Red-shifted mutants of green fluorescent protein: reversible photoconversions studied by hole-burning and high-resolution spectroscopy

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Abstract

Mutants of green fluorescent protein (GFP) are usually designed to absorb and emit light as “one color” systems, i.e. with a single, photostable conformation of the chromophore. We have studied two red-shifted GFP-mutants (S65T and EYFP) by means of hole-burning and high-resolution optical spectroscopy at low temperature, and compare the data to those previously reported for RS-GFP. We prove that these GFP-mutants are not “one color” systems because they can be reversibly phototransformed from one conformation into another. The results are rationalized in terms of energy-level schemes that are similar to that previously derived by us for wild-type GFP. In these schemes, each mutant can be interconverted by light among at least three conformations that are associated with the protonation-state of the chromophore. The results have relevance for the study of protein–protein interactions by fluorescence resonance energy transfer (FRET), where GFP-mutants of different colors are used as labels in donor–acceptor pairs. Furthermore, we present a detailed mechanism that explains the “on–off” and “blinking” behavior of single GFP-molecules with the proposed energy-level diagrams. © 2002 Elsevier Science B.V. All rights reserved.

1. Introduction

Autofluorescent proteins, such as green (GFP) [1–3] and red (DsRed) [4–7] fluorescent proteins are important in molecular and cell biology as markers for gene expression and protein localization, and for visualization of dynamic events inside living cells, like the study of protein–protein interactions by fluorescence resonance energy transfer (FRET) [8–11]. GFP from the jellyfish Aequorea victoria was the first protein discovered to possess an
intrinsic, bright fluorescence without the need of external cofactors [1,12]. After it was cloned in 1992 [12] and expressed in another organism in 1994 [13], its crystal structure was determined with a resolution of 1.9 Å in 1996 [14,15]. Based on the crystal structure, many mutants have been designed with the aim of improving the optical properties of wild-type (wt)-GFP (e.g. photostability and fluorescence quantum yield) and to expand its spectral range for multicolor labeling [1–3,16–18]. Neither the denatured GFP nor the GFP-chromophore by itself emits fluorescence. The DNA sequence coding for GFP can be fused to the gene of a desired target protein for which the expression or cellular location is of interest. The expressed GFP folds independently of the protein to which it is fused and acts as an in vivo fluorescence label.

GFP is a small, single-chain protein consisting of 238 amino acids organized in eleven anti-parallel β-strands forming a compact, cylindrical barrel-like structure [12,14,15]. Inside the barrel, an α-helix contains the sequence from which the chromophore is derived. The chromophore, a p-hydroxybenzylideneimidazolinone [1,16,19], originates from an autocatalytic cyclization and subsequent oxidation of three amino-acid residues in the chain: serine (Ser)-65, tyrosine (Tyr)-66 and glycine (Gly)-67 [16,20]. It is completely protected from the bulk solvent and rigidly held within the barrel, forming a fluorescent p–π* system.

The absorption spectrum of wild-type GFP (wt-GFP) at room temperature is characterized by two maxima that have been associated with different protonation-states of the chromophore: a neutral A-form absorbing at ~398 nm and an anionic or deprotonated B-form absorbing at ~478 nm [1–3,16,21]. From previous studies at room temperature and at 77 K it was concluded that the interconversion from the A- to the B-form should occur through an intermediate-state I* involving a very fast excited-state proton transfer reaction, followed by a slow solvation process [22,23]. In [22] it has also been proposed that I* is responsible for the strong, green fluorescence at 508 nm [2,3,21]. However, the intermediate-state I had only been conjectured but not observed, and the details of the photoconversion remained unclear [22,23].

Recently, we identified the I-form of wt-GFP by means of high-resolution spectroscopy at liquid-helium temperature [24]. In that study we also located the 0–0 transitions of the three A-, B- and I-forms by hole-burning and determined vibrational frequencies of their ground- and excited-states. In addition, we unraveled the pathways of photoconversion between these three forms. The results were summarized in an energy-level scheme [24]. Compared to room-temperature spectroscopy, laser spectroscopy at low temperature has the advantage that many of the thermally induced conversions are blocked, and the discrimination of individual species is made easier. In addition, at low temperature the spectra become more structured and highly resolved vibrational information is obtained. Furthermore, energy-level schemes derived from low-temperature experiments set limits to the interpretation of room temperature results.

