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几个荧光蛋白发色团双光子吸收性质的理论研究

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摘要: 实验测得的荧光蛋白的单、双光子吸收光谱在低频和高频区域都表现出明显不同的特征。为了揭示这些不同点的起源和研究荧光蛋白的构-效关系, 我们详细研究了三种荧光蛋白发色团(一种增强型蓝绿色荧光蛋白的中性发色团和两种红色荧光蛋白的阴离子发色团)的单、双光子吸收特性, 分别计算了纯的和振动分辨的电子谱。计算结果表明: 光谱线形与计算采用的交换相关密度泛函及谱截面计算所采用的近似关系密切; 如果在计算光谱截面时, 我们利用长程修正的交换相关泛函CAM-B3LYP来计算几何和电子结构参数, 然后把Franck-Condon (FC)效应和包含Herzberg-Teller (HT)效果的电-声耦合效应都考虑进去, 理论计算的光谱与实验测定的光谱可以很好地符合; 对于两种离子态的发色团, HT电-声耦合效应使得对应于基态到第一激发态跃迁的双光子吸收最强峰相对于单光子吸收的最强峰发生了蓝移, 但HT电-声耦合效应对高频的双光子吸收谱没有太大的影响; 分子内电荷转移是导致高频区的双光子吸收明显强于单光子吸收的主要原因。

关键词: 双光子吸收; 荧光蛋白发色团; 振动光谱; 含时密度泛函理论

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Two-Photon Absorption Properties of Chromophores of a Few Fluorescent Proteins: a Theoretical Investigation

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Abstract: The experimentally-measured two-photon absorption (TPA) spectra of fluorescent proteins (FPs) show quite different characteristics with one-photon absorption (OPA) spectra in both the low- and high-frequency regions. To reveal the mechanism that results in the discrepancies between OPA and TPA spectra, and to obtain the fundamental structure–property relationships of FPs, here we conduct a theoretical study of OPA and TPA properties of three FP chromophores, including a neutral chromophore in enhanced cyan fluorescent protein (ECFP) and two anionic FP chromophores in DsRed2 and TagRFP. Both the pure electronic and vibrationally-resolved TPA spectra have been calculated. The calculated spectra were found to be highly dependent on the density functional theory exchange-correlation functional used. The experimental spectral lineshapes of vibronic spectra can be well produced when the Franck-Condon (FC) scattering and Herzberg-Teller (HT) vibronic coupling effects were taken into account and the structure parameters produced by CAM-B3LYP were applied in the theoretical calculations. The HT effects affect the low-frequency absorption bands corresponding to the electronic transition from S_0 to S_1 for two anionic chromophores, leading to a blue-shift of the TPA maximum relative to OPA maximum, while the HT effect is insignificant in the higher-frequency region of the TPA spectra. The intramolecular charge-transfer

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character of higher-lying excited states explains why the TPA spectra in the higher-frequency region are much stronger than those in the low-frequency region.

Key Words: Two-photon absorption; Fluorescent protein chromophore; Vibronic spectrum; Time-dependent density functional theory

1 Introduction

In recent years, the two-photon absorption (TPA) phenomena of fluorescent proteins (FPs) have attracted a lot of interests¹⁻⁵. The main reason is that the TPA spectra of FPs have fascinating applications in bioimaging and medical technology as biomarker, biosensor^{6,7}, and now even in communication technology⁸. Especially, FPs are widely used in two-photon laser scanning microscopy, which can penetrate deeper tissue with less damage⁹. Therefore, the scientists have performed a large amount of experimental studies to investigate which proteins are the brightest and what are the best excitation wavelengths. However, due to competing optical processes such as stimulated emission and scattering that arise in high-intensity regime inherent in these experiments, it is difficult to experimentally measure the absolute TPA cross sections¹⁰. In biological systems, there are additional calibration difficulties leading to significant discrepancies in the reported measurements of absolute TPA cross sections². It is thus essential to theoretically calculate the TPA cross sections to assist experimental studies for describing the microscopic excited-state dynamics, explaining the experimental results and designing new materials with desired non-linear optical properties as well¹¹.

Numerous computational studies of TPA phenomena have been reported in the past couple of decades within the framework of time-dependent density functional theory (TDDFT)¹²⁻²⁰, a lower-level couple cluster method²¹ or the equation-of-motion coupled-cluster method¹¹. However, most of theoretical works were focused on the calculations of pure electronic spectra. The vibrational motions and the vibronic coupling effect are not incorporated into the spectra. Compared with the pure electronic spectra, vibrationally-resolved electronic spectra show the detailed characteristic of molecular vibrational motions and reveal more reliable molecular structure-property relationship. In this work, we thus simulate the vibrationally-resolved OPA (VROPA) and TPA (VRTPA) spectra of a few FP chromophores to obtain the fundamental structure-property relationships, unveil the origin of the significant discrepancies between OPA and TPA spectra, and reach a close comparison with the experimental spectra.

