Photochromic Proteins

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Hydrogen Bond Fluctuations Control Photochromism in a Reversibly Photo-Switchable Fluorescent Protein

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Abstract: *Reversibly switchable fluorescent proteins (RSFPs) are essential for high-resolution microscopy of biological samples, but the reason why these proteins are photochromic is still poorly understood. To address this problem, we performed molecular dynamics simulations of the fast switching Met159Thr mutant of the RSFP Dronpa. Our simulations revealed a ground state structural heterogeneity in the chromophore pocket that consists of three populations with one, two, or three hydrogen bonds to the phenolate moiety of the chromophore. By means of non-adiabatic quantum mechanics/molecular dynamics simulations, we demonstrated that the subpopulation with a single hydrogen bond is responsible for off-switching through photo-isomerization of the chromophore, whereas two or more hydrogen bonds inhibit the isomerization and promote fluorescence instead. While rational design of new RSFPs has so far focused on structure alone, our results suggest that structural heterogeneity must be considered as well.*

Fluorescence microscopy with fluorescent proteins is playing an ever-increasing role not only in revealing how living systems function, but also in understanding diseases and finding new strategies to fight them.[1] Recent developments have even pushed the spatial resolution of this technique beyond the diffraction limit.[2] One strategy for achieving nanometer resolution in cells is to use reversibly photoswitchable fluorescent proteins (RSFPs),[3] whose fluorescence can be turned on and off repeatedly with different wavelengths. However, achieving the full potential of this technique for high resolution in vivo imaging requires significant improvements of these photochromic protein labels.

Proteins of the photochromic Dronpa family have promising switching characteristics,[4] but the achievable resolution is limited due to low photostability. Their application in fluorescence nanoscopy is further limited by the fact that photo-switching and fluorescence occur at the same wavelength. Although transient spectroscopy,[5] X-ray crystallography,[6] and NMR[7] have provided important information about the switching, these insights have so far not been sufficient to overcome the limitations of RSFPs through rational protein design. A notable exception is Dreiklang, which has different wavelengths for fluorescence and switching,[8] but the blue-shift into the UV limits its use in vivo.

The main obstacle of structure-based optimization approaches is that the photo-switching is essentially a dynamic process. Optimizing the switching therefore requires a complete understanding of the underlying molecular dynamics and the influence of the protein environment. As the relevant time and spatial resolution are notoriously hard to access by experiment, we have used hybrid quantum mechanics/molecular mechanics (QM/MM) simulations to reveal that photochromicity is controlled by structural fluctuations that have so far not been considered in the protein optimization process.

Because the low quantum yield of photo-switching in Dronpa would require far too many trajectories for a systematic investigation of the switching mechanism, we performed our simulations on the M159T mutant instead (Figure 1),[9] which has a much higher quantum yield of off-switching.[10] Replacing the bulky methionine by a threonine increases the volume around the chromophore.[9] In previous work, we speculated that this mutation reduces the steric hindrance during photo-isomerization and thereby enhances off-switching.[9] Spectroscopic investigations of this mutant have furthermore established that the chromophore is deprotonated in the fluorescent on-state.[5] Because the chromophore is also a strong photo-acid,[5c] no protonation was observed in the excited state.[5b] Therefore, our working hypothesis is that the chromophore is deprotonated before and during the excited state dynamics.

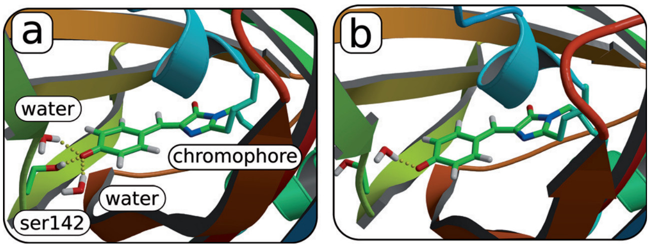

Figure 1. Close up of the chromophore pocket in the M159T mutant of Dronpa, with an intact (a) and a transiently broken hydrogen bond network (b).

