Controlling the fluorescence of ordinary oxazine dyes for single-molecule switching and superresolution microscopy

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Fluorescent molecular switches have widespread potential for use as sensors, material applications in electro-optical data storages and displays, and superresolution fluorescence microscopy. We demonstrate that adjustment of fluorophore properties and environmental conditions allows the use of ordinary fluorescent dyes as efficient single-molecule switches that report sensitively on their local redox condition. Adding or removing reductant or oxidant, switches the fluorescence of oxazine dyes between stable fluorescent and non-fluorescent states. At low oxygen concentrations, the off-state that we ascribe to a radical anion is thermally stable with a lifetime in the minutes range. The molecular switches show a remarkable reliability with intriguing fatigue resistance at the single-molecule level: Depending on the switching rate, between 400 and 3,000 switching cycles are observed before irreversible photodestruction occurs. A detailed picture of the underlying photoinduced and redox reactions is elaborated. In the presence of both reductant and oxidant, continuous switching is manifested by “blinking” with independently controllable on- and off-state lifetimes in both deoxygenated and oxygenated environments. This “continuous switching mode” is advantageously used for imaging actin filament and actin filament bundles in fixed cells with subdiffraction-limited resolution.

electron transfer | molecular switch | sensor | single-molecule spectroscopy

Molecular switches are single-molecule devices, and hence they are key building-blocks for future, bottom-up nanotechnological devices in computers, data storages, (bio-) sensors, and displays. Furthermore, they are exciting molecules for triggered drug-release or for superresolution imaging (1–3). Molecular switches possess at least 2 stable states and can be converted between these states by external stimuli. These external stimuli can essentially be any kind of physical or chemical trigger, such as light, electricity, or certain chemical reactions. Fluorescence is a preferred transduction mechanism because of its ease of noninvasive detection with ultimate sensitivity (i.e., single-molecule sensitivity). For future devices and for superresolution microscopy, it is also important that the molecular switches can be operated at the level of single molecules. This requirement imposes further demands with respect to reliability, reproducibility, response time, and fatigue resistance. Although the single-molecule approach provides detailed information about the properties and mechanisms, only a few examples of fluorescent switches have been demonstrated (4, 5). Among the fluorescent switches, there are systems that use 2 different wavelengths for switching (photoswitchable molecules) (6–13), those that switch the efficiency of photoinduced electron transfer (14, 15) and those that respond to pH changes (15, 16).

Here, we show that based on a detailed understanding of their photophysics, ordinary, fluorescent dyes can act as single-molecule switches and sensors. By adapting the energy levels of the fluorescent dye to the redox conditions of the environment, we achieve exquisite control of the fluorescence properties of the dyes and the ability to switch the dyes on and off. This is achieved by using generic dark states, i.e., radical anion states, as reversible off-states that are stable in the absence of electron acceptors.

Commonly, unwanted and uncontrolled switching between bright “on” and dark “off” states is observed in single-molecule fluorescence transients and is referred to as “blinking.” Besides photobleaching, dark states represent a main concern in many biomolecular single-molecule studies. Dark states often originate from stochastic transitions to the triplet state (17), or from radical ion states formed as a result of electron transfer reactions (18–21). Recently, an improved understanding of the underlying (redox) processes has led to ways to suppress photobleaching as well as triplet- and redox-blinking (22, 23). To gain control over blinking, we used oxazine dyes with low energy of the first reduced state and high energy of the first oxidized state, which stabilizes the reduced state and minimizes the influence of molecular oxygen that we cannot completely exclude in the experiments. We presumed that a reduced state could then be thermally stable. Switching is achieved for single immobilized DNA–oxazine constructs (oxazines: ATTO655, ATTO680, or ATTO700) in aqueous buffers through changing the redox environment from reducing for switching off to oxidizing for turning back on. We used single-molecule fluorescence spectroscopy (SMFS) and fluorescence correlation spectroscopy (FCS) to elucidate the photophysical pathways of these oxazine dyes and obtained a comprehensive model through correlation with thermodynamic data. Besides an intriguing switching performance, the dependence of the switching kinetics is used as a single-molecule redox-sensor (SMRS) for oxidants and reductants.

