PHYSICAL REVIEW E **70**, 030901(R) (2004)

Multiphoton switching dynamics of single green fluorescent proteins

G. Chirico,[1] F. Cannone,[1] A. Diaspro,[2] S. Bologna,[3] V. Pellegrini,[4] R. Nifosì,[4] and F. Beltram[4]
[1]*INFM and Department of Physics, University of Milano Bicocca, Piazza della Scienza 3, 20126 Milano, Italy*
[2]*INFM and Department of Physics, University of Genoa, Via Dodecaneso 33, 16146 Genova, Italy*
[3]*Department of Biochemistry and Molecular Biology, University of Parma, 43100 Parma, Italy*
[4]*NEST-INFM and Scuola Normale Superiore, Piazza dei Cavalieri 7, I- 56126 Pisa, Italy*
(Received 19 February 2004; published 21 September 2004)

Multi-photon driven photo-switching between dark and bright (fluorescent) states of a green fluorescent protein (GFP) mutant is demonstrated. A single-protein investigation shows the existence of two distinct bright states that display sharp two-photon cross-section bands peaked at 780 nm and at 870 nm. Fluorescence of these two species can be independently switched on and off. These results highlight a new photoconversion pathway for photochromic GFPs and can have significant applications in multi-photon confocal microscopy and in optical data-storage architectures.

DOI: 10.1103/PhysRevE.70.030901 PACS number(s): 82.37.Vb, 87.14.Ee, 87.64.Vv

Intrinsically fluorescent proteins triggered a revolution in molecular biology and allowed *in-vivo* monitoring of gene expression and protein function. Their discovery and diffusion also lead to a large effort aimed at understanding and exploiting their photophysical and photochemical properties down to the single-protein level. Among these optically-active biomolecules, the green fluorescent protein (GFP) of the Aequorea Victoria jellyfish has emerged in recent years as a unique fluorescent label with a vast impact on biological studies [1,2].

GFP is a fluorescent protein (238 amino acids) with a cylinder-shaped nanometric three-dimensional structure and the chromophore located in its center [3]. The chromophore consists of two consecutive rings, the phenol-type ring of amino-acid Tyr66 and a five-membered heterocycle (imidazolidinone) formed by Tyr66, Ser65, and Gly67. In the folded protein it is positioned close to several polar and aromatic residues. These and some water molecules establish a peculiar hydrogen-bond network around the chromophore that largely influences GFP photophysical properties. Single and multiple mutations involving the chromophore amino-acids and its local environment induce marked variations in GFP properties. This complexity still makes theoretical modeling a challenge and is stimulating significant experimental and simulation efforts [4,5].

Wild-type GFP exhibits two absorption bands with maxima at 395 and 480 nm. These are associated to a neutral (labeled state A) and anionic (labeled state B) forms of the chromophore, respectively [6,7]. In wild-type GFP and several mutants, efficient excited-state proton-transfer processes occur. These processes lead to population of an intermediate form (labeled I) in which an amino-acid in the chromophore environment (Glu222) is protonated [8,9]. Transitions from I to B are believed to be rare and associated to an additional displacement of amino-acid threonine at position 203 of the protein sequence [6,10]. These photoconversion pathways impact the fluorescence properties. In particular, following excitation of state A, both weak emission at around 440 nm and at 510 nm are observed. The former is associated to direct optical A-state recombination, the latter to optical recombination from state B or I. GFP fluorescence dynamics is also characterized by transitions between bright and dark (nonfluorescent) states which, at the single-protein level, lead to reversible fast turning on and off (*blinking* or *flickering*) and ultimately to switching off (*photobleaching*) of the emission, the latter process occurring typically after few seconds under intense excitation [11]. The molecular nature of these dark states and of the photoconversion pathways are open issues of great interest. This is particularly true for what concerns the *dark* state associated to photobleaching since this process sets a limit for the use of these biomolecules as fluorescent tags in optical imaging.

