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Structural characterization of the photoswitchable fluorescent protein Dronpa-C62S ☆

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Abstract

The photoswitching behavior of green fluorescent proteins (GFPs) or GFP-like proteins is increasingly recognized as a new technique for optical marking. Recently, Ando and his colleagues developed a new green fluorescent protein Dronpa, which possesses the unique photochromic property of being photoswitchable in a non-destructive manner. To better understand this mechanism, we determined the crystal structures of a new GFP Dronpa and its mutant C62S, at 1.9 Å and 1.8 Å, respectively. Determination of the structures demonstrates that a unique hydrogen-bonding network and the sulfur atom of the chromophore are critical to the photoswitching property of Dronpa. Reversible photoswitching was lost in cells expressing the Dronpa-C62S upon repetitive irradiation compared to the native protein. Structural and mutational analyses reveal the chemical basis for the functional properties of photoswitchable fluorescent proteins and provide the basis for subsequent coherent engineering of this subfamily of Dronpa homolog's.

Keywords: Dronpa; Real-time imaging; Photoswitchable; Dronpa-C62S; Isomerization

Fluorescent proteins have become a popular tool for studying gene expression, subcellular localization, protein–protein/protein–DNA interactions, biosensors, single-molecule studies, etc. These proteins have wide usage in molecular and cell biology and play a vital role in cell system research [1]. There have been various difficulties due to *in vivo* and *in vitro*—photobleaching, however, resulting in low quantum yields. Recently, a new green fluorescent protein cloned from coral, termed Dronpa, was developed by Ando et al. [2]. These investigators identified a unique photochromic property of Dronpa i.e. the protein could be excited, erased, and excited again in a non-destructive manner. The absorption/emission peaks were at 503/518 nm, and Dronpa could be converted into a dim state by intense irradiation at 488 nm and switched back to the original emissive state by minimal irradiation at 405 nm. By using this new reversibly photoswitchable green fluorescent protein (GFP), the monitoring of fast protein dynamics, such as nucleocytoplasmic shuttling of a signaling protein becomes possible *in vivo* [2].

Habuchi et al. [3] recently, demonstrated the reversible photoswitching behavior of Dronpa at the single-molecule level and proposed a model consisting of several states. Reversible photoswitching was possible at over 100 repetitions with the response time dependent on the irradiation

☆ Coordinates: Coordinates have been deposited in the Protein Data Bank (Accession Codes pdb2GX0 and pdb2GX2).

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power in the order of milliseconds [3]. Using subsequent X-ray analysis, Andresen et al. [4] demonstrated that the chromophore of asFP595-A143S protein, another photoswitchable protein with low quantum yield and comparatively slow switching kinetics, isomerizes from a *trans* (off) to a *cis* (on) state upon stimulation. These investigators used molecular dynamics calculations to suggest that the isomerization adopts a bottom hula twist mechanism (HT^{bot}) through rotation of both bonds of the chromophoric methionine ring bridge [4], which implies that a large structural change of the molecule is required for the switching behavior. While this manuscript was under review the bright-state structures of Dronpa were reported [5,6]. These reports suggest that the isomerization of the chromophore may be key in photoactivation. To better understand the molecular mechanism of photoswitchable proteins, crystal structures of Dronpa from *Echinophyllia* sp. *SC22* were determined by X-ray crystallography. In this study, we present the structures of Dronpa-apo and Dronpa-C62S and describe the chemical basis for the non-destructive reversible photoswitching characteristics of Dronpa. In addition, we have explored the photoswitching behavior of Dronpa-C62S in cells by fluorescence microscopy to confirm the postulated role of the sulfur atom of the chromophore in Dronpa. These structural studies contribute to an improved understanding of the basis for photoswitching, spectral tuning, and oligomerization in Dronpa. Our results are expected to provide valuable information for the rational engineering of this new molecular marker for real-time imaging.

