm).

These calculations resulted in the shift (Δλ) in the position of absorption maximum when passing from the gas phase to the protein environment of about +23 nm for the anionic chromophore, and about +51 nm for the neutral chromophore. Such computed values for the excitation energies cannot pretend for a great accuracy, but we stress that apparently the shifts Δλ due to the protein environment are substantial.

(vi) The Y67H and Y67W mutants of GFP

Additional arguments in favor of our statement that the absorption maximum of the true neutral GFP chromophore in the gas phase should be closer to 360 nm than to 400 nm may be put forward when considering the GFP variants (called monomeric teal FP, mTFP1) in which the tyrosine-derived chromophore is replaced by the histidine (Y67H) and tryptophane (Y67W) derived moieties. Campbell et al. [31] reported, in particular, absorption maxima for the corresponding proteins as 369 nm for Y67H and 432 nm for Y67 W (the latter value refers to the mean wavelength of two close "humps" at 424 and 440 nm typical for a tryptophane-derived chromphore [31]. A detailed description of the simulations for the mTFP1 protein and its chromophores will be presented elsewhere. Here we consider only the results for the absorption maximum wavelengths estimated for the isolated chromophores in the ZINDO//B3LYP/6-31 + G(d,p) approximation.

Combined with the experimental absorption maximum for GFP at 400 nm, we have at hand a set of reference wavelengths (369, 400, 432 nm) of three neutral chromophores (Y67H, GFP and Y67 W) inside the protein matrices. Structures of these three chromophore molecules are illustrated in Fig. 5. In calculations, we optimized the equlibrium geometry parameters of the gas phase chromophores in the neutral state in the B3LYP/6-31 + G(d,p) approximation. In legend to Fig. 5 we also point out the mean polarizabilities of the molecules, $\alpha = 1/3(\alpha_{XX} + \alpha_{YY} + \alpha_{ZZ})$, computed in the B3LYP/6-31 + G(d,p) approximation by using the G03 program [26]. When placing the chromophore moieties in the protein environment the shifts Δλ in the absorption maximum from the gas phase to the protein must correlate with the polarizabilites of the guest species.

For the series of chromophores illustrated in Fig. 5 the ZINDO//B3LYP/6-31 + G(d,p) approximation gives 355, 360 and 380 nm, correspondingly. When combining these values with the above cited experimental data for the proteins we obtain the shifts Δλ (14, 40 and 52 nm) placed in the graph of Fig. 6.

Apparently, the value for the gas phase absorption wavelength maximum at 360 nm for the neutral GFP chromophore perfectly matches the correlation Δλ versus chromophore polarizability, but the value 399 nm [8] does not.

Table 2
The computed wavelengths of the S_0–S_1 transition.

Species	ZINDO	MRMP2
True neutral GFP (Fig. 1b)	360	364
"Neutral+", conformation, higher energy (Fig. 2a)	418	408
"Neutral+", conformation, lower energy (Fig. 2b)	436	413
Cationic form of the GFP chromophore	411	401

4. Conclusion

In this paper we present evidences that raise doubts on the estimated wavelength for the gas phase GFP chromophore in the neutral form at 399 nm as estimated [8] through the measurements

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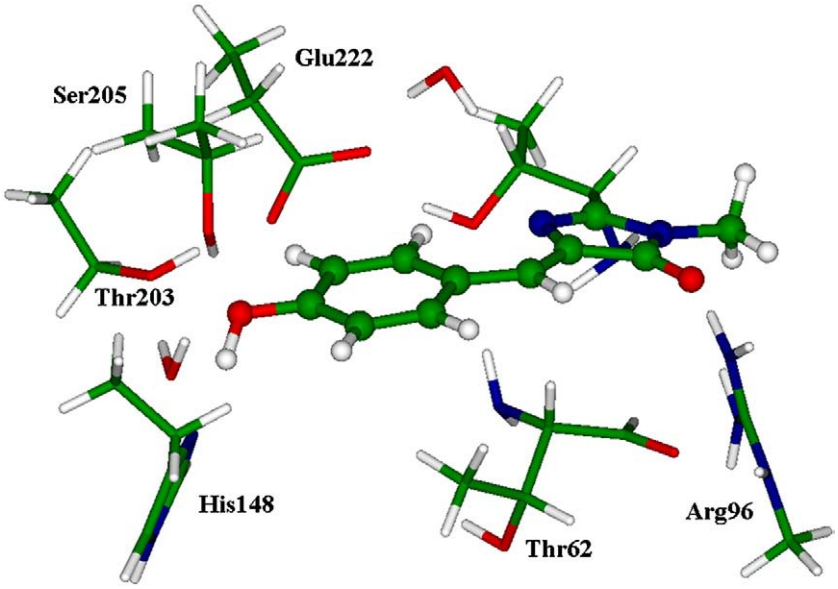

Fig. 4. The molecular cluster for simulating properties of the neutral chromophore inside the protein.