An understanding of the photophysics and conformational interconversions occurring in GFP and its mutants is crucial for their utilization as in vivo labels. GFPs are also challenging from a theoretical chemistry point of view because the origin of the intrinsic fluorescence is still unclear [25–27]. Particularly interesting for FRET experiments are “one color” GFP-mutants (i.e. with only one, photostable conformation), in contrast to wt-GFP, which exhibits at least three conformations. The various mutants, when used for FRET, should absorb and emit light at different wavelengths. Most of the successful GFP-mutations reported in the literature involve a change of one or more amino-acid residues within, or close to the chromophore [1,3,18,20]. For example, a mutation of tyrosine (Tyr)-66 to histidine (His) or tryptophan (Trp) leads to a blue shift of the spectra with respect to wt-GFP [16], whereas mutations on serine (Ser)-65 result in a red shift [17,20].

Here we present new results on the photophysics of two red-shifted mutants of GFP: S65T and EYFP, and compare these to the data previously reported by us for RS-GFP [28]. All data were obtained by high-resolution optical spectroscopy at liquid-helium temperature. In the S65T mutant, Ser-65 is replaced by threonine (Thr) [1,15,17,20].
the latter having an extra methyl group, which results in a major absorption peak at 490 nm (Fig. 1). This peak is shifted to the red of that of the B-form (472 nm) of wt-GFP (Fig. 1). More than one mutation in the vicinity of the chromophore often leads to a shift of the absorption maximum further to the red. For example, RS-GFP has a triple mutation at positions 64, 65 and 69 [1,3,17] and absorbs at 495 nm (Fig. 1), whereas EYFP absorbing at 520 nm (Fig. 1) has four amino acids replaced at positions 65, 68, 72 and 203 [1,15,29]. These red-shifted mutants, which appear to be lacking the protonated A-form (at ~400 nm), have simpler spectra than wt-GFP (Section 3.1). The maxima of S65T and RS-GFP (Fig. 1) are very close to the maximum of the I-form of wt-GFP (495 nm) [24]. This similarity challenged us to investigate whether these mutants are in the B-form as proposed for S65T [18,30], or in the I-form as suggested by our own results on wt-GFP [24].

In contrast to current views, we demonstrate that the red-shifted mutants S65T, RS-GFP and EYFP are not photostable and can be reversibly phototransformed between various conformations (Section 3.2). Our results are relevant for studies of protein–protein interactions by means of FRET experiments in which pairs of GFP-mutants are fused as donors and acceptors to the proteins of interest. The energy-level schemes that result from our experiments (Section 3.3) yield an interpretation for the “on–off” and “blinking” behaviour observed in single molecules of GFP-mutants at room temperature (Section 3.4) [29,31,32].

2. Experimental

2.1. Samples

Expression plasmids for S65T and RS-GFP were gifts of Dr. R. Rivera-Pomar (MPIbpc, Göttingen, Germany), while that for EYFP was a gift of Dr. D. Piston (Vanderbilt University, Nashville, TN, USA). The mutants carry the following changes in the protein sequence: S65T (Ser 65 → Thr), RS-GFP (Phe 64 → Met, Ser 65 → Gly, Glu 69 → Leu) and EYFP (Ser 65 → Gly, Val 68 → Leu, Ser 72 → Ala and Thr 203 → Tyr). The recombinant proteins with a 6-histidine tag at the amino terminus were expressed and purified on a Ni-chelating resin (Ni-NTA-Agarose, Qiagen, Hilden, Germany) using standard procedures [33]. All proteins were dissolved in 10 mM Na-phosphate buffer, pH 7, containing 50% (v/v) spectroscopic grade glycerol. Protein concentrations as determined from the respective chromophore extinction coefficients [34] were ~20 μM (S65T and RS-GFP) or ~5 μM (EYFP).

2.2. Hole-burning and high-resolution optical spectroscopy

All spectroscopic measurements were performed at 1.6 K in Leiden. The samples were
placed in a cuvette (thickness 3 mm) and introduced into a 4He-bath cryostat that was filled with liquid N\textsubscript{2}. After a few minutes, the liquid N\textsubscript{2} was blown out, the cryostat filled with liquid He and, subsequently, pumped down to 1.6 K.

