Ground-State Structures and Vertical Excitations for the Kindling Fluorescent Protein asFP595

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Geometry configurations of a large fraction of the kindling fluorescent protein asFP595 around the chromophore region were optimized by using the effective fragment potential quantum mechanical−molecular mechanical (QM/MM) method. The initial coordinates of heavy atoms were taken from the structure 1XMZ from the Protein Data Bank archive corresponding to the dark-adapted state of the Ala143 → Gly mutant of asFP595. Optimization of geometry parameters was performed for all internal coordinates in the QM part composed of the chromophore unit and the side chains of His197, Glu215, and Arg92 as well as for positions of effective fragments constituting the MMpart. The structures corresponding to the anion trans, anion cis, and zwitterion trans moieties were considered among various alternatives for the chromophore unit inside the protein matrix. The QM/MM simulations show that the protein environment provides stabilization for the trans-zwitterion isomer compared to the gas-phase conditions. By using the multiconfigurational CASSCF and the time-dependent density functional theory calculations, we estimated positions of spectral bands corresponding to vertical S_0-S_1 transitions. The results of simulations support the assumption that the dark state of asFP595 corresponds to the anionic or zwitterionic trans-conformation, while the kindled state corresponds to the anionic cis-conformation.

Introduction

Fluorescent proteins of the green fluorescent protein (GFP) type are widely used in molecular biology and medicine as biological markers.[1,2] A newly discovered GFP-like protein from the sea anemone *Anemonia sulcata* (*A. sulcata*) asFP595 is initially nonfluorescent, but in response to intense light irradiation at 568 nm it becomes brightly fluorescent (kindles) with emission at 595 nm at room temperature.[3] The mechanism of kindling is not clear, and presently substantial efforts are being performed to understand the intriguing properties of asFP595.[4−8] The hypothesis about an involvement of cis−trans isomerization of the protein chromophore as an origin of the kindling mechanism has been formulated by the discoverers of asFP595.[9] This possibility has been discussed in recent papers describing the crystal structures of asFP595.[4−6] An analysis of chromophore contacts with the neighboring residues in asFP595 and considerations on the possible flexibility of the nearby chains, especially of His195 and Glu215 (the numbering corresponds to the structure 1XMZ[4] from the Protein Data Bank (PDB) archive), allowed the authors to conclude that cis−trans isomerization could be readily accommodated within the chromophore cavity.[4] Similar considerations are presented in the study of the far-red fluorescent protein HcRed, in which the chromophore has been found in the trans-configuration,[10] as well as in two recent experimental studies on the DsRed protein.[11,12] Andersen and colleagues[6] presented arguments in favor of the asFP595 isomerization from a trans (dark) to a cis (fluorescent) state. The authors obtained crystal structures for both isomers for the wild type and mutated species and characterized photoswitching of the samples by using fluorescent microscopy. From the classical molecular dynamics simulations the authors concluded that the trans−cis isomerization responsible for switching could occur through the hula-twist mechanism.[6] Molecular dynamics simulations of the cis−trans isomerization of the chromophore of the E^2GFP variant were also reported in the most recent paper of Nifosi and Tozzini.[13]

A widely quoted 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.[14] The authors used the NMR spectroscopy to characterize conformations of 4-hydroxynenzylidene-1,2-dimethylimidazoline (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 cationic, neutral, and anionic states, respectively. The corresponding activation barriers for trans-cis isomerization were estimated as 11.7, 13.1, and 13.1 kcal/mol for cationic, neutral, and anionic forms.[14] Theoretical estimates[10,15−19] for the GFP-like chromophores in vacuo and in solution resulted in a conclusion that the cis isomers possessed lower energy than the trans form or zwitterionic species. The semiempirical barriers for cis−trans isomerization[15,16] were strongly dependent on the protonation status of the chromophore. The calculations of ref 16 are usually cited as a reason the high barrier hula-twist rotational mechanism should be excluded from consideration.