We demonstrate the potential of the molecular switch for far-field superresolution microscopy that is based on the subsequent localization of single molecules. Recently, the importance of dark states for far-field superresolution fluorescence microscopy using for example switchable molecules has been realized (2, 24, 25). The oxazine molecular switch offers some advantageous because the number of photons emitted during 1 on-time can be controlled, and the fluorescence control can be achieved in the presence of oxygen with impressive photostability. In this context, superresolution images of actin filaments immobilized on glass slides and actin bundles in fixed cells are presented.

Results and Discussion

Basics of Controlling Single-Molecule Fluorescence. To switch ordinary dye molecules we intended to use generic radical ion states as controllable off-states. Therefore, single oxazine dyes were attached to biotinylated double-stranded DNA that itself was immobilized on BSA/BSA-biotin-streptavidin coated cover slides according to published procedures (26). Measurements were carried out in aqueous buffers, which allowed enzymatic oxygen removal and

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addition of ascorbic acid (AA) as reductant and methylviologen (MV) as oxidant.

Although it is comparatively simple to prepare a fluorophore in its radical ion state through photoinduced electron transfer with a reductant or oxidant in solution (23, 25), it is challenging to stabilize the radical anion and prevent thermal return to the ground state. This requires careful removal of all electron acceptors from solution. By using enzymatic oxygen scavenging, the potential oxidant oxygen is removed to only low micromolar concentrations (27). We therefore selected oxazine derivatives with high reduction potential, i.e., the reduced state has very low energy so that reoxidation by oxygen with $E_{\text{red}} = -0.57$ V becomes inefficient (28). To describe the photophysical properties of single fluorophores taking into account the interaction with reducing and oxidizing agents, we used a 5-level scheme adapted to the thermodynamic parameters of the oxazine derivative ATTO655 (Fig. 1A). The energy levels were obtained by cyclic voltammetry as well as from steady-state absorption and fluorescence spectra [see [supporting information \(SI\) Text](#) for details]: The zero-zero energy $E_{0,0} = 1.86$ eV, the first 1-electron reduction potential $E_{\text{red}} = -0.42$ V (vs. SCE), and the first 1-electron oxidation potential $E_{\text{ox}} = 1.31$ V (vs. SCE). The exact energy of the triplet state is not known, and it is assumed to be ≈ 0.3 eV lower than the singlet state (i.e., at ≈ 1.56 eV).

After placing a molecule in the laser focus, adding AA (100 μM) as reductant and removing oxygen, single-molecule fluorescence transients such as in Fig. 1B are obtained. A short fluorescent spike is observed terminated by rapid apparent photobleaching. In the presence of AA and MV (both 100 μM), however, single ATTO655 molecules show pronounced blinking with typical on- and off-times in the millisecond range (Fig. 1C). The fact that the fluorescence of ATTO655 resumes in the presence of the oxidant supports the idea that the fluorophore in Fig. 1B did not photobleach but instead entered a dark and—under these conditions stable—reduced state. The appearance of blinking for the investigated oxazines is attributed to inefficient recovery from the reduced state by MV although the presence of both reductant and oxidant have successfully removed blinking for some other fluorophores (23). Accordingly, the oxidant MV, which exhibits a reduction potential $E_{\text{red}} = -0.69$ V, is not able to efficiently depopulate the reduced state because of a calculated free energy for reoxidation of $\Delta G = 0.27$ eV. By using oxygen rather than MV, the off-times are considerably longer with $\tau_{\text{off}} = 1,800 \pm 1,000$ ms.

Switching Single Oxazine Molecules. To test the hypothesis that ATTO655 in the presence of only AA is not photobleached but indeed switched off to a stable reduced state, we scanned surfaces of immobilized dsDNA doubly labeled with Cy3B and ATTO655 using alternating laser excitation (Fig. 2) (29). Half of the time per pixel, the sample is excited with 533-nm light for excitation of the green dye Cy3B, and half of the time the red dye ATTO655 is excited by 640 nm. The first scan (Fig. 2A) was carried out in PBS containing ambient concentrations of oxygen, in which ATTO655 shows stable emission (see [Fig. S1](#)), to locate the doubly labeled DNAs. Molecules containing only Cy3B (Fig. 2, green), only ATTO655 (red), or both green and red dyes (yellow) were observed.

