Abstract

We propose a new scheme of the photoactivation and the photobleaching for Dronpa with molecular dynamics method and density functional theory. These processes can be explained by considering cis-trans isomerization in neutral state of chromophore. The proton transfer from anionic to neutral chromophore makes cis-trans isomerization possible via hula-twist rotation process, since the space for cis-trans isomerization is opened at around the region near chromophore by moving out of the imidazole ring on H193 from the position below the phenol ring on chromophore. Then the cis-trans isomerization can occur through the hula-twist process. The contributions from the protein environment around CRO, especially S142 and H193, are indispensable for photoconversion of Dronpa.

keyword: Dronpa, Photoactivatable fluorescent proteins, Proton transfer, cis-trans isomerization

1. Introduction

In 1960’s Shimomura et al.1 discovered green fluorescent protein (GFP) from jellyfish (Æquorea victoria). Nowadays, many fluorescent proteins are applied to a biological marker in the wide area such as molecular biology, medicine, and cell biology. In particular, photoactivatable fluorescent proteins (PAFPs)1 draw attention as a useful tool to directly observe the movement of some biomolecules at multiple time points in individual cells. The PAFPs are capable of changes in their spectral properties in response to irradiation with light of a specific wavelength and intensity.

Recently, Ando et al.4 purified Dronpa from a coral protein. Dronpa has a property of reversible photoconversion from a green fluorescent state (bright state) to non fluorescent state (dark state). Such property of Dronpa is applied to the analysis of the heretofore unexplored regulation of fast protein dynamics, and there are also some reports to enhance the fluorescent characters for Dronpa5, 6. In the bright state, Dronpa has an absorption major peak at 503 nm (2.46 eV), and an emission peak at 518 nm (2.39 eV) with a very high fluorescence quantum yield (φf=0.85).
Intense irradiation with blue light (470-510 nm) for bright state converts to the dark state with the absorption peak at 390 nm (3.18 eV). This photophysical behavior is called photobleaching. In contrast, irradiation with UV laser (390-410 nm) for the dark state converts to bright state, and such photophysical behavior is called photoactivation. Ando et al.\(^7\) reported that the phenol (hydroxyphenyl) moieties on chromophore (CRO) of Dronpa in bright and dark states are anionic and neutral forms, respectively, by NMR analysis. Andersen et al.\(^8\) reported that the CROs in each state are cis and trans forms, respectively, by X-ray structural analysis. These reports mean that both the excited-state proton transfer (ESPT) and the cis-trans isomerization are important for the photoconversion in Dronpa.

To explore the mechanism of such reversible photoconversion of Dronpa, some experimental and theoretical papers were already reported\(^9\)-\(^12\). Nam et al.\(^9\) pointed out that there was no space at around the region near CRO in the structures of bright state, and a large conformational change was indispensable for the cis-trans isomerization of the CRO. Recently, Miyawaki et al.\(^11\) suggested the photoconversion scheme of Dronpa. In this scheme, photobleaching occurred from cis form of anionic CRO (B state) into trans form of neutral CRO (A\(_2\) state), while the photoactivation occurred from the A\(_2\) to the B states via non-fluorescent intermediate form (I state). They proposed that some species in the excited B state does not turn into the A\(_2\) state, but into the "unknown dark-state (D state)", and then converts into the B state spontaneously. They also suggested that the A\(_2\) state converts into the I state slowly in "ground state".

There are some reports for the calculation of model structures of CRO, since it is very difficult to calculate large molecules such as whole proteins in excited state. For instance, absorption energies were compared between theoretical and experimental values for many CROs\(^13\),\(^14\). In addition, some studies about production and decay of the fluorescent state were reported, and the decay channel along a space-saving hula-twist process through a conical intersection was proposed.\(^15\) To our knowledge, however, the mechanism for photoconversion in Dronpa is not known, yet.

In this paper, thus, we have performed molecular dynamics (MD) and density functional theory (DFT) calculations to qualitatively understand the reaction path considering the effect of conformation change for whole Dronpa and to propose the new scheme of photoconversion (photoactivation and photobleaching) in Dronpa. In Section 2, we describe the computational details of MD and DFT calculations. In Section 3, some results of our calculations are reported. First, MD calculations were performed to explore the difference between neutral and anionic structures of CRO in cis form. Second, to elucidate the mechanism of cis-trans isomerization, we performed some MD and DFT calculations of cis, trans, and corresponding approximate transition state structures for CRO model and whole protein. From our results, we would propose a new scheme of the photoswitching on Dronpa. Finally, we would like to draw our conclusions in Section 4.