Materials and methods

Expression and protein purification. The expression culture was grown at 37 °C in LB supplemented with kanamycin (50 μg/mL) and induced for 18 h at 22 °C by adding 1 mM IPTG. After centrifugation at 5000*g* for 30 min, the harvested cells were suspended in lysis buffer (50 mM Tris–HCl, pH 8.0; buffer A) and sonicated. The supernatant was heat-treated at 50 °C for 5 min, and then centrifuged at 20,000*g* for 30 min. The supernatant was loaded onto a His Trap column (Amersham Biosciences) pre-equilibrated with buffer A. The protein was eluted using a linear gradient of 0.5 M imidazole in buffer A. Dronpa was further purified on a Hi-Load 16/60 Superdex 200 prep-grade column (Amersham Biosciences) in buffer composed of 10 mM Tris, pH 8.0, 2 mM DTT. Homogeneity was confirmed to be more than 95% by SDS–PAGE analysis. A similar procedure was followed to express and purify the Dronpa-C62S.

Crystallization and data collection. All crystals suitable for X-ray data collection were obtained at 20 °C by the hanging drop vapor diffusion method by mixing equal volumes of the protein solution (10 mM Tris, pH 8.0, 2 mM DTT) and a reservoir solution composed of 20% (w/v) PEG 4000 and 0.1 M Tris–HCl (pH 8.5), 100 mM $MgCl_2$. They were transferred to a cryoprotectant solution containing 20–30% (v/v) ethylene glycol and then flash cooled in liquid nitrogen at 100 K. A Dronpa and Dronpa-C62S data set indexed in the space group $P2_12_12_1$ and $P1$ were measured at the Pohang Light Source (PLS) on beamline 4A and at the Photon Factory (PF) on beamline 5A, respectively. The images were processed and the reflections scaled using the program HKL2000, Scalepack, Denzo [13].

Structure determination and refinement. The structure was solved by molecular replacement in CNS [14], using a monomer of KikG structure (PDB ID 1XSS) and four translated positions were found within the asymmetric unit. Multiple cycles of editing and adjustment of the model into sigma A weighted $2F_o-F_c$, F_o-F_c, and $2F_o-F_c$ composite omit maps were performed using the program Coot [15]. Further, simulated annealing, energy minimizations, and individual isotropic B factor refinement was carried out in CNS. The final models were validated with PROCHECK [16], with 100% of the residues lying within the allowed regions of a Ramachandran Plot for the Dronpa and Dronpa-C62S structures. The primary model of the Dronpa-C62S was built based on the phase obtained from the molecular replacement solution with the native Dronpa structure using CCP4 [17]. The structure refinement statistics are summarized in Table 1.

Plasmid, cell culture, and transfection. The Dronpa-C62S was generated by site-directed PCR mutagenesis by using the QuickChange mutagenesis kit (Stratagene) from CoralHue™ Donpa-Green1 (pDG1-MN1) (MBL, Japan). COS-7 cells were maintained in Dulbecco's modified Eagle medium (Invitrogen) with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37 °C in 5% CO_2. Transient transfection of the cells was performed using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. Cells were passaged 24 h prior to transfection and imaged at 48 h post-transfection. Cells expressing the native and the Dronpa-C62S had similar expression levels and phenotypes.

Fluorescence microscopy. The prepared cells were placed in a temperature-controlled chamber in a Delta Vision RT imaging system (Applied Precision) imaging analysis. The microscope was equipped with an IL-70 (Olympus, Tokyo, Japan), an oil immersion objective lens (1.4 numerical aperture, 60×), a CoolSnap HQ digital camera from Roper Scientific (Tucson, AZ) and optical filter sets from Omega Optical (Brattleboro, VT). The intermediately bright cells expressing native or Dronpa-C62S were chosen for the imaging experiments. A 100 W mercury arc lamp with a 436 ± 10 nm and a 490 ± 20 nm filter was used for viewing photoswitching. Cells were irradiated with a 436 ± 10 nm excitation filter for 200 ms and the fluorescence was quenched totally by continuous irradiation with the 490 ± 20 nm filter for 20 s. This procedure was repeated 100

