PROGRAMMA DI RICERCA STM

Istituto di afferenza: **Istituto Nanoscienze CNR**

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con qualifica: **Ricercatore livello III**

Titolo del programma:

Fluorescent proteins for super-resolution imaging: A molecular modeling approach

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1. Introduction

The research program of my three-week visit in Prof. Claudia Filippi's group at the University of Twente, Enschede, regarded the computational modeling of some aspects of fluorescent proteins optical properties, in particular focusing on the photoswitching effect, at the basis of applications of fluorescent proteins in super-resolution microscopy. The chromophore in fluorescent proteins is the chemical group absorbing and emitting light (see Figure 1). The protein amino acids surrounding the chromophore have critical effects in i) tuning the excitation and emission spectra [1] and ii) determining the fluorescent protein photophysics. Regarding the latter point, a particularly stunning example is how the single E222Q mutation is able to turn non-photoswitchable GFP mutants into photoswitchable ones [2]. It is known that photoswitching in GFP and related proteins involves *cis-trans* photoisomerization of the chromophore and change of its protonation state [2].

2. Aims

Our investigation was aimed at i) finding a theoretical/computational framework sufficiently accurate to describe the tuning of fluorescent proteins ii) devising ways to understand the effects of residues in modulating the photoswitching ability, with particular regards to the E222Q mutation.

3. Results

3.1 Tuning of fluorescent proteins

Different mutants and homologues of the Green Fluorescent Proteins, containing the same chromophore in the anionic protonation state, display a range of excitation wavelengths from ~450 to ~515 nm (variation in excitation energy of ~ 0.35eV). Describing this "fine tuning" effect requires an accurate description of the (mainly electrostatic) interactions between chromophore and surrounding residues. A computationally feasible way to treat the problem is to use different levels of theory for the light-active part (the chromophore) and the rest of the protein. The former is described by quantum mechanics (QM), the latter with empirical molecular mechanics force fields (MM).

Non-polarizable QM/MM coupling only partially (~0.1 eV) reproduces the tuning [Amat2012]. The inclusion of classical polarizable embedding through induced dipoles (QM/MMpol) was recently shown by Filippi and co-workers to yield excitation energies in agreement with the experimental measurements for the wild type GFP [3]. We extended this QM/MMpol approach to 2 proteins of the GFP family using CAM-B3LYP TD-DFT QM methods (calculations with CAS-SCF/CASPT2 are on-going). The results are reported in Table 1. With respect to the unpolarizable model (nopol) the MMpol excitation energies are red shifted (though still blue shifted with respect to experiment, as generally expected from CAM-B3LYP TD-DFT), and the 0.3 eV blue shift from Dronpa to MTFP0.7 is better reproduced (0.2 eV with MMpol against 0.1 eV in the non-polarizable model).

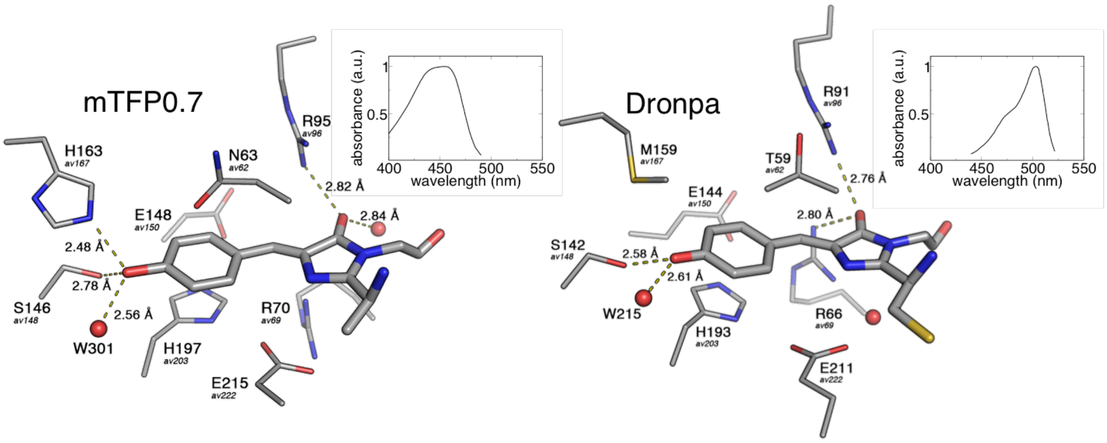

Figure 1: Chromophore and immediate environment of mTFP0.7 and Dronpa. The insets report the corresponding absorption spectrum (Figure modified from ref. [1]).

