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Permalink

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Journal

Journal of the American Chemical Society, 131(51)

ISSN

0002-7863

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Publication Date

2009-12-30

DOI

10.1021/ja904588g

Peer reviewed

Photoswitching Mechanism of Cyanine Dyes

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Photoswitchable fluorescent probes have been used in recent years to enable super-resolution fluorescence microscopy by single-molecule imaging.^{1–6} Among these probes are red carbocyanine dyes, which can be reversibly photoconverted between a fluorescent state and a dark state for hundreds of cycles, yielding several thousand detected photons per switching cycle, before permanent photobleaching occurs.^{7,8} While these properties make them excellent probes for super-resolution imaging, the mechanism by which cyanine dyes are photoconverted has yet to be determined. Such an understanding could prove useful for creating new photoswitchable probes with improved properties.

The photoconversion of red cyanine dyes into their dark states occurs upon illumination by red light and is facilitated by a primary thiol in solution,^{7,9} whereas agents with a secondary thiol do not support photoswitching. These observations suggest that the reactivity of the thiol plays a key role in the photoswitching process. On the basis of this hypothesis, we tested for a cyanine–thiol reaction using single-molecule imaging and mass spectrometry. We have shown that the rate of switching to the dark state depends on the concentration of deprotonated thiol in solution. Upon conversion to the dark state, a new product forms with a mass spectrum and fragmentation pattern consistent with thiol attachment to the polymethine bridge of the fluorophore. These results indicate that the stable dark state is formed by thiol addition to an excited state of the cyanine.

To characterize the spectral properties of the dark state, we photoconverted hydrolyzed Cy5-*N*-hydroxysuccinimide ester (Cy5-COOH) in a buffer containing a primary thiol agent, β -mercaptoethanol (β ME) [Figure 1A; see the Supporting Information (SI) for details]. The sample was illuminated with red laser light (633 or 647 nm), which led to a substantial reduction in the original absorption peak at 650 nm and a concurrent increase of a new peak at approximately half the original absorption wavelength (~ 310 nm) (Figure 1B). While we have shown previously that reactivation of the cyanine dye by visible light can be facilitated by a nearby chromophore that efficiently absorbs the activation light,⁷ the observation of UV absorption by dark-state Cy5 prompted us to try its direct reactivation with UV light without an assisting chromophore. Indeed, illuminating the dark-state product at wavelengths near the UV absorption peak led to efficient reversion of the molecules back to the fluorescent state at a drastically higher rate than that of thermal reactivation (Figure 1C and Figure S1 in the SI). Fluorescence measurements showed a corresponding loss

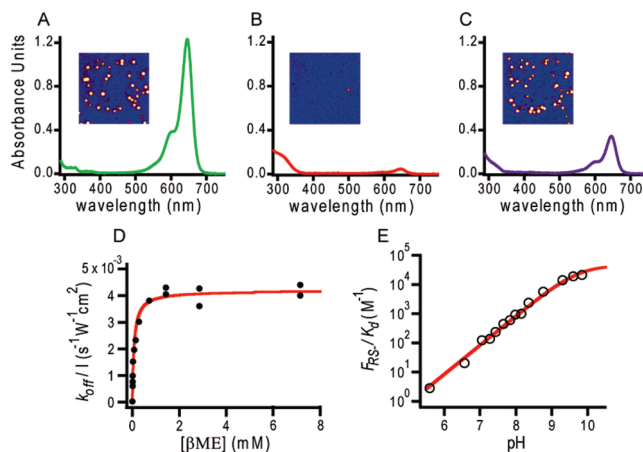


Figure 1. Spectral and kinetic analyses of the photoconversion of Cy5 in the presence of β ME. (A) Absorption spectra of Cy5-COOH. The inset shows fluorescence of single Cy5 molecules anchored to a surface. (B) After excitation by a red laser, the Cy5 fluorescence disappears (inset), and the absorption at 650 nm is diminished, while a new peak at 310 nm appears. (C) Following UV illumination, the 650 nm absorption of the Cy5 solution is only partially recovered because of photobleaching of some Cy5 molecules. In the single-molecule-imaging assay with antiphotobleaching buffer, nearly all of the Cy5 molecules recover to the fluorescent state (inset). (D) The rate constant (k_{off}) for switching the dye off, normalized by the red (633 nm) laser intensity (I), is plotted as a function of $[\beta$ ME] at pH 9.85 (black dots); the fit to eq 4 in the SI (red line) is also shown. (E) The fraction of deprotonated thiol normalized by the dissociation constant of the encounter complex (F_{RS}/K_D) is shown as a semilogarithmic plot against pH. The red curve shows the fit of the data to eq 5 in the SI.

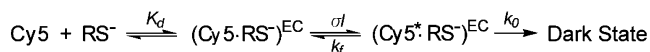
of fluorescence after red excitation and recovery following UV illumination (data not