This study presents a step toward theoretical simulations of photophysical properties of the asFP595 chromophore inside

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the protein matrix. Recent publications[20,21] describe applications of standard quantum chemical calculations for the asFP595 chromophore in vacuo[20,21] and in solutions.[21] We apply here an advanced quantum mechanical−molecular mechanical (QM/MM) theory based on the effective fragment potential (EFP) methodology.[22] The use of hybrid QM/MM methods[23−30] to characterize properties of large molecular systems and to model chemical reactions in condensed media has gained increasing attention in recent years. A number of successful realizations of the idea to describe a central part of the entire molecular system at the QM level and the environmental part by the MM force fields are extensively presented in the literature. The EFP-based QM/MM technique[22] is an approach which allows one to perform calculations close to an ab initio treatment of the entire molecular system.

In this communication we describe modeling properties of asFP595 in the ground electronic state taking the available protein crystal structure reported in ref 4 as a starting point. We concentrate our efforts on possible arrangements of molecular groups around the chromophore region for a fairly large fraction of the protein and compare structures corresponding to trans and cis chromophore conformations of the chromophore. For the computed geometry configurations we estimate positions of spectral bands with large oscillator strengths corresponding to vertical transitions to the S_1 excited state.

Calculation Approaches

The QM/MM method used in optimization of geometry parameters of a large fraction of the asFP595 protein is based on the effective fragment potential theory,[22] implemented in the GAMESS(US) program system.[31] This is an approach which allows one to perform calculations close to an ab initio treatment of the entire molecular system. In this scheme, molecular groups assigned to the MM part are represented by effective fragments which contribute their electrostatic potentials expanded up to octupoles to the quantum Hamiltonian. These one-electron electrostatic potentials are obtained in preliminary quantum chemical calculations by using ab initio electron densities. The exchange-repulsion potentials to be combined with the electrostatic terms can be also created in preliminary ab initio calculations. Thus, all empirical parameters are entirely contained in the MM subsystem. Compared to the original EFP-based QM/MM methodology,[22] we apply here a modified approach,[32,33] in which the peptide chains of the protein are described as flexible compositions of small effective fragments, and fragment−fragment interactions are computed with conventional force fields.

Each EFP is written as follows:

$$V_\mu(r) = \sum_{k=1}^{K} V_{\mu,k}{}^{\mathrm{ELEC}}(r) + V_\mu{}^{\mathrm{REP}}(r) \qquad (1)$$

The electrostatic potential $V^{\mathrm{ELEC}}(\mathrm{r})$ acting on the external QM subsystem is represented by distributed multipoles centered at each atom and each bond midpoint of the fragment μ. The multipole expansions are extended from charges up to octupoles, and the corresponding parameters can be created in preliminary ab initio calculations using the GAMESS(US) program. The exchange-repulsion interaction between an effective fragment and a quantum subsystem is modeled by one-electron potentials, which have the form of Gaussian functions located at each atomic center

$$V^{\mathrm{REP}}(r) = \sum_{m=1}^{M}\sum_{k=1}^{k_{\max}} c_{mk} \exp(-\alpha_{mk} r_m{}^2) \qquad (2)$$

where M is the total number of atoms. The corresponding parameters c_{mk} and α_{mk} should be optimized by a fitting procedure. In eqs 1 and 2, r denotes electronic coordinates originating from the corresponding expansion points in the μth effective fragment, K is the number of such expansion points for a distributed multipolar analysis. We use all atomic centers and midpoint points as the expansion points. Two Gaussian functions ($k_{\max} = 2$ in eq 2) have been optimized for each repulsive point. The terms of eq 1 are added to the one-electron operators in the Hamiltonian of the ab initio subsystem.