2. Computational Details

2.1. Molecular dynamics simulation

The MD calculations were performed for the system which contains whole Dronpa and 6819 water molecules, as shown in Figure 1, in truncated octahedral cell with the periodic boundary condition using AMBER\(^16\) program package. We calculated cis structures with anionic (anionic cis) and neutral (neutral cis) CRO forms, and trans structure with neutral (neutral trans) CRO form. The AMBER\(^17\),\(^18\) and TIP3P\(^19\) force fields were used for whole Dronpa and water molecules, respectively. For CRO, in particular, a general AMBER force field (GAFF)\(^18\) was used. We have already checked the validation of the GAFF for CRO in our previous work.\(^20\)

The initial coordinates were picked up from 2Z1O\(^7\) PDB, and hydrogen atoms were added to the anionic cis and the neutral cis forms, based on the structures proposed by Mizuno et al.\(^7\) As the initial coordinate for the trans form, we substituted trans CRO for cis CRO in the 2Z1O PDB structure. The energy minimization was carried out from these three structures. Then, we performed the NVT-MD simulation at 300 K from minimized structures to obtain the thermally averaged structures. The time step and total simulation time are 2.0 fs and 4.0 ns (2,000,000 steps), respectively.
2.2. Density functional theory calculations

We calculated cis, trans, and corresponding transition state (TS) for anionic and neutral 4-hydroxy-benzylidene-1,2-dimethylimidazoline (HBDI) known as a model structure of CRO, as shown in Figure 2, using Gaussian 03 program package. We assume the TS structure with the constraint of two dihedral angles of $\phi$ and $\tau$ in Figure 2, that is, $(\phi, \tau) = (0.0, \pm 90.0)$ and $(\pm 90.0, \pm 90.0)$ are fixed for TS structures via simple $\tau$ (ST) and hula-twist (HT) rotations, respectively, because of the limitation of our computational facilities. The equilibrium structures were optimized with B3LYP/6-31G* level for $S_0$ state, and calculated the energies of $S_1$ state under these structures with TD-B3LYP/6-31G*. It is reported that the B3LYP functional calculations can be reproduced qualitatively the excited energies for some CROs of some fluorescent proteins.

We have also performed the single point ONIOM calculation of whole Dronpa and 2021 water molecules for the structures minimized with AMBER force field. We defined the CRO corresponding to HBDI, water46, and the parts from $\alpha$-carbon on H193 to $\alpha$-carbon on I195 as high-level layer with B3LYP or TD-B3LYP/6-31G*, and the other residues and water molecules as low-level layer with AMBER force field.

Fig. 2. HBDI (4-hydroxybenzylidene-1,2-dimethylimidazoline) model structure for CRO. The dihedral angles $\phi$ and $\tau$ are defined.
3. Results and Discussion

3.1. Protein structures with the neutral cis and the anionic cis CRO with MD calculations

MD calculations of whole Dronpa with 6819 water molecules were performed to analyze the difference between the neutral cis and the anionic cis forms. The energy of the anionic cis form is 70.0 kcal/mol lower than that of the neutral cis form. We focus on the three hydrogen-bonding distances between oxygen atom on phenol ring at CRO and oxygen atom on hydroxyl group at S142 (R1), oxygen atom on peptide group at S142 and nitrogen atom on peptide group at H193 (R2), and nitrogen atom on peptide group at S142 and oxygen atom on peptide group at H193 (R3) as shown in Figure 3(a).

Figure 4 shows the distributions of these distances for (a) the anionic cis and (b) the neutral cis forms, respectively. The neutral cis form is a proton transferred structure from the anionic cis form. The considerable difference between the anionic and the neutral structures is found for the distributions of R1, each peak are observed around 2.7 Å and 4.0 Å, respectively. Our result clearly supports the experimental results by NMR7, where S142 does not form a hydrogen-bonding to CRO in neutral form, while S142 forms a hydrogen-bonding in the anionic form.

![Fig. 3](image3.png)

**Fig. 3.** The top-view (a) and side-view (b) of the part of hydrogen bonding system around CRO in Dronpa. We defined the center of the double carbons (X) as the point between the carbon on CH group and 4-th carbon on imidazole ring. The five distances are also shown.; The hydrogen-bonding distances between oxygen atom on phenol ring at CRO and on oxygen atom on hydroxyl group at S142 (R1), oxygen atom on peptide group at S142 and nitrogen atom on peptide group at H193 (R2), and nitrogen atom on peptide group at S142 and oxygen atom on peptide group at H193 (R3), The distance between a center of imidazole ring on H193 and a center of phenol ring on CRO (R4) or X point (R5).