Table 1
Data collection and refinement statistics

	Dronpa	Dronpa-C62S
Data collection		
Space group	$P2_12_12_1$	$P1$
Cell dimensions		
a, *b*, *c* (Å)	73.59 103.59 122.32	73.09 106.160 110.07
α, β, γ (°)	90.00 90.00 90.00	61.17 70.8586.21
Resolution (Å)	50–1.9	50–1.8
R merge	4.3 (14.3)	6.0 (20.07)
$I/\sigma I$	25.4 (4.3)	20.3 (2.7)
Completeness (%)	91.8 (66.2)	94.4 (81.9)
Redundancy	5.2 (3.6)	3.1 (2.3)
Refinement		
Resolution (Å)	20–1.9	20–1.8
No. reflection	74,266	252,038
R_{work}/R_{free} (%)	17.79/21.05	18.18/20.94
No. of atoms		
Protein	7015	20,953
Ion		64 (Mg^{2+})
Water	639	2548
B-factors		
Protein	23.4	24.6
Ligand/ion		44.8
Water	21.0	18.9
RMS deviations		
Bond lengths (Å)	0.006	0.006
Bond angles (°)	1.4	1.4

Highest resolution shell is shown in parenthesis.

times. As in the wild type Dronpa, irradiation at around 490 nm appeared to bleach the Dronpa-C62S, and the bleached fluorescence was completely regained at around 400 nm. Images were taken with a color charge-coupled device camera by using the same detection filter and analyzed with the SoftWoRx® program from Applied Precision.

Results and discussion

The structures of Dronpa and its derivatives Dronpa C62S have the classic 11-stranded "β-can" with a central α-helix, nearly identical to other GFP structures [1,5–8] (Fig. 1). In the crystal structures, Dronpa exists as a tightly associated tetramer and the two dimers are related by a non-crystallographic two-fold pseudosymmetry axis perpendicular to β-can. Tetramer formation decreases the accessible surface by 2282.4 $Å^2$ per monomer, which amounts to 13.6% of the total surface of the monomer. Ando et al. engineered a monomeric Dronpa by introducing both random and rational mutations [2,9]. The three residues (N102, H194, and E218) of their mutations are located at the tetramer interface. Among the tetramer interface residues of Dronpa, 32% are hydrophobic, 68% hydrophilic, and 42% charged, indicating that the majority of the tetrameric interface is formed by polar interactions. In general, the oligomerization of fluorescence proteins has the effect on slow maturation, stability to changes in external pH, and FRET between subunits [8]. The tetramer interface of Dronpa is very similar to that of another tetrameric red fluorescent protein DsRed [8] (average root mean square deviation of 1.32 Å for 11-218 pairs of aligned Cα atoms) (PDB code: 1GGX). The structural comparisons with a GFP from jellyfish *Aequorea victoria* (PDB code: 1EMC), an asFP595-A143S from *Aequorea sulcata* (PDB code: 2A53), and a KikG from (PDB code: 1XSS) provide a likely explanation for differences in photochromic characteristics. A chromophore of Dronpa consists of the unique tripeptide, Cys62-Tyr63-Gly64, arranging a chromophoric, conjugated (5*Z*)-2-(1-amino-2-mercaptoethyl)-5-(4-hydroxybenzylidene)-3-(2-hydroxyethyl)-3,5-dihydro-4H-imidazole-4-one. The chromophore is positioned at the core α-helix of Dronpa like other GFP family members, either as Cys62-Tyr63-Gly64 (CYG) in the native or Ser62-Tyr63-Gly64 (SYG) in the Dronpa-C62S. The hydrogen-bonding network between chromophore and the surrounding residues correlates well with maintaining the stabilization of protonation/deprotonation in the chromophore [10]. Interestingly, the CYG chromophore of Dronpa in our crystal structure adopts the *cis* conformation, while asFP595-A143S adopts the *trans* conformation in the off-state of the chromophore [4]. Andreson et al. suggested that the key event in asFP595-A143S is a HT^{bot} mechanism resulting in a *trans–cis* isomerization of the chromophore, however, there is no room for isomerization of the chromophore in the Dronpa structures. Therefore, we also suggest that the isomerization of the chromophore of Dronpa will require a large conformational change [5,6]. In comparison with other GFP proteins, we suggest two possibilities to explain the photoswitchable property of the Dronpa molecule. First, the hydrogen-bonding network of the chromophore may play a role in photoswitch-

