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 photo-activatable 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 (asCF; asulCP) isolated from the sea anemone Anemona 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 phototransitioned, 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 (ΦFL = 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 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 phototransitioned, 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 (ΦFL = 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].

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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
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 (Ni²⁺-nitriiotriacetate) affinity chromatography and subsequent size-exclusion chromatography according to standard procedures. The purified proteins were concentrated to ∼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 His₆-tag by sitting drop vapour diffusion at room temperature (22 °C), by employing a reservoir of 22 mM NaCl (pH 7.5) for crystallization. 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.

**RESULTS**

**Crystallization and overall structure of Dronpa**

N-terminally His₆-tagged Dronpa was expressed in *E. coli*, purified to apparent homogeneity and crystallized. In solution Dronpa adopts, at thermodynamic equilibrium, the on-state as Dronpa on. We refer to this state as 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, ∼0.3 W·cm⁻²) and ‘UV light’ (405/10 nm excitation filter, ∼0.2 W·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 1 s (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 Analysetechnik).

**Relaxation kinetics**

The determination of the relaxation half-time $t_{1/2, \text{relax}}$ 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, ∼0.75 W·cm⁻²) for ∼15 min (Dronpa) or ∼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·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.

**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 $F_{\text{rel}}$ relative to the reported values of Dronpa [9].
Table 1 Properties of Dronpa, rsFastLime and Dronpa-M159T

<table>
<thead>
<tr>
<th></th>
<th>Absorption maximum (nm)</th>
<th>Emission maximum (nm)</th>
<th>Molar absorption coefficient (M⁻¹·cm⁻¹)</th>
<th>Fluorescence quantum yield (Φ₁)*</th>
<th>Switch-off half-time t₁/₂off (s)</th>
<th>Switch-on half-time t₁/₂on (s)</th>
<th>Off-state relaxation half-time t₁/₂relax (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dronpa</td>
<td>503</td>
<td>522</td>
<td>95 000</td>
<td>0.85</td>
<td>263.00</td>
<td>0.10</td>
<td>840</td>
</tr>
<tr>
<td>rsFastLime</td>
<td>496</td>
<td>518</td>
<td>39 094</td>
<td>0.77</td>
<td>5.00</td>
<td>0.11</td>
<td>8</td>
</tr>
<tr>
<td>Dronpa-M159T</td>
<td>489</td>
<td>515</td>
<td>61 732</td>
<td>0.23</td>
<td>0.23</td>
<td>0.05</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Φ₁ was calculated relative to Φ₁-Dronpa, Φ₁-Dronpa was taken from [9].

**Figure 1 Characteristics of Dronpa**

(A) Dronpa* fluorescence emission spectra (excitation at 488 nm). Displayed is the normalized emission spectrum of Dronpa* in solution (dotted line) and of Dronpa* protein crystals (solid line) a.u., absorbance units. (B) Overall structure of Dronpa*. 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/R₉₅ values converged at 18.5/21.6 %. Residues 2–219 of Dronpa* 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 2Fᵣ–Fᵣ 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* 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* exhibits a slightly non-coplanar cis chromophore**

The chromophore is a [2-(1-amino-2-mercapto-ethyl)-4-[1-(4-hydroxy-phenyl)meth-(E)-ylidene]-5-oxo-4,5-dihydro-imidazol-1-yl]-acetaldheyde moiety that is formed spontaneously from the tripeptide Cys⁶²–Tyr⁶³–Gly⁶⁴ 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*, CYG adopts a cis isomeric state (Figure 2A). The normals to the planes of the five- and six-membered rings span an angle of ∼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-hydroxypbenyl ring of CYG stacks on the His¹⁹³ side chain and the hydrogen-bonding potential of the polar groups is completely saturated (Figure 2B). The apical hydroxy group of the p-hydroxypbenyln ring forms a direct hydrogen bond with the side chain of Ser¹⁶² and a water-mediated contact with the backbone carbonyl group of Glu¹⁴⁸. The imidazolinone ring forms an array of seven hydrogen bonds (direct interactions with the side chains of Thr⁵⁹, Arg⁶⁶, Arg⁹¹ and Glu²¹¹; water-mediated interactions with the side chains of Gln³⁸, Glu²¹¹ and the backbone carbonyl group of Asn¹⁰¹). 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,
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 $\beta 7$ and $\beta 10$ on the surface of the $\beta$-barrel (indicated by the dotted red line in Figures 3A and 3C). The opening is particularly noticeable in Dronpa™, 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™ 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<sup>on</sup> 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<sup>on</sup> the protein backbone is intact, in asFP595 there is a chain break immediately preceding the chromophore. Second, 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<sup>193</sup> 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 chromophores in their ground states.