We obtained parameters c_{mk} and α_{mk} of the repulsion potentials of eq 2 for the most typical fragments representing amino acid side chains by the following procedure. For the biomolecules, description of hydrogen bonding seems to be of a primary importance, and therefore, the water molecule can serve as a probing vehicle in the adjustment procedure. We considered a variety of directions along which the water molecule could reach an effective fragment and carried out ab initio calculations in order to provide reference data. We utilized the computer code REPGEN of W. Stevens to perform the least-squares optimization of parameters for the exchange-repulsion potentials. The restricted Hartree−Fock (RHF) approximation with the conventional 6-31G** basis sets was used systematically: (i) for the reference calculations, (ii) for creation of multipole expansion parameters in $V^{\mathrm{ELEC}}(r)$, and (iii) for the fitting procedure. We verified that the parameters of $V^{\mathrm{REP}}(r)$ fitted with the RHF/6-31G** procedure can be used in subsequent QM/MM calculations with other basis sets and with other quantum chemical methods. The sets of parameters c_{mk} and α_{mk} of the repulsion potentials of eq 2 for the effective fragments representing amino acid side chains are presented as Supporting Information to this paper.

We consider the MM subsystem as connected chains of small rigid effective fragments, calculate their interactions with the QM part as in the original EFP method,[22] but replace fragment−fragment interactions by interactions dictated by MM force fields. A combination of the molecular modeling programs PC GAMESS (Granovsky, A. A. URL http://lcc.chem.msu.ru/gran/gamess), which presents an Intel-specific version of the GAMESS(US)[31] program system, and TINKER (Ponder, J. W. URL http://dasher.wustl.edu/tinker) provides a technical realization for this QM/MM scheme.

The treatment of the QM/MM boundary across the $C_\alpha-C_\beta$ bonds[32,33] is essentially based on the concept of effective fragments. The key issue of the present method is an introduction of a buffer fragment as a group of atoms belonging to both QM and MM subsystems. In many cases the -CH_2- group may be assigned to the buffer. We employ the usual maneuver to saturate the broken valence and add the closing (link) hydrogen atom to complete the QM subsystem. In the QM part, we distinguish the buffer (often, CH_3) as a special group of the quantum subsystem. The same geometry configuration of the buffer fragment with the frozen internal coordinates is assumed in the MM part. In the MM subsystem, which is a collection of effective fragments, the buffer is a special fragment as well. The position of the link atom is formally considered as an additional expansion point (analogous to midbond points in "normal" effective fragments), which actually holds no multipoles. This trick, essentially based on the GAMESS(US) implementation of the EFP method, helps us to keep the link atom precisely along the broken C−C bond during geometry

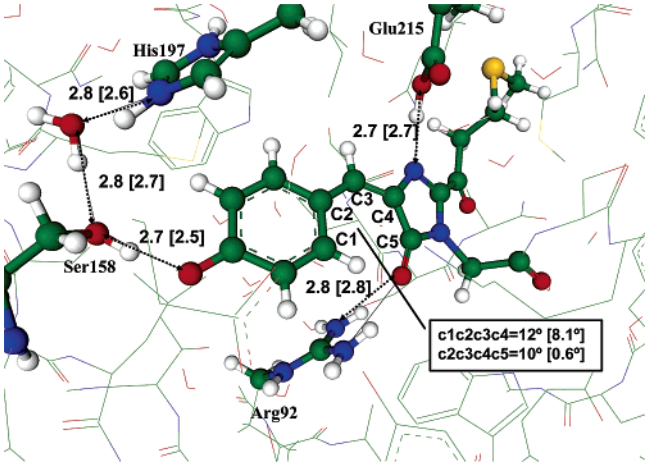

Figure 1. Computed trans anion structure in the vicinity of the chromophore unit. Distances between heavy atoms are given in angstroms; the values in square brackets refer to the crystal structure 1XMZ.[4] Specified values of dihedral angles C1C2C3C4 and C2C3C4C5 indicate strong nonplanarity of the chromophore unit consistent with the experimental data.[4]