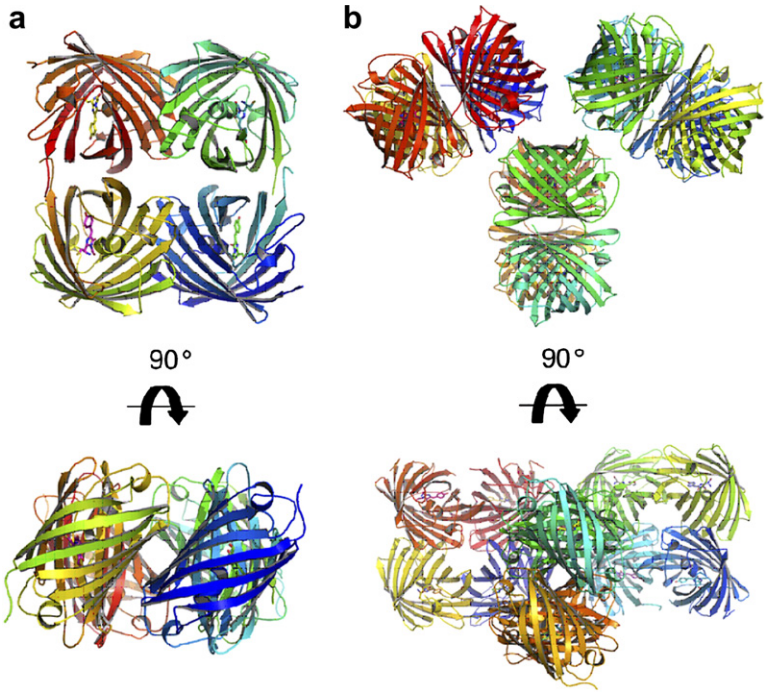

Fig. 1. Overall structures of wild type Dronpa (a) and mutant C62S Dronpa (b). Schematic drawing (a) four molecules of Dronpa native and (b) twelve molecules of mutant dronpa (C62S) in the asymmetric unit. The chromophores are represented as a stick. The figures were drawn by the program PYMOL.

