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1.8 Å bright-state structure of the reversibly switchable fluorescent protein Dronpa guides the generation of fast switching variants

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RSFPs (reversibly switchable fluorescent proteins) may be repeatedly converted between a fluorescent and a non-fluorescent state by irradiation and have attracted widespread interest for many new applications. The RSFP Dronpa may be switched with blue light from a fluorescent state into a non-fluorescent state, and back again with UV light. To obtain insight into the underlying molecular mechanism of this switching, we have determined the crystal structure of the fluorescent equilibrium state of Dronpa. Its bicyclic chromophore is formed spontaneously from the Cys^{62}–Tyr^{63}–Gly^{64} tripeptide. In the fluorescent state, it adopts a slightly non-coplanar *cis* conformation within the interior of a typical GFP (green fluorescent protein) β-can fold. Dronpa shares some structural features with asFP595, another RSFP whose chromophore has previously been demonstrated to undergo a *cis–trans* isomerization upon photoswitching. Based on the structural comparison with asFP595, we have generated new Dronpa variants with an up to more than 1000-fold accelerated switching behaviour. The mutations which were introduced at position Val^{157} or Met^{159} apparently reduce the steric hindrance for a *cis–trans* isomerization of the chromophore, thus lowering the energy barrier for the blue light-driven on-to-off transition. The findings reported in the present study support the view that a *cis–trans* isomerization is one of the key events common to the switching mechanism in RSFPs.

Key words: asFP595, chromophore, Dronpa, photoswitching, reversibly switchable fluorescent protein.

INTRODUCTION

Fluorescent proteins such as the avGFP (green fluorescent protein from *Aequorea victoria*), used as non-invasive markers, have revolutionized cell biology [1–3]. RSFPs (reversibly switchable fluorescent proteins) are an emerging new class of fluorescent proteins. These photochromic proteins adopt either a fluorescent (on) or a non-fluorescent (off) state; they may be selectively and reversibly transferred between these two states by irradiation with visible light of appropriate different wavelengths.

The ability to be repeatedly switched on and off is a distinct advantage of RSFPs over the more established, irreversibly photoactivatable or photoconvertable fluorescent proteins. The latter may be activated once from a non-fluorescent into a fluorescent form or their emission spectra may be converted unidirectionally after irradiation with light of appropriate power and wavelength respectively [2,4]. Thus only RSFPs will enable repeated measurements of protein movements in single cells, for example. Moreover, RSFPs have been demonstrated to be suitable for attaining spatial resolution far below the diffraction barrier in fluorescence microscopy [5,6]. Likewise they may open a new pathway to high-density data storage applications [7,8] for which reversibility is important if not mandatory.

Currently, with Dronpa from the coral *Pectiniidae* [9], asFP595 (asCP, asulCP) isolated from the sea anemone *Anemonia sulcata* [10], and their respective variants, two RSFPs have been described. Both proteins may be repeatedly highlighted, erased and highlighted again. Currently, structural information is only available for asFP595. It is an obligate tetramer whose subunits adopt a GFP (green fluorescent protein)-like structure, i.e. an 11-stranded β-barrel surrounding a central α-helix that contains the chromophore [7,11,12]. By using asFP595 protein crystals, in which the chromophores were quantitatively and reversibly photoconverted, it was demonstrated that a *cis–trans* isomerization of the bicyclic chromophore is a key event in the switching process of asFP595 [7]. As predicted previously [13], the fluorescent on-state corresponds to the *cis* conformation, whereas the chromophore in the non-fluorescent off-state adopts a *trans* conformation. These structural changes are apparently accompanied by different protonation states of the chromophore that determine the fluorescent properties of the protein [7]. However, Dronpa is currently the RSFP of choice for most cell biology applications, mainly because it is monomeric and because it exhibits a number of favourable spectroscopic properties. In equilibrium, Dronpa is in its on-state, displaying bright green fluorescence with an emission maximum at 518 nm. Its fluorescence quantum yield is high ($\Phi_{FL} = 0.85$) [9]. For excitation, laser light at 488 nm is conveniently used; intense light of the same wavelength transfers the protein to the non-fluorescent off-state. Subsequent minimal irradiation at around 400 nm transfers the chromophore back to the on-state and restores fluorescence [9]. Not surprisingly, this protein has already been employed in several studies to monitor the intracellular mobility of fusion proteins [9,14–16].

Despite its large potential for cellular applications, little is known about the structural basis of photochromic switching in Dronpa. In the present study we have solved the X-ray structure of the fluorescent equilibrium state of Dronpa at 1.8 Å (1 Å = 0.1 nm) resolution. In the fluorescent equilibrium state, we find

Abbreviations used: Φ_{FL}, fluorescence quantum yield; avGFP, green fluorescent protein from *Aequorea victoria*; CYG, Dronpa chromophore; GFP, green fluorescent protein; MYG, asFP595 chromophore; RSFP, reversibly switchable fluorescent protein; NCS, non-crystallographic symmetry.

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that the chromophore adopts exclusively the *cis* isomeric form. Surprisingly, the chromophore environment of Dronpa is almost identical to that of KikG, a fluorescent protein that does not exhibit photoswitching [17]. Furthermore, we find structural similarities to asFP595 that point to a *cis–trans* isomerization of the chromophore as a key event in photochromic switching. Based on this assumption and the obtained structural information we generated rsFastLime (Dronpa-V157G) and Dronpa-M159T, two variants that exhibit strongly accelerated switching kinetics. Our findings support the view of a photoinduced *cis–trans* isomerization of the chromophore in Dronpa.

