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Triple-Decker Motif for Red-Shifted Fluorescent Protein Mutants

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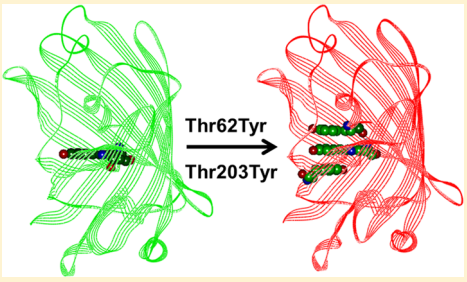
Supporting Information

ABSTRACT: Among fluorescent proteins (FPs) used as genetically encoded fluorescent tags, the red-emitting FPs are of particular importance as suitable markers for deep tissue imaging. Using electronic structure calculations, we predict a new structural motif for achieving red-shifted absorption and emission in FPs from the GFP family. By introducing four point mutations, we arrive to the structure with the conventional anionic GFP chromophore sandwiched between two tyrosine residues. Contrary to the existing red FPs in which the red shift is due to extended conjugation of the chromophore, in the triple-decker motif, the chromophore is unmodified and the red shift is due to π-stacking interactions. The absorption/emission energies of the triple-decker FP are 2.25/2.16 eV, respectively, which amounts to shifts of ~40 (absorption) and ~25 nm (emission) relative to the parent species, the I form of wtGFP. Using a different structural motif based on a smaller chromophore may help to improve optical output of red FPs by reducing losses due to radiationless relaxation and photobleaching.



SECTION: Molecular Structure, Quantum Chemistry, and General Theory

Genetically encoded fluorescent labels from the green fluorescent protein (GFP) family[1−7] enable in vivo imaging of protein localization and interactions. In the context of biomedical applications, deep tissue imaging calls for proteins with absorption/emission above 600 nm[8−10] (or below 2.07 eV). Most of the existing red fluorescent proteins (RFPs) share a DsRed-like chromophore in which the π-system of the GFP chromophore is extended by an additional *N*-acylimine moiety.[11] One of the challenges in RFP engineering is their relatively low optical output[8−10,12] and photobleaching. Lower quantum yield is attributed to an extended range of motion of the chromophore;[12,13] thus, using a structural motif based on a smaller (and more rigid) chromophore may help to develop a new family of RFPs with improved properties. Some of the RFPs' shortcomings (including photobleaching) are likely due to the tetrameric nature of the parent DsRed,[11] which is blamed for a structural weakness in the β-barrel of the mFruit series.[14] A traditional approach would be to remedy this weakness by directed mutagenesis of DsRed-derived RFPs;[8−10] however, starting from a different parent structure that does not have this problem to begin with might be more fruitful. The successful design of FPs with customized properties ultimately requires experimental realization and directed molecular evolution combined with high-throughput cell sorting techniques; however, computational chemistry is a useful tool for testing the effect of key mutations on the optical properties of the FP in order to provide the initial structural motif for mutagenesis studies.

A bathochromic shift can also be achieved by π-stacking interactions. For example, a replacement of the Thr203 residue in GFP by Tyr has led to the yellow FP (YFP)[15,16] featuring a 20 nm red shift in absorption/emission relative to the parent species. As discussed in ref 15, the Thr203Tyr variant led to the most noticeable emission red shift among the mutants in which Thr203 was replaced by amino acid residues with planar aromatic rings (Phe, His, Trp, Tyr). One of the recent examples[17] of in silico protein design has predicted red-shifted (up to 26 nm relative to the parent protein) mutants of mCherry; the theoretical predictions were confirmed experimentally. The authors[17] applied the Thr to Tyr mutation, similar to that used in YFP,[15] along with the modification of the residues around the acylimine oxygen and the phenolate moiety of the chromophore.[17] The π-stacking motif has also been exploited in TagRFP567[18] in which the red chromophore is stacked with histidine (mutation at position 69), yielding the excitation/emission maxima at 611/657 nm.[10,18]

Here, we outline a strategy for increasing the bathochromic shift in the GFP-like proteins by further extending π-stacking interactions of the chromophore with two nearby aromatic amino acid residues. We computationally constructed GFP variants in which the conventional anionic GFP chromophore, 4-hydroxybenzylidene-imidazoline, is sandwiched between the two tyrosine residues, resulting in a "triple-decker" motif. To this end, we performed a few point mutations, in addition to the known modification Thr203Tyr.[15,16] The critical issue is to replace Thr62 by Tyr and to make additional changes in the