ing of Dronpa or second, the hydrogen-bonding inability of the Sγ atom of Cys62 has a critical role in the photoswitchable character of Dronpa. The superposition of the atomic coordinates of the Dronpa monomer with those of GFP (PDB code: 1EMC), eqFP611 (1UIS), zFP538 (1XAE), asFP595 (2A50), asFP595-A143S (2A53) and KikG (1XSS) result in Cα r.m.s.d. of 1.37, 0.85, 0.81, 0.89, 0.89, and 0.46 Å, respectively. We speculate that the hydrogen-bonding network around the chromophore is different between Dronpa and the other GFP family members (Fig. 2). Although our results reveal a clear structural similarity between Dronpa and KikG with a high sequence identity of 80.2%, there is no photoswitchable characteristic in KikG, which is a member of the photoconversion GFP family. In prior studies, a tripeptide of chromophore and its environment are crucial to the photophysical behavior of GFP [11]. In the native structure, the Nε1 atom of Gln38 is 3.92 Å and the Oε1 atom of Glu211 is 4.35 Å distant from the Sγ atom of Cys62. However, in the Dronpa-C62S structure, the Nε1 atom of Gln38 is 3.32 Å and the Oε1 atom of Glu211 is only 2.77 Å away from the Oγ atom of Ser62 (Fig. 3). The basis for a non-(or weak) interaction was deduced from the chemical property of atoms in each residue. Electronegativity measures show how strongly an atom attracts the electrons in a chemical bond. The electronegativity of the sulfur atom (Pauling value $\chi = 2.5$) is lower than that of the oxygen atom ($\chi = 3.5$), which is similar to that of a carbon atom ($\chi = 2.5$), and implies that the sulfur atom is unlikely to participate in a hydrogen-bonding network with the surrounding residues. In addition, a previous study confirmed that the hydrogen-bonding ability of oxygen atoms is more favorable than that of sulfur atoms in intramolecular interactions and resonance inducing character [12]. Upon UV irradiation, the photoswitching network of Dronpa-C62S may be rearranged by the hydrogen-bond of the Oγ atom of Ser62 with the surrounding residues. In contrast, Cys62 in the chromophore of the native Dronpa does not have this hydrogen-bonding interaction, which implies that the surrounding residues will continuously interact and the chromophore will be independent from surrounding molecular interactions. Therefore, we suggest that the hydrogen-bonding inability of the Sγ atom of Cys62 has a critical role in determining the photoswitchable character of Dronpa.

To confirm the role of the sulfur atom of the chromophore in Dronpa, we explored the photoswitching behavior of Dronpa-C62S in cells by fluorescence microscopy. The COS-7 cells expressing Dronpa-C62S were irradiated repeatedly (more than 100 times) with bleaching each time. Like the native Dronpa, irradiation at around 490 nm appeared to bleach the Dronpa-C62S, and the bleached fluorescence returned completely at around 400 nm. Fluorescence could be switched on and off repeatedly, however, the intensity of Dronpa-C62S rapidly decreased during the