In eight out of twelve QM/MM simulations, the chromophore remains planar after photo-excitation, and no decay from the excited state (S_1) to the ground state (S_0) was observed within 50 ps (Supporting Information, Figure S12 and Table S1). Instead, the S_1-S_0 energy gap is $292 \pm 16\,\text{kJ}\,\text{mol}^{-1}$ on average and never falls below $110\,\text{kJ}\,\text{mol}^{-1}$ (Figure S13). Although 50 ps is much shorter than the

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fluorescence lifetime in this protein, we consider these systems fluorescent because the planar conformation of the chromophore is apparently stabilized by the protein environment.

In the remaining trajectories, a simultaneous rotation of the τ and ϕ torsions was observed within 1 ps, bringing the system to a hula-twist conical intersection[11] with S_0 (Figure 2). The existence of this intersection and the pathway leading to it were confirmed at the correlated xMCQDPT2 level of theory (Figure S8).[12] In the protein environment, the hula-twist photo-isomerization[11] is favored over a single bond flip around τ or ϕ, because it avoids a steric clash with the histidine side chain that lies stacked on top of the chromophore and is held firmly in place by two ionic hydrogen bonds with glutamate side chains (Figure S18).

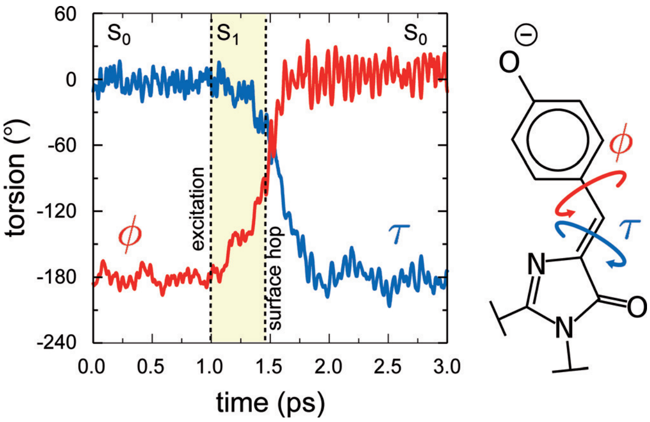

Figure 2. Time evolution of the methine bridge torsions after photo-excitation in a QM/MM trajectory initiated from a configuration with a single hydrogen bond to the phenolate moiety (Figure 1 b).

After reaching the S_1/S_0 intersection, the system decays to S_0, restoring the *cis* conformation of the chromophore in two out of four cases. In the other two cases, the chromophore quickly relaxes into the *trans* configuration. In line with previous experimental[6] and computational studies,[13] we associate the *trans* chromophore with the non-fluorescent state of Dronpa. Although de-activation of fluorescence also requires protonation, Van Thor and co-workers[5b] showed that this occurs after the isomerization and at timescales beyond reach of our QM/MM simulations. We therefore will address the protonation process in the future, using either a classical approach for proton transfer,[14] a combination of empirical valence bond theory[15] and the QCFF/PI model,[16,17] or by means of QM/MM pK_a calculations.[18]

Inspection of the starting geometries revealed that rapid photo-isomerization occurs only if the phenolate oxygen of the chromophore accepts at most one hydrogen bond from the environment, whereas no deactivation was observed if there are two or three hydrogen bonds (Table S1). Upon relaxing from the Frank–Condon region to the hula-twist conical intersection, about −0.3 e charge migrates from the phenolate ring onto the imidazolinone ring (Figure S15). By stabilizing the negative charge on the phenolate ring, the hydrogen bonds oppose this charge transfer and thus introduce a barrier for the photo-isomerization process. Therefore, reducing the number of hydrogen bonds is necessary to remove this barrier and unlock ultra-fast access to the conical intersection (Figure S15).