From the theoretical point of view, TPA is a non-linear optical process with a quadratic dependence of the absorption strength on the intensity of the incident light. Since two-photon transitions become allowed only in the second order of perturbation theory, TPA cross sections are usually much small. However, in TPA spectra of FP chromophores², it is observed that the TPA spectra in high-frequency region are stronger than

that in low-frequency region, while the OPA spectra are extremely weak². It is thus urge to know the intrinsic physical mechanism which is called quantum mechanical effect known as resonant enhancement by some researchers²². We here conduct a theoretical calculation of both the TPA and OPA spectra for a few FP chromophores. The time-dependent approaches to model OPA and TPA cross sections with inclusion of Franck-Condon (FC) scattering and Herzberg-Teller (HT) vibronic coupling effects will be used^{23,24}. Besides the efficient dynamic method, the accurate structure parameters are further needed to predict OPA and TPA cross sections. In the electronic structure calculations, the ground-state properties can be easily obtained, and the main difficulty is to provide a good description on the resonant excited-state potential energy surfaces. In principle, high-accuracy wavefunction-based correlated *ab initio* methods can provide an accurate description of excited electronic states. However, these techniques are currently computationally intractable when applied to molecules of practical interests. Alternatively, time-dependent density functional theory (TDDFT) approach has been proposed to calculate the excited-state properties. The successful realization of the analytical excited-state Hessian within the framework of TDDFT²⁵⁻²⁹ has made the large-scale numerical calculations feasible on the excited-state vibrational frequencies and other related physical quantities, such as the geometrical derivatives of the transition dipole moment for the medium-sized systems.

Three model FP chromophores in gas phase and water solution are investigated, which include a neutral chromophore in photoactive enhanced cyan fluorescent protein (ECFP)³⁰ and two anionic FP chromophores in DsRed2 and TagRFP³¹. As a result, the environment-dependent characteristics of OPA and TPA spectra of those model chromophores are exhibited. As the representation of neutral chromophore, ECFP shows an apparent overlap between the OPA spectra and TPA spectra in the longest-wavelength absorption band, corresponding to the electronic transition from the ground state (S_0) to the first singlet excited state (S_1). However, as the representation of anionic chromophores, Dsred2 and TagRFP show a distinct blue-shift of the low-energy TPA maxima relative to the OPA maxima. However, in higher-frequency regions, the OPA and TPA spectra of two-types of FP chromophores exhibit similar characteristics. The TPA is very strong while the OPA is extremely weak. Those strong resonant enhancements^{32,33} appeared in TPA spectra in the short wavelength definitely have crucial relationship with the characters of higher-lying excited states³⁴. It is thus essential to present a profound insight to address the issue.

2 Computational details

On the basis of the perturbation theory, the transition rate of one-photon (two-photon) transition from the initial state $|I\rangle$ to the final state $|F\rangle$ is proportional to $|\langle F|\mathbf{M}|I\rangle|^2 g(\Delta w)$. Here, $g(\Delta w)$ is the lineshape function with $\Delta w = w_i - w_{FI}$ ($\Delta w = w_1 + w_2 - w_{FI}$) in one-photon (two-photon) transition. Here w_i , w_1 , and w_2 denote the incident photon frequencies, the one-photon and two-photon transitions are separately expressed as follows:

$$\mathbf{M} = \boldsymbol{\mu}, \quad (1)$$

$$\mathbf{M} = \sum_L [\boldsymbol{\mu} \cdot \mathbf{e}_2 |L\rangle \langle L| \boldsymbol{\mu} \cdot \mathbf{e}_1 / (w_1 - w_{LI}) + \boldsymbol{\mu} \cdot \mathbf{e}_1 |L\rangle \langle L| \boldsymbol{\mu} \cdot \mathbf{e}_2 / (w_2 - w_{LI})] \quad (2)$$

where $\boldsymbol{\mu}$ denotes the transition dipole moments between two electronic states. $\mathbf{e}_1$, $\mathbf{e}_2$ represent the direction of the corresponding transition. $|L\rangle$ is the intermediate states. In Born Oppenheimer (BO) approximation, $|I\rangle$, $|F\rangle$, and $|L\rangle$ can be separated into electronic and vibrational states as $|L/I/F\rangle = |\Phi_{L/I/F}^e\rangle |v_{l/i/f}\rangle$ with the energy $w_{L/I/F} = E_{L/I/F} + \varepsilon_{v_{l/i/f}}$. Here $E_{L/I/F}$ denotes the energy of the corresponding electronic state and $\varepsilon_{v_{l/i/f}}$ denotes the vibrational energy.

At the microscopic level, the spectral differential cross section of OPA can be explicitly written as

$$\sigma_{\text{OPA}}(w_i) = (4\pi^2 w_i / 3c) \cdot \text{Im} \sum_{i_1} (\langle v_i | \mu_{i_1}^{IF} | v_f \rangle)^2 / (w_{FI} - w_i - i\gamma_F') \quad (3)$$

and the TPA cross section (σ_{TPA}) for the two identical linearly polarized photons with the energy different from the molecular electronic excitation can be written as³⁵⁻³⁸

$$\sigma_{\text{TPA}}(w_i) = (4\alpha^2 \pi^3 \hbar^3 w_i^2 / 15e^4) \cdot \sum_{i_1, i_2} \text{Im} [\gamma_{i_1 i_2 i_2 i_1} (-w_i; w_i; w_i; -w_i) + \gamma_{i_1 i_2 i_1 i_2} (-w_i; w_i; w_i; -w_i) + \gamma_{i_1 i_2 i_1 i_2} (-w_i; w_i; w_i; -w_i)] \quad (4)$$

