Light-induced conformational changes and energy transfer in red fluorescent protein

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Abstract

Reversible conformational changes have been photo-induced in the red fluorescent protein DsRed at low temperature by wavelength-selective laser irradiation. We have found two new fluorescent forms: a shifted-red (SR-) and a new green (G0-) form that absorb and emit, respectively, ~14 nm to the red and ~80 nm to the blue of the ‘mature’ red (R-) form present in an un-illuminated sample of DsRed. Further, we have identified the 0–0 transitions of the various forms by spectral hole burning and estimated their ground-state energy differences and barrier heights by means of temperature-dependent excitation and fluorescence spectroscopy between 1.6 and 295 K. We have also proven that ‘downhill’ energy transfer takes place between these forms within the tetrameric structure of DsRed.

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1. Introduction

The range of fluorescent labels for molecular and cell biology has been significantly expanded with the recent cloning of red fluorescent proteins from non-bioluminescent reef corals [1]. In particular, DsRed from Discosoma sp. has absorption and emission spectra that show a large shift to the red compared to green fluorescent proteins (GFPs). DsRed thus has great potential to complement the widely used GFPs [2] as an expression marker for simultaneous multicolour tracking and as an acceptor in donor–acceptor pairs for fluorescence resonance energy transfer (FRET) [3,4]. Moreover, the advantage of DsRed is that its strong, orange-red emission at 583 nm (with a fluorescence quantum yield of ~0.7 [5]) does not overlap with the green autofluorescence of cells when used as a marker inside living organisms and, unlike most GFPs, the spectra show no dependence on pH when this is varied between 5 and 12 [5].

The structure of DsRed consists of a 28 kDa ‘β-barrel’ protein with 225 amino-acid residues [1,5], very similar to that of GFPs [2], but its ‘β-barrels’ are oligomerized into tetramers [5–9] through the protein interfaces [8]. Oligomerization appears to take place even at nanomolar concentrations [10,11]. The large spectral red shift of ~80 nm with respect to wt-GFP originates from a chromophore, buried within the ‘β-barrel’, that is formed from residues Gln66, Tyr67 and Gly68 in
the protein chain [1]. During maturation of DsRed, which proceeds via a GFP-like green intermediate to the final red species [6], one backbone bond undergoes oxidation and dehydrogenation at Gln66 by which the conjugated π-electron system is modified and enlarged (see Section 3.1, Fig. 1a) [6,8,9]. A peptide bond with cis–trans isomerization has been suggested as a possible step in this process [8,9].

Because maturation leading to the red form (extinction coefficient \( e \approx 75,000 \text{ M}^{-1} \text{ cm}^{-1} \)) is slow and incomplete, DsRed always shows an additional green fluorescence at about 500 nm emitted by an ‘immature’ green (G-) form [5,6]. The two drawbacks, slow maturation and oligomerization, cause energy transfer (ET) from the G- to R-form at room temperature [5,6,10–12]. These undesirable properties for biotechnological applications are being addressed through systematic mutagenesis [13,14].

To get a better understanding of the intricate photophysics of DsRed, we carried out a study of this red fluorescent protein at 1.6K and as a function of temperature up to 295K by high-resolution optical spectroscopy. At liquid-helium temperature, much better-structured spectra and considerably narrower bands are obtained than at higher temperatures because thermal broadening is suppressed. In this way, information is revealed that otherwise remains hidden in the spectra. Although it has been reported that DsRed is more photostable than GFPs [5], we have discovered that DsRed undergoes reversible photo-conversions at 1.6 K. Thus, conformational changes take place in this protein in a similar way as previously found by us for wt-GFP [15] and three of its mutants [16,17]. In addition to laser-induced spectral changes, we have observed ‘downhill’ ET between these forms within the tetramer of DsRed. We have further identified the excitation and fluorescence spectra of the various conformations of DsRed and determined their \( S_1 \rightarrow S_0 \) 0–0 transitions by means of spectral hole-burning experiments. From the temperature dependence of wavelength-selected, laser-excited emission spectra we estimated the ground-state energy differences and energy-barrier heights between these forms.