EXPERIMENTAL PROCEDURES

Protein production and purification

The expression plasmid pRSETb-Dronpa was a gift from A. Miyawaki (Riken Brain Science Institute, Japan). Proteins were expressed in the *Escherichia coli* strain HMS 174 (DE3) and purified by Ni-NTA (Ni^{2+}-nitrilotriacetate) affinity chromatography and subsequent size-exclusion chromatography according to standard procedures. The purified proteins were concentrated to $\sim$23 mg/ml by ultrafiltration and taken up in 20 mM Tris/HCl and 120 mM NaCl (pH 7.5) for crystallization.

Crystallographic analyses

Dronpa was crystallized without removal of the N-terminal His_6-tag by sitting drop vapour diffusion at room temperature (22 °C), by employing a reservoir of 22 % (w/v) PEG (poly[ethylene glycol]) monomethyl ether 3350 and 0.14 M $Mg(NO_3)_2$. Crystals appeared within 2 days and continued to grow for several weeks. They could be frozen in liquid nitrogen after transfer into perfluoro-polyether. Diffraction data were collected at the PXII beamline of the Swiss Light Source at 100 K using a MarResearch CCD detector. The data were processed with the HKL package [18].

The crystal structure of the equilibrium state of Dronpa was solved by molecular replacement with MOLREP [19] using the structure co-ordinates of asFP595 (PDB ID 2A50; [7]) without the chromophore and water molecules. The sequence was manually adjusted and fitted to the $2F_o - F_c$ and $F_o - F_c$ electron densities with COOT [20]. The chromophore was added into an unambiguous and unoccupied patch of the $F_o - F_c$ electron density. Subsequently, water molecules were placed with the computer program Arp/wArp [21]. The water structure was completed manually. Refinement in Refmac5 [22] was initially restrained by the NCS (non-crystallographic symmetry). In later refinement cycles NCS-restraints were removed. Ultimately, TLS (translation-libration-screw) refinement cycles were included, by which individual anisotropic temperature factor corrections for each of the four crystallographically independent molecules were introduced [23]. During the entire refinement procedure, 5 % of randomly selected reflections were set aside for monitoring of the R_{free} factor [24] (see Table 1 of supplementary data at http://www.BiochemJ.org/bj/402/bj4020035add.htm).

Spectroscopic analyses

Absorption spectra were recorded with a Varian Cary4000 UV/VIS Spectrometer. A Varian Cary Eclipse fluorescence spectrometer with excitation at 457 nm (10 nm slit) was used for measuring emission spectra. Within the cuvettes the proteins were irradiated with 405 nm (405 ± 5 nm filter) light to quantitatively maintain them in the on-state. We determined the molar absorption coefficients and the Φ_{FL} relative to the reported values of Dronpa [9]. Protein concentrations were measured using the 280 nm peak of the absorption spectra.

Mutagenesis

Point mutations were inserted by site-directed mutagenesis using the QuikChange™ Site Directed Mutagenesis Kit (Stratagene) according to the manufacturers' instructions.

Optical switching

Photoswitching experiments were performed using a modified computer-controlled fluorescence microscope (Leica) equipped with a 40 × NA 0.6 air objective lens. The microscope was equipped with two 100 W Hg lamps delivering 'blue light' (488/10 nm excitation filter, $\sim 0.3\ W \cdot cm^{-2}$) and 'UV light' (405/10 nm excitation filter, $\sim 0.2\ W \cdot cm^{-2}$). Irradiation was performed in alternate turns for 32 min (Dronpa), 33 s (rsFastLime) or 2.25 s (Dronpa-M159T) with blue light and for 4 s (Dronpa), 2 s (rsFastLime) or 1s (Dronpa-M159T) with UV light together with blue light respectively. The fluorescence was detected through the same objective lens and recorded by a photomultiplier tube (HR9306-0, Hamamatsu, Hamamatsu City, Japan) using a 500 nm longpass detection filter (HQ 500 LP, AHF Analysentechnik).

Relaxation kinetics

The determination of the relaxation half-time $t_{1/2}^{relax}$ from the off-state into the equilibrium was performed using the following protocol. Proteins were expressed in *E. coli* and the cells were suspended in 1 % low melting-point agarose. A 1.5 μl aliquot of this suspension was placed in a microtitre well, covered by a coverslip and sealed with Vaseline. After complete switching into the off-state with 'blue light' (488/10 nm excitation filter, $\sim 0.75\ W \cdot cm^{-2}$) for $\sim$15 min (Dronpa) or $\sim$20 s (rsFastLime and Dronpa-M159T) respectively, the relaxation into the equilibrium state was followed at room temperature in the dark by consecutive short measurements with $0.01\ W \cdot cm^{-2}$ blue light (every 10 min, 20 s or 10 s for Dronpa, rsFastLime or Dronpa-M159T respectively). We note that it is of paramount importance to perform this experiment in complete darkness.

RESULTS

Crystallization and overall structure of Dronpa^{on}

N-terminally His_6-tagged Dronpa was expressed in *E. coli,* purified to apparent homogeneity and crystallized. In solution Dronpa adopts, at thermodynamic equilibrium, the on-state and exhibits a fluorescence emission maximum at 522 nm (Table 1). We have found that Dronpa protein crystals display similar fluorescence emission spectra to the protein in solution (Figure 1A). The fluorescence emission of Dronpa crystals was not further enhanced by irradiating the crystals with light of 405 nm, a wavelength at which off-state Dronpa is converted into the on-state. Hence we conclude that in the protein crystals obtained Dronpa exclusively adopts the on-state. We refer to this state as Dronpa^{on}.