here, i_1 and i_2 represent the Cartesian coordinate directions x , y , z . α is the fine structure constant. With the exploit of the approximation $w_{LI} = E_{LI} + v_l - \varepsilon_{v_m} \approx E_{LI}$ (E_{LI} is the energy gap between $|L\rangle$ and $|I\rangle$, v_l , ε_{v_m} is the vibrational energy of the corresponding vibrational states), and the closure relation of vibrational states $|v_l\rangle$, the second-hyperpolarizability becomes³⁹

$$\gamma_{i_1 i_2 i_3 i_4} (-w_i; w_i; w_i; -w_i) = \sum_f \langle v_i | S_{i_1 i_2}^{IF} | v_f' \rangle \langle v_f' | S_{i_3 i_4}^{FI} | v_i \rangle / (E_{FI} + \varepsilon_{v_f'} - \varepsilon_{v_i} - 2w_i - i\gamma_F) \quad (5)$$

where $S_{i_1 i_2}^{FI}$ is the two-photon transition tensor, defined by

$$S_{i_1 i_2}^{FI} = \sum_L (\mu_{i_1}^{FL} \mu_{i_2}^{LI} + \mu_{i_2}^{FL} \mu_{i_1}^{LI}) / (E_{LI} - w_i) \quad (6)$$

here $\mu^{FI} = \langle \Phi_F^e | \boldsymbol{\mu} | \Phi_I^e \rangle$ is the electronic transition dipole moment between the initial and final electronic states. γ_F is introduced to represent the lifetime of the final excited states $|F\rangle$,

the adiabatic energy gap is $w_{FI} = E_{FI} + \varepsilon_{v_f'} - \varepsilon_{v_i}$. For the absorption process, the molecule starts in the ground electronic state, and usually also in the vibrational ground state $v_i = 0$ because at ordinary temperatures the energy necessary for vibrational excitation is larger compared with the average thermal energy.

One can calculate the cross sections in Eqs.(3) and (4) by means of the sum-over-state method straightforwardly or by the time-dependent approach. The time-dependent expressions for the OPA and TPA cross sections with inclusion of Duschinsky rotation (mode-mixing), FC and HT vibronic coupling effects have been derived in our previous works^{23,24,40} in detail. The mode-mixing effect accounts for the differences between ground- and excited-state normal-mode coordinates and vibrational frequencies. The HT vibronic coupling effect accounts for the dependency of transition dipole moments on the mode coordinates, $\mu_{eg} = \mu_0 + \sum_i \partial \mu_{eg} / \partial Q_i + \dots$. Therefore, to predict the OPA and TPA spectra, one is required to accurately compute the structure parameters, such as the equilibrium geometries and vibrational frequencies of resonant states, the excitation energies, the transition dipole moments, and the two-photon transition tensors as well as their geometric derivatives, etc. Even though, the ground-state properties can be easily obtained, it is difficult to calculate the excited-state properties. To avoid the difficulty, in this paper, we adopt the vertical gradient (VG) approximation^{41,42} which has been simply described in our previous paper^{23,24}.

In VG approximation, physically, the excited-state potential energy surface is just a mere shift relative to the ground-state potential surface without any scrambling of the normal coordinates or a change in the harmonic frequencies, i.e., $w_j^g = w_j^e$ and $L_g = L_e$. Here w denotes the vibrational frequencies and L corresponds to the transform matrix, which is obtained by the diagonalization of the mass weighted Hessian matrix $\mathbf{H}$ as $\mathbf{L}^T \mathbf{H} \mathbf{L} = \mathbf{w}^2$. The displacements which are related to the structural change along the normal mode Q_j upon excitation of the molecule read

$$(\partial E / \partial Q_j) = (w_j^i)^2 \bar{\Delta}_j \quad (7)$$

here $\partial E / \partial Q_j$ is the first-order derivative of the vertical excitation energy with respect to the j -th normal-mode coordinate Q_j , and $\bar{\Delta} = (L_e)^T d$. $d = x^g - x^e$ denotes the corresponding shift, where x^g and x^e represent the mass-weighted coordinates of equilibrium ground and excited electronic states, respectively. Although the VG approximation could not give finer details like resolved vibrational spacings, it is quite adequate for the overall band lineshape following multi-dimensional vibronic excitations, i.e., the envelop and width of the profile. Once the required structure parameters are acquired, one can use the time-dependent approaches^{23,24} to calculate the VROPA and VRTPA spectra with inclusion of FC and HT effects. The broadening of the spectra is described by a Lorentzian lineshape function.

From the bank of protein data, the crystal structures of ECFP³⁰ and two red fluorescent proteins³¹ are obtained. And then, we get the initial structures of the chromophores by rational extraction and modification. All the concerned structure parameters except the two-photon transition tensors and their geometrical derivatives are calculated by DFT or TD-DFT methods within the Gaussian 09 software package⁴³. The two-photon transition tensors and their geometric derivatives are evaluated within the Dalton programme⁴⁴. The finite-difference methods are applied to calculate the geometric derivatives of transition dipole moments and two-photon transition tensors. The hybrid DFT exchange-correlation (XC) functional B3LYP and the long-range-corrected DFT XC functional CAM-B3LYP are adopted. The basis sets 6-31G* and 6-311+G** are applied. The effect from the surrounding protein environment on molecular geometries and on spectroscopic properties has been inspected by the integral equation formalism polarizable continuum media model (PCM)^{45,46}.

