Reversible molecular photoswitches: A key technology for nanoscience and fluorescence imaging

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In recent years, molecular switches have attracted considerable interest because they hold great promises as molecular electronic and photonic devices. In contrast to commonplace switches that turn electric appliances on and off, molecular switches enable the storage of information on a molecular level, and their application in nanotechnology, biomedicine, and computer chip design opens up whole new horizons. Usually, molecular switches are addressed by an electrical field, a scanning tunneling microscope tip, or a chemical or electrochemical reaction to specifically switch the physical properties between two states (1–3). Alternatively, molecules might be switched optically between two stable forms, and quite an effort has been put into the synthesis of reversibly photoswitchable fulgides and diarylethenes (4). A photoswitch exhibits two stable and selectively addressable states, a fluorescent and a nonfluorescent, which can be conveyed into another in a reversible fashion upon irradiation with different wavelengths of light. Although highly reproducible optical switching of individual chromophores could be achieved in liquid helium temperature experiments (5), the synthetic approach based on a photoswitchable diarylethene derivative was crowned with success only recently (6, 7). Very recently (8, 9), it was demonstrated that even conventional unmodified carbocyanine derivatives such as Cy5 can function as efficient reversible single-molecule photoswitches.

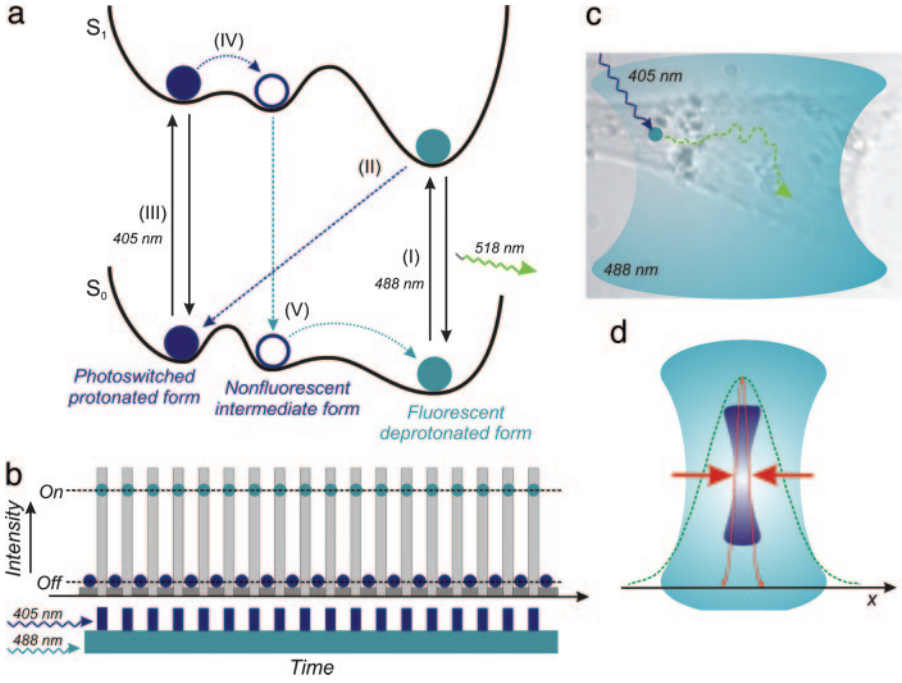

Fig. 1. Molecular photoswitches might be advantageously used for the study of protein trafficking in living cells and ultrahigh-density optical data storage at the single-molecule level. (*a*) Schematic of the operating mode of the reversible and highly reliable GFP-like photoswitch Dronpa. Upon excitation of the deprotonated form at 488 nm, it converts into the ground state of the photoswitched protonated form with a switching yield Φ_{SW} of 3.2×10^{-4} (II). Excitation of the protonated form at 405 nm (III) results in excited state proton transfer to an intermediate state (IV), which converts into the deprotonated ground state with a switching yield Φ_{SW} of 0.37 (V). (*b*) Using simultaneous illumination at 488 nm and applying a 30-ms pulse of 405-nm laser light every second, Habuchi *et al.* (10) achieved up to 170 photoswitching events at the single-molecule level with high reliability. (*c*) Fluorescence of tagged proteins can be erased by applying 488-nm light and switched on at specific locations by using 405-nm irradiation to directly visualize protein movement routes. (*d*) For nanoscale writing, a 405-nm laser beam could be focused to a diffraction-limited focal spot to induce photoswitching to the bright deprotonated form. A second 488-nm laser beam could switch the molecules at the outer parts of the focus back to the dim protonated form by using a phase mask.

In this issue of PNAS, Habuchi *et al.* (10) report about photoswitching of a mutant of a GFP-like fluorescent protein that was cloned from the coral Pectiniidae. They demonstrate reversible photoswitching between dim and bright states of individual molecules embedded in poly(vinyl alcohol) by using 488 and 405 nm laser light with a response time in the millisecond range and a repeatability of >100 times (Fig. 1). So far, only a few successful reversible photoswitching events have been reported for a GFP mutant with a response time of several minutes (11). The intriguing switching performance of the new GFP-like fluorescent protein led to the naming "Dronpa," after "dron," a ninja term for vanishing, and "pa," which stands for photoactivation (12). Because GFP and its fluorescent homologous provide direct genetic encoding of strong visible fluorescence, they have become invaluable tools for *in vivo* imaging of cells and tissue (13, 14). In combination with photobleaching techniques, fluorescent proteins can also provide information on the movement of intracellular proteins. However, photobleaching techniques do not allow direct visualization of protein movement routes within a living cell. Therefore, photoswitchable fluorescent proteins such as Dronpa are strongly desired for improved protein tracking in living cells (14, 15).