2. Experimental

Fluorescence excitation spectra between \( \sim 350 \) and 700 nm were obtained by irradiating the sample with the light of a Xe lamp that was passed through a 1 m monochromator (SPEX 1802, resolution \( \sim 40 \text{ cm}^{-1} \)). The power density at the sample was \( \sim 50 \mu \text{W/cm}^2 \). The fluorescence signal was detected at 90° with respect to the excitation beam through a 0.85 m double monochromator (SPEX 1402, resolution \( \sim 30 \text{ cm}^{-1} \)) at a fixed wavelength, with a cooled photomultiplier (PMT, EMI 9658 B) and an electrometer (Keithley, 610 CR). The excitation spectra were corrected by dividing the intensity at every wavelength by that of the excitation beam at the corresponding wavelength (detected with a second PMT, Hamamatsu 931A).

Emission spectra were obtained by exciting the sample at a given wavelength with one of the three lasers: (1) a pulsed N$_2$-pumped (Molelectron UV22) dye laser (Molelectron DL200, 10 pps, bandwidth \( \sim 1 \text{ cm}^{-1} \)) between \( \sim 400 \) and 600 nm using various dyes; (2) a CW Ar$^+$-ion laser at 514 nm (Coherent Innova 300) or (3) a CW Ar$^+$-ion-pumped dye laser (Coherent 599-21, without ICA, bandwidth \( \sim 1 \text{ cm}^{-1} \)) at 575 nm (dye Rhodamine 110). The power density at the sample was \( \sim 1–5 \mu \text{W/cm}^2 \). The fluorescence was detected at 90° with respect to the excitation through a 0.75 m spectrograph (Acton Research Pro 750, resolution \( \sim 20 \text{ cm}^{-1} \)) with a liquid-N$_2$-cooled CCD camera (Princeton Instruments, LN/CCD-1340PB). Emission spectra were taken in about 5 s.

Holes were burnt for about 15 min with the same N$_2$-pumped dye laser (bandwidth \( \sim 1 \text{ cm}^{-1} \)). Burning power densities were \( \sim 2–5 \mu \text{W/cm}^2 \). This corresponds to burning fluences \( Pt_b/A \sim 1.8–4.5 \text{ J/cm}^2 \) (with \( P \) the power of the laser, \( A \) the area of the laser beam on the sample and \( t_b \) the burning time). After burning, the holes were probed in fluorescence excitation through the same 0.85 m double monochromator and PMT as used for recording the excitation spectra, with the laser power attenuated by a factor of \( \sim 20 \). The widths of the holes were limited by twice the laser bandwidth.
Conformational changes were induced by laser irradiation at various wavelengths. The relatively photo-stable ‘mature’ red (R-) form was photo-converted into a shifted-red (SR-) and a new green (G0-) form by irradiating it with the 514nm line of the CW Ar+-ion laser with power densities \( P/A = 30–300 \text{mW/cm}^2 \) and \( t_b = 1–90 \text{min} \). Burning fluences were used from \( P_{tb}/A = 10–2000 \text{J/cm}^2 \). The back reactions were induced by irradiation of the G0-form with the pulsed dye laser at 430nm with \( P = 4–5 \text{mW/cm}^2 \) for \( 1 \text{h} \), i.e. \( P_{tb}/A = 15–20 \text{J/cm}^2 \). The SR-form was transformed back by irradiating it with the CW Ar+-pumped dye laser at 575nm with \( P/A = 5–20 \text{mW/cm}^2 \) for \( 20–90 \text{min} \), i.e. \( P_{tb}/A = 5–20 \text{J/cm}^2 \). Care was taken to overlap the beams of the three lasers at 514, 430 and 575nm at the same spot of the sample by means of dichroic mirrors.