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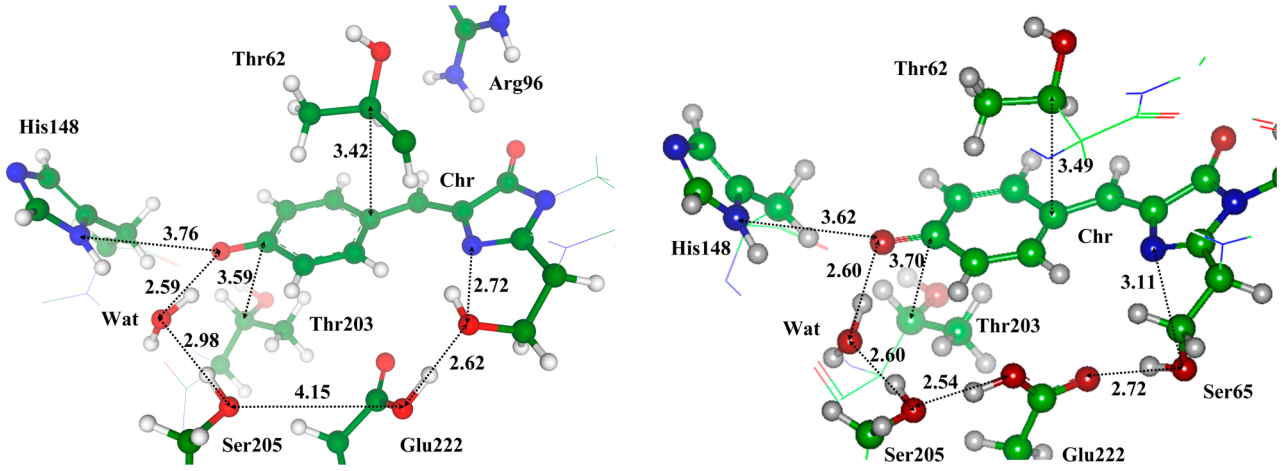

Figure 1. Fragments of the QM/MM-optimized model structures representing the B (left) and I (right) forms of wtGFP with the anionic chromophore. Here and below, the carbon atoms are colored in green, oxygen is in red, and nitrogen is in blue; the distances between the heavy atoms are given in Å.

Table 1. Wavelengths (nm, bold), Electronic Energy Differences (in parentheses, eV), and Computed Oscillator Strengths (in brackets) of the Electronic Transitions for the B and I Forms of wtGFP

	S_0–S_1 (absorption)			S_1–S_0 (emission)		
model structure	XMCQDPT	SOS-CIS(D)	exp.	XMCQDPT	SOS-CIS(D)	exp.
B form	**484** (2.56)	**489** (2.53)	**475** (2.61)[26,27]	**539** (2.29)	**536** (2.31)	
	[1.00]	[1.58]	**472** (2.62)[28]	[0.87]	[1.41]	
I form	**493** (2.51)	**492** (2.52)	**495** (2.50)[28]	**543** (2.28)	**532** (2.33)	**508** (2.44)[26−28]
	[0.97]	[1.50]		[0.87]	[1.36]	

structure resulting in up to four modifications, which were required to achieve structures compliant with the Ramachandran plot.

The computational strategy used in this work was validated by computing structures and spectra of wtGFP with the anionic chromophore. Then the same approach was applied for modeling the mutated GFP with the triple-decker motif of the chromophore pocket. For full computational details, see the Supporting Information for this Letter. In brief, we used the flexible effective fragment version[19,20] of the quantum mechanics–molecular mechanics (QM/MM) approach[21] to optimize the equilibrium structures of the ground and excited states of the FP. The stationary points on the ground-state potential energy surface were located using QM(PBE0/6-31G*)/MM(AMBER). Optimization of the S_1 excited-state geometry was performed by the QM(CASSCF(10/9)/6-31G*)/MM(AMBER) method by using complete active space self-consistent field (CASSCF). Calculations of the vertical $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_0$ energy differences were performed using the extended multiconfigurational quasi-degenerate perturbation theory (XMCQDPT2) method,[22] which has been shown to yield accurate excitation energies in complex fluorophores (e.g., see ref 23). Further validation of the computed energies was carried out by another ab initio approach, the configuration interaction singles method augmented by perturbative doubles correction within the scaled-opposite-spin framework, SOS-CIS(D).[24] As shown below, both approaches yield quantitatively consistent results.