The samples were excited with a dye-laser (Molectron DL 200, bandwidth $\sim1\text{ cm}^{-1}$) pumped by a pulsed N\textsubscript{2}-laser (Molectron UV 22) [24]. Continuous tunability between 355 and 535 nm was achieved using 10 dyes. Absorption spectra were obtained by scanning the laser and detecting the transmission through the sample with a photomultiplier (type EMI9658B) and an electrometer (Keithley 610CR). Excitation-, emission- and hole-burning (resolution $\sim1\text{ cm}^{-1}$) spectra were detected in fluorescence at 90$^\circ$ with respect to the excitation beam through a 0.85 m double monochromator (SPEX 1402, resolution 5 cm$^{-1}$) with the same photomultiplier and electrometer.

3. Results and discussion

In this paper, we present a detailed hole-burning and high-resolution spectroscopy study at low temperature of the red-shifted GFP-mutants S65T and EYFP and a comparison to RS-GFP [28]. The results indicate that these three mutants act similarly in many respects. In order to stress the similarities and differences among these mutants, the spectra of S65T and EYFP are presented in conjunction with those of RS-GFP, even though most of the data for the latter have been reported elsewhere [28]. We compare the results to those previously obtained by us for wt-GFP [24].

3.1. Absorption spectra at 1.6 K

Fig. 1 shows the absorption spectra at 1.6 K of wt-GFP and the three red-shifted mutants. They are significantly more structured than at room temperature [22–24]. The two strong bands observed in the spectrum of wt-GFP at 407 and 472 nm are attributed (see above) to the neutral or protonated A-form and the anionic or deprotonated B-form of the GFP-chromophore. By combining room- and low temperature spectroscopy with hole-burning, we recently discovered the I-form of wt-GFP as the intermediate and located its 0–0 transition at 495 ± 1 nm [24]. We also found that in thermodynamic equilibrium (i.e., in a previously non-illuminated sample) the intermediate I-form is present at room temperature but absent at low temperature. This is illustrated in Fig. 1 (top), where only the A- and the B-forms are present at 1.6 K. The I-form, however, can be photoinduced at low temperature by exciting either the A- or the B-form with a laser. In the same study, we identified the 0–0 transition of A at 434 ± 1 nm and that of B at 477 ± 1 nm and found that the photoreactions between A and I, and between I and B are reversible, i.e. $A \leftrightarrow I \leftrightarrow B$. Since we did not observe a direct interconversion between A and B, we concluded that I is a real intermediate species [24].

All three red-shifted GFP-mutants have a strong absorption maximum at their red edge and weaker bands towards the blue (Fig. 1). The spectra appear to lack the A-form (at $\sim400$ nm) characteristic for wt-GFP. As noted above, it is interesting that S65T and RS-GFP have their maxima in the same spectral region as the photoinduced I-form of wt-GFP (495 nm) [24]. This similarity suggests that the mutants are in a conformation corresponding to the I-form of wt-GFP and not to the B-form, as claimed in a study of S65T [18,30] (see below). The absorption spectrum of S65T at 1.6 K shows relatively strong bands on the blue side of its main peak (490 nm) as compared to the spectra of EYFP and RS-GFP. The latter two are similar, although shifted with respect to each other (see Fig. 1). We prove here that the bands in the spectrum of S65T correspond mainly to other conformations of the chromophore and, to a lesser extent, to vibrational bands of the conformation absorbing at 490 nm.