A \(^4\)He-bath cryostat was pre-cooled to \( \sim 150 \text{K} \) before introducing the sample. The cryostat was then filled with liquid \(^4\)He and pumped down to 1.6 K. For the temperature-dependent experiments, the temperature in the cryostat was slowly increased at a speed of \( \sim 10–15 \text{K/h} \). Excitation and emission spectra were measured between 1.6 and 295 K every \( \sim 5 \text{K} \) for \( T < 85 \text{K} \) and every \( \sim 10–20 \text{K} \) for \( T > 85 \text{K} \).

### 3. Results and discussion

#### 3.1. Fluorescence excitation and emission spectra at 1.6 K

The fluorescence excitation spectra at 1.6 K of DsRed (detected at 615 nm) and of wt-GFP (detected at 482 nm [15]) are shown in Fig. 1a, for comparison. These spectra are far better resolved and, therefore, contain more vibrational information than the spectra at room temperature [1,2,5]. The chromophores of the two autofluorescent proteins (depicted at the top of the figure) form part of the amino-acid residue chain and are buried inside the protein ‘β-barrel’. The two extra double bonds C=N–C=O in DsRed are responsible for the extended π-electron system and, thus, for the red shift of the spectra with respect to GFP [6,8,9]. The maxima of the excitation spectra at 1.6 K of DsRed at 555 nm and of wt-GFP at 472 nm (its deprotonated form [15]) are blue shifted by 3–5nm relative to those at room temperature.

The emission spectrum of DsRed at 1.6 K is shown in Fig. 1b (right) between 480 and 700 nm.
700 nm. It is much narrower and more structured than the corresponding spectrum at ambient temperature. Two regions are clearly distinguished: one in the green with very weak intensity between 490 and 550 nm, and another one in the orange-red with a strong maximum at 572 nm and a band at about 616 nm. The maximum at 572 nm is about 11–12 nm shifted to the blue as compared to the maximum at ambient temperature [1, 5]. The shape and position of the fluorescence spectrum is independent of excitation wavelength for $\lambda_{\text{exc}} \sim 490–560$ nm. The green emission spectrum, which appears when exciting at wavelengths shorter than 484 nm, corresponds to the ‘immature’ G-form [5, 6] and is similar to that of wt-GFP. Its excitation spectrum (Fig. 1b, left), detectable between 490 and 530 nm, has bands at 450 and 478 nm and is mirror symmetric with respect to the corresponding part in the emission spectrum. We have observed that the intensity of the green fluorescence decreases with the degree of ‘maturation’ of the sample (not shown) but, to our knowledge, no ‘pure red’ emitting samples have been reported yet. It has been proposed in the literature that the nature of this ‘immature’ G-form might be a precursor polypeptide similar to wt-GFP [6]. The fact that on excitation of the G-form at $\lambda < 484$ nm, the weak green emission is always accompanied by a strong red emission, independent of the degree of maturation of the sample, is striking. It will be demonstrated below that this effect is the consequence of ‘downhill’ ET within the DsRed tetramer.

The spectral origins of the red (R-) and green (G-) forms are roughly given by the wavelengths of the intersections of the excitation and emission spectra. A more accurate determination of $S_1 \leftarrow S_0$ 0–0 transitions can be obtained by hole-burning spectroscopy [18]. In a similar way as previously found by us for wt-GFP [15] and its mutants [16, 17], we have identified the 0–0 transition of the red (R-) form at 563 ± 1 nm. It proved impossible to burn holes in the ‘immature’ G-form, an indication that this form is photo-stable. The width of the holes was not determined by the homogeneous line width $\Gamma_{\text{hom}}$, but limited by twice the laser bandwidth of $\sim 1 \text{ cm}^{-1}$ [18]. Recently, however, we succeeded in burning very narrow holes in DsRed with MHz resolution from which we indeed obtained the value of $\Gamma_{\text{hom}}$ [19]. This is the first time, to our knowledge, that the homogeneous line width and optical dephasing have been determined in an autofluorescent protein, demonstrating the potential of the hole-burning method for the study of protein dynamics.