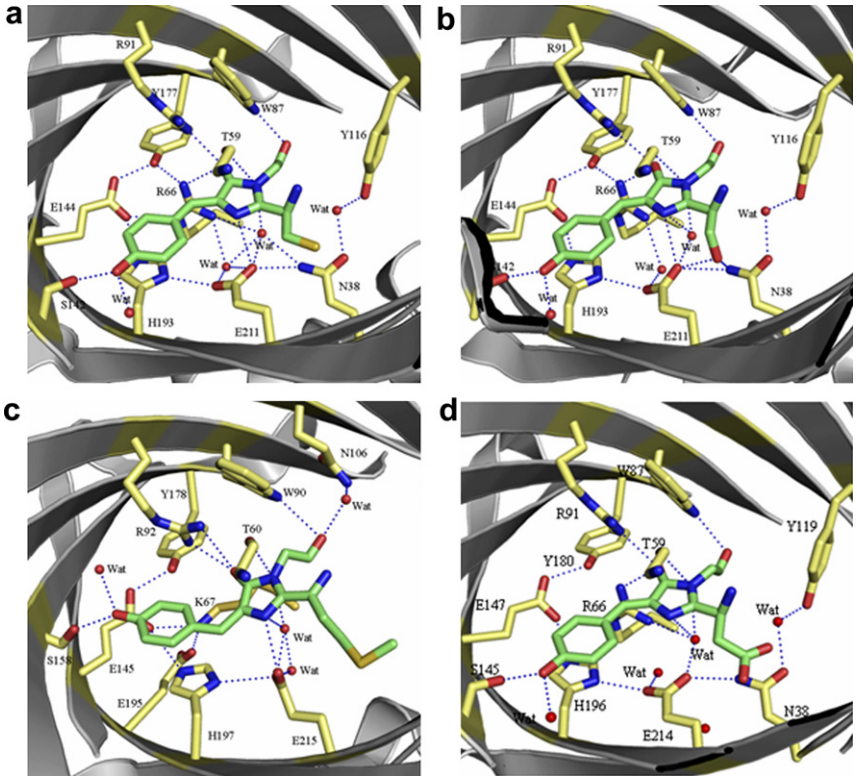

Fig. 2. Comparison of the hydrogen-bonding network surrounding the chromophore with other fluorescent proteins. Representative views of the hydrogen-bonding network between protein and chromophore (a) wild type Dronpa (b) Dronpa-C62S (c) asFP595-A143S (2A53) (d) KikG (1XSS). Red spheres, ordered waters; dashed line, possible hydrogen bonds in distances 3.5 Å; yellow, protein carbons; red, oxygen; blue, nitrogens; yellow, sulfur; green, chromophore. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

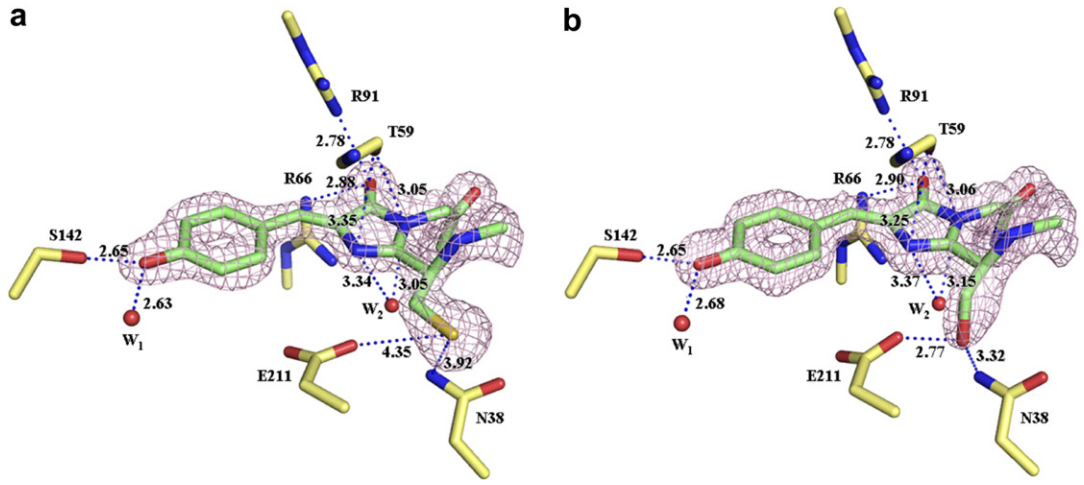

Fig. 3. Differences between wild type Dronpa (left) and Dronpa-C62S (right) in their crystal structures. A $2F_o-F_c$ electron density map contoured at 1.5σ is shown in magenta around a chromophore of wild type (a) Dronpa and (b) Dronpa-C62S, with a view of the interactions of Dronpa with the chromophore. Red spheres, ordered waters; dashed line, main hydrogen bonds in distances 3.5 Å; gray, protein carbons; red, oxygen; blue, nitrogens; yellow, sulfur; green, chromophore; red circle, S or O in the first residue of chromophore. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