3.2. Excitation-, emission- and hole-burning spectra

3.2.1. S65T

A series of excitation- and fluorescence spectra at 1.6 K of a previously non-illuminated S65T sample are depicted in Fig. 2. The positions and shapes of the spectra depend markedly on the wavelengths of excitation ($\lambda_{\text{exc}}$) and detection ($\lambda_{\text{em}}$), indicating the presence of more than one form or
Fig. 2. S65T. Left: Excitation- and emission spectra at 1.6 K. Their intersections, which depend on the excitation- and detection wavelength, determine the rough positions of the 0–0 transitions of the three forms, I (a), B (b) and A (c). The approximate vibrational frequencies (within ~50–100 cm⁻¹) corresponding to the bands shown in the spectra (averaged over the ground- and excited-states) for the three forms are: (a) I-form: 205, 815 and 1520 cm⁻¹, (b) B-form: 260 and 1500 cm⁻¹, (c) A-form: 270 and 1780 cm⁻¹. Right: Excitation spectra before (-----) and after (---------) burning into the 0–0 transition of a given form. (a) Hole burnt into $I_{0-0}$ at 495 ± 1 nm; the I-form photoconverts into A and B. (b) Hole burnt into $B_{0-0}$ at 478 ± 1 nm; B transforms into I. (c) Hole burnt into $A_{0-0}$ at 429 ± 1 nm; A transforms into I. Thus, $A \rightarrow I \rightarrow B$ (see also Fig. 6(a)).
species in S65T. By varying $\lambda_{\text{exc}}$ and $\lambda_{\text{em}}$, three pairs of spectra are observed (left-hand column of Fig. 2) displaying three intersections which we ascribe to the 0–0 transition regions of three forms (I, B and A, from top to bottom). This behavior is similar to that previously observed for wt-GFP [24]. All three forms are already populated at $T = 1.6$ K, because they fluoresce independently when excited individually (Fig. 2, left). From the wavelengths of the intersections we obtain a rough estimate of the spectral positions of the 0–0 transitions, which by hole-burning we could accurately locate: $I_{0,0}$ at 495 ± 1 nm, $B_{0,0}$ at 478 ± 1 nm and $A_{0,0}$ at 429 ± 1 nm. The burnt holes are narrow (Fig. 2, right), with a width that is not given by the homogeneous linewidth [35] but equal to twice the laser bandwidth of $\sim 10^3$ cm$^{-1}$. In general, holes burnt in vibronic bands are difficult to detect because they are very broad (a factor of $\sim 10^3$ compared to holes in 0–0 transitions) due to the short (picoseconds) vibronic-state lifetimes [35].

If the assignments of the three I-, B- and A-forms in S65T are correct, we expect the photo-conversions $I \rightarrow A$ and $I \rightarrow B$ to occur. This is indeed observed (Fig. 2(a), right): when burning a hole into $I_{0,0}$, both the red wing and the vibronic bands of I decrease in intensity. Simultaneously, the intensities of the spectral regions assigned to the A- and B-forms increase slightly. If a hole is subsequently burnt into $B_{0,0}$ (Fig. 2(b), right), the vibronic bands of B decrease and the excitation spectrum of I increases (not shown), proving that B is converted into I and vice versa. Similarly, when a hole is burnt into $A_{0,0}$ (Fig. 2(c), right), the intensity of the total A-band slightly decreases and the intensity of I increases (not shown). We conclude that the I- and A-forms, and the I- and B-forms reversibly photoconvert, i.e. $I \rightarrow A$ and $I \rightarrow B$. These reactions occur through radiationless pathways (probably the triplet-state) during decay from the excited-state of one form to the ground-state of the other, for example, $I^* \rightarrow A$ and $A^* \rightarrow I$ (see also Fig. 6(a)). Photoconversions do not seem to take place in the excited singlet-state because all three forms fluoresce when excited individually (Figs. 2(a)–(c), left).

We conclude that the I-form is the predominantly populated conformation in S65T at low temperature. This is in contrast to the result obtained for wt-GFP, where the A- and the B-forms (Fig. 1, top) are the species populated at 1.6 K, not the I-form. This has significant consequences when trying to relate the conformations with the structures reported in the literature [14,15].

By similarity of wavelengths and spectral shapes, we associate the structure reported for S65T absorbing at ~490 nm [15,30] with the I-form $I_{0,0} = 495 \pm 1$ nm and not with the B-form $B_{0,0} = 478 \pm 1$ nm) of wt-GFP as suggested in [18,30]. The spectral position of $A_{0,0}$ at 429 ± 1 nm is also close to that found for $A_{0,0}$ in wt-GFP (434 nm) [24]. The approximate vibrational frequencies corresponding to the bands observed in the excitation- and emission spectra of Figs. 2(a)–(c) (left column) are mentioned in the caption. By means of ultra-high-resolution experiments we recently observed more than 30 sharp vibrational lines for each of the mutants studied. An analysis of the corresponding frequencies is in progress.