In recent years, some yellow-shifted GFP mutants were shown to recover their fluorescence properties after photobleaching upon blue-UV irradiation [12–15]. In one of these mutants, E^2GFP (with mutations F64L, S65T and T203Y), some of us showed that it is possible to recover fluorescence emission in single proteins by relatively brief laser irradiation at 350 nm [13]. It was also shown that following the excitation of A or B bands E^2GFP solutions displayed a characteristic absorption band at 365 nm [16]. While the molecular mechanisms of these reversible photoconversion processes still await a full clarification, *ab-initio* and molecular dynamics simulations were used to associate this behavior to a dark-state neutral configuration [16,17].

In this Letter we demonstrate controlled photo-switching of single E^2GFP proteins under two-photon excitation and show an unexpectedly complex photoconversion pattern. The two-photon cross-section bands associated with A and B states are reported here for the first time and display a very narrow linewidth. This property allows us to *selectively* address and photoswitch proteins in state A *or* B.

Conversions between states A and B are shown to be rare. Surprisingly *distinct* recoverable dark states are found associated to optically-active A and B states. Single-protein experiments and the spectroscopic UV-shifted characteristics of the recovery spectra suggest that these dark states share the same protonation and have chromophore environments resembling those of the bright A and B states to which they are connected. After photobleaching, fluorescence can be recovered with high efficiency. The excitation spectrum leading to fluorescence reactivation is remarkably sharp and peaked at

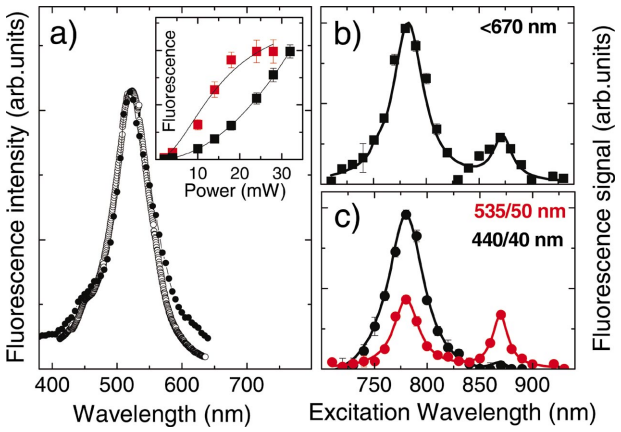

FIG. 1. (Color) (a) Fluorescence emission spectra of E^2GFP in solutions (protein concentration 1 μM, *p*H=7) upon single-photon excitation at 400 nm (filled circles) and two-photon excitation at 780 nm (open circles). The inset reports the emission for the A state (red squares; excitation at 780 nm, emission filter 440/40), and for the state B (black squares; excitation at 870 nm, emission filter 535/50) versus incident power. Solid lines are best fits as described in the text. (b) Two-photon excitation spectra in solution. Fluorescence is observed through a short-pass IR filter below 670 nm. (c) Two-photon excitation spectra detected through a 440/40 (black) and 535/50 emission (red) filters. Solid lines represents the fit to a sum of Gaussians.

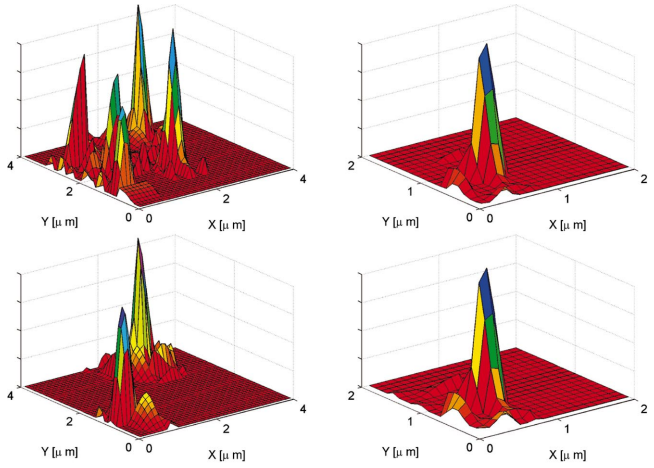

FIG. 2. (Color) Upper panels: (left) fluorescent image of single proteins excited in the A band (excitation at 780 nm emission at 440 nm); (right) the same field of view but on the B channel (excitation at 880 nm emission at 535 nm). Lower panels: (left) fluorescent image in the A channel after irradiation at 710 nm (2 mW and 50 ms); (right) the same field of view but on the B channel and after irradiation at 720 nm (2 mW and 50 ms). Color levels of the left panels are five times those in the right panels

720 nm. These results suggest that A and B forms of E^2GFPs behave as two distinct species that can be independently photoswitched by near-infrared irradiation.