To understand the nature of electronic excitations, we analyse the excited states by constructing the natural transition orbitals (NTOs)⁴⁷ within the Gaussian program package. The NTOs are defined by transformations of the occupied and unoccupied molecular orbitals *via* the matrices *U* and *V* obtained by singular value decomposition of the transition density matrix *T*, i.e., $UTV^+ = \lambda$, respectively. The matrices *U* and *V* are unitary and λ is diagonal.

3 Results and discussion

In order to unveil the structure–property relationship, and reveal the impacts of molecular external environments and DFT

XC functional on the electronic structure and optical properties of FPs, we here investigate three model FP chromophores: the neutral ECFP chromophore, the anionic DsRed2 and TagRFP chromophores in different molecular environments. The structure parameters which enter the expressions of spectral cross sections are calculated by DFT or TD-DFT with XC functional B3LYP and CAM-B3LYP. From the bank of protein data, the crystal structures of concerned FPs are obtained, and then, we get the initial structures of the chromophores by rational extraction and modification.

3.1 Neutral ECFP chromophore

Fig.1(a) shows the geometry of ECFP chromophore. At the optimized geometry of *S*₀, we calculate the vertical excitation energies and corresponding oscillator strengths of OPA and TPA as shown in Table 1. Then, both the pure electronic and vibronic spectra are simulated. Here we only show the low-frequency OPA spectra since the OPA transition is forbidden in the high-frequency region as Table 1 shows. The experimental TPA spectrum of ECFP, in Fig.1(b), shows three TPA bands centered at around 857, 640, and 550 nm with different spectral intensities. The theoretical spectra, in Fig.1(c–f), are calculated with respect to the geometric and electronic structure parameters produced by different DFT XC functionals at different molecular environments. It is found that the calculated OPA and TPA peaks by TD-B3LYP/PCM coincide rather well with the experimental spectra in the long wavelength region. Obviously, TD-CAM-B3LYP overestimates the excitation energies and subsequently blue-shifts the spectra compared with the experimental measurement. The solvent effect decreases the excitation energies. From the calculated pure electronic spectra of

Table 1 Basic parameters of ECFP

Method	ES	$f_{\text{OPA}}/(\text{a.u.})$	$f_{\text{TPA}}/(\text{a.u.})$	VEE/eV
in vacuo B3LYP	<i>S</i> ₁	0.6(0.6)	1647.6(1693.5)	3.4(3.4)
	<i>S</i> ₂	0.0(0.0)	0.0(0.1)	3.8(3.8)
	<i>S</i> ₃	0.0(0.0)	687.4(844.3)	3.9(3.9)
	<i>S</i> ₄	0.0(0.0)	6100.5(6214.9)	4.1(4.1)
in water B3LYP	<i>S</i> ₁	0.9(0.9)	7560.9(7563.7)	3.1(3.1)
	<i>S</i> ₂	0.0(0.0)	3.5(3.0)	3.8(3.9)
	<i>S</i> ₃	0.0(0.0)	2342.6(2536.3)	3.9(3.9)
	<i>S</i> ₄	0.0(0.0)	36906.6(36859.5)	4.0(4.0)
in vacuo CAM-B3LYP	<i>S</i> ₁	0.7(0.7)	1440.3(1461.7)	3.8(3.7)
	<i>S</i> ₂	0.0(0.0)	0.0(0.0)	4.2(4.3)
	<i>S</i> ₃	0.0(0.0)	3831.3(4042.0)	4.7(4.7)
	<i>S</i> ₄	0.0(0.0)	2357.9(1809.7)	4.9(4.8)
in water CAM-B3LYP	<i>S</i> ₁	1.0(0.7)	6748.1(6843.7)	3.4(3.4)
	<i>S</i> ₂	0.0(0.0)	0.0(0.1)	4.3(4.2)
	<i>S</i> ₃	0.0(0.0)	27502.5(30046.0)	4.6(4.7)
	<i>S</i> ₄	0.0(0.0)	10431.8(6686.4)	4.8(4.8)
Expt. peak/eV		2.9(TPA,OPA)	3.8(TPA)	4.6(TPA)

The calculated vertical excitation energies (VEE), oscillator strengths of one-photon absorption (f_{OPA}) and two-photon absorption (f_{TPA}) transitions of low-lying singlet excited states (ES) for ECFP chromophore at theoretical levels of TD-B3LYP and TD-CAM-B3LYP with basis sets 6-31G* and 6-311+G**, respectively. The data in the parentheses are the results with 6-311+G** (Tables 2, 3 are the same with it).

