Combined Study by Crystallography and NMR Reveals Structural Basis for a Reversible Switchable Fluorescent Protein

As affirmed by the 2008 Nobel Prize in Chemistry recognizing the initial discovery of green fluorescent protein (GFP) and a series of influential developments related to its biological applications, GFP and GFP-like proteins are now ubiquitous tools. The proteins enable us to visualize interesting, but otherwise invisible, phenomena such as gene expression, subcellular localization of protein, and protein-protein/DNA interaction in living cells. In recent years, interest has grown in the development of reversible switchable fluorescent proteins (RSFPs) that can be selectively and reversibly switched between a fluorescent (bright) state and nonfluorescent (dark) state by irradiation with light of appropriate wavelengths. Such ability promises more localized and/or precise studies of rapid protein movements within living cells. A successful example of RSFP includes Dronpa from the coral *Pectiniidae* [1]. Whereas Dronpa emits green fluorescence, strong irradiation at 488 nm can convert the protein into a dark state (Dronpa^{D}). The protein can be switched back to the original bright state (Dronpa^{B}) from the Dronpa^{D} by brief and minimal irradiation at 403 nm. Despite the exquisiteness of the protein for not only biological application but also realization of a nondestructive read-out system, the structural basis for the reversible switching in Dronpa is still poorly understood.

To unravel the mechanisms, we determined the crystal structures of Dronpa^{B} using beamlines **BL26B1**, **BL44B2** and **BL45XU** PX-station as well as 22 G, the obligate tetrameric precursor protein of Dronpa. The overall structures of both proteins were nearly identical to other GFP structures, namely, an 11-stranded β-barrel with a coaxial central α-helix holding a chromophore (so-called "β-barrel fold") (Fig. 1(b)). The chromophore formed spontaneously from the internal tripeptide Cys62-Tyr63-Gly64. The resulting 4-(*p*-hydroxybenzylidene)-5-imidazolinone moiety is typical for green-emitting GFP-like proteins. The chromophore adopted *cis* and coplanar conformation and the phenolate moiety hydrogen-bonded to Ser142. The imidazole ring of His193 was parallel to the phenolate ring of the chromophore, which is representative of a π-π stacking interaction. The structural comparison between Dronpa^{B} and 22 G indicated little difference in the chromophore environment between the two proteins (Figs. 1(c) and 1(d)). This is in line with the fact that Dronpa acquired the reversible switchable ability during a mutagenesis study aimed at the monomerization of 22 G, and thus, the substituted six residues do not make direct contacts with the chromophore. Therefore, it was hypothesized that the monomeric property is essential for engineering RSFPs.

Next, we determined the crystal structure of Dronpa^{D}. Although the crystal of Dronpa^{B} can be converted to Dronpa^{D} by irradiation with a 514.5 nm argon laser at ambient temperature and turned back to the Dronpa^{B} by irradiation with violet light, the electron density corresponding to the chromophore and the vicinity such as His193 in the Dronpa^{D}

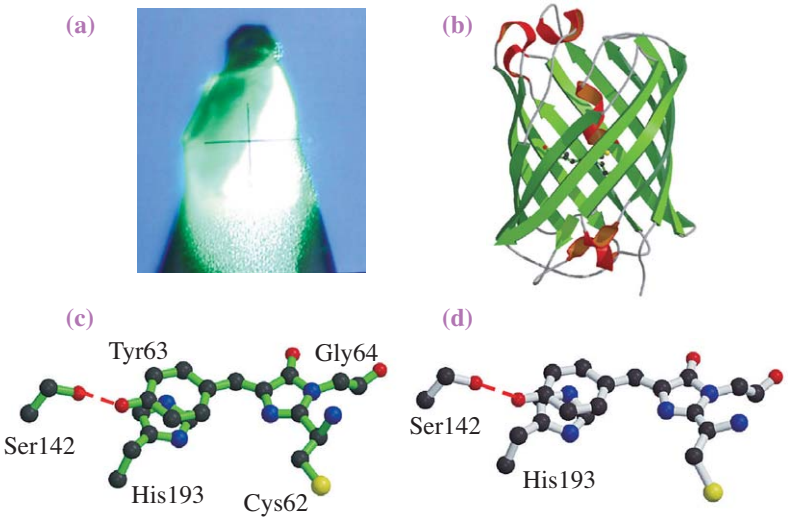

Fig. 1. **(a)** Crystal of Dronpa^{B} on beamline BL44B2. **(b)** Overall structure of Dronpa^{B}. The chromophore is represented as a ball-and-stick model. The chromophore environments of Dronpa^{B} **(c)** and 22G **(d)**. 22G is the precursor protein of Dronpa.