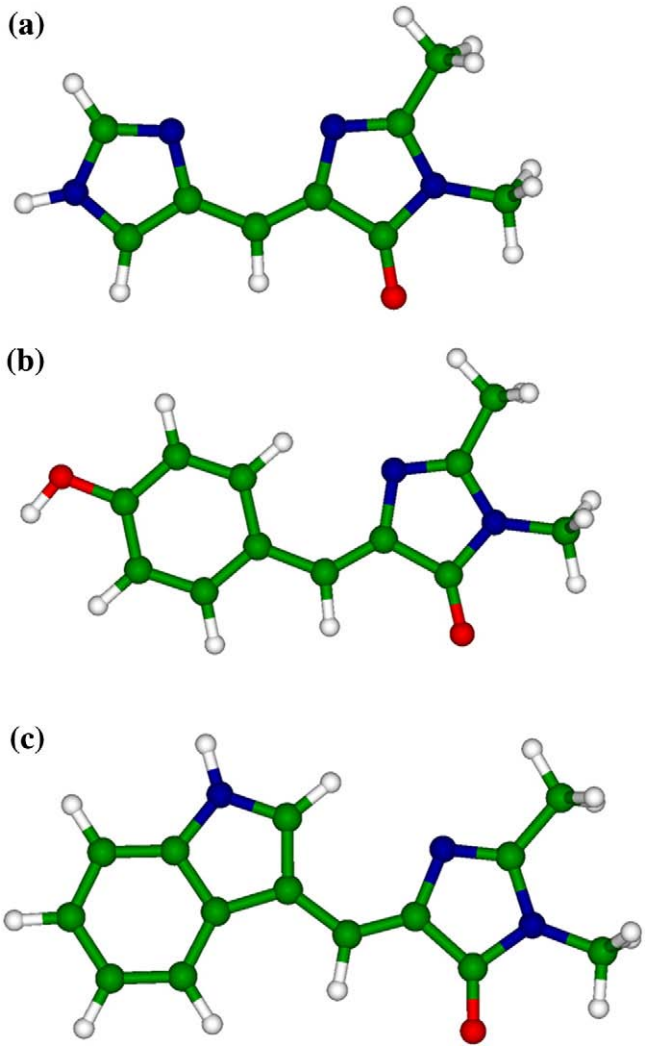

Fig. 5. Calculated structures of the neutral GFP and GFP-related chromophores and their mean polarizabilities in atomic units: (a) Y67H ($\alpha = 125$), (b) GFP ($\alpha = 194$), (c) Y67W ($\alpha = 221$).

for the related "neutral$^+$" species (Fig. 2). This conclusion is based on the analysis of several model systems and different simulation approaches. We believe that for the isolated neutral GFP chromophore the absorption maximum should be closer to 360 nm. Therefore, the protein matrix in GFP accounts for a fairly large shift of about 40 nm in the S_0–S_1 absorption band compared to the gas phase.

When this paper was under review, a new publication of Filippi et al. [32] appeared which provided a strong support to our conclusions from the side of the high level *ab initio* calculations. The authors also raised doubts on the interpretation of photodestruction spectroscopy experiments for the neutral GFP chromophore [8]. An agreement between the results of the modeling approaches applied in this work and those of the *ab initio* based methods for GFP encourage us to consider other fluorescent proteins, for which new experimental results have been published recently, in particular, [33–35], with the same modeling tools. These results which will appear shortly demonstrate a good correlation between the theoretical and experimental findings.

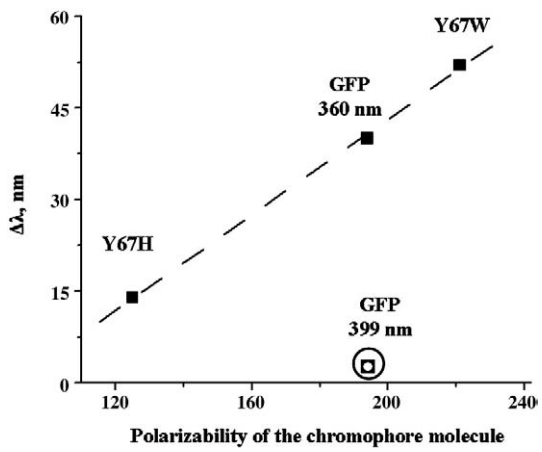

Fig. 6. Correlation between the shifts in absorption maxima wavelength when comparing the gas phase and protein absorption of the chromophores shown in Fig. 5. The circled symbol shows the position of the GFP species if one assumes the gas phase value 399 nm [8] instead of 360 nm.

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