Fig.1 Calculated and experimental spectra of ECFP chromophore in gas phase and water solution based on the structure parameters produced by B3LYP and CAM-B3LYP with the basis set 6-31G*

The dark and blue lines denote the VROPA spectra and the pure electronic spectra of TPA, respectively; the sky blue and red lines denote the VRTPA spectra at FC and FC + HT approximations, respectively (here VG approximation is applied); the green lines denote VRTPA spectra with inclusion of FC, HT, and mode-mixing effects. (a) The structure of ECFP chromophore; (b) the experimental OPA and TPA spectra²; (c) and (d) the calculated spectra in gas phase and water solution based on the structure parameters produced by B3LYP and B3LYP/PCM, respectively; (e) and (f) the calculated spectra in gas phase and water solution based on the structure parameters produced by CAM-B3LYP and CAM-B3LYP/PCM, respectively. For the comparison, the actual excitation wavelength in one-photon absorption is multiplied by a factor of 0.5. The damping factor of 200 cm⁻¹ and the lorentzian line width of 10 cm⁻¹ are applied. VR: vibrationally resolved, NVG: non vertical gradient

TPA (the blue lines in Fig.1), we observe that the relative order of peak intensities of TPA spectra calculated with respect to structure parameters by TD-CAM-B3LYP/PCM is almost consistent with the experimental TPA spectrum if we redshift the pure electronic TPA spectrum about 100 nm. However, it is obvious that the spectral width cannot be well produced by the simply broadening by means of the line-shape function. We thus calculate the vibronic spectra at different approximations. It is obvious that the calculated vibronic spectra with inclusion of FC and HT effects provide us more abundant information about vibrations and vibronic coupling, which actually matches the experimental spectral lineshape quite well. For example, in

the long wavelength region, the first absorption band corresponds to the electronic transition of $S_0 \rightarrow S_1$ and both OPA and TPA show a board band width of about 200 nm. Our simulation verifies that the boarding comes from the vibrational transitions between different electronic states. The second TPA band centered at 650 nm corresponds to the electronic transitions of $S_0 \rightarrow S_3$ and S_4 , and the third intensive band centered at around 550 nm comes from the absorption from $S_0 \rightarrow S_5$ or higher energy excited electronic states.

In addition, for ECFP chromophore, we observe that no obvious blue-shift phenomenon appears at TPA maximum of the neutral ECFP chromophore and the spectra under FC and

FCHT approximations are nearly same, which indicates that the HT vibronic coupling does not have large contribution to the vibronic spectra of low-frequency band. The calculated TPA spectra with VG approximation and without VG approximation show slight differences, especially the peak intensities (see Fig.1(c, d)), which indicates that to correctly predict the VRTPA spectra, it seems important to include the difference of the potential energy surfaces between the resonant states. Due to the expensive computational cost of the excited-state Hessian calculation, in the later calculations, we thus adopt VG approximation.

Moreover, to further understand the nature of the excited states, we make an analysis on the NTOs corresponding to electronic transitions from the ground state to the low-lying singlet excited states for ECFP chromophore in water solution. Comparing the hole/particle NTO pairs for the transitions corresponding to three low-lying excited states as shown in Fig.2, we observe the obviously photon-induced intramolecular charge-transfer CT character in the later two excitations, while the intramolecular CT character accompanying with the first excited state is not visible. The character of excited states indeed explains why OPA is allowed for the transition of $S_0 \rightarrow S_1$, but it is forbidden for the transitions from S_0 to higher excited states,

Fig.2 Electronic densities of hole and particle NTO pairs with largest values of λ for the dipole-allowed singlet excited states of ECFP chromophore

and it also explains why CAM-B3LYP can yield the correct order of excited states and better TPA spectral lineshapes compared with the experiments although it overestimates the excitation energies.

3.2 Anionic DsRed2 chromophore

Then we calculate the anionic DsRed2 chromophore⁴⁸ with the same procedure for the neutral ECFP chromophore. The calculated electronic structure parameters are listed in Table 2,

Table 2 Basic parameters of DsRed2

Method	ES	$f_{\text{OPA}}/(\text{a.u.})$	$f_{\text{TPA}}/(\text{a.u.})$	VEE/eV
in vacuo B3LYP	S_1	0.9(0.9)	1660.5(2363.7)	2.4(2.4)
	S_2	0.0(0.0)	0.1(0.1)	2.6(2.7)
	S_3	0.0(0.0)	22.4(358.8)	3.3(3.3)
	S_4	0.0(0.0)	173.8(6.2)	3.3(3.3)
in water B3LYP	S_1	1.2(1.2)	27896.4(27628.0)	2.2(2.2)
	S_2	0.0(0.0)	0.0(0.2)	2.9(3.0)
	S_3	0.1(0.1)	155017.1(171032.5)	3.5(3.5)
	S_4	0.0(0.0)	502.7(2427.3)	3.5(3.5)
2H ₂ O + water B3LYP	S_1	1.2(1.2)	39461.5(39017.4)	2.3(2.3)
	S_2	0.0(0.0)	530.5(162.3)	3.4(3.5)
	S_3	0.0(0.0)	112368.0(108672.2)	3.6(3.6)
	S_4	0.0(0.0)	4216.6(2587.9)	3.6(3.6)
in vacuo CAM-B3LYP	S_1	1.1(1.1)	3714.7(4230.3)	2.6(2.6)
	S_2	0.0(0.0)	0.0(0.0)	3.5(3.5)
	S_3	0.0(0.0)	783.9(2259.8)	3.9(3.9)
	S_4	0.2(0.2)	8412.2(14960.7)	4.0(4.0)
in water CAM-B3LYP	S_1	1.4(1.4)	28402.9(31492.3)	2.4(2.4)
	S_2	0.0(0.0)	0.5(1.6)	3.8(3.9)
	S_3	0.3(0.3)	569339.7(315452.5)	4.1(4.1)
	S_4	0.0(0.0)	3004.8(18162.8)	4.2(4.2)
2H ₂ O + water CAM-B3LYP	S_1	1.4(1.4)	48030.3(48682.9)	2.6(2.6)
	S_2	0.0(0.0)	344.2(2205.8)	4.1(4.2)
	S_3	0.1(0.1)	88241.8(154167.6)	4.2(4.2)
	S_4	0.0(0.0)	2972.6(6421.4)	4.4(4.5)
Expt. peak/eV		2.2(OPA)	2.4(TPA)	3.5(TPA)