Crystals of Dronpa^{on} belong to a centered orthorhombic crystal system with four molecules per asymmetric unit (see Table 1 of supplementary data at http://www.BiochemJ.org/bj/402/bj4020035add.htm). The crystal structure was solved by molecular replacement using the structure co-ordinates of asFP595 (PDB ID 2A50;[7]) as a search model and omitting the chromophore. The structure was refined at an effective resolution of 1.8 Å

Table 1 Properties of Dronpa, rsFastLime and Dronpa-M159T

	Absorption maximum (nm)	Emission maximum (nm)	Molar absorption coefficient ($M^{-1} \cdot cm^{-1}$)	Fluorescence quantum yield (Φ_{FL})*	Switch-off half-time $t_{1/2}^{off}$ (s)	Switch-on half-time $t_{1/2}^{on}$ (s)	Off-state relaxation half-time $t_{1/2}^{relax}$ (min)
Dronpa	503	522	95 000	0.85	263.00	0.10	840
rsFastLime	496	518	39 094	0.77	5.00	0.11	8
Dronpa-M159T	489	515	61 732	0.23	0.23	0.05	0.5

* Φ_{FL} was calculated relative to Φ_{FL}-Dronpa, Φ_{FL}-Dronpa was taken from [9].

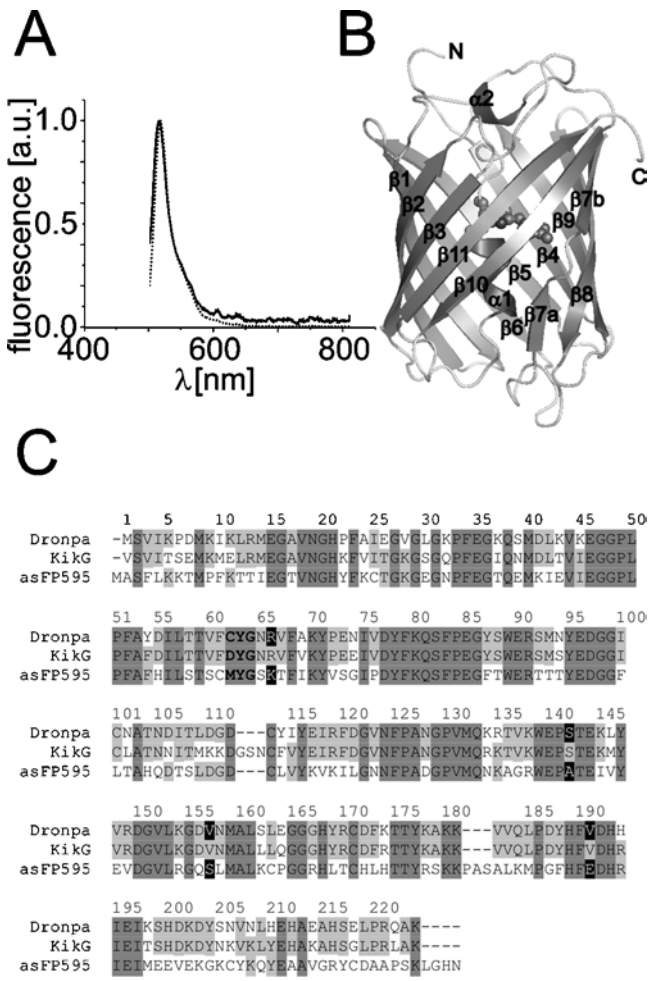

Figure 1 Characteristics of Dronpa

(**A**) Dronpa^{on} fluorescence emission spectra (excitation at 488 nm). Displayed is the normalized emission spectrum of Dronpa^{on} in solution (dotted line) and of Dronpa^{on} protein crystals (solid line) a.u., absorbance units. (**B**) Overall structure of Dronpa^{on}. The chromophore is shown as a ball-and-stick model. N- and C-termini and secondary structure elements are labelled. All structure figures were prepared with PyMOL (http://pymol.sourceforge.net/). (**C**) Amino acid sequence alignment of Dronpa, KikG and asFP595. Amino acid numbering according to Dronpa. The residues forming the chromophore are highlighted by bold letters. Identical residues are shaded in dark grey, positions at which two of the proteins share identical amino acids are shaded in light grey. Distinct amino acid residues in the immediate chromophore environments of Dronpa and asFP595 are shaded in black.

maintaining good stereochemistry (PDB ID 2IOV; see Table 1 of supplementary data at http://www.BiochemJ.org/bj/402/bj4020035add.htm). R/R_{free} values converged at 18.5/21.6 %. Residues 2–219 of Dronpa^{on} could be unequivocally assigned in all four copies of the protein and the final models exhibited uninterrupted electron density for the entire main chains and the vast majority of side chains. Only a few surface-exposed side chains were not covered by the final $2F_o - F_c$ electron density. The four crystallographically independent molecules adopted highly similar structures [pairwise rmsd (root mean square deviations) 0.15–0.31 Å for 215–218 matching Cα atoms].

We have found that Dronpa^{on} exhibits a typical GFP-like fold, comprising an eleven-stranded β-barrel with a co-axial, partially helical element between strands β3 and β4, that bears the chromophore (Figure 1B).

Dronpa^{on} exhibits a slightly non-coplanar *cis* chromophore

The chromophore is a [2-(1-amino-2-mercaptho-ethyl)-4-[1-(4-hydroxy-phenyl)meth-(E)-ylidene]-5-oxo-4,5-dihydro-imidazol-1-yl]-acetaldehyde moiety that is formed spontaneously from the tripeptide Cys^{62}–Tyr^{63}–Gly^{64} of the precursor protein (all residue numbering in this manuscript is according to the Dronpa nomenclature; see Figure 1C). We refer to the chromophore as 'CYG'. The electron density showed unambiguously that in Dronpa^{on}, CYG adopts a *cis* isomeric state (Figure 2A). The normals to the planes of the five- and six-membered rings span an angle of $\sim$15°. Hence the five- and six-membered rings of CYG are slightly non-coplanar.