### 3.2.2. RS-GFP

As already mentioned, most of the results on RS-GFP have been reported elsewhere [28] but, for completeness sake and for comparison to the results obtained on other GFPs, we recapitulate them here. In Fig. 3, a series of emission spectra taken at various excitation wavelengths is shown for a previously non-illuminated RS-GFP sample. In contrast to S65T, the spectra are all identical regardless of $k_{\text{exc}}$, proving that only one form is present in RS-GFP at 1.6 K before burning. We tentatively assign this spectrum to the I-form. This assignment is supported by (1) the emission- and excitation spectra of Fig. 4(a), which show mirror symmetry about their intersection at ~499 nm, and (2) the fact that no other intersection was observed while exciting and detecting at various wavelengths. By comparison to wt-GFP [24] and S65T (see above), the wavelength of the intersection and the similarities of the shape and spectral positions of the emission bands strongly suggest that RS-GFP exists here in the I-form. Because it further proved possible to burn a narrow hole at 499 ± 1 nm (Fig. 4(b), right), we ascribe this wavelength to the 0–0 transition of I.
By burning into the I-form of RS-GFP, not only a 0–0 hole appears but, simultaneously, the absorption band at \(495\) nm (I-form) decreases and the region between \(460–475\) nm increases (Fig. 4(b)), indicating that a second conformation has been photoinduced. Since a hole could subsequently be burnt at \(476\) nm into the new photoproduct (Fig. 4(b)), and by similarity to wt-GFP [24], we associate this hole with the 0–0 transition of the B-form. Thus, the reaction \(I^* \rightarrow \text{B}\) occurs through a radiationless pathway (probably via the triplet-state). Conversely, when the newly formed B is excited, fluorescence is observed from \(I^*\) but not from from \(B^*\) (not shown), implying that the reaction \(B^* \rightarrow I^*\) is barrierless. The short excited-state lifetime of \(\approx 1.1\) ns obtained at room temperature may be related to this process [36]. By burning a hole into \(B_{0-0}\), the intensity of the vibronic region of I at \(495\) nm increases (not shown), from which we conclude that B can be reversibly photoconverted into I.

By burning into the I-form of RS-GFP, both the B-form (Fig. 4(b)) and the A-form (Figs. 4(c) and (d)) are produced. The excitation spectrum in Fig. 4(c) (Spectrum 1) shows the absorption region of the expected A-form while detecting at \(\approx 450\) nm (the emission region of A). A hole can be burnt on the red wing of A at \(A_{0-0} = 434 \pm 1\) nm (Fig. 4(c), right). Simultaneously, the entire A-band decreases (Spectrum 2) and the I-form increases (not shown). When subsequently burning into I, the A-band in turn increases (Spectrum 3) proving that the photoreaction \(I \rightarrow A\) is reversible. This is confirmed by looking at the fluorescence spectrum of the A-region excited at \(395\) nm, before and after burning into I (Fig. 4(d)). The region between 440 and 475 nm, where the A-form is expected to emit, shows no fluorescence signal before burning into I, but exhibits three bands at \(\approx 439, 449\) and \(473\) nm after burning. We conclude that the radiationless reaction \(I^* \rightarrow A\) takes place and that \(A^*\) emits light after having been produced and excited (see Fig. 6(b)).

Finally, after having burnt the I-form, the excitation spectrum of A (absorption region at \(\approx 350–450\) nm) does not increase when detected in the emission region of I (Fig. 4(b)), indicating that the excited-state reaction \(A^* \rightarrow I^*\) does not occur, in contrast to what is observed in wt-GFP. Thus, the reaction \(A^* \rightarrow I\) proceeds radiationlessly, probably through the triplet-state.

### 3.2.3. EYFP

Although the most red-shifted mutant EYFP at \(1.6\) K shows a single strong absorption maximum at \(520\) nm (Fig. 1), it is already present in three conformations. This is revealed by the emission- and excitation spectra of Figs. 5(a) and (b) that show bands that have to be attributed to all three A-, B- and I-forms. When exciting at \(\approx 400\) nm, the region in which the A-form is expected to absorb, we observe very weak emission bands at \(\approx 450, 462\) and \(476\) nm and much stronger ones between \(500\) and \(550\) nm (Fig. 5(a), bottom). The intensity of the emission bands at \(507\) and \(\approx 540\) nm decrease when \(\lambda_{\text{exc}}\) is tuned towards the red, indicating that these bands, by analogy to wt-GFP [24], belong to the I-form that is populated via the excited \(A^*\)-form (see also Fig. 6(c)). For \(\lambda_{\text{exc}}\) on the low-en-
ergy side of A₀₀, the A-form is no longer excited. In addition to some weak emission from I’ originating by direct excitation of I, only strong bands of the B-form at 527 and 547 nm remain (Fig. 5(a)). Thus, the reaction A → I takes place in EYFP in a similar fashion as in wt-GFP. Simultaneously, by exciting A, a very weak emission from A’ → A is observed (see also Fig. 6(c)). Direct excitation of the I-form at ~490 nm yields only a weak I’ → I emission, whereas the reactions I’ → B and I’ → A occur radiationlessly, probably through the triplet-state. Excitation into B only (500 nm < λexc ≤ 524 nm) leads to a B’ → B emission and to a radiationless reaction from B’ → I (see Fig. 6(c)).