calculated vertical excitation energies, oscillator strengths of OPA and TPA transition from S_0 to the low-lying singlet excited states for anionic DsRed2 chromophore

and the calculated and experimental TPA spectra of DsRed2 chromophores are shown in Fig.3(b–f).

By inspecting into Fig.3(a), it is easily found that the experimental TPA maxima are centered at 700 and 1050 nm, while the low-energy OPA maximum is centered at 1100 nm. Obviously, the first TPA maximum has a blue-shift of 50 nm relative to the OPA maximum. Comparing the theoretical spectra calculated at different levels as shown in Fig.3(b–f), TD-B3LYP/PCM produces best consistent peak positions with the experimental profiles. The VRTPA spectrum based on the structure parameters produced by B3LYP/PCM gives two evident TPA bands whose main peaks are located at 712 and 1120 nm and VROPA spectrum shows one main band located at 1138 nm. Obviously, the blue-shift value between the first main

TPA and OPA maxima are not exactly yielded by the vibronic spectra calculated with respect to the structure parameters from B3LYP/PCM. The calculated vibronic spectra with respect to the structure parameters from CAM-B3LYP/PCM show the exact value of experimentally-measured blue-shift. The HT vibronic coupling effect is extremely significant on TPA spectra of the DsRed2 anionic chromophore in the low-frequency region, which results in the blue-shift of the first TPA maximum relative to OPA maximum.

To well analyze the TPA spectra, the pure electronic TPA spectra are simulated, too. The correct relative intensities of absorption bands are produced by the pure electronic spectra. In Fig.3(d), we also show the result (the green line) with a different molecular geometry which is obtained by optimizing the

Fig.3 Calculated and experimental spectra of anionic DsRed2 chromophore in gas phase and water solution

(a) the experimental OPA and TPA spectra; (b) the calculated spectra of 2H₂O + Dsred2 chromophore at B3LYP/6-31G* in water solution; (c) and (d) the calculated spectra of Dsred2 chromophore at B3LYP/6-31G* in gas phase and water solution, respectively; (e) and (f) the calculated spectra of Dsred2 chromophore at CAM-B3LYP/6-31G* in gas phase and water solution, respectively.

neutral chromophore and discarding a proton from the optimized geometry without further optimization. With this geometry, the calculated relative intensities and locations of TPA peaks agree better with the experimental measurement. It is obvious that the resonant forms of geometric structure determine the properties of anionic chromophore.

Combining vibronic spectrum and pure electronic spectrum, we assign the first band in the experimental spectrum coming from the electronic transition of $S_0 \rightarrow S_1$. As for the second band, it may come from the contribution of two electronic transitions because the third and fourth excited states are nearly degenerate. From Fig.3(d), the transition of $S_0 \rightarrow S_3$ makes dominant contribution to the second band and the contribution from the transition of $S_0 \rightarrow S_4$ is small.

As we all know, the experimental TPA spectra of those chromophores are acquired under the protein environment, which includes water molecules, counter ions, nearby charges, or polar amino acids. Therefore it is necessary to take the perturbation of protein environment to the photoactive part into considerations. To simplify the calculations, in the above work we just focus on the solvent effect. The implicit solvent PCM has been applied for the single chromophores. Here we consider complexes which are formed by the anionic chromophores with two

Fig.4 Electronic densities of hole and particle NTO pairs with largest values of λ for the dipole-allowed singlet excited states of DsRed2 chromophore in solution (a) and the complex formed by $2H_2O$ and DsRed2 chromophore (b)