The experiments reported here exploit an inverted optical microscope equipped with a pulsed Ti-Sapphire laser yielding on-sample pulsewidth of 280 fs [18] and 80 MHz repetition rate. Bulk spectroscopic studies were performed in phosphate buffered saline solutions, while single-protein experiments were carried out with E^2GFP proteins trapped in silica gels. Wet silica gels allow the observation of immobile single proteins with no detectable influence on their folding. 10 μm$\times$10 μm images were acquired [19]: single-protein spots could be identified on the basis of their temporal dynamics and spectroscopic characteristics [20]. All experiments were performed at room temperature.

Figure 1(a) shows E^2GFP emission spectra in solution at *p*H=7 after one-photon (filled circles) and two-photon (open circles) excitation at 400 nm and 780 nm, respectively. Both spectra show two main (and composite) emission bands, at around 440 nm and 530 nm, the former associated to A-state optical recombination. The presence of mutation T203Y in the amino-acid sequence of E^2GFP, leads to a red-shift in the main emission band (from 510 to 530 nm) compared to wt-GFP and EGFP [3].

Two-photon excitation (TPE) spectra were recorded with the insertion of three different filters in order to select (i) integrated emission (short-pass filter cutting wavelengths above 670 nm, Fig. 1(b)), (ii) emission from state A [filter centered at 440±40 nm, Fig. 1(c), black curve] or (iii) emission from state B [filter at 535±50 nm, Fig. 1(c), red curve]. Two well-separated and rather sharp bands can be identified centered at 780 nm and 870 nm whose relative amplitudes depend on the emission window selected. These values together with the spectral evolution as a function of *p*H (data not shown) support their assignment to A and B states, respectively. TPE and one-photon absorption spectra present marked qualitative differences. With respect to what might be expected by simply doubling the wavelength in one-photon experiments, TPE A and B spectra are blue shifted, much sharper and show a different relative intensity of the two bands. A similar behavior was previously reported for other mutants [21,22]. The presence of the 780 nm peak in the 535/50 nm emission spectrum in Fig. 1(c) and the absence of the 870 nm peak in the 440/40 nm spectrum could be due to different conversion efficiencies between the neutral and the anionic excited states [23]. The inset of Fig. 1(a) reports the fluorescence signal versus excitation power P at emission wavelength 440 nm (black circles, excitation at 780 nm) and 535 nm (red circles, excitation at 870 nm). As expected, in both cases the behavior is proportional to $P^2/(1+(P/P_{sat})^2)$, with $P^A_{sat}=15\pm2$ mW and $P^B_{sat}\gtrsim50$ mW for states A and B, respectively. The saturation excitation power of B, estimated by assuming a cross-section of 40×10^{-58} m^4 s (similar to that of EGFP at 870 nm) and using the measured E^2GFP fluorescence lifetime of 3.3 ns (data not shown) is 45 mW, in reasonable agreement with our experimental observation. State A is characterized by a two-photon cross section ≈30 times greater [24] and a lifetime ≈1 ns (data not shown). Our estimate of a saturation excitation power of 17 mW is in agreement with the data.

We now turn to single-protein experiments. We performed fluorescence kinetics studies by recording 400 images in a total acquisition time of 100s. The plot of the fluorescence emission versus time on each spot was obtained as described in Ref. [25]. The time interval between two consecutive excitations of each protein was 250 ms and the illumination time per single-protein spot was 1 ms. Blinking events at 870

nm and 780 nm were observed for times shorter than the bleaching time (T_B). A detailed analysis will be reported elsewhere.