The excitation spectra of the A-, I- and B-forms of EYFP selectively detected at 470, 510 and 530 nm, respectively (Fig. 5(b)) show vibronic bands extending from ~400 to ~520 nm. Holes can be burnt into the three forms, with A₀₀ at 446 ± 1 nm, I₀₀ at 496 ± 3 nm and B₀₀ at 524 ± 1 nm. We note that the 0–0 transitions of the A- and B-forms of EYFP are red-shifted by about 10 and almost 50 nm with respect to the 0–0 transitions of wt-GFP [24], S65T and RS-GFP (see above and Fig. 6). This strong red shift is probably related to the T203Y mutation in EYFP, which is responsible for the π–π stacking between the phenol rings of Tyr-203 and the chromophore (Tyr-66) [37]. We assign the most red-shifted form of EYFP to the
B-form because it is photoconverted into I, and not into A.

3.3. Energy-level diagrams

The results obtained for the three mutants are summarized in the energy-level schemes of Fig. 6. These schemes exhibit a general pattern similar to that deduced for wt-GFP [24], but differ in the details. They show the 0–0 transitions of the A-, I- and B-forms and the pathways of photoconversion between them. Reversible phototransformations occur between A and I, and between B and I, but not directly between A and B.

For S65T (Fig. 6(a)), we found that the I-form is the most populated. It is represented by the strongest band absorbing at ~490 nm (Fig. 1). Since the A- and B-forms are also present at 1.6 K, but with considerably less intensity, we assume that their ground-state levels lie a few tens of cm⁻¹ above that of the I-form. This is contrary to what occurs in wt-GFP, where the I-form is not populated at 1.6 K and can only be photoinduced from the A- and/or from B-forms. Excited-state photoconversions in S65T seem to occur neither between A' and I', nor between B' and I'; the barriers between these levels are probably high (a few 1000 cm⁻¹). However, the photoconversions

![Energy-level diagrams for EYFP mutants](image_url)
A → I, I → A, I → B and B → I, still occur radiationlessly (presumably through the triplet-state).

The ground-state levels of the A- and B-forms of RS-GFP at 1.6 K are not populated in a previously non-illuminated sample, but they become populated as a consequence of steady illumination (burning) of the I-form. Thus, they have to be at least 100 cm\(^{-1}\) higher in energy than the ground-state level of the I-form (see Fig. 6(b)). Judging from the fluorescence spectra, however, these levels are populated at room temperature [36]. After B is formed radiationlessly from I → B, it can be excited directly leading to the reaction B → I → I. Since no emission is observed from B → B, we conclude that there is no barrier between B and I. In contrast, by exciting A, the reaction A → I does not take place, but emission from A → A is observed. The barrier between A and I, thus, has to be high (probably a few 1000 cm\(^{-1}\)). The transformations A → I, I → A and I → B in RS-GFP occur radiationlessly.

In EYFP (Fig. 6(c)), the three A-, I- and B-forms are already present at 1.6 K. The ground-state level of the B-form is now the lowest-lying level so that the B-form is the most populated one, in contrast to the situation in S65T and RS-GFP. By excitation into A, only very weak fluorescence is observed from A → A, but strong fluorescence from I', as in wt-GFP [24]. Thus, the process from A → I' involves a very small energy barrier. Since excitation into I' does not lead to emission from B', the barrier between I' and B' has to be high (at least a few 1000 cm\(^{-1}\)). When exciting into B, there is no reaction between B' and I'. We conclude that I and B reversibly photoconvert through radiationless pathways.

3.4. Single GFP-molecules: interpretation of their “on–off” and “blinking” behavior

Based on the energy-level schemes of Fig. 6, we propose a tentative explanation for the “blinking” and “on–off” behaviour observed in single mutant GFP-molecules at room temperature [29,31,32]. The experiments of [29,31] were performed on a mutant of GFP similar to our EYFP, whereas those of [32] were done on S65T. By continuous
illumination with a laser at 488 nm, the individual molecules showed fluorescence (“on”-state) during intervals of hundreds of microseconds to several seconds (“blinking”) until a dark, long-lived (“off”)-state was reached after a few minutes. In the case of EYFP, the “on”-state could be recovered from the “off”-state by irradiation with a lamp at 405 nm [29]. For both EYFP and S65T it was reported that the “on”-periods were dependent on laser excitation intensity, becoming shorter at higher intensities, whereas the “off”-periods were independent of light intensity at 488 nm.