Table 3 Basic parameters of TagRFP

Method	ES	$f_{OPA}/(a.u.)$	$f_{TPA}/(a.u.)$	VEE/eV	
in vacuo	S_1	1.2(1.2)	2288.5(3317.6)	2.6(2.6)	
B3LYP	S_2	0.0(0.0)	0.3(0.3)	2.7(2.8)	
	S_3	0.0(0.0)	3201.2(5385.9)	3.3(3.3)	
	S_4	0.00(0.0)	38.0(17.1)	3.3(3.4)	
	in water	S_1	1.6(1.6)	43988.6(40238.5)	2.3(2.3)
B3LYP	S_2	0.0(0.0)	1.1(1.1)	3.0(3.1)	
	S_3	0.0(0.0)	369598.4(393683.5)	3.4(3.5)	
	S_4	0.0(0.0)	245.1(4779.9)	3.6(3.7)	
	2H ₂ O + water	S_1	1.5(1.5)	55332.6(49756.9)	2.4(2.4)
B3LYP	S_2	0.0(0.0)	1887.8(295.9)	3.5(3.5)	
6-31G(<i>d</i>)	S_3	0.0(0.1)	361847.6(252960.6)	3.5(3.6)	
	S_4	0.0(0.0)	7012.60(108824.5)	3.6(3.7)	
	in vacuo	S_1	1.4(1.4)	4210.2(5270.9)	2.7(2.7)
	CAM-B3LP	S_2	0.0(0.0)	0.0(0.1)	3.5(3.6)
S_3		0.0(0.0)	3904.9(46752.5)	3.9(3.9)	
S_4		0.0(0.0)	34920.5(1419.9)	3.9(4.0)	
in water		S_1	1.7(1.6)	30491.0(32070.9)	2.5(2.5)
CAM-B3LP	S_2	0.0(0.0)	15.0(9.2)	3.8(3.9)	
	6-31G(<i>d</i>)	S_3	0.0(0.0)	784399.0(479099.9)	4.0(4.1)
	S_4	0.0(0.0)	787.9(24382.9)	4.3(4.3)	
	2H ₂ O + water	S_1	1.5(1.5)	47020.8(47982.9)	2.7(2.7)
CAM-B3LP	S_2	0.0(0.0)	95.7(543.9)	4.2(4.2)	
	S_3	0.0(0.0)	19674.8(12546.0)	4.3(4.3)	
	S_4	0.0(0.0)	13197.9(374822.8)	4.4(4.4)	
	Expt. peak/eV	2.2(TPA)	2.3(OPA)	2.3(TPA)	3.3(TPA)

calculated vertical excitation energies, oscillator strengths of OPA and TPA of low-lying singlet excited states for anionic TagRFP chromophore

explicit solvent water molecules. Then the hydrogen-bond complexes are calculated at the theoretical level of B3LYP/PCM. Two water molecules are put around the phenyl ring of the chromophores to form double hydrogen bonds with the anionic oxygen atom^{28,49}. Compared with the results of the anionic DsRed2 chromophore without explicit solvent molecules, the vertical excitation energy of the first excited state increases 0.1 eV and the energy spacing between the third excited state and the fourth excited state still keeps relatively small. With the smaller energy space, the coupling between the third and the fourth excited states may play an important role in the TPA spectrum. As we observe from Fig.3(b, d), the calculated TPA

spectra show distinct difference on the higher energy region and the first absorption bands are not influenced much except the peak locations. Additionally, in Fig.4, we can not only observe the same intramolecular CT in the higher excited states with ECFP but also find visible intermolecular CT in the higher excited states when the two water molecules are taken into account. It reveals that the charge transfer phenomena occurring in the higher excited states make great contribution to the enhancement of the intensities of the TPA peaks at the high-frequency domain.

3.3 Anionic TagRFP chromophore

Finally, we move to anionic TagRFP chromophore⁵⁰. The cal-

Fig.5 Calculated and experimental spectra of anionic TagRFP chromophore in gas phase and water solution

(a) the experimental OPA and TPA spectra; (b) the calculated spectra of $2\text{H}_2\text{O} + \text{TagRFP}$ chromophore at B3LYP/6-31G* in water solution; (c) and (d) the calculated spectra of TagRFP chromophore at B3LYP/6-31G* in gas phase and water solution, respectively; (e) and (f) the calculated spectra of TagRFP chromophore at CAM-B3LYP/6-31G* in gas phase and water solution, respectively.

Table 4 Calculated transition dipole moments between the different states at the different theoretical levels

Chromophore	Transition dipole	Dipole moment/(a.u.)					
		A	B	C	D	E	F
ECFP	μ_{01}	7.5	12.4	7.8	11.9		
	μ_{12}	0.0	2.4	0.0	0.0		
	μ_{13}	6.7	0.0	5.7	4.8		
	μ_{14}	12.3	12.7	0.2	0.2		
DsRed2	μ_{01}	15.7	23.3	22.3	17.5	24.5	22.2
	μ_{12}	0.0	0.0	0.1	0.0	0.0	0.0
	μ_{13}	0.2	31.3	23.8	0.1	2.5	2.9
	μ_{14}	35.5	0.1	1.4	8.7	0.0	0.0
TagRFP	μ_{01}	19.3	28.6	26.8	21.3	26.8	23.4
	μ_{12}	0.0	0.0	0.1	0.0	0.0	0.0
	μ_{13}	43.7	36.9	35.5	0.5	2.7	0.6
	μ_{14}	1.1	0.0	1.0	11.6	0.0	0.0

A: B3LYP, B: B3LYP/PCM, C: CAM-B3LYP, D: CAM-B3LYP/PCM, E: B3LYP for the model of chromophore + 2H₂O, F: CAM-B3LYP for the model of chromophore + 2H₂O