CAM-B3LYP MM (nopol)						**Exp**
Protein	**Exc (eV)**	**f**	**μ GS (x,y,z) (Debye)**	**μ ES (x,y,z) (Debye)**	**|Δμ| (Debye)**	**Exc (eV)**
Dronpa	3.17	1.05	0.7,17.4, 13.9	0.9,17.1,13.5	0.5	2.46
MTFP0.7	3.27	0.98	0.6,22.9,18.2	1.4,21.5,16.9	2.1	2.74
CAM-B3LYP MMPol						
Dronpa	2.94	1.23	-0.9,19.3,16.9	-0.4,18.1,15.9	1.6	2.46
MTFP0.7	3.13	1.15	-0.7,24.5,20.5	0.5,22.3,18.4	3.3	2.74

Table 1: Excitation energies with MMPol and nopol schemes. Ground and excited state dipole moments are reported (the latter in the CIS approximation), together with their difference in module.

Our conclusion is that the MMpol model for the inclusion of electrostatic polarization effects substantially improves the prediction of excitation tuning by the surrounding residues, at a very modest additional computational cost with respect to the non-polarizable QM/MM coupling. Thereby, the MMpol framework can be applied also to study the tuning of GAF domain proteins where it is important to understand the mechanisms of chromophore tuning in order to design mutants with larger spectral separation between ON and OFF states.

3.2 Photoswitching and mutations

We first selected two pairs of GFP mutants differing for the E222Q mutation. The first couple is EGFP (F64L and S65T, with respect to wild type GFP) and EGFP-Q (EGFP+E222Q). The second pair is F64L/T203Y (E1GFP) and F64L/T203Y/E222Q (also known as EYQ1). We performed 100-ns molecular dynamics simulations using the Amber99sb* force field (see ref. [4] for other details of the simulation). The aim of these MD simulations was to point out differences in the H-bonding network due to the E222Q mutation.