![Fig. 4](image4.png)

**Fig. 4.** The distributions of hydrogen bonding distance of R1, R2, and R3 in the anionic form (a), and the neutral form (b).
b) Neutral cis form

Next, we focus on the two coordinates, which are the distances from the center of imidazole ring on H193 to the center of phenol ring on CRO (R₄) and to the center (X) between the carbon on CH group and 4-th carbon on imidazole ring (R₅), as shown in Figure 3(b). The distributions of R₄ and R₅ are shown in Figure 5(a) (b), where R₄ in the neutral cis form tends to be shorter than that in the anionic cis form, while R₅ in the neutral cis form tends to be longer than that in the cis anionic form. These results show that, in the case of neutral cis form, the space for cis-trans isomerization is opened at around the region near CRO by moving out of the imidazole ring on H193 from the position below the phenol ring on CRO. Figure 6 shows the two-dimensional distributions of dihedral angles, defined in Figure 2, in the anionic cis (a) and the neutral cis (b) forms. The distribution along τ is around 0 deg. in the anionic cis form. Meanwhile, we notice that, in the neutral cis form, the distribution becomes broader having negative values of both φ and τ dihedral angles at around -17 and -7 deg., respectively. This results means that the structures of neutral form tend to be distributed in the region of the hula-twist (HT) process, compared with the structures of the anionic form.
As the results of such different distributions between the anionic and the neutral forms in Figure 4, 5, and 6, the thermally averaged snapshots for both forms are shown in Figure 7. As shown in Figure 5(a) (b), we found the stacking between the phenol ring on CRO and imidazole ring on H193 in the anionic cis form, while it is broken in neutral cis form. We have found that the space between H193 and CRO, as shown in Figure 7(b), is enough to isomerization between cis and trans forms of CRO via HT rotational process. We can suggest that the protein environment in neutral form has possibilities for CRO to isomerization between the cis and trans forms via HT rotation.

3.2. CRO isomerization from the neutral cis to the neutral trans form with MD calculations

To elucidate the isomerization from the neutral cis to the neutral trans form, we performed the MD calculation for the neutral trans structure that we substituted trans CRO for cis CRO of the neutral cis structure. Figure 8 shows the representative snapshot for the neutral trans form. As shown in the neutral cis form, the stacking between the phenol ring on CRO and imidazole ring on H193 are found and the hydrogen bonding between CRO and S142 does not exist in the neutral trans form. In addition, a new hydrogen bonding network is formed between CRO and E144 and between CRO and w242 in Figure 8. We notice here that our minimized neutral trans structure is very similar to the 2POX° PDB structure.
We found the minimized anionic cis structure is 28.0 kcal/mol lower than the neutral trans structure. The energy barriers from cis to trans forms are estimated as 112.0 and 75.0 kcal/mol via ST and HT rotations, respectively. The energy barrier via HT rotation is 37.0 kcal/mol lower than that through ST rotation in the isomerization, because the ST isomerization requires more space according to the larger conformational change than the HT isomerization.

### 3.3. Cis-trans isomerization with density functional theory

Next, we performed DFT and TD-DFT calculations with B3LYP functional. Table 1 shows the vertical absorption energies of Bright and Dark states and their energy shifts for HBDI and whole Dronpa. Though the absolute values of the absorption energies are not in agreement with the corresponding experimental values, our energy shift of ONIOM calculation is 0.73 eV, which is in reasonable agreement with the corresponding experimental value of 0.72 eV. Thus, we adapted B3LYP functional calculations for the qualitative discussion of the Dronpa photoconversion.

To estimate the energy barriers for cis-trans isomerization via ST and HT rotations on S0 and S1 states in Dronpa, at first, we performed DFT and TD-DFT calculations for the simple HBDI model. Table 2 shows the relative energies of each form in S0 and S1 states, where the origin is set to the energy of the cis form in S0 state. The energy barriers from cis to trans forms in S0 state are 29.5, 42.4, 36.6, and 40.8 kcal/mol for ST and HT isomerization process in the anionic and the neutral forms, respectively, while those in S1 state are 6.1, 34.5, 14.0, and 28.3, respectively. The energy barriers in S1 state are lower than those in S0 state in all processes, and the energy barriers via ST processes are lower than those via the HT processes. We again address here that the isomerization via ST rotation requires a big cavity around CRO region. There is, however, no space for CRO to ST isomerization in the environment of whole Dronpa.