The crystal structure of the M159T mutant[9] suggests three hydrogen bonds: one with Ser 142, and two with two waters molecules (Figure 1 a). Although this hydrogen bond pattern remains stable on average throughout a 100 ns classical MD simulation, several fluctuations were observed in which one or two hydrogen bonds are transiently broken (Figure 1 b and Figure 3). Not only are the internal water molecules exchanging with bulk solvent, but the hydrogen bond with Ser 142 also breaks and reforms repeatedly (Figure 3). Thus, if, as the QM/MM simulations suggest, two of the hydrogen bonds are required to break in order to activate off-switching, the MM trajectory shows that such events are sufficiently frequent to ensure a significant isomerization quantum yield.

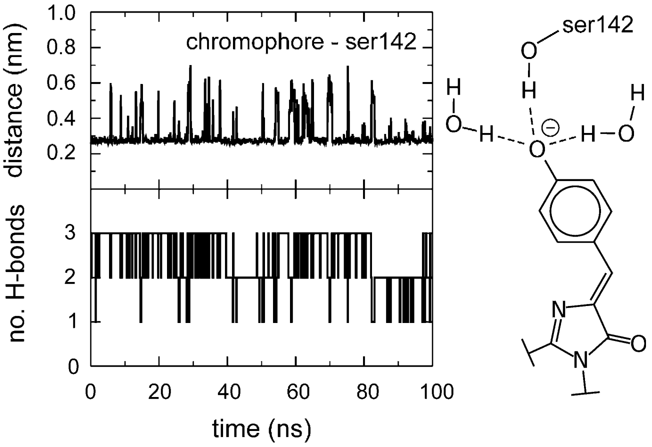

Figure 3. Time evolution of the distance between the hydroxy group of Ser 142 and the chromophore (top) and of the total number of hydrogen bonds that the phenolate moiety forms (bottom) in a classical MD simulation.

The notion that photo-isomerization is only possible during structural fluctuations has, to our knowledge, not been considered to explain why these proteins are photochromic. Our results not only underscore the necessity to include ground state equilibration in QM/MM simulations of reactive events,[19] but also reveal a major weakness in focusing solely on the X-ray structure when optimizing the properties of RSFPs.

Furthermore, our findings suggest an alternative strategy to control the switching. The heterogeneity responsible for photochromism may be exploited if the fluorescent and switchable populations can be excited selectively by choosing an appropriate excitation wavelength. Calculations at the xMCQDPT2/cc-pVDZ level[12] on a larger QM system demonstrate that the absorption wavelength increases with the number of hydrogen bonds (Table S2). These small differences in excitation energies between the populations suggest that such approach may indeed be possible and could be used not only to verify our predictions, but also to achieve higher resolution in nanoscopy.

In summary, we found that structural heterogeneity in the hydrogen bond network around the chromophore divides the protein ensemble into fluorescent and non-fluorescent populations. To keep the chromophore planar in the excited state and promote fluorescence, two or three hydrogen bonds are required. In contrast, a single hydrogen bond is too weak to prevent isomerization and promotes photo-switching by a hula-twist mechanism instead.

Experimental Section

In total 12 QM/MM surface hopping simulations were initiated from a 100 ns ground state MM trajectory and run for at most 50 ps in the excited state. In these simulations, the chromophore was described at the SA(2)-CASSCF(6,6)/3-21G level of theory,[20] while the remainder was modeled with the Amber03 force field.[21] *Diabatic* surface hopping was used to model deactivation at the conical intersection.[22] Further details of the simulations are given in the Supporting Information. Prior to the simulations, the active space was validated by comparing the energies of stationary points to higher levels of theory. We also recomputed the energy profiles along the trajectories at the highly accurate xMCQDPT2//SA(3)-CASSCF(12,11)/cc-pVDZ level[12] with a much larger QM system. Details of these and further validation studies, including the effect of incorporating water molecules into the QM region, are described in the Supporting Information.

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