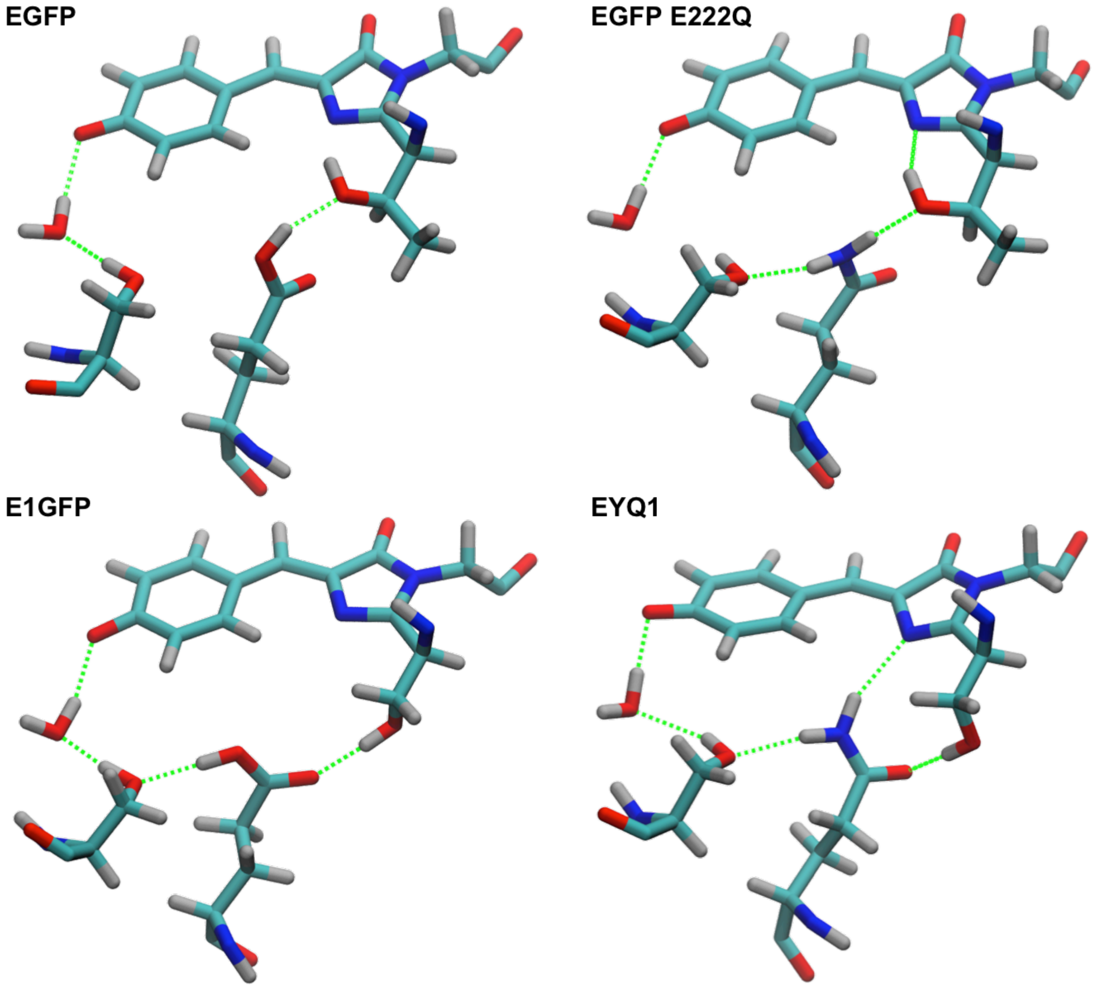

Figure 2: Representative snapshots from MD simulations showing the chromophore and residue E/Q222, S205 and the water molecule close to the chromophore phenolate oxygen. Green dashed lines indicate the presence of H-bond configurations.

The analysis of H-bond network (Figure 2) does not directly suggest a mechanism for photoswitching promotion by the Q222 mutants. However Q222 has a similar configuration in EGFP E222Q and EYQ1. This may result in a similar electrostatic modulation of the energy profile for *cis-trans* isomerization. This suggestion will be further investigated.

We analyzed another possible mechanism, based on excited-state proton transfer from protonated E222 to the imidazolidinone N of the chromophore. Indeed with respect to Gln (Q), protonated Glu (EH) can more easily donate its proton and become anionic. Once this putative proton transfer happens, the chromophore *cis-trans* isomerization pathway may be suppressed by competing de-excitation pathways.

To evaluate the feasibility of this pathway we evaluated the energy of this excited-state reaction:

$$\mathrm{EH+Cro\text{-}N^{-}\rightarrow E^{-}+Cro\text{-}NH}$$

The two excited-state optimized structures are shown in Figure 3. The atoms linking the chromophore and E222 to the rest of the protein were constrained, together with the two dihedral angles around the bonds linking the imidazolidinone ring to the rest of the protein.