The single-protein emission spectrum obtained by averaging over about a hundred proteins with the insertion of the short-pass (670 nm) filter is very similar to that obtained with E^2GFPs in solution [Fig. 1(b)]. Remarkably, however, when the same field of view is excited in the A (excitation at 780 nm, emission at 440 nm) or B bands (excitation at 870 nm, emission at 535 nm), *different* individual proteins are detected. An example is given in Fig. 2, upper panels. We never observed a single-protein spot in both A and B channels over a sample of 60 proteins. These results indicate that conversion between A and B (both in ground and excited states) is strongly inhibited in E^2GFP [26]. This separation between A and B states is somewhat unexpected. In fact the mutated tyrosine at 203, unlike threonine in wild type GFP, cannot perform a stabilizing function by existing in two different conformations each peculiar of one of the two states A and B. It must be noted, however, that following A excitation significant emission is detected also at 535 nm [see the red curve in Fig. 1(c)]. These data suggest an efficient photoconversion channel between A and I states similarly to what observed in wild-type GFP [6,9]. This indicates that similar photoconversion pathways are shared between wild-type GFP and E^2GFP.

Several tens of images of single E^2GFP proteins could be collected in the IR region 770–890 nm before photobleaching. Once photobleached, however, proteins did not recover spontaneously their fluorescence emission even when left in the dark for several hours. The measured photobleaching rate $1/T_B$, shows a Gaussian distribution, as found with simple dyes [25]. The average value of T_B depends on the excitation power (P) as P^{α} where $\alpha = 2.5 \pm 0.5$ for both states [see the inset of Fig. 3(c)] confirming that a two-photon process is involved in the absorption [27].

Proteins photobleached in either of the two states recover their emission after laser irradiation at around 720 nm. This property was verified on a sample of 100 single proteins. A representative example is shown in Fig. 2 for proteins emitting in state A (left upper panel) and then photoactivated by laser irradiation at 710 nm after photobleaching (left lower panel) or in state B and photoactivated at 720 nm (right panels). The recovered fraction depends on laser wavelength, irradiation time and energy released (the product of power and irradiation time): data are displayed in Fig. 3. At 720 nm, in particular, 100% recovery efficiency could be obtained with 16.5 ms-long excitation at a power of 5.7 mW (i.e., an energy release of 94 μJ). The recovery efficiency dropped to 50% for an energy release of approximately 65 μJ: its behavior can be very well described by a fourth power dependence as indicated by the solid line in Fig. 3(b). Remarkably single proteins photobleached in either of the two bands recover their emission in the *same* band with similar recovery spectra (data not shown). This was verified on a sample of 50 proteins in state A and B (for a total of 100 observed proteins) at different excitation power and irradiation times. We tested a second S65T mutant, gfp-mut2 [17,27] observing (data not shown) no recovery over a wide range of irradiation wavelengths (700–900 nm).