A model system mimicking wtGFP was constructed by using the coordinates of heavy atoms from the crystal structure of the S65T mutant of GFP with the anionic chromophore (PDB id: 1EMA entry[25]). We restored the side chain of Ser at position 65 and added hydrogen atoms by assigning standard protonation states of polar residues, that is, positively charged Lys and Arg and negatively charged Glu and Asp.

Fragments of the QM/MM-optimized chromophore-containing domain corresponding to two known conformations of GFP with the anionic chromophore (the B and I forms[26,27]) are shown in Figure 1. The main difference between these forms is attributed to different conformations of the Glu222 side chain (the syn position in the B form and the anti position in the I form). The green fluorescence of wtGFP at 508 nm (2.44 eV) is assigned to the $S_1 \rightarrow S_0$ transition from the excited state of

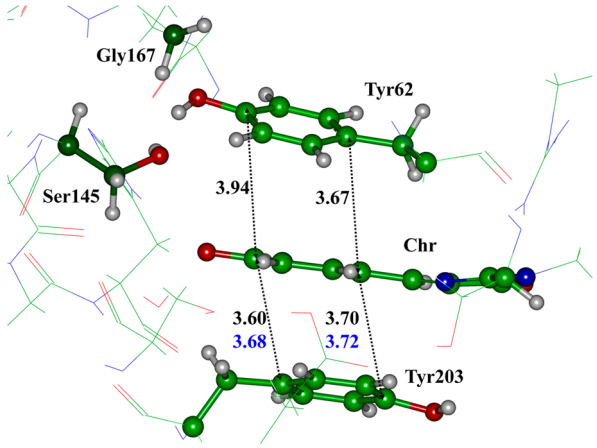

Figure 2. Fragment of the Thr62Tyr/Tyr145Ser/Ile167Gly/Thr203Tyr mutant showing all four replaced residues. Selected distances are given in Å; the bottom values for the Chr–Tyr203 pair refer to the crystal structure PDB id: 1YFP[15] of the yellow mutant of GFP (Thr203Tyr/Ser65Gly/Val68Leu/Ser72Ala).

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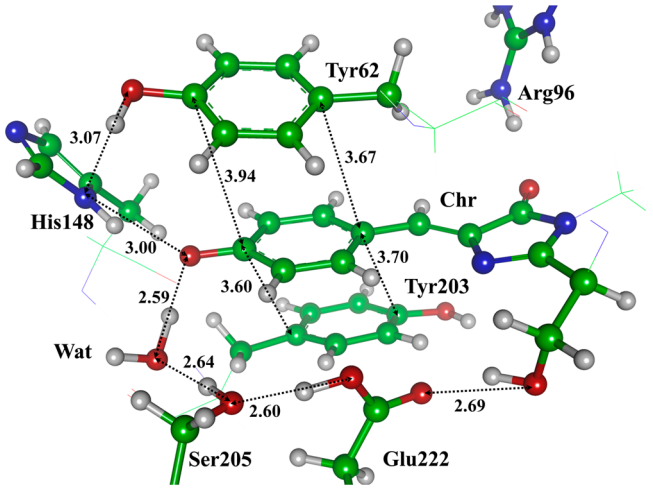

Figure 3. Model system with the triple-decker chromophore motif derived from wtGFP by introducing four mutations Thr62Tyr/Tyr145Ser/Ile167Gly/Thr203Tyr.

the I form,[26−28] while the absorption at 475−472 nm (2.61−2.63 eV) is attributed to the B form,[26−28] and the slightly red-shifted band at 495 nm (2.51 eV) is attributed to the I form.[28]

The computed excitation energies and the corresponding experimental band maxima are collected in Table 1. First of all, we note an almost perfect correlation between the computed and experimental results for the absorption bands, which even reproduce subtle differences between the B and I forms. Slightly larger discrepancies are observed for emission bands; however, the differences (∼0.1 eV) are within the established error bars of the QM and QM/MM protocols when applied for electronic transition energies of the protein-bound biochromophores.[23,29−34]

Below, we compare electronic transition energies of the mutated model protein with those of the I form of wtGFP (Figure.1, right panel).