3.2. Photo-induced reversible conformational changes

When burning the R-form, we observed not only holes but also conformational changes: two photo products were created, one to the red side of the R-form that we call the shifted-red (SR-) form, and the other one in the same spectral region as the ‘immature’ G-form that we call the green (G') form.

To get a better insight into the photo-induced conformational changes taking place in DsRed, we have recorded excitation and emission spectra at 1.6 K as a function of excitation and detection wavelength before and after prolonged burning of the red (R-) form. In a second step, we looked whether the photo-induced reactions were reversible. Examples are shown in Fig. 2. An excitation spectrum (detected at 590 nm) is depicted in Fig. 2a (left) before (black) and after (grey) irradiation in the red (R-) form of DsRed at 514 nm for 60 min with a power density of $\sim 200 \text{ mW/cm}^2$. After irradiation, the intensity of the bands between $\sim 480$ and 560 nm corresponding to the R-form have decreased, while those at $\lambda_{\text{exc}} < 480$ nm and $\lambda_{\text{exc}} > 560$ nm have increased. We attribute these spectral changes to photo-convertions from the R-form into the shifted-red (SR-) form absorbing at $\sim 575$ nm and into the new green (G')-form absorbing at $< 480$ nm. It proved possible to burn holes into the 0–0 transitions of these photoproducts at 577 ± 1 nm in SR and at 484 ± 1 nm in G' (Fig. 2a).

Fig. 2b shows three series of fluorescence spectra before and after irradiation into the R-form at 514 nm for a time of 30, 60 and 90 min. In the figure at the top, excitation occurs at $\sim 575$ nm, a wavelength at which the SR-form absorbs. As a function of irradiation time, a new emission spectrum grows in, which is red-shifted with
respect to that of the R-form by 14 nm (cf. Fig. 2a). The emission spectrum of the SR-form shows a peak at 586 nm (vs. 572 nm in the R-form, Fig. 1b) and a weak band at 635 nm (vs. 616 nm in the R-form, Fig. 1b). We have found it to be mirror symmetric with respect to its excitation spectrum, which has a maximum at 568 nm (Fig. 2a). Moreover, the 0–0 transition lies at 577 ± 1 nm (see hole in Fig. 2a). The nature of this conformation will be discussed in Section 4.

The middle spectra in Fig. 2b have been excited in the R-form at 514 nm. Before irradiation in the R-form, a strong red fluorescence is present (see also Fig. 1b) that decreases as a function of irradiation time. During irradiation of R, the maximum of the emission spectrum broadens and shifts towards the red. We attribute the decrease of the fluorescence signal and the change in the shape of the spectrum to, respectively, a decrease of the number of proteins in the R-form (see also Fig. 2a) and to ‘downhill’ ET from the R-form (donor) to the SR-form (acceptor) within a tetramer of DsRed (for further discussion, Section 3.3). We think that the slight shift to the red of the maximum of the emission band as a function of irradiation time originates from an increase of the...
contribution of the SR-form (the acceptor, Fig. 2b top) to the fluorescence intensity.