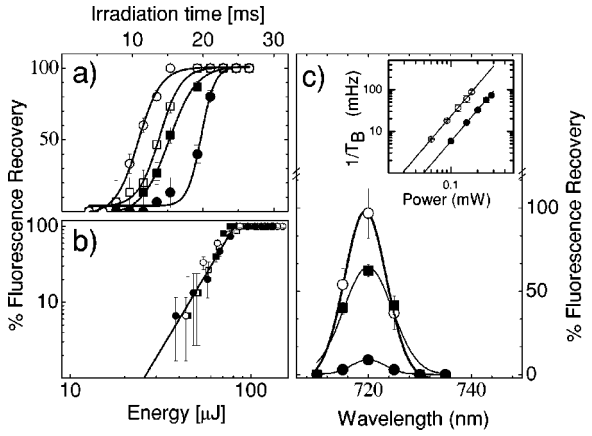

FIG. 3. (a) Percentage of single-protein fluorescence recovery versus the illumination time at 720 nm for different excitation powers: 3.34 mW (solid circles), 4.34 mW (solid squares), 5 mW (open squares) and 5.7 mW (open circles). The solid lines are best fit to a sigmoidal function. (b) Recovery efficiency with laser excitation at 720 nm versus the product of the illumination time with excitation power: 3.34 mW (solid circles), 4.34 mW (solid squares), 5 mW (open squares) and 5.7 mW (open circles). The solid line is the best-fit power law with exponent 3.8 ± 0.2. (c) Recovery efficiency at fixed illumination time of 16.5 ms versus the excitation wavelength at 5.7 mW (open circles), 4.34 mW (filled squares) and 3.34 mW (filled circles). The error bars are the statistical errors due to the number of events ν observed ($100 \cdot \nu^{-0.5}$). The inset shows the photobleaching rate $1/T_B$ versus excitation power for the two states (A, open squares; B, filled squares).

The independent photoswitching of A and B forms of E^2GFP is the central result of this work. This unexpected observation was made possible by the narrow TPE spectra and is consistent with the limited conversion observed between the A and B forms. The similar characteristics of the two recovery spectra with the marked blue shift of the absorption with respect to the optically active form suggest that photoswitching is accompanied by protonation of the chromophore. The configuration of the chromophore and of the surrounding residues in the dark state favors nonradiative decay pathways from the excited state, at the expense of the radiative decay [28]. Such pathways involve twisting around the bonds connecting the two rings of the chromophore and eventual crossing of a conical intersection. The dynamics through a conical intersection can lead either back to the original ground-state configuration or, arguably with smaller probability, to a different isomer, like in the *cis*/*trans* or *trans*/*cis* photoisomerization. This should be the mechanism for on/off switching in the A state [16], and could then be the molecular basis for the photochromicity of both A and B [29]. The full separation of the two photoconversion processes are naturally ascribed to the chromophore environment. The latter would keep memory of the initial state in the photoswitching process. Different microscopic configurations can be linked to this behavior: recent molecular dynamics simulations, for instance, suggest the existence of *two* minimum-energy chromophore + environment configurations, characterized by different orientations of Thr65 side chain [30]. These calculations also indicate the presence of a large

energy barrier separating these two states. While one configuration was observed in the x-ray structure of the B state in the GFP mutant S65T [6], the anionic form of Glu222 in the A state is expected to favor the other configuration. The relatively high energy barrier between these states may cause these two configurations to remain unaffected by photoswitching leading to the observed memory effect. Alternatively, this effect could be associated with the recently observed photo-decarboxylation of Glu222 [31]. The photoreaction leaves Glu222 with a methyl group in place of the carboxyl, thus preventing the residue from acting as a proton donor. In this view, the single E^2GFP proteins observed in the B-state channel are those with decarboxylated—hence neutral—Glu222, whereas those observed in the A-state channel contain intact Glu222. Indeed the neutral chromophore in the A state requires anionic Glu222. The precise microscopic identification of the processes discovered in this work, however, requires further theoretical work.

Recovery spectra are very narrow and their widths do not change appreciably with the excitation power [Fig. 3(c)]. Maximum recovery efficiency occurs at 720 nm. This corresponds to twice the peak wavelength of the spectrum of the dark state obtained in single-photon experiments [16]. The fourth-power dependence of the recovery shown in Fig. 3(b) indicates that two two-photon transitions are required to overcome the energy barrier from a dark state to the optically-active form to which it is connected. The second transition is likely to bring the system from the excited dark state (or its relaxed form) to a higher excited level. Detuning between the two transitions may cause the observed sharp recovery spectrum. Vibrational-electronic couplings could also contribute to reduce the width of the recovery spectrum [32,33].

In conclusion, we demonstrated independent and highly non-linear photoswitching processes for the A and B forms of a GFP mutant. Our two-photon data provide new insight into the complex photophysics of GFPs and show promising directions for the exploitation of photochromic mutants and TPE in the development of single-protein-based optical memory arrays.

This research was funded by the FIRB project "Nanobiotechnology," by the COFIN project 2004–2005 to G.C., and by a grant of the Human Frontier Research Program to V.P. We thank Enrico Gratton and Valentina Tozzini for useful discussions and Caterina Arcangeli, Mauro Giacca, and Arianna Sabo for preparation of the E^2GFP mutants.