In Dronpa<sup>on</sup>, the hydroxy group of the p-hydroxyphenyl ring forms a hydrogen bond to the side chain of Ser<sup>142</sup> (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<sup>on</sup>, it appears that the bulky Val<sup>157</sup> and Arg<sup>66</sup> sterically hinder the chromophore from adopting a trans conformation (Figure 5A). In asFP595 the analogous positions are occupied by the slightly smaller residues Ser<sup>157</sup> and Lys<sup>66</sup>, which might explain the lowered energy barrier for the cis–trans isomerization. Furthermore, in ground-state asFP595, Ser<sup>157</sup> hydrogen bonds with the apical hydroxy group of the chromophoric tyrosyl ring and thereby stabilizes the trans conformation. Val<sup>157</sup> in Dronpa<sup>on</sup> does not exhibit the hydrogen-bonding capacity to interact similarly with the chromophore. Indeed, exchange of asFP595 Ser<sup>157</sup> 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<sup>on</sup>.

rsFastLime: a fast switching RSFP

In their fluorescent-states, the chromophores of both Dronpa<sup>on</sup> 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<sup>on</sup> Val<sup>157</sup> 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, \( t_{1/2}^{\text{off}} \), is reduced by a factor of \( \sim 50 \) in Dronpa-V157G (\( t_{1/2}^{\text{off}} = 5.0 \text{ s} \)) compared with Dronpa (\( t_{1/2}^{\text{off}} = 263.0 \text{ s} \)) (Table 1, Figure 5B). The actual values of \( t_{1/2}^{\text{off}} \) depend on the light intensities employed. Nonethe-
Dronpa-M159T (blue line) fit into one switching cycle of Dronpa (black line). Inset: (protein. Main image: 56 switching cycles of rsFastLime (red line) and 650 switching cycles of off-state. Optical settings were identical; the duration of irradiation was adjusted to the respective proteins into the fluorescent on-state or with blue light to switch them into the non-fluorescent Colonies were irradiated in alternate turns with UV-light together with blue light to switch the Dronpa and rsFastLime. (Figure 5B). Because of the favourable combinations of proper-

Flexible anchoring of the chromophore is required for reversible switching

DISCUSSION

In the present paper we have determined a high-resolution equilibriumb structure of the RSFP Dronpa. In the fluorescent equilibrium state, the CYG chromophore adopts a cis conforma-

The chromophore environment of Dronpa 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 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–Tyr–Gly tripeptide. In KikG, the carboxy group of Asp maintains four water-mediated hydrogen bonds to Gln, Phe, Tyr and Leu, thus firmly stabilizing this part of the chromophore, probably inhibiting considerable relocations of the chromophore. Comparable linkages are missing in Dronpa, since the thiol group of Cys does not engage in any hydrogen bonds. Rather, this portion of the CYG chromophore 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 in Dronpa. Furthermore, in asFP595 flexibility is further increased by a main chain break between Cys 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 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 for a serine or alanine residue (results not shown). However, this increase in the switching kinetics is accompanied by a reduction in the half-life (Table 1). In Dronpa-M159T, is halved already after 20 switching cycles, whereas rsFastLime undergoes 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).
of the on-state asFP595. After photoswitching the fluorescent state of asFP595 has a half life of only $\sim 7$ 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 $\sim 14$ 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$^{\text{TM}}$ 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 $\Phi_R$ and its moderately accelerated switching behaviour, render it a very promising protein for several applications, in particular for breaking the diffusion 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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**REFERENCES**


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