Before taking the second scan of the same area, oxygen was removed, and 100 μM AA was added. Under these conditions, the scanned area shows only green spots because ATTO655 rapidly enters a long-lived dark state (Fig. 2B). The green spots exhibit some dark pixels because of the transient formation of radical anions (23). Because the sample can slightly move upon buffer exchange, the green dye merely served to identify the same molecule positions as in the previous scan. After all red dyes are switched off, the buffer is exchanged again by washing the surface 3 times with PBS, which contains oxygen. In the subsequent scan (Fig. 2C), all ATTO655 molecules reappear. This procedure can be repeated several times and 3 such cycles with perfect switching of ATTO655 (see circled molecules) are shown in Fig. 2A–G.

Fig. 1. Photoinduced processes of the fluorophore ATTO655. (A) Scheme. After excitation to the first excited singlet state S_1 , fluorescence is emitted at rate k_{Fl} (neglecting nonradiative processes). Intersystem crossing competes with fluorescence and leads to the infrequent formation of triplet states T_1 with the rate constant k_{ISC} . The triplet state is depopulated by intersystem crossing with rate constant k_{T} or by electron transfer. This may occur either through oxidation by methylviologen (MV) forming a radical cation F^{++} or through reduction by AA yielding a radical anion F^- . The radical ions could be recovered to singlet ground-state fluorophores by the complementary redox reaction. Direct electron transfer from the singlet manifold (k_{RedS} , k_{OXS}) has to be taken into account at higher redox-agent concentration. (B and C) Fluorescence transients of ATTO655-labeled dsDNA immobilized in aqueous environment after oxygen removal, addition of 100 μM AA (B) and additionally 100 μM MV (C), respectively. The transients are binned in 10 ms. Samples were excited at 640 nm with an average excitation intensity of 1.5 kW/cm^2 .

Alternatively, MV can be used to switch ATTO655 on again, evidencing that it is the oxidation that turns ATTO655 back on. However, the efficient removal of MV from the chamber is more difficult and requires many more washing steps. The described switching procedure was also conducted with other oxazine dyes such as described in [Fig. S2](#).

The data of Fig. 2 indicate that oxazines act as molecular switches where, in photodynamic equilibrium, the fluorescence is switched off under reducing conditions and switched on by using MV or oxygen. Important properties of molecular switches are (i) their bistability (i.e., the stability of the 2 states), (ii) the reliability and response time of switching, and (iii) their fatigue resistance (repeatability). Obviously, one critical issue is the stability of the reduced state. The observation of very long off-times of the reduced

Fig. 2. Confocal fluorescence images ($6 \times 6 \mu\text{m}$) of ATTO655-Cy3B-labeled dsDNA. The colors green and red encode for the overall fluorescence intensity after green and red excitation, respectively. dsDNA bearing the 2 dyes are hence visible as yellow spots. (A) The first image was acquired in PBS. (B, D, F) The second scan shows the same part of the cover slide after the buffer was deaerated and $100 \mu\text{M}$ AA was added. (C, E, G) Rinsing of the surface and refilling the chamber with standard PBS switches the molecules back into their fluorescent form. This cycle of reversible redox-switching of single ATTO655-molecules was repeated several times, 3 cycles are shown in A–G.

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Fig. 3. Dependence of the on- and off-state lifetime τ_{on} and τ_{off} on the concentration of the reductant AA and the oxidant MV in the absence and presence of oxygen. (A and D) AA dependence of switching at constant 100 μM MV concentration. (B and E) MV dependence of switching at constant 100 μM AA concentration. (C and F) The on- and off-times with simultaneous change of MV and AA. The first row shows data measured without oxygen, whereas all data shown in the second row were measured in the presence of oxygen. The error bars indicate the standard deviation from the mean. The data were fitted by using an allometric function ($f(x) = ax^b$) to guide the eye.

obtained for the longer-wavelength oxazines ATTO680 and ATTO700 (see Figs. S2 and S7), emphasizing the general applicability.