The bottom spectra in Fig. 2b have been excited at 430 nm, a wavelength at which the G'-form absorbs. In this spectral region also the ‘immature’ G-form absorbs. Two effects are observed: (1) the fluorescence intensity in the green region (between 490 and 550 nm) increases as a function of irradiation time (in the R-form). This is understandable because the number of photo-induced proteins in the G'-form increases. We notice further that the spectrum builds up on the already existing emission spectrum of the ‘immature’ G-form. (2) Simultaneously with the increase of the fluorescence of the G'-form, the fluorescence of the red region (between 560 and 700 nm) increases and changes its shape, with the maximum of the emission spectrum broadening and shifting to the red. This is a surprising result because the number of proteins in the R-form decreases with irradiation time (while that in the G'-form increases). We interpret the time evolution of these spectra in terms of ‘downhill’ ET from the G'-form (the donor) to the R- and SR-form (the acceptors, see also Section 3.3). The inset in Fig. 2b (bottom) shows an amplification of the emission spectrum in the green region, normalized to the band at 490 nm before (black) and after (grey) irradiation of the R-form for 90 min. The fluorescence spectrum before irradiation is that of the ‘immature’ G-form (the G'-form is not present yet), whereas the emission spectrum of the photo-induced G'-form builds up on top of that of the G-form with emission bands at the same wavelengths (490 and 525 nm), but with slightly different relative intensities. The spectra of the G- and G'-form also resemble that of the deprotonated form of wt-GFP [15] (see Section 4).

3.3. ‘Downhill’ ET within the tetramer of DsRed

We interpret the fluorescence spectra of Fig. 2b (bottom), where an increase of the emission spectrum of the green G'-form is observed together with a simultaneous increase and spectral red shift of the red fluorescence as a function of the irradiation time of the R-form, in terms of ‘downhill’ ET within the DsRed tetramer. To understand this, we first consider Förster’s ET mechanism based on non-radiative dipole–dipole interaction [20]. In order for ET to occur, three conditions have to be fulfilled: (1) spectral overlap should exist between the emission spectrum of the donor (D) and the absorption spectrum of the acceptor (A); (2) the distance \( R \) between donor and acceptor should be smaller than or equal to the Förster radius \( R_0 \), the distance at which the rate of ET, \( k_{ET} = 1/\tau_0(R/R_0)^6 \), is equal to the reciprocal of the fluorescence lifetime \( \tau_0 \) of the donor in absence of ET; (3) the transition dipole moments of D and A should not be perpendicular to each other.

In Fig. 3, the emission spectrum of the G-form, which is almost identical to that of the G'-form (see Fig. 2b, inset), is shown together with an absorption spectrum of the R-form at 1.6 K. The fact that the two spectra overlap is proof that condition (1) is fulfilled. With condition (2), however, there is a problem unless we make specific assumptions. The Förster radius for DsRed is \( R_0 \sim 5–7 \text{ nm} \) [21,22]. Since the concentration of our sample is \( c \sim 1 \mu \text{M} \), the average
distance \( R \) between DsRed proteins is larger than 100 nm. Moreover, there is no diffusion between the proteins in our experiments at 1.6 K. This would mean that \( R \gg R_0 \). From our results we deduce that the only way to fulfil condition (2) is that the individual ‘\( \beta \)-barrel’ proteins (which have a size of \( \sim 3 \times 4 \) nm\(^2\)) are very close together, i.e. they have to form at least dimers or oligomers. It was found by biochemical [5,6] and X-ray diffraction [8,9] methods that DsRed indeed forms tetramers at room temperature. Therefore, the condition \( R \ll R_0 \) is also fulfilled. This is schematically illustrated in the inset of Fig. 3, where a tetramer with three ‘\( \beta \)-barrel’ subunits in the R-form and one in the G-form is sketched. Finally, condition (3) that the relative orientation of donors and acceptors should not be perpendicular is also fulfilled, because from the X-ray structure of DsRed [8,9] it is known the ‘\( \beta \)-barrels’ within the tetramer are nearly parallel to each other.