Aiming to design a structure in which an almost planar chromophore is sandwiched between two planar π-systems, we manually prepared several structures by introducing four mutations in the wtGFP sequence. Our starting point was the model system mimicking the I form of wtGFP described above (Figure 1, right panel). The key idea was to replace Thr62 and Thr203 by Tyr residues and to narrow the spatial separation between the planar species in the triple-decker motif Tyr−Chr−Tyr. To accommodate the Tyr side chain at position 62, we had to introduce a few more mutations; the minimal set of four replacements is as follows: Thr62Tyr/Tyr145Ser/Ile167Gly/Thr203Tyr. Then the optimization of the ground-state structure was carried out using QM/MM. Figure 2 shows this model system with all four replaced residues. Note that the packing of the Chr−Thr203 pair in the computationally designed system resembles the corresponding motif in the crystal structure PDB id: 1YFP of the Thr203Tyr/Ser65Gly/Val68Leu/Ser72Ala GFP mutant.[15,16]

This model system is shown in Figure 3 from another angle allowing for direct comparison with wtGFP. We see that the most important features of the wtGFP machinery are not disturbed; the conservative Glu222 and Arg96 residues remain at their positions, and the hydrogen bond wire (Chr−Wat−Ser205−Glu222) is not disrupted.

The computed excitation energies for this mutant are collected in Table 2. The XMCQDPT2 values are computed with and without the field from the MM subsystem given in the columns marked "with EFP" and "no EFP", respectively.

Despite the slight variation in the computed values due to different approaches, the data in Table 2 clearly show that the mutant's optical bands are red-shifted (relative to the I form of wtGFP) by ∼0.2 (∼40 nm) and ∼0.1 eV (∼25 nm) for absorption and emission, respectively, amounting to 2.25 eV absorption and 2.16 eV emission. At all levels of theory, we observe that mutations do not affect oscillator strengths significantly, and both transitions remain bright.

As discussed in the introductory part of this Letter, a stacked motif with a tyrosine near the chromophore has been exploited in several studies.[15−17] A similar idea has been used in development of TagRFP567[10,18] in which the red chromophore is stacked with histidine (mutation at position 69). One can also consider using another aromatic residue, Trp; however, its larger size could disrupt folding. We note that for practical applications properties beyond spectral shifts, such as brightness and photostability, are crucially important. While first-principle modeling of these properties is beyond the scope of this study, we note the experimental evidence[16] that high quantum yields (e.g., >0.7 in some citrines) are attainable in π-stacked FPs.

In conclusion, a novel aspect of this study is a prediction of mutated structures in which the native GFP chromophore is sandwiched between two tyrosine residues. According to high-level calculations, these mutants featuring triple-decker stacked chromophores should exhibit noticeable red-shifted absorption and emission. The proposed motif can be used in directed mutagenesis studies aiming at engineering new RFP variants with improved optical properties.[8−10] In particular, this motif can be used to achieve a large Stokes shift via the eximer-type relaxation, that is, without excited-state proton transfer that leads to lower quantum yields. Thus, our study presents a

Table 2. Computed Wavelengths (nm, bold), Electronic Transitions Energies (eV, in parentheses), and Oscillator Strengths (in brackets) of the Triple-Decker Mutant of GFP and the Shifts of the Transition Energies Relative to the I Form of wtGFP (see Table 1)

	S_0-S_1			S_1-S_0		
	XMCQDPT2		SOS-CIS(D)	XMCQDPT2		SOS-CIS(D)
	with EFP	no EFP		with EFP	no EFP	
mutated protein	**527** (2.35)	**550** (2.25)	**530** (2.34)	**555** (2.23)	**572** (2.16)	**569** (2.18)
	[0.91]	[0.89]	[1.11]	[0.89]	[0.87]	[1.09]
shifts from wtGFP-I, eV	−0.16	−0.22	−0.17	−0.05	−0.12	−0.15
wtGFP-I	**493** (2.51)	**501** (2.47)	**492** (2.51)	**543** (2.28)	**542** (2.28)	**532** (2.33)
	[0.97]	[1.01]	[1.50]	[0.87]	[0.88]	[1.36]

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starting point for a new route toward new RFPs featuring smaller chromophores in a tight environment.

■ ASSOCIATED CONTENT

Supporting Information

Detailed description of the computational protocols; Cartesian coordinates for the optimized structures; and relevant molecular orbitals. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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