After clarifying the independent role of AA and MV and the absence of oxidized states, we focused on the rate of reduction of the excited singlet state k_{RedS} and the rate for reduction of the excited triplet state k_{RedT} as well as the reoxidation rate $k_{\text{OX'}}$, which are the remaining rates in Fig. 1A. Because of the long lifetime of the triplet state compared with the lifetime of the first excited singlet state, it is expected that k_{RedT} is responsible for the formation of radical anions at low reductant concentrations, whereas k_{RedS} becomes important in the millimolar range. The following discussion assumes that the number of photons, emitted during 1 on-time (on-counts N_{on}), is independent of excitation conditions. The on-counts can be described as the ratio of the sum of the rates depopulating the S_1 and T_1 states toward the ground state to the sum of rates that depopulate the S_1 and T_1 states yielding the radical anion:

$$N_{on}([AA]) = \frac{k_{Fl} + k_{Nr} + k_{Is} \cdot \left(\frac{k_T}{k_T + k_{\text{RedT}} \cdot [AA]} \right)}{k_{\text{RedS}} \cdot [AA] + k_{Is} \cdot \left(\frac{k_{\text{RedT}} \cdot [AA]}{k_T + k_{\text{RedT}} \cdot [AA]} \right)} \cdot \eta_{det} \cdot \phi_{fl}. \quad [1]$$

The term for N_{on} is additionally weighted with the detection efficiency η_{det} and the fluorescence quantum yield ϕ_{fl} . With the fluorescence lifetime without additional quenching τ and the relation $\tau^{-1} = k_{Fl} + k_{nr} + k_{Is}$, Eq. 1 can be rewritten as:

$$N_{on}([AA]) = \frac{\tau^{-1} + k_{Is} \cdot \left(\frac{k_T}{k_T + k_{\text{RedT}} \cdot [AA]} - 1 \right)}{k_{\text{RedS}} \cdot [AA] + k_{Is} \cdot \left(\frac{k_{\text{RedT}} \cdot [AA]}{k_T + k_{\text{RedT}} \cdot [AA]} \right)} \cdot \eta_{det} \cdot \phi_{fl}. \quad [2]$$

Fig. 4 shows a plot of the on-counts N_{on} versus the AA concentration obtained from data such as in Fig. 3A.

The data could be well fitted by using Eq. 2 (Fig. 4, black line) with the ATTO655 fluorescence lifetime $\tau = 3.69$ ns, the intersystem crossing rate $k_{Is} = (1.2 \pm 0.2) \times 10^5 \text{ s}^{-1}$ and the triplet rate constant $k_T = (5.5 \pm 2.5) \times 10^3 \text{ s}^{-1}$, yielding rate constants of

$k_{\text{RedS}} = (9.3 \pm 5.2) \times 10^7 \text{ s}^{-1} \text{M}^{-1}$, $k_{\text{RedT}} = (4.6 \pm 2.4) \times 10^7 \text{ s}^{-1} \text{M}^{-1}$ and the product $\eta_{det} \cdot \phi_{fl} = 0.08 \pm 0.03$. The faster rate constant from the singlet compared with the triplet is in accordance with expectations from Marcus theory because the driving force for the reaction is larger from the singlet state (33). Qualitatively, the S-shape of the graph can be understood by the reduction of the triplet state at low AA concentration that saturates at $\approx 500 \mu\text{M}$ AA. At higher AA concentrations, quenching of the singlet state becomes important and bends the graph downward again. The overall small values of the rate constants for the direct reduction of the singlet and triplet states is in good agreement with the fact that the addition of AA does not reduce the fluorescence lifetime up to 100 mM AA. By using reduced states formed from both singlet and triplet states enables an even broader dynamic range of dark state formation than shown in Fig. 3 with the possibility to generate dark states after, for example, 1 emitted photon, which is important for some superresolution microscopy approaches (34).

Finally, we determined the reoxidation rate $k_{\text{OX'}}$ by MV from a fit to the τ_{off} graph in Fig. 3B to $k_{\text{OX'}} = (1.1 \pm 0.1) \times 10^5 \text{ s}^{-1} \text{M}^{-1}$ because $\tau_{off} = (k_{\text{OX'}} \cdot [\text{MV}])^{-1}$. All rates determined are summarized in Table S1.