To illustrate qualitatively our interpretation, we make use of Fig. 2c, which symbolizes a tetramer of DsRed. Let us assume that in an un-illuminated ‘mature’ DsRed sample there are many more proteins in the R-form than in the ‘immature’ G-form. This is represented by the left square with three ‘\( \beta \)-barrel’ subunits in the R-form and one in the G-form. In a real sample, however, there will be a statistical distribution of tetramers with many of them having four or three subunits in the R-form and one in the G-form and probably less tetramers with two, three or even four subunits in the G-form. By irradiation of the R-form at 514 nm, some subunits in the R-form will be converted to the G'-form and others to the SR-form (see Fig. 2c, right). Since the number of green G'-donors will increase as a function of irradiation time, the probability of ‘downhill’ energy transfer from G' to R on the subsequent excitation at 430 nm will increase (as long as the number of R-acceptors is larger than that of the G'-donors). This, in turn, will cause an increase of the red fluorescence on excitation in the green. Simultaneously, a photo-conversion from R to SR takes place, by which the number of SR-acceptors will increase with irradiation time as well as the ET probability from G' to SR. Thus, the R- and SR-forms will emit simultaneously while the SR-emission increases with irradiation time. The result is not only an increase but also a red shift of the red fluorescence (see Fig. 2b, bottom).

We have verified that DsRed acts as an optical switch at 1.6 K because the photo-induced conformational changes are reversible. After producing the G'- and SR-form, these photo-products were irradiated, respectively, at 430 and 575 nm and the R-form was recovered. To check whether our qualitative interpretation of the photo-conversions and ‘downhill’ energy transfer is correct and to quantify our results, we have developed a kinetic model, to be presented elsewhere [23].

### 3.4. Temperature dependence of the fluorescence intensity

We have estimated the values of the energy-level differences and energy-barrier heights in the ground states of the R-, the SR- and the G'-forms of DsRed from their excitation and emission spectra measured between 1.6 K and ambient temperature. In Fig. 4, the intensity of the fluorescence of the SR- and G'-forms are plotted as a function of temperature. At 1.6 K, the R-form was irradiated and photo-converted into the SR-form. After the irradiation was stopped, the fluorescence intensity at 586 nm was followed while the temperature of the sample was increased (open circles in Fig. 4a). Up to \( T \sim 60 \) K the SR-form is thermally stable, at \( \sim 65 \) K it rapidly disappears and gradually reappears again for \( T \geq 90 \) K. We interpret the behaviour up to 65 K in terms of an Arrhenius-activated process with an energy barrier \( E_A \) between SR and R and a rate to cross over the barrier \( k = k_A \exp(-E_A/kT) \). We measured the rate of disappearance of the SR-form of \( \sim (10–30 \) min\( )^{-1} \) at \( T \sim 65 \) K and assuming a pre-exponential factor of \( k_A \sim 10^{11}–10^{12} \) s\(^{-1} \), we estimate a barrier height \( E_A \sim 1500 \pm 100 \) cm\(^{-1} \) (see Fig. 5). In a second experiment, we followed the fluorescence intensity at 586 nm as a function of temperature without previously having photo-induced the SR-form. We observe no emission up to 90 K; above 90 K the fluorescence slowly appears and its intensity increases with temperature (filled-in circles in Fig. 4). If we assume that Boltzmann equilibrium is established between the
R- and SR-form at temperatures $T \geq 90\,\text{K}$, i.e. $I_{\text{SR}}/I_{\text{R}} \sim \exp(-\Delta E/kT)$, with $I_{\text{SR}}$ and $I_{\text{R}}$ the intensities of the SR- and R-forms, respectively, and if we further assume that $I_{\text{SR}}/I_{\text{R}} \sim 0.01$ at 90 K (just above the noise level), we estimate the energy-level difference between the ground state of the R-form and that of the SR-form to be $\Delta E \sim 250 \pm 50\,\text{cm}^{-1}$ (see Fig. 5).