Let us assume that the energy-level scheme for the EYFP mutant of [29,31] is similar to that of our EYFP (Fig. 6(c)). Illumination at 488 nm would then excite both the I- and the B-form. If a single molecule, for example in the B-form, is excited, it will fluoresce (“on”) intermittently (“blinking”) from the B-C3-state. “Blinking” in GFPs is most probably due to intersystem-crossing cycles to the triplet-state, where the molecule remains microseconds to milliseconds before it returns to the ground-state and is excited again [38,39]. Eventually, the molecule will be photoconverted into the I-form. While in the I-form, the molecule can either be excited into I’ at 488 nm and emit light (from an “on”-state with “blinking”) or be photoconverted into the A- or B-form. If the molecule returns to B, it will be excited again at 488 nm and emit light (from an “on”-state with “blinking”). If it proceeds to A, the molecule will not be excited at 488 nm because the energy of the 0–0 transition of A (446 nm) exceeds that of the laser. The A-form thus becomes a dark (“off”) -state until the molecule goes either thermally over the ground-state barrier to the I-state (see Fig. 6(c)) or is illuminated at a wavelength \( \lambda < 446 \) nm (the 0–0 transition of A), for example at 405 nm as in [29]. If the thermal reaction occurs, the length of the “off”-period should be independent of light intensity as indeed observed, but should depend on temperature (still to be demonstrated). However, when the A-form is excited at 405 nm into A’, it will be photoconverted into I’, from where emission (from an “on”-state with “blinking”) occurs. Once back in the I-form, the molecule will be excited again into I’ at 488 nm and the “blinking” and “on–off” cycle resumes.

We interpret the behavior of single S65T-molecules [32] in a similar way as for EYFP. For example, if a molecule is in the I-form, it will be excited into I’ at 488 nm and will emit light intermittently from I’ (“on”-state and “blinking” due to intersystem-crossing cycles to the triplet-state) until it is photoconverted into A or B (see Fig. 6(a)). While in the A- or B-form, the molecule will not be excited at 488 nm because the 0–0 transition energies of these forms are larger than the energy of the laser. It will thus remain in the ground-state of the A- or B-form (dark, “off”-states for excitation at 488 nm) until it crosses thermally over the energy barrier back into the I-form. The length of this “off”-period should therefore not depend on light intensity as indeed observed, but should only depend on temperature (still to be verified). Once back in the I-form, the molecule can be excited again into I’ at 488 nm and recommence the “blinking” and “on–off” cycle.

4. Conclusions

We have proven here by means of low temperature laser spectroscopy and hole-burning that the red-shifted GFP-mutants S65T and EYFP, analogously to RS-GFP [28], are not “one color”, photostable proteins, as assumed in the literature. They photoconvert reversibly among at least three (protonated and deprotonated) forms, A, I and B, in a manner similar to that previously demonstrated by us for wt-type GFP.

In addition to identifying the 0–0 transitions of the various forms within a specific mutant, we have determined the pathways of interconversion and interpreted the results in terms of energy-level schemes. Such reversible photoreactions between different forms of a GFP molecule probably represent a more general phenomenon also occurring in other GFP-mutants, as well as in similar types of autofluorescent proteins such as the red fluorescent protein DsRed [40].

As mentioned earlier [24,28], our results have important consequences for applications of GFP-mutants in molecular and cell biology, such as the study of protein–protein interactions by fluorescence resonance energy transfer (FRET). In this
technique a pair of differently absorbing GFP-mutants, one as donor and the other as acceptor, are used. A change in color of the fluorescence is generally interpreted as a sign for energy transfer, and thus for an interaction between different proteins. We have demonstrated, however, that a change in color may also originate from a photo-induced reaction among conformations within a given GFP-mutant.

The localization of the 0–0 transitions by hole-burning will allow us now to determine their homogeneous linewidths and get information on dynamical processes, like excited-state proton transfer reactions and chromophore–protein interactions at low temperature. The energy-level diagrams, furthermore, provide detailed explanations of the “on–off” and “blinking” behavior of specific single mutant GFP-molecules at room temperature.

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