Since CYG is buried deeply in the protein core, it engages in multiple interactions with neighbouring residues. The *p*-hydroxyphenyl ring of CYG stacks on the His^{193} side chain and the hydrogen-bonding potential of the polar groups is completely saturated (Figure 2B). The apical hydroxy group of the *p*-hydroxyphenyl ring forms a direct hydrogen bond with the side chain of Ser^{142} and a water-mediated contact with the backbone carbonyl group of Glu^{140}. The imidazolinone ring forms an array of seven hydrogen bonds (direct interactions with the side chains of Thr^{59}, Arg^{66}, Arg^{91} and Glu^{211}; water-mediated interactions with the side chains of Gln^{38}, Glu^{211} and the backbone carbonyl group of Asn^{65}). While the central part of the chromophore engages in many polar interactions, van-der-Waals contacts prevail in the peripheral portions of the chromophore. Thus the chromophore is firmly attached via its imidazolinone ring but not with its peripheral parts.

An opening to the chromophore

In Dronpa the bulk solvent pH has been shown to elicit a strong influence on its absorption and emission spectra by influencing the protonation state of the chromophore [9,25,26]. Hence, although the CYG resides inside the β-barrel, it is not fully shielded from the protein environment. We identified a water-filled opening at the mid-section of the otherwise closed β-barrel, between strands β7 and β10 that connects the CYG with the bulk solvent (Figure 3). The opening possibly relays environmental conditions,

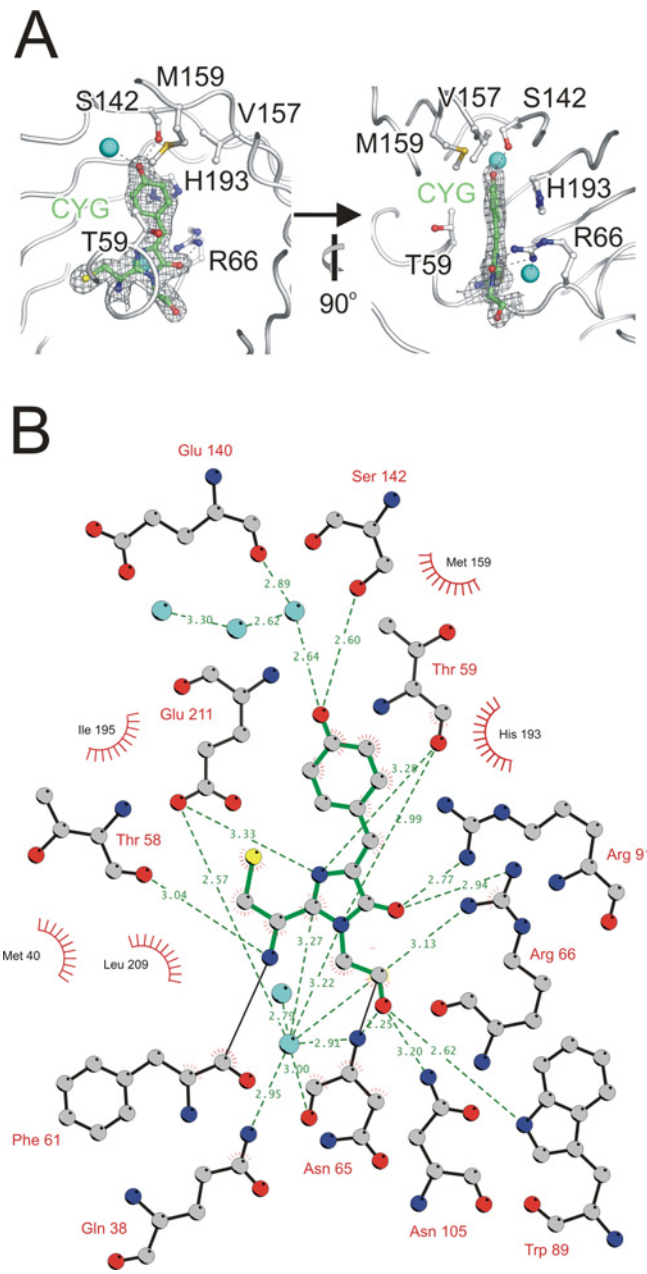

Figure 2 Details of the Dronpa^{on} chromophore environment

(**A**) Representation of the chromophore and selected residues in its neighbourhood in two orthogonal views. The chromophore and selected residues in the neighbourhood are shown as a ball-and-stick model, colour-coded by atom type (carbon, green; oxygen, red; nitrogen, blue; sulfur, yellow; carbon neighborhood residues, grey) and labelled. The protein backbone is shown as a grey tube. Water molecules are displayed as cyan spheres and hydrogen bonds are indicated by broken lines. The final $2F_o - F_c$ electron density around the chromophore is contoured at the 1σ level and displayed in grey. Single letter amino acid codes are used. (**B**) LIGPLOT diagram of the chromophore interacting with its immediate environment. Chromophore bonds are shown in green and bonds within the chromophore environment are shown in black. Hydrogen bonds are represented as green broken lines with indicated distances (in Å). The atoms are colour-coded as before. Residues in hydrophobic contact with the chromophore are represented by red semi-circles with radiating spokes.