In a similar way as for the SR-form, we have followed the fluorescence signal of the G'-form at 490 nm as a function of temperature (Fig. 4b). In a first experiment, the G'-form was photo-induced at 1.6 K and, after the irradiation was stopped, its emission intensity was measured as a function of temperature. A strong decrease of the signal is
observed at $T \sim 45$ K (open circles in Fig. 4b). In contrast to the SR-form, the fluorescence of the G'-form did not reappear at higher temperatures. Assuming the same pre-exponential factor and rate of disappearance as for the SR-form, but now for $T \sim 45$ K, we estimate a barrier height between the G'- and R-form of $E_A \sim 1100 \pm 100$ cm$^{-1}$ (see Fig. 5). Since the emission of the G'-form does not reappear up to ambient temperature ($T \sim 295$ K), the energy difference $\Delta E$ between the ground-state levels of the G'- and R-form has to be at least 800 cm$^{-1}$ (see Fig. 5). To check whether our estimates of the energy barriers and energy-level differences in the ground states of the various forms of DsRed are correct and to get more accurate values, we have developed a model that takes into account the shapes of the spectral bands and their thermal broadening and shifts. This model and the results we obtained from it will be presented elsewhere [24].

4. Conclusions: energy-level scheme of DsRed

Our findings are summarized in the two diagrams of Fig. 5. The first diagram in Fig. 5a represents the ground and electronically excited states of the three photo-convertible R-, SR- and G'-forms of DsRed. It also shows the $S_1 \rightarrow S_0$ 0–0 transitions of these forms, which we identified by means of spectral hole burning at 1.6 K, and the energy-barrier heights and energy-level differences in the ground state between these forms which we obtained from temperature-dependent fluorescence spectra between 1.6 and 295 K. The scheme in Fig. 5b summarizes the reversible photo-conversions and the ‘downhill’ energy-transfer processes taking place between the G'-, R- and SR-forms which we determined from wavelength-selected excitation and emission spectra as a function of irradiation time in each of the forms at 1.6 K [23]. It should be mentioned that at room temperature, using high excitation power ($\sim 3$ W/cm$^2$) and prolonged irradiation (a few hours) at 514 nm, we observed that the R-form irreversibly photo-converts into a red-shifted form with similar spectral characteristics as the SR-form studied here. Simultaneously, a blue form appears at $\sim 390$ nm. After $\sim 12$ h irradiation, the red-shifted form disappears and the blue- and the G-forms remain (data not shown). Similar irreversible photo-conversions have been reported in Refs. [25,26].

Although we do not know what the SR- and G'-forms are, we may speculate on their nature. We think that the chromophore in the shifted-red (SR-) form is chemically the same or very similar as that in the red (R-) form, but differs slightly in the conformational arrangement of the surrounding hydrogen-bonding network. We base our argument on the fact that the 0–0 transitions of R and SR are only $\sim 430$ cm$^{-1}$ (14 nm) apart and that the shape of their emission spectra are very similar. With respect to the G'-form, we conjecture that the structure of its chromophore is almost identical with that of the ‘immature’ G-form and the deprotonated form of wt-GFP (see Fig. 1 top, left) since the shape and position of its spectral bands are very similar to those of these species. The species G’, however, is thermally unstable because it disappears at temperatures above 45 K. It might be that on irradiation of the R-form, the C=N bond in DsRed breaks and water is added at this position, in a similar way as has been proposed for a mildly denaturated form of DsRed [6]. Alternatively, G’ might be the result of protonation of R, in analogy to wt-GFP [15]. Quantum-chemical calculations may help to decide the issue. Whatever G’ is thought to be, one has to take into account that it can be photo-induced back to the R-form.

As mentioned, our spectroscopic results can only be understood if, in addition to photo-conversions, ‘downhill’ ET takes place and this, in turn, is only possible if DsRed forms oligomers. The photo-induced conformational changes may have detrimental consequences when used for multicolour tracking, because DsRed under illumination may change its colour. Moreover, the ‘intra-tetrameric’ energy transfer process may become a problem when DsRed is used as acceptor in combination with GFP as donor in FRET experiments, for example, to study protein–protein interactions. In fact, excitation in the 400–480 nm region may cause ‘downhill’ ET from G’ (or G) to R or to SR within the tetramer of DsRed, instead
of the wanted FRET signal between GFP and DsRed.

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