Fig.6 Electronic densities of hole and particle NTO pairs with largest values of λ for the dipole-allowed singlet excited states of TagRFP chromophore (a) and 2H₂O + TagRFP chromophore (b)

culated electronic structure parameters are listed in Table 3. The experimental spectra in Fig.5(a) show three low-energy TPA bands centered at around 600, 759, and 1100 nm and two OPA bands centered at around 700 and 1100 nm. The lowest-energy TPA and OPA bands centered at 1100 nm correspond to electronic transition of $S_0 \rightarrow S_1$. Overall, the calculated vibronic spectral lineshapes based on the structure parameters produced by CAM-B3LYP/PCM coincide with the experimental data although the peak positions are overestimated. Like anionic DsRed2 chromophore, a distinct blue-shift can be found between the TPA maximum and OPA maximum within the first absorption band, which is ascribed to HT vibronic coupling effect. However, HT effect is not so evident in the high-energy region since there is no large discrepancy between the pure electronic spectrum with the spectral boarding and the vibronic spectrum, and the calculated spectral lineshapes are almost same with the experimental one. The second TPA band corresponds to the electronic transition of $S_0 \rightarrow S_3$ and the third TPA band at around 600 nm comes from the electronic transition from the ground state to higher excited electronic states. For B3LYP/PCM, the dominant contribution to the third band is

from the electronic transition of $S_0 \rightarrow S_8$ while for CAM-B3LYP/PCM, the dominant contribution is from the electronic transition of $S_0 \rightarrow S_5$. Like DsRed2 chromophore, the same CT character can be found from Fig.6 for TagRFP and again strengthens the significance of it in the enhancement of the TPA peaks corresponding to the higher excited states. Additionally, comparing the structures of two anionic chromophores, it is easily found that the DsRed2 chromophore is *cis* conformation, while the TagRFP chromophore is *trans* conformation⁵¹⁻⁵³. Even though they have same atom composition, their TPA spectra show different characters mainly ascribing to the difference of their geometric structures. Our calculations demonstrate that the conformation of a chromophore affects its TPA spectrum to some extent.

It is noted that the transition dipole moments from S_0 to S_1 (μ_{01}) and from S_1 to S_n ($n > 1$) can also do us a favor to understand the nature of resonant enhancement of TPA corresponding to the electronic transition from S_0 to the high-lying excited states. The transition dipole moments of three FP chromophores have calculated and shown in Table 4, which have been calculated by B3LYP/PCM since B3LYP can reproduce more consistent experimental peak locations. From our calculations we know the transitions from S_0 to S_n ($n > 1$) are weak and some transitions from S_1 to S_n ($n > 1$) are strong. The transition dipole moments from S_1 and S_n ($n > 1$) actually play a key role in deciding the intensities of TPA peaks in high-frequency domain since in the three-level approximation, the TPA cross section can be written as $\sigma_2(w) \propto w_I^2 |\mu_{n1}|^2 |\mu_{10}|^2 g(w) / (w_{10} - w_I)^2$. For ECFP chromophore, the ratio of $\mu_{01} : \mu_{14}$ can directly predict the relative intensities of TPA peaks in low-frequency domain. For DsRed2 and TagRFP, the ratio of $\mu_{01} : \mu_{13}$ can also give the right relative intensities in TPA.

4 Conclusions

In this work, we have investigated the OPA and TPA proper-

ties of three FP chromophores in gas phase and water solution. Both the pure electronic and vibrationally-resolved electronic spectra have been calculated. We focus not only on the lowest-energy excited state but also on the transition from the ground state to the high-lying excited states. Comparing the calculated and measured spectra, all the absorption peaks are analyzed and characterized. The transitions occurring at different wavelengths make different contribution to the TPA peaks which are illuminated qualitatively. The corresponding electronic excitations have been characterized by the hole/particle pairs of natural transition orbitals. The DFT XC functional effect on the OPA and TPA spectra has been checked. In the calculations, the structure parameters which enter the spectral cross sections are obtained by DFT or TDDFT. Two functionals, B3LYP and CAM-B3LYP, have been applied.

The following conclusions have been achieved by this investigation. (1) The calculated electronic structures are functional-dependent. TD-B3LYP usually produces the excitation energies closer to the experiment while TD-CAM-B3LYP overestimates the excitation energies. However, the vibronic spectral lineshapes based on the structure parameters produced by CAM-B3LYP/PCM are more consistent with the experimental measurement even though one has to redshift the vibronic spectra 100 nm. (2) The experimental spectral lineshapes can be exactly produced by the calculated vibronic spectra with inclusion of FC and HT effects. The blue-shift phenomenon of TPA maximum in lowest-energy band for anionic chromophores is ascribed to significant HT vibronic coupling effect. However, the HT effect on the TPA spectra of high-frequency region is insignificant. (3) The high-lying excited states possess intramolecular CT character which indeed explains why TPA spectra in higher-frequency region are much stronger than the OPA spectra.

In this work, we just utilize the implicit or implicit + explicit solvent model to model the protein environmental effect which is not enough. In future work, we expect to use QM/MM method²⁸ to deal with the micro environment of the chromophore and more perturbations like counter ions, nearby charges, or polar amino acids and so on will be taken into account in order to better reproduce the experimental result and investigate the relationship between the intrinsic electronic structures and the TPA spectra. We expect that the data and information received from this theoretical work can provide a comprehensive guide for the researchers to choose the right FP and excitation wavelength for two-photon application of FPs.

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