The fact that most fluorescent proteins known nowadays exhibit slow response time to light and irreversible switching behavior highlights the importance of the discovery of Dronpa, a genetically encoded monomeric fluorescent protein that can be addressed reversibly for repeated measurements of protein behavior. Similar to GFP derivatives and other GFP-like proteins, Dronpa shows two absorption peaks, a major peak at 503 nm and a minor peak at 388 nm, which can be attributed to the deprotonated and the protonated form of the chromophore, respectively. Although the deprotonated form shows bright fluorescence at 518 nm with a quantum yield Φ_{fl} of 0.85 and a monoexponential fluorescence decay time of 3.6 ns, independent of the detection wavelength deactivation of the excited state of the protonated form is dominated by nonradiative relaxation. Therefore, the

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protonated form shows only weak fluorescence at 450 nm with a quantum yield Φ_{fl} of 0.02. The existence of a protonated and deprotonated form in Dronpa suggests that photoswitching might occur through excited state proton transfer (11, 16).

Single-Molecule Fluorescence Spectroscopy Uncovers the Photoswitching Mechanism

Habuchi *et al.* (10) studied in detail the photophysical characteristics of Dronpa at ensemble and single-molecule levels and approved a direct connection between the protonated and deprotonated forms through photoswitching. They demonstrate that the absorption of the deprotonated form can be completely recovered after a single round of photoswitching. Furthermore, their data suggest that both protonation and deprotonation reactions occur through a one-photon excitation process. Studying the fluorescence characteristics of single Dronpa molecules embedded in poly(vinyl alcohol) under various excitation conditions, Habuchi *et al.* could shed light on the complicated photophysics behind reversible photoswitching (Fig. 1*a*). They uncovered a photoswitched protonated form that undergoes photo-induced excited state proton transfer to a nonfluorescent intermediate with a lifetime of a few picoseconds before it finally converts into the ground state of the deprotonated bright form (17). The achievements reported by Habuchi *et al.* in this issue of PNAS emphasize the importance of single-molecule fluorescence spectroscopy for an improved understanding of molecular photoswitches. A refined understanding of photoswitching mechanisms, including the identification of all states and intermediates involved, is key for the development of new improved molecular photoswitches.

The fast and reliable reversible photoswitching of Dronpa at the single-molecule level is in contrast with other GFP mutants (11) and diarylethene derivatives (6, 7). This achievement could be explained by the fact that switching requires only minimal structural changes of the chromophore (protonation/deprotonation instead of ring closure reactions). On the other hand, photoswitching of individual carbocyanine dyes (Cy5, Alexa 647) in aqueous solution revealed a repeatability and reliability similar to Dronpa (9). Therefore, carbocyanine dyes might be considered as alternative competitors for *in vivo* applications, yet efficient photoswitching required oxygen removal and the addition of a triplet quencher that certainly limits its application. Because the chromophore is buried in the protein β-barrel of Dronpa, environmental influences are unexpected, and it can be anticipated that Dronpa exhibits similar photoswitching properties even under physiological conditions. Thus, the Dronpa photochromism promises important biological applications such as the direct observation of individual key signaling molecules shuttling between the cytoplasm and nucleus of a living cell (Fig. 1*c*).

The Dronpa Photoswitch Possesses All Fundamental Requirements to Be Potentially Useful for Ultrahigh-Density Optical Data Storage

Furthermore, photoswitches such as Dronpa might be potentially useful for ultrahigh-density optical data storage and far-field optical writing at the nanoscale (18). Because Dronpa reversibly undergoes light-induced transitions between two thermally stable states (thermal recovery occurs in the order of days at room temperature) (10), and the transitions are optically saturable, a spatial intensity distribution of two laser wavelengths (405 and 488 nm) featuring a local minimum might allow writing and readout of structures at the nanoscale (Fig. 1*d*). Conceptually analog to stimulated emission depletion microscopy where quenching of the fluorescent state is realized by saturation through stimulated emission a spatial resolution of <30 nm could be within reach (19, 20). To achieve nanoscale writing, Dronpa molecules could be immobilized on a surface and switched quantitatively into the protonated dim form upon intense irradiation at 488 nm. Then, a 405-nm laser beam could be focused to a diffraction-limited focal spot to induce photoswitching to the bright deprotonated form. Simultaneously, a second 488-nm laser beam saturates the transition to the dim protonated form by using, e.g., a phase mask that redistributes the energy of the 488-nm beam, yielding a null intensity (node) at the exact focus but preserving the wings of the beam. Therefore, the deprotonated form could be written only in a spatially confined region.

One potential drawback of such photoswitches constitutes the fact that readout (probing the state of the switch) is affected by photoswitching. Thus, far-field nanoscale fluorescence imaging is not yet accessible. However, readout of the nanoscale structure without diffraction limit could be realized when an additional transition from the deprotonated form into a permanent fluorescent form could be initiated (18). Alternatively, near-field techniques such as near-field scanning optical microscopy could be used to attain subdiffraction spatial resolution for both writing and readout (21). In addition, even though switching and probing can be realized applying different excitation intensities, a third independent wavelength would certainly be more useful for probing the state of the photoswitch (22). Another practically limiting factor may be the number of reversible transitions that a GFP-like fluorescent protein can undergo before removed from the process by photobleaching. Semiconductor quantum dots with unique optical properties such as brightness, photostability, and spectral tunability (23), for example, are ideal candidates onto which to base the development of future photoswitches. Although there is ample scope for alternative improvements, the run for the perfect molecular photoswitch has just begun.

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