The presented results demonstrate an exquisite control of the oxazine fluorescence that even allows switching for a well-adapted

Fig. 4. Plot of the detected photons during an on-cycle, on-counts N_{on} , obtained from the fluorescence transients, versus the AA concentration. The error bars indicate the standard deviation from the mean. A fit using Eq. 2 is shown as black solid line.

The adjustability and the possibility to work in oxygenated environments are important advantages of the switchable oxazine fluorophores for superresolution microscopy. Additionally, these fluorophores are much more photostable in the presence of oxygen than, for example, rhodamine or cyanine derivatives, which is also likely related to their redox properties: Because of their high oxidation potential they are not efficiently oxidized by molecular oxygen, which is considered to be a key step in photobleaching pathways (41). Recalling that living cells themselves provide redox

active agents such as the reductants glutathione and ascorbic acid as well as oxidants such as quinone derivatives and oxygen, super-resolution under in vivo conditions may become possible on the basis of the switchable molecules presented.

Conclusions

Recent advances in understanding fundamental processes governing the fluorescence of individual molecules are key to the success of many approaches in fluorescence sensors and super-resolution microscopy. In this work, we demonstrate how fine adjustment of fluorophores and environmental conditions can be used to achieve exquisite control of the properties of single fluorophores and even to switch ordinary fluorophores on and off. Switching of single-oxazine dyes such as ATTO655, ATTO680, and ATTO700 was carried out with good stability of the dark state, fast response, unique reliability, and high photostability enabling ≈ 400 – $3,000$ switching cycles with $>260,000$ detected photons. The generic nature of the dark state, i.e., the anionic state, indicates the universal character of the switching mechanism and suggests that for the development of switchable molecules, the interaction with the environment might be as important as the design of the molecular switch itself. By using oxidizing and reducing agents simultaneously, continuous switching was demonstrated and used for the elaboration of a detailed kinetic scheme. Importantly, the oxidant and the reductant represent 2 independent variables to control off- and on-times, respectively. This control of fluorescence was even carried out in the presence of oxygen and further used for superresolution blink microscopy of actin filaments immobilized on glass slides and actin bundles in fixed cells. Knowing the rules, it will only be a matter of time until fluorophores exhibiting adjusted redox blinking under the specific redox conditions of

living cells will be demonstrated, further extending the toolbox of superresolution microscopies. The single-molecule-sensing capability can be used for localized redox sensing and has also been exploited to resolve the antiblinking mechanism of the vitamin E analogue Trolox as shown in refs. 22 and 42.

Materials and Methods

Details concerning fluorescent probes, e.g., sequences of DNA oligonucleotides and sample preparation are provided in *SI Text*.

Fluorescence transients of single dye conjugates and images were recorded by using a confocal scanning microscope equipped with 2 spectrally separated detectors. Excitation was carried out by a supercontinuum laser (SuperK Extreme; Koheras) in combination with acousto-optical tunable filters (AOT-Fnc-VIS; AA Optoelectronic). The spectral positions at 533 nm (spectral width of ≈ 2 nm, excitation of Cy3B) and 640 nm (spectral width of ≈ 2 nm, excitation of ATTO655) were chosen as excitation wavelengths. Average light intensities for acquisition of confocal fluorescence images were 1.5 kW/cm^2 at 533 nm and 4 kW/cm^2 at 640 nm. The laser beam was coupled into a single-mode fiber and subsequently into an oil-immersion objective (60 $\times$, N.A. 1.35, UPLSAPO 60XO; Olympus). The fluorescence was collected by using the same objective. The signal was spatially and spectrally filtered by using a 50- μm pinhole and a dichroic beam splitter (Dualband z533/633; AHF Analysentechnik), respectively. After spectrally splitting by a dichroic beam splitter (640DCXR; AHF Analysentechnik) and filtering (Brightline HC582/75, and ET-Bandpass 700/75, respectively; both AHF Analysentechnik), the fluorescence was detected by using 2 avalanche photodiodes (APD, SPCM-AQR-14; PerkinElmer). Further details about image scanning, measurement of intensity transients, and redox switching are provided in *SI Text*.

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