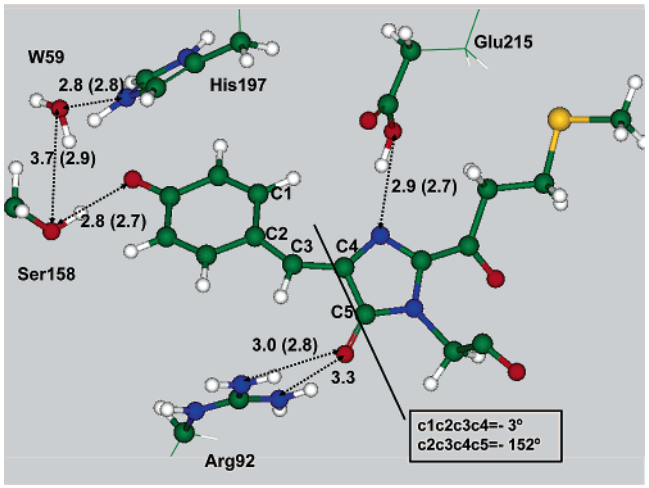

Figure 2. Computed cis anion structure in the vicinity of the chromophore unit. Distances between heavy atoms are given in angstroms; the values in parentheses refer to the trans anion configuration (Figure 1).

optimizations of the entire system. In our scheme, this empty expansion point and the neighboring CH group of the MM peptide chain form an effective fragment, which interacts with the buffer fragment according to the MM force fields and, as a consequence, the link atom cannot leave the C−*C* axis. Conservation of the total charge is maintained with a high accuracy in this scheme. Reference 33 contains descriptions of various tests of this QM/MM approach, as well as the details of the link atom approach to design the QM and MM interface.

In the present application the atoms of the MM-subsystem were grouped in 615 effective fragments whose positions were optimized along with the internal coordinates of the quantum subsystem. The AMBER force field parameters were used for the MM subsystem.

Our model for the QM/MM calculations includes a fairly large fraction of the protein with 2122 explicit atoms (linear size ∼ 40 Å). The entire chromophore unit, 2-acetyl-4-(*p*-hydroxybenzylidene)-1-methyl-5-imidazolone, and the side chains of the neighboring His197, Glu215, and Arg92 amino acids are assigned to the 50-atomic QM part. His and Glu are expected to participate in proton transfers from and to the chromophore unit, while the charged residue Arg near the subsystem of primary interest should be assigned to QM according to the previous experience of QM/MM simulations.[34]

The Structures

We performed optimization of geometry parameters when starting from the coordinates of heavy atoms referred to the structure of the A143G variant of asFP595 with the chromophore in the trans form (PDBID: 1XMZ) solved at 1.38 Å resolution and 100 K.[4] This entry corresponds to the dark-adapted state of asFP595 with a kindled half-life that is much longer than that of wild type. Before QM/MM calculations, hydrogen atoms were added manually and their coordinates were corrected by using the MM optimization with the AMBER force field parameters. In QM/MM optimization, positions of the molecular groups from the protein further than ∼10 Å from the chromophore unit were kept frozen to maintain the correct shape of the model protein. This strategy guarantees, in particular, positions of the backbone C_α atoms in the model structures close to those in the crystal moiety.

Figure 1 shows an arrangement of the key molecular groups in the computed equilibrium geometry configuration of the trans anion chromophore inside the protein. Analysis of the 1XMZ structure allows us to assume that the phenolic proton from the chromophore was transferred to His197, presumably through the hydrogen bond network of Ser158 and Wat59. The corresponding pathway should have low activation energy even in the ground electronic state as shown for the GFP.[35] The assumption of the anionic (or possible zwitterionic) form of the chromophore also follows from the observed small Stokes shift of the dark species.[8]

The short O(Glu215)−N(Cro65) distance 2.7 Å observed in 1XMZ urged us to introduce the proton between this pair of atoms. Along with the trans anion structure shown in Figure 1 where this proton belongs to Glu215, another stationary point has been located corresponding to the trans zwitterion form of the chromophore in which the proton is bound to nitrogen. Other geometry parameters of the trans zwitterions conformation are close to those of the trans anion structure.

The structure with the cis anion isomer of chromophore was derived from the previously obtained trans isomer by a manual rotation of the benzyl group and complete re-optimization of the geometry parameters. Figure 2 illustrates the equilibrium configuration of the cis anion form inside the protein. One can see certain changes in the intermolecular distances compared to the trans form. Both trans and cis conformations are characterized by strong nonplanarity of the chromophore unit as illustrated in Figures 1 and 2 by the values of torsional angles C1C2C3C4 and C2C3C4C5.