[1] M. Chalfie *et al.*, Science **263**, 802 (1994).
[2] K. Sullivan and S. Kay, in *Green Fluorescent Proteins* (Academic, San Diego, 1999).
[3] M. Ormö *et al.*, Science **273**, 1392 (1996).
[4] R. Cinelli *et al.*, Phys. Rev. Lett. **86**, 3439 (2001).
[5] M. Marques *et al.*, Phys. Rev. Lett. **90**, 258101 (2003).
[6] K. Brejc *et al.*, Proc. Natl. Acad. Sci. U.S.A. **94**, 2306 (1997).
[7] S. B. Nielsen *et al.*, Phys. Rev. Lett. **87**, 228102 (2001).
[8] M. Chattoraj *et al.*, Proc. Natl. Acad. Sci. U.S.A. **93**, 8362 (1996).
[9] T. Creemers *et al.*, Nat. Struct. Biol. **6**, 557 (1999).
[10] M. Lill *et al.*, Proc. Natl. Acad. Sci. U.S.A. **99**, 2778 (2002).
[11] D. W. Pierce *et al.*, Nature (London) **388**, 338 (1997); M. F. Garcia-Parajo *et al.* Proc. Natl. Acad. Sci. U.S.A. **97**, 151 (2000).
[12] R. Dickson *et al.*, Nature (London) **388**, 355 (1997).
[13] R. Cinelli *et al.*, Appl. Phys. Lett. **79**, 3353 (2001).
[14] W. Moerner, J. Phys. Chem. B **106**, 910 (2002).
[15] G. Patterson and J. Lippincott-Schwartz, Science **297**, 1873 (2002).
[16] R. Nifosì *et al.*, J. Phys. Chem. B **107**, 1679 (2003).
[17] T. Laino *et al.*, Chem. Phys. **298**, 17 (2004).
[18] F. Cannone *et al.*, J. Microsc. **210**, 149 (2003).
[19] A. Diaspro, *Methods in Cellular Imaging*, edited by A. Periasamy (Oxford University Press, New York, 2001).
[20] R. A. G. Cinelli *et al.*, Photochem. Photobiol. **71**, 771 (2000); G. Chirico *et al.* Protein Sci. **11**, 1152 (2002).
[21] G. Blab *et al.*, Chem. Phys. Lett. **350**, 71 (2001).
[22] A. Kummer *et al.*, Chem. Phys. **237**, 183 (1998).
[23] Although the chromophore, lying in a plane, is symmetrical with respect to reflection in the molecular plane, the surrounding protein residues break even this symmetry so that no particular selection rule is expected in the case of GFP. This considerably reduces the effect of the different parity of the ground and excited states in one-photon (odd) and two-photon excitation (even) on the excitation spectra.
[24] This value is calculated from a best-fit Gaussian analysis of single- and two-photon excitation and emission spectra. A detailed analysis will be reported elsewhere.
[25] G. Chirico *et al.*, Biophys. J. **84**, 588 (2003).
[26] Flip events from state A to B and *vice versa* are rare. In a time window of 4 hours only 2 flip events were observed on a total of 30 single molecules. This suggests that a large energy barrier separates the ground states of A/I from B and that excited-state proton transfer is also strongly suppressed.
[27] G. Patterson *et al.*, Biophys. J. **78**, 2159 (2000).
[28] W. Weber *et al.*, Proc. Natl. Acad. Sci. U.S.A. **96**, 6177 (1999).
[29] The origin of the dark state cannot be ascribed to a transition to a triplet state whose lifetime is typically few microseconds, much shorter than the observed dark state lifetime (several hours).
[30] R. Nifosí *et al.*, Proteins **51**, 378 (2003).
[31] J. van Thor *et al.*, Nat. Struct. Biol. **9**, 37 (2002).
[32] G. He *et al.*, J. Phys. Chem. B **106**, 11081 (2002).
[33] P. Macak *et al.*, J. Chem. Phys. **113**, 7055 (2000).