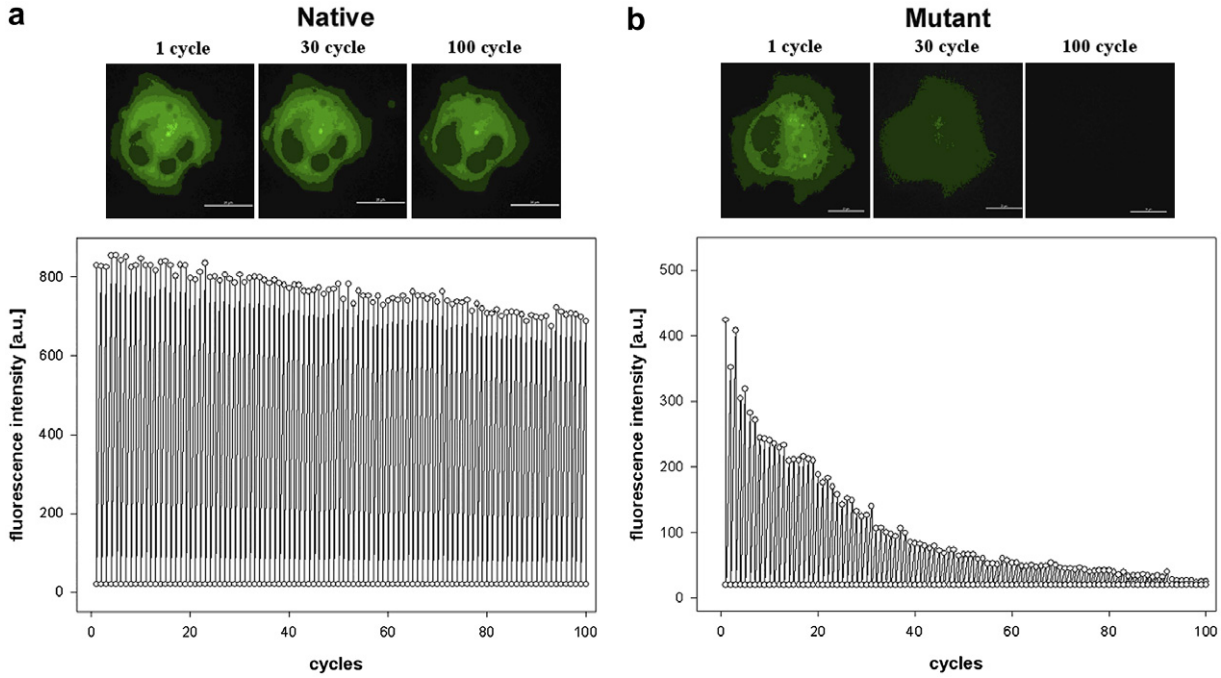

Fig. 4. Differences between wild type Dronpa (left) and Dronpa-C62S (right) in their fluorescence images. The reversible photoswitching behavior of wild type (a) Dronpa and (b) Dronpa-C62S under a fluorescence microscope. (Upper) The fluorescence images of a COS-7 cell expressing wild type and Dronpa-C62S at the 1st, 30th, 100th cycle. (Lower) Fluorescence on/off cycles of the same cells are shown in the upper panel.

on/off switching cycles (Fig. 4). After 30 cycles, the fluorescence intensity of Dronpa-C62S was less than 30% of the original level. In direct imaging, Dronpa-C62S was obviously being quenched, but, the fluorescence intensity of native Dronpa approximated 80% of the original level after 100 cycles as previously reported [2]. Overall, the mutant C62S does exhibit photoswitching, however, it does not survive for as many cycles. This result supports the notion that the cysteine amino acid substitution of the Dronpa chromophore may affect reversible photoswitching.

In summary, Dronpa maintains its photoswitchable fluorescence characteristics with the lasting reversibility due to a special molecular structure which is unique compared to other GFP proteins. First, Dronpa has a strong hydrogen-bonding network between the chromophore and the surrounding residues sufficient to stabilize the π-

conjugating system of chromophore when the protein is in an excited or an emissive state at a specific energy and second, the sulfur atom of the chromophore has low electronegativity such that the hydrogen-bonding inability may play a role in the reversibility of photoswitching. The chromophore of Dronpa is chemically very different from that found in the other GFP family members and there are significant differences in the size and shape of the chromophore cavities. These structural and functional studies support the major mechanistic conclusions reached on the basis of the other Dronpa structures [5,6]. We believe that these structural results will assist in the designing of new and effective molecular markers for real-time imaging in molecular and cellular biology.

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