As known from experimental[14] and theoretical[10,15−19] estimates for the GFP-like chromophores in vacuo and in solution, the cis isomers possess lower energy than the trans forms or zwitterionic species. In our case the total energies of the 2122-atomic system computed in the QM(RHF/6-31G*)/MM(AMBER) approximation correspond to the lowest energy trans anion, followed by trans zwitterion (+1.4 kcal/mol) and cis anion (+1.6 kcal/mol). However, if the quantum subsystem with the same geometry parameters as found in QM/MM optimizations is extracted from the MM environment, the recomputed energies are in the opposite ordering: the cis anion is the lowest structure followed by the trans anion and the trans zwitterion species. For the separated chromophore in vacuo, the conventional quantum chemical approaches result in planar equilibrium

TABLE 1: Energies of the Vertical S_0-S_1 Transition, ΔE, Corresponding Wavelengths, λ, and Oscillator Strengths, f, Computed in the TDDFT(B3LYP/6-31+G*) Approximation

structure		ΔE, eV	λ, nm	F
chromophore in vacuo[a]	trans anion	2.74	452	1.06
	trans zwitterion	2.55	486	0.94
	cis anion	2.59	479	0.83
chromophore and neighboring residues in protein[b]	trans anion	2.72	455	0.79
	trans zwitterion	2.71	457	0.63
	cis anion	2.50	495	0.67

[a] B3LYP/6-31+G* optimized geometry. [b] QM/MM optimized geometry.

TABLE 2: Energies of the Vertical S_0-S_1 Transition, ΔE, Corresponding Wavelengths, λ, Computed in the CASSCF(10/10)/6-31+G* Approximation

structure		ΔE, eV	λ, nm
chromophore in vacuo[a]	trans anion	3.13	396
	trans zwitterion	3.21	386
	cis anion	2.85	435
chromophore in protein[b]	trans anion	3.60	344
	trans zwitterion	3.70	335
	cis anion	3.44	360

[a] B3LYP/6-31+G* optimized geometry. [b] QM/MM optimized geometry.

geometry configurations for all species with the cis form being the lowest in energy at all computational levels (see Supporting Information to this paper). Therefore, we can conclude that the protein matrix provides stabilization for the trans conformation of the chromophore.

Apparently, the RHF approximation cannot guarantee an accuracy high enough to characterize the relative energies of the structures within 1−2 kcal/mol, and in this respect, the reported estimates should be considered only as qualitative ones. However, they are consistent with the values computed at a higher level theoretical for the similar chromophores. In particular, according to the B3LYP/6-31++G**//B3LYP/6-31G** calculations performed for the HcRed chomophore isolated from the protein, the cis conformer is 1.7 kcal/mol lower in energy than the trans isomer.[10]

Vertical Excitations

In Table 1 we present the results of time-dependent density functional theory (TDDFT) calculations for the parameters of the vertical S_0-S_1 transition carried out with Gaussian03.[36] We compare properties of the separated planar chromophore, whose geometry parameters have been optimized in vacuo, and those of the nonplanar chromophore at the geometry configurations found in the QM/MM optimization inside the protein. In the latter case the model system used in TDDFT calculations included the His and Glu residues along with the chromophore unit (see Figures 1 and 2).

The computed values of excitation energies are higher than those expected from experimental studies, 2.18 eV (568 nm) for absorption and 2.08 eV (595 nm) for emission. Evaluation of possible errors of this method applied to the GFP-like chromophores in comparison to other recent theoretical approaches[17−19,21,37−44] (see Supporting Information to this paper) allows us to ascribe approximately 0.55 eV overestimation in energy to inaccuracies in geometry parameters and to errors of the TDDFT approximation. Taking this correction into account, we can assign the absorption band to the trans anion (or trans zwitterion) and the emission band to the cis anion structures. Calculations reveal a strong effect of the protein matrix on the zwitterion construct (Table 1): as opposite to the chromophore in vacuo for the system in protein environment, we cannot distinguish the trans anion and trans zwitterion states by their spectral properties. Another effect of the protein matrix is a noticeable reduction of the oscillator strengths of the bands.