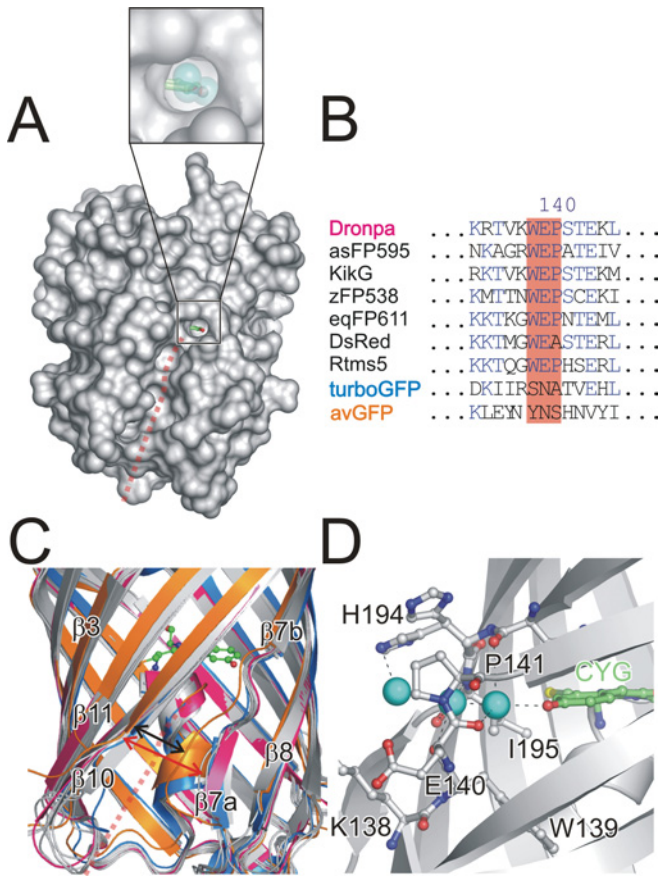

Figure 3 Opening in the β-barrel

(**A**) Surface view of Dronpa^{on} displaying an apparent opening in the β-barrel connecting the *p*-hydroxyphenyl moiety of the chromophore (as a ball-and-stick model; colour-coded by atom type as in Figure 2) with the bulk solvent. Compared with Figure 1(C), the molecule has been rotated 25° about the horizontal axis (top to back). The cleft on the protein surface is highlighted by a broken line. The inset shows a magnification of the opening filled with three well-ordered water molecules. (**B**) Partial sequence alignment of various fluorescent proteins. The sequence Trp–Glu–Pro (WEP) within strand β7 appears to be conserved in order to generate the cleft. (**C**) The cleft (indicated by a broken line) near to strand β7 is conserved. Displayed are overlayed ribbon plots of the fluorescence proteins listed in (**B**). Colour coding for Dronpa^{on}, TurboGFP and avGFP as indicated in (**B**). The red and the black double headed arrows indicate the width of the cleft in the case of Dronpa^{on} and avGFP respectively. (**D**) Details of the opening to the chromophore. Water molecules are shown as cyan spheres, building a hydrogen-bonded connection between the chromophore and the aqueous environment. The protein region around the opening and the chromophore is shown as a ball-and-stick model. Colour-coding is as before. Broken lines indicate hydrogen bonds. His^{194} adopts alternative conformations on the surface of the protein.

such as pH, to the chromophore. It may also serve to discharge harmful oxygen radicals, which are generated at the chromophore, from the immediate chromophore environment. The opening resides within a cleft-like structure between strand β7 and β10 on the surface of the β-barrel (indicated by the dotted red line in Figures 3A and 3C). The opening is particularly noticeable in Dronpa^{on}, whereas it is not a general feature of GFP-like fluorescent proteins. However, the cleft-like structure itself is conserved in most GFP-like proteins (Figure 3C). In fluorescent proteins isolated from *Cnidaria* species a largely conserved sequence motif (Trp–Glu–Pro) appears to be responsible for a pronounced cleft. This motif, and with it the cleft, are absent in avGFP (Figures 3B and 3C).

Remarkably, an opening structurally similar to that identified in Dronpa^{on} has recently been described for the engineered fluorescent protein TurboGFP, which has been derived from a fluorescent protein of an evolutionarily distant marine *Copepoda* species (Figure 3) [27]. In line with our findings, it has been suggested that in TurboGFP the opening facilitates oxygen conveyance to the premature chromophore, thus speeding up maturation [27].

Structural analysis of the chromophore environments of Dronpa^{on} and asFP595 points to an analogous switching mechanism

Dronpa, asFP595 and their derivatives are the only RSFPs described to date [9,10]. Besides their colour and their tetramerization tendency, the two proteins differ in three obvious properties. First, whereas in Dronpa^{on} the protein backbone is intact, in asFP595 there is a chain break immediately preceding the chromophore. Secondly, the first chromophoric residues differ in Dronpa and asFP595 (CYG and MYG respectively). And thirdly, at thermal equilibrium the Dronpa chromophore adopts a fluorescent *cis* conformation, whereas the asFP595 chromophore is in a non-fluorescent *trans* conformation.

Despite these differences both chromophores exhibit a similar stacking interaction between the *p*-hydroxyphenyl ring and the His^{193} side chain. The immediate chromophore environments of Dronpa and asFP595 differ only in four residues, i.e. positions 66, 142, 157 and 191. Three of these exchanges (positions 66, 142 and 157) apparently bring about the different isomerization states observed for the two chromphores in their ground states.

In Dronpa^{on}, the hydroxy group of the *p*-hydroxyphenyl ring forms a hydrogen bond to the side chain of Ser^{142} (Figures 4A and 4B), thus stabilizing the chromophore in the *cis* state. In asFP595, position 142 is occupied by an alanine residue that does not facilitate such a hydrogen bond. Exchange of this alanine residue for a serine residue yields an asFP595 variant in which a substantial proportion of the chromophore population (10–20 %) is at thermal equilibrium in the fluorescent state. Furthermore, structural analysis verified that in asFP595-A142S the *cis* position is stabilized by a new hydrogen bond (Figure 4A) [7].