At the next step, we performed the ONIOM calculations. Table 3 shows the relative energies in the processes of ST and HT rotations for the neutral form of CRO in S0 and S1 states. The energy barriers in S1 state are 28.8 and 14.0 kcal/mol via ST and HT rotations, while those in S0 state are 100.3 and 60.5 kcal/mol, respectively. We found that the energy barriers via HT rotation are lower than those via ST rotation in the whole protein environment. The result of ONIOM calculation is similar to that of MD calculation, where the energy barrier for the cis-trans isomerization via HT process is lower than that via ST process. Our ONIOM result suggests that the energy barrier for HT process in the S1 state is about a quarter of the barrier in the S0 state, though our S1 energy profiles were evaluated with vertical excitation energies.

| Table 1 | Vertical absorption energies and their shifts for HBDI and whole Dronpa with TD-B3LYP/6-31G* and ONIOM(TD-B3LYP/6-31G*:AMBER), and experimental values. The unit is in eV |
|---------|-------------------------------------------------------------------------------------------------|
| Our calculations | Exptl. |
| HBDI model | ONIOM calc | gas | protein |
| anionic-cis (Bright state) | 3.19 | 3.12 | 2.59 | 2.46 |
| neutral-trans (Dark state) | 3.45 | 3.85 | (3.12) | 3.18 |
| Absorption energy shift | 0.26 | 0.73 | (0.53) | 0.72 |

| † This value is an absorption peak for neutral-cis form of HBDI in gas phase. |

| Table 2 | The relative energies of cis-trans isomerization via simple r rotation (ST) and hula-twist (HT) rotation for HBDI model with the anionic form and the neutral form. The unit is in kcal/mol |
|---------|-------------------------------------------------------------------------------------------------|
| anionic form | neutral form |
| cis form | TS form | trans form | cis form | TS form | trans form |
| S0 | 0.0 | 29.5 | 42.4 | 2.5 | 0.0 | 36.6 | 40.8 | 2.4 |
| S1 | 73.7 | 79.8 | 108.1 | 76.4 | 81.5 | 95.5 | 109.8 | 82.2 |
Table 3. The relative energies of cis-trans isomerization via simple $\tau$ rotation (ST) and hula-twist (HT) rotation for whole Dronpa protein with ONIOM calculations. The unit is in kcal/mol

|       | neutral structure | TS form | trans form |
|-------|-------------------|---------|------------|
| cis form | $S_0$  | 0.0 | 100.3 | 60.5 | 20.4 |
| trans form | $S_1$  | 95.9 | 124.7 | 109.9 | 109.1 |

3.4. New proposed scheme of photoswitching for Dronpa

We propose a new scheme of the photoswitching on Dronpa based on our and previous results in Figure 9. In this mechanism, the reversible photoswitching mechanism of Dronpa can explain with only three CRO states of the neutral trans form (A state), the neutral cis form (I state), and the anionic cis form (B state).

Our MD results show that the B state is most stable structure, and the I state is metastable structure. From our ONIOM results, the I state is more stable than the A state, and the energy barrier from the I to the A state in $S_1$ state is extremely lower than that in $S_0$ state.

Fig 9. Proposed schematic diagram of photoconversion for Dronpa.
In the process of photobleaching, the ESPT from the anionic to the neutral forms of cis CRO occurs at the first step. Then, the cis-trans isomerization can be induced by the HT rotation, and the state of Dronpa changes from S₁ to S₀ states as the second step. After that, some species turn into either the A or I states. As coming to the I state, a proton transfer from the neutral cis to the anionic cis form will be easily occurred in S₀ state. In the photoactivation of Dronpa, at the first step, the cis-trans isomerization of CRO occurs by HT rotation in S₁ state, and the state of Dronpa changes to S₁ state. Then, the CRO changes to the B state via the I state. The reason why the A and the I states via S₁ are non-fluorescent process may be the existence of the conical intersection\(^{15}\) on CRO in the transition from S₁ to S₀. The I state in our scheme can be corresponding to the D state in Miyawaki’s scheme.

The cis-trans formation of CRO following ESPT is indispensable to explain the first step of the mechanism of the photoactivation and photobleaching of Dronpa. Our scheme is very similar to that of the reversible photoswitching mechanism of asFP595.\(^{24}\) Our results suggest that the contributions from the protein environment around CRO, especially S₁₄₂ and H₁₉₃, are very important in the cis-trans isomerization process of Dronpa. The simulation of whole Dronpa in S₁ state with ONIOM method, including these residues as high-level layer, would be an interesting subject to obtain the dynamical information for the photoconversion of Dronpa, and such study is now preparing in our group.

4. Conclusions

We propose a new scheme of the photoactivation and the photobleaching for Dronpa with molecular dynamics method and density functional theory. These processes can be explained by considering cis-trans isomerization in neutral state. In photobleaching process, the excited-state proton transfer (ESPT) makes cis-trans isomerization of the CRO possible via HT rotation process, since the space for cis-trans isomerization is opened at around the region near CRO by moving out of the imidazole ring on H₁₉₃ from the position below the phenol ring on CRO. The contributions from the protein environment around CRO, especially S₁₄₂ and H₁₉₃, are indispensable for the photobleaching and photoactivation process of Dronpa. Now, we are preparing the dynamics of whole Dronpa in excited state with ONIOM treatment.

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