The anonymous reviewer of this paper pointed out that the use of TDDFT method at the RHF optimized geometry configurations could lead to serious errors for excitation energies (see Supporting Information to this paper). We recomputed the data collected in Table 1 by using the complete active space self-consistent field (CASSCF) method (Table 2). In these applications the multiconfigurational expansions corresponded to distributions of 10 electrons over 10 active orbitals, i.e., to the CASSCF(10/10) approach. The usual state averaging technique with equal weights for the ground and excited states was applied to compute the CASSCF excitation energies. Selection of initial active π and π^* orbitals of the chromophore unit was performed manually by inspecting the graphs of the RHF orbitals. For the chromophore in protein, the effective fragment potential QM/MM method was used to evaluate contributions from the protein environment to the excitation energies in the QM part.

As expected, the simple CASSCF estimates for the excitation energies lacking dynamical correlation effects resulted in even larger discrepancies with the experimental band positions. However, these data are completely consistent with the qualitative conclusions from the TDDFT calculations, namely, the cis anion structure is characterized by longer wavelengths than trans anion or trans zwitterions species.

Discussion and Conclusions

The most recent experimental studies on the wild-type (dark state) and the mutated A143S variant of asFP595 protein resulted in conclusion that the chromophore could undergo isomerization from a trans (dark) to a cis (fluorescent) state.[4,6] It should be noted that the analysis of crystal structures reported in ref 4 and in refs 5 and 6 shows an apparent difference in positions of the key residue His197 near the chromophore unit, expanding far beyond differences in possible locations of His197 discussed in ref 4. The simulations described in this work are relied on the structure 1XMZ reported in ref 4. In particular, the structure with the cis anion isomer of chromophore was derived from the previously obtained trans isomer without rotations of the side chain of His197, but adjusting its position when optimizing the geometry parameters. QM/MM-based examination of molecular arrangements initiated by other crystal structures reported in refs 5 and 6 is a subject of our current studies.

Other choices for a subdivision of the protein to the QM and MM subsystems may be also of importance. In particular,

Chudakov et al.[9] tentatively suggested that Ser and Val residues near the phenolic ring of the chromophore might be important in kindling. However, analysis of the crystal structure 1XMZ does not favor an assignment of these residues, especially, Val, to the QM subsystem at least for the first estimates. In the present model the Ser and Val residues are included to the MM part, but contribute essentially to the quantum Hamiltonian as effective fragments.

For more quantitative conclusions of these simulations certain improvements of the calculation scheme are desirable, including (i) the use of quantum chemistry methods with the electron correlation effects in QM for more precise relative energies, (ii) optimization of geometry parameters for the excited states for better evaluation of fluorescence maximum, and (iii) the use of more accurate methods for excitation energies. All these issues will require extremely expensive QM/MM type computations. The main purpose of the present work was to perform pilot calculations of the properties of the asFP595 chromophore *inside the protein matrix* in order to rationalize the hypotheses formulated in experimental studies.[4,6,9] The next steps should include scans of the ground- and excited-state potential energy surface along the coordinates of internal rotation of the chromophore inside the protein, since the mode of trans-cis izomerization is a subject of debates.[6,16]

In summary, the results of this work provide a theoretical support based on quantum calculations to the hypothesis on the possibility of trans-cis izomerization of the chromophore in the mechanism of kindling proposed in experimental studies.[4,6,9] The system can absorb light in the trans anion (or trans zwitterion) form and emit at longer wavelength in the cis anion form. Simulations emphasize the crucial role of the protein environment in modeling structure, energetics, and spectral properties of the chromophore.

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Supporting Information Available: Tables of parameters of the effective fragment potentials, energies and coordinates of the chromophore in vacuo, and estimates of possible errors in calculations of the S_0-S_1 transitions in the TDDFT approximation. This material is available free of charge via the Internet at http://pubs.acs.org.

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