If we model a *trans* conformation of the CYG without adjusting the protein matrix of Dronpa^{on}, it appears that the bulky Val^{157} and Arg^{66} sterically hinder the chromophore from adopting a *trans* conformation (Figure 5A). In asFP595 the analogous positions are occupied by the slightly smaller residues Ser^{157} and Lys^{66}, which might explain the lowered energy barrier for the *cis*–*trans* isomerization. Furthermore, in ground-state asFP595, Ser^{157} hydrogen bonds with the apical hydroxy group of the chromophoric tyrosyl ring and thereby stabilizes the *trans* conformation. Val^{157} in Dronpa^{on} does not exhibit the hydrogen-bonding capacity to interact similarly with the chromophore. Indeed, exchange of asFP595 Ser^{157} for a valine residue has been demonstrated to permanently trap the chromophore in the fluorescent *cis* state (Figure 4B) [7,13]. Hence the asFP595-S157V mutant seems to be reminiscent of the situation observed in Dronpa^{on}.

rsFastLime: a fast switching RSFP

In their fluorescent-states, the chromophores of both Dronpa^{on} and on-state asFP595 adopt a *cis* isomerization state. Given the overall similarities of the chromophore environments of both proteins it is tempting to assume that off-state Dronpa, like off-state asFP595, adopts a *trans* conformation.

As described above, in Dronpa^{on} Val^{157} seems to sterically oppose a *trans* conformation of the chromophore (Figure 5A). We reasoned that removal of the valine side-chain would be more compatible with a *trans* chromophore and consequently might reduce the energy barrier for photochromic switching in Dronpa. To test this hypothesis, we generated the Dronpa variant V157G. In line with the prediction, the switching speed of Dronpa-V157G is increased (Figure 5B). Under the light intensities employed the blue light-induced switch-off half-time, $t_{1/2}^{off}$, is reduced by a factor of ~50 in Dronpa-V157G ($t_{1/2}^{off} = 5.0$ s) compared with Dronpa ($t_{1/2}^{off} = 263.0$ s) (Table 1, Figure 5B). The actual values of $t_{1/2}^{off}$ depend on the light intensities employed. Nonetheless, we find a striking difference between $t_{1/2}^{off}$(Dronpa-V157G) and $t_{1/2}^{off}$(Dronpa) for a broad range of analysed light intensities. The observed acceleration of the overall switching kinetics of this variant is almost entirely due to differences in $t_{1/2}^{off}$, since the (already fast) switch-on kinetics of Dronpa ($t_{1/2}^{on} = 0.10$ s) are practically unchanged in Dronpa-V157G ($t_{1/2}^{on} = 0.11$ s) (Table 1). Dronpa-V157G adopts an intermediate equilibrium state; in the equilibrium state a bulk solution of Dronpa-V157G displays ~50 % of the fluorescence measured after complete conversion into the on-state. The absorption and emission spectra of both proteins are very similar (see Figures 1 and 2 of supplementary data at http://www.BiochemJ.org/bj/402/bj4020035add.htm). In the complete dark, Dronpa-V157G displays an off-state relaxation half time ($t_{1/2}^{relax}$) from the off-state to the equilibrium state of ~8 min, whereas Dronpa is basically bistable with $t_{1/2}^{relax}$

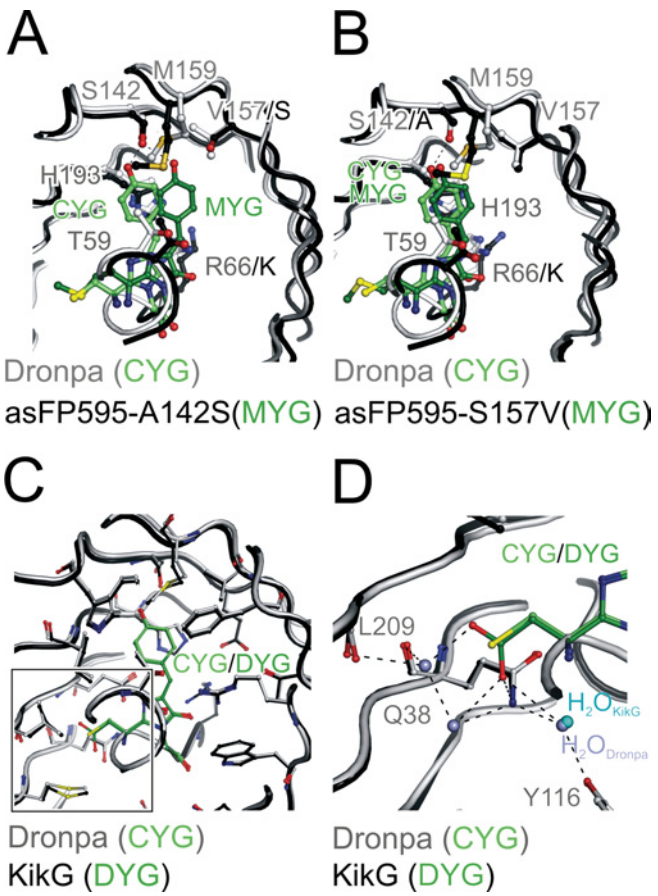

Figure 4 Comparison of Dronpa^{on} with asFP595 variants and KikG

(**A**) Comparison of the chromophore environment of Dronpa^{on} with on-state asFP595-A142S (PDB ID 2A56; [7]) after global superimposition of the proteins. Backbone tube and carbon atoms (except the chromophore) of Dronpa^{on} are in grey, those of asFP595 are in black. Selected residues are labelled in grey. asFP595 residues distinct from Dronpa are indicated in black. Carbon atoms of the Dronpa^{on} (CYG) and the asFP595 (MYG) chromophores are in light and dark green respectively. Otherwise, the colour coding is as before. The view is identical to that of Figure 2(A) (left side). (**B**) Similar comparison of Dronpa^{on} with asFP595-S157V (PDB ID 2A52; [7]) after global superimposition of the proteins. (**C**) Comparison of the chromophore environments of Dronpa^{on} (protein/CYG-chromophore; grey/light green) with KikG (PDB ID 1XSS; protein/DYG-chromophore; black/dark green) after global superimposition of the proteins. All residues in the direct vicinity of the chromophores are identical and are shown as a ball-and-stick model. Water molecules are omitted for clarity. The only significant structural discrepancy is due to the first position in the respective chromophores, as highlighted by the rectangle. (**D**) Representation of the boxed region in (**C**). Some residues are omitted for clarity. Hydrogen bonds are indicated by broken lines. Water molecules are indicated as cyan (Dronpa^{on}) and blue (KikG) spheres. Single letter amino acid codes are used.