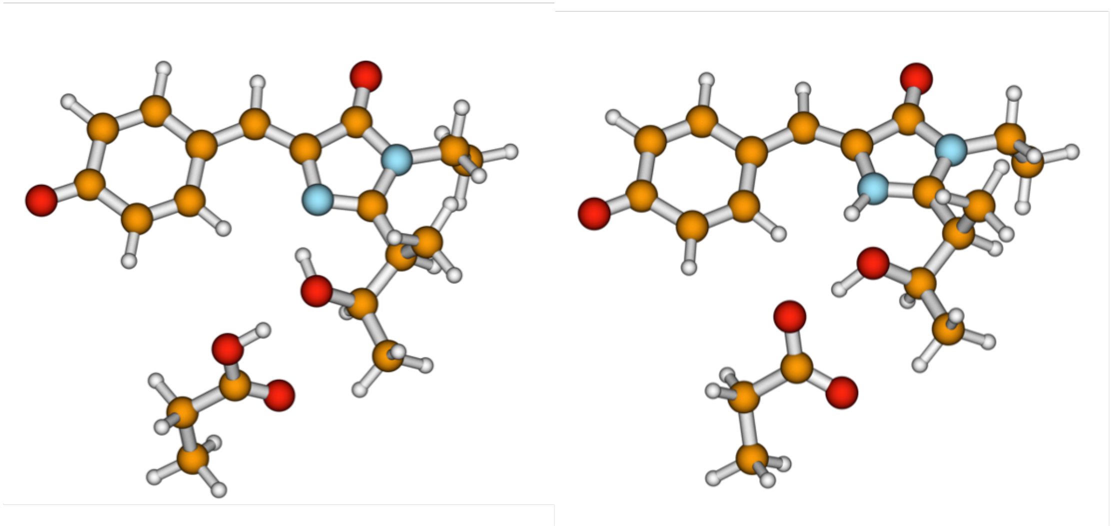

Figure 3: Chromophore and Glu222 in the two analyzed states before (left) and after (right) proton transfer from protonated Glu (EH) to the chromophore imidazolidinone.

The energies of the optimized excited-state structures are reported in Table 2. The proton-transferred configuration is about 6-7 kcal/mol higher in energy. This value is not so high as to exclude that it may be lowered to the thermal regime (0.5 kcal/mol) by the interactions of the chromophore with the other surrounding amino-acids and water molecules. Further analysis including the rest of the protein in a QM/MM approach is needed to elucidate these effects. It should be added that analogous calculations on the chromophore Gln222 system did not result in any stable minima for the proton-transferred state, indicating that only Glu222 can donate its proton.

EH+Cro-N$^-$(a.u.)	**E$^-$+Cro-NH (a.u.)**	**|ΔE| (Kcal/mol)**
B3LYP		
-1224.7025	-1224.6930	6.00
CAM-B3LYP		
-1224.0951	-1224.0833	7.39
LC-BLYP		
-1221.4359	-1221.4237	7.70

Table 2: Optimized excited-state energies before and after proton transfer of the two states in Fig. 3 evaluated with the reported exchange and correlation DFT potentials.

4. Conclusions and further considerations

My stay at Prof. Claudia Filippi group was fruitful and led to the results described above, which will be further refined and extended thanks to the established collaboration. In addition to the already reported activity, during thorough discussions with Claudia and her group, we analyzed on-going QM/MM simulations of two other reversibly switchable fluorescent proteins, Dronpa and Padron. These simulations were performed with the CPMD program, and extensive work was needed to calibrate the system, in particular regarding the interactions between the QM and MM parts (i.e. the cut-off schemes for electrostatics). Only after thoughtful consideration of the various parameters was it possible to obtained accurate simulations in terms of energy conservation and acceptable behavior of the (fictitious) electronic temperature. These QM/MM MD simulations will allow us to investigate ground-state proton equilibria in Dronpa and Padron at a reasonable computational cost. In addition, extension of the excited-state proton transfer analysis (described above) to the dynamical regime will be pursued.

References

[1] P Amat, R Nifosì , Spectral “fine” tuning in fluorescent proteins: The case of the GFP-like chromophore in the anionic protonation state. J Chem Theo Comput 9, 497-508. (2012).

[2] R Bizzarri, M Serresi, F Cardarelli, S Abbruzzetti, B Campanini, et al. Single amino acid replacement makes Aequorea victoria fluorescent proteins reversibly photoswitchable. J Am Chem Soc 132, 85-95 (2009).

[3] C Daday, C Curutchet, A Sinicropi, B Mennucci, C Filippi. Chromophore–Protein Coupling beyond Nonpolarizable Models: Understanding Absorption in Green Fluorescent Protein. J Chem Theo Comput 11, 4825-4839 (2015).

[4] M Drobizhev, PR Callis, R Nifosì, G Wicks, C Stoltzfus, L Barnett, et al. Long-and Short-Range Electrostatic Fields in GFP Mutants: Implications for Spectral Tuning. Scientific reports 5, 13223 (2015).