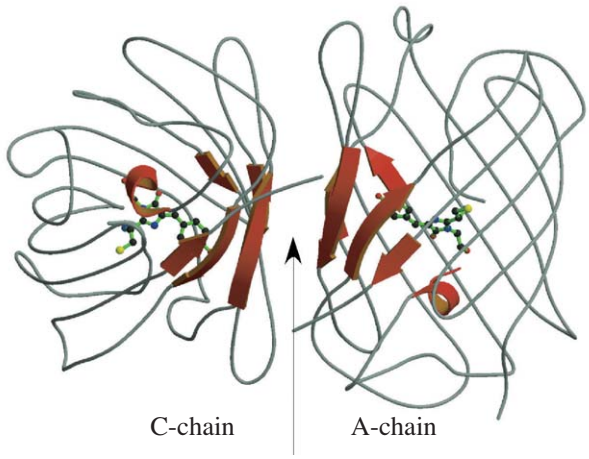

Fig. 2. Mapping of dimer interface, in which the residues in red were not sssigned to any peaks in NMR spectra of Dronpa^{D}, on the Dronpa^{B} crystal structure. The chromophores are represented as a ball-and-stick model.

structure was not well resolved, suggesting a disorder, i.e., the chromophore and surroundings may adopt various conformations in Dronpa^{D}.

Although the monomeric state of Dronpa was confirmed in solution, the crystal structure of Dronpa^{B} revealed that the crystallization of Dronpa enforced a dimer-of-dimer formation on the protein. The tetramer formation in the crystal was considered to be inappropriate for further investigation. Thus, we performed NMR analysis of Dronpa in solution at ambient temperature in combination with the crystallographic study. The NMR data conclusively demonstrated the structural flexibility around the dimer interface, where the peaks for Dronpa^{D} could not be assignable (Fig. 2). The ^{1}H-^{15}N heteronuclear sequential quantum correlation (HSQC) spectra also indicated the flexibility of a β-strand containing His193 that participated in the dimer interface. In addition, a peak for the proton assigned to the Ser142 hydroxyl group at 14.0 ppm was present in the ^{1}H NMR spectrum for Dronpa^{B} but not for Dronpa^{D}, indicating the loss of the hydrogen bond between Ser142 and the chromophore of Dronpa^{D}. Altogether, we proposed a structural basis for the reversible photoswitching in Dronpa as shown in Fig. 3; the deprotonated chromophore (phenolate form) tightly adopted the *cis*/coplanar conformation mainly because of non-covalent interactions from Ser142 and His193 in Dronpa^{B}. Upon irradiation, the chromophore is protonated (phenol form) and becomes free from the β-barrel in Dronpa^{D}. As a result, the chromophore and part of the β-barrel become flexible. The proposed mechanism focusing on the flexibility of the protein is perfectly consistent with the initial hypothesis, in which the monomeric property is essential for engineering RSFPs.

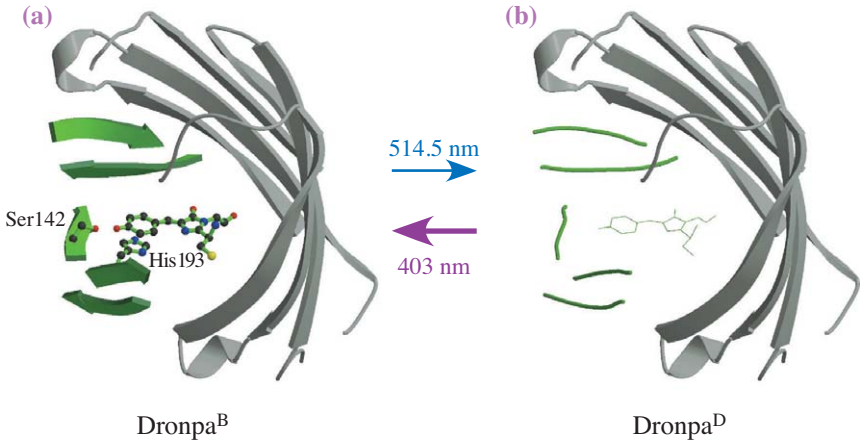

Fig. 3. Proposed mechanism for the reversible switching in Dronpa. The key to the reversible switching is a light-dependent regulation of the structural flexibility of the chromophore and part of the β-barrel in the protein. In Dronpa^{D}, strands highlighted with lime green and the chromophore are flexible **(b)**, while they have a rigid structure that is characteristic of GFP proteins in the Dronpa^{B} **(a)**, probably due to noncovalent interactions between the chromophore and surroundings such as Ser142 and His193.

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References

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