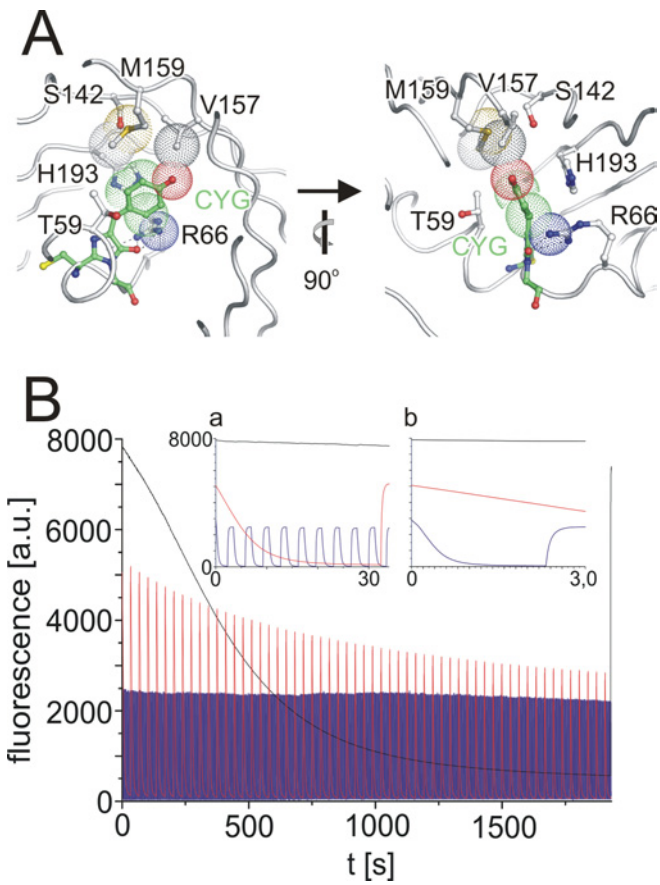

Figure 5 The fast switchable proteins rsFastLime and Dronpa-M159T

(**A**) Representation of a hypothetical *trans* chromophore modelled into the Dronpa^{on} structure. Without major adjustments in the protein backbone or side chain conformations, the side chains of Arg^{66} and Val^{157} would sterically interfere with the chromophore in *trans* as indicated by the dotted van-der-Waals spheres. The views are identical to Figure 2(A). (**B**) rsFastLime and Dronpa-M159T exhibit accelerated switching compared with Dronpa. The time courses of fluorescence intensities were recorded on live *E. coli* colonies expressing the respective proteins. Colonies were irradiated in alternate turns with UV-light together with blue light to switch the proteins into the fluorescent on-state or with blue light to switch them into the non-fluorescent off-state. Optical settings were identical; the duration of irradiation was adjusted to the respective protein. Main image: 56 switching cycles of rsFastLime (red line) and 650 switching cycles of Dronpa-M159T (blue line) fit into one switching cycle of Dronpa (black line). Inset: (**a**) (Partial) switching cycles of Dronpa and Dronpa-M159T overlaid with one switching cycle of rsFastLime. (**b**) One switching cycle of Dronpa-M159T overlaid with a fraction of the switching cycles of Dronpa and rsFastLime.

$\sim$14 h (Table 1, and Figure 3 of supplementary data at http://www.BiochemJ.org/bj/402/bj4020035add.htm). Φ_{FL} of Dronpa is 0.85 [25], whereas Dronpa-V157G exhibits a Φ_{FL} of 0.77 (Table 1). This accounts for the only slightly reduced overall fluorescence intensity of Dronpa-V157G when the protein is expressed in cells (Figure 5B). Because of the favourable combinations of properties (namely fast switching kinetics and high Φ_{FL}), which render Dronpa-V157G very promising for several applications, we decided to denote Dronpa-V157G as rsFastLime.

Val^{157} is not the only residue that potentially interferes with a *cis–trans* isomerization. Met^{159} is localized slightly above the *p*-hydroxyphenyl ring (Figure 5A). We hypothesized that Met^{159} may interfere with the movement of this six-ring upon a light-induced *cis–trans* isomerization. Indeed, we find that exchange of Met^{159} by the smaller threonine residue results in a dramatic reduction of the switch-off half-time [$t_{1/2}^{off}$(Dronpa-M159T) = 0.23 s], thus accelerating the switching kinetics compared with Dronpa by a factor of more than 1000. We note that similar acceleration of the switching kinetics is also achieved by exchanging Met^{159} for a serine or alanine residue (results not shown). However, this increase in the switching kinetics is accompanied by a reduction in the Φ_{FL} (Table 1). In Dronpa-M159T, Φ_{FL} is reduced to 0.23, potentially rendering this variant less attractive for many applications.

For most imaging applications, low photobleaching is mandatory. An adequate measure of the photostability of RSFPs in the bulk is the number of cycles they may undergo until the fluorescence intensity in the on-state is halved. Switching whole *E. coli* colonies, we find that the duration of a switching cycle is $\sim$0.5 h for Dronpa and $\sim$0.5 min for rsFastLime at $I_{blue\ light} \approx 0.3\ W \cdot cm^{-2}$. (Figure 5B). We find that the Dronpa fluorescence is halved already after $\sim$20 switching cycles, whereas rsFastLime undergoes $\sim$75 switching cycles until the same level of photobleaching is reached. Hence, by this criterion, in rsFastLime the photobleaching is reduced by a factor of almost 4. In the Dronpa-M159T variant, photobleaching is negligible, even after 650 switching cycles (Figure 5B).

DISCUSSION

In the present paper we have determined a high-resolution equilibrium state structure of the RSFP Dronpa. In the fluorescent equilibrium state, the CYG chromophore adopts a *cis* conformation. Relying on structural comparisons to another RSFP, namely asFP595 and its variants, we predict that a *cis–trans* isomerization may also accompany the switching off of Dronpa. Based on this prediction we have generated two fast switching variants, in which the switch off half-time $t_{1/2}^{off}$ is reduced by a factor of $\sim$50 (rsFastLime) or by more than 1000 (Dronpa-M159T) compared with the original Dronpa.

Flexible anchoring of the chromophore is required for reversible switching

The chromophore environment of Dronpa^{on} exhibits a striking structural similarity to that of KikG, a non-switchable fluorescent GFP-like protein from the stony coral *Favia favus* (PDM ID 1XSS; [17]). All residues in the direct neighbourhoods of the two chromophores are identical in Dronpa^{on} and KikG (Figures 4C and 4D). The single major structural difference within the immediate chromophore environments are the chromophores themselves. In contrast with the CYG chromophore of Dronpa, the KikG chromophore is formed from an Asp^{62}–Tyr^{63}–Gly^{64} tripeptide. In KikG, the carboxy group of Asp^{62} maintains four water-mediated hydrogen bonds to Gln^{38}, Phe^{61}, Tyr^{116} and Leu^{209}, thus firmly stabilizing this part of the chromophore, probably inhibiting considerable relocations of the chromophore. Comparable linkages are missing in Dronpa^{on}, since the thiol group of Cys^{62} does not engage in any hydrogen bonds. Rather, this portion of the CYG chromphore is primarily anchored by van-der-Waals interactions, which may allow movement of the chromophore within the protein matrix.

In line with this interpretation, molecular dynamics calculations on asFP595 demonstrated that structural flexibility of the chromophore facilitates photochromism [7]. In asFP595, residue 62 is a methionine that is not capable of forming a stabilizing hydrogen bond, similar to Cys^{62} in Dronpa^{on}. Furthermore, in asFP595 flexibility is further increased by a main chain break between Cys^{61} and the MYG. This increased flexibility appears to further reduce the energy barrier for a *cis–trans* isomerization and thus for photoswitching, which may be reflected in the lowered stability

of the on-state asFP595. After photoswitching the fluorescent state of asFP595 has a half life of only ~7 s before it reverts back into the non-fluorescent equilibrium state [13], whereas Dronpa is almost bistable, with the non-fluorescent state exhibiting a half-life of ~14 h (Table 1 and Figure 3 of supplementary material at http://www.BiochemJ.org/bj/402/bj4020035add.htm). In summary, the ability to make a flexible adjustment of the chromophore position with respect to the protein matrix appears to be essential for efficient reversible switching.

Interplay of factors for reversible switching

We propose that several preconditions have to be met before efficient reversible photoswitching may occur in a fluorescent protein: (i) the chromophore should exhibit sufficient flexibility to allow a light-driven *cis–trans* isomerization; (ii) the chromophore environment must provide sufficient space to allow the chromophore to accommodate both isomerization states; and (iii) the chromophore environments in the *cis* and the *trans* position should be sufficiently distinct to influence the fluorescence properties of the chromophore. In Dronpa, the chromophore exists in an equilibrium between protonated non-fluorescent and deprotonated fluorescent forms [9,25]. Therefore while a *cis–trans* isomerization appears to be a key event that accompanies photochromic switching in RSFPs, chromophore isomerization itself is not sufficient to fully explain the phenomenon of photochromism in RSFPs. Currently it is difficult to predict whether a specific isomerization state is fluorescent or not. It will be enlightening to understand the detailed interplay of chromophore planarity, environment and protonation state, which are all crucial for fluorescence, with the *cis–trans* isomerization of the chromophore.

Fast switchable protein rsFastLime

Modelling of a *trans* chromophore into the Dronpa^{on} structure immediately revealed several spatial constraints, primarily imposed by the side chains of Arg^{66} and Val^{157}. If there is a photoinduced *cis–trans* isomerization in Dronpa, it requires the movement of Arg^{66} and Val^{157}, which in turn might induce spatial rearrangements of other residues. Therefore the structural changes taking place in Dronpa upon photoswitching might be more complex than previously reported for asFP595. Still, the fact that exchange of either Val^{157} or Met^{159} by smaller residues accelerates switching supports the view that a *cis–trans* isomerization of the chromophore is a key event in photochromic switching of Dronpa.

Irrespective of the exact details of the mechanism, the outstanding properties of rsFastLime, namely its high Φ_{FL} and its moderately accelerated switching behaviour, render it a very promising protein for several applications, in particular for breaking the diffraction barrier in far-field fluorescence microscopy. For example, for RESOLFT (reversible saturable optical fluorescence transitions) super-resolution microscopy techniques using switchable fluorescent proteins, fast switching kinetics are mandatory [5,28]. The same applies to the recently proposed methods using individual molecule switching [29,30].

In Dronpa and its variants the wavelengths required for probing fluorescence also induce the on-to-off state conversion. For protein tracking applications in live cells, this property may prove to be a constraint for some applications, whereas it may be a virtue for others. The speed of this transition is expedited in rsFastLime. Hence, for long-term protein tracking approaches which do not require a fast switching behaviour, the almost bistable Dronpa is probably better suited, whereas for faster movements requiring fast switching, rsFastLime might be advantageous.

Note added in proof (received 7 December 2006)

While this manuscript was under review, a second Dronpa bright-state structure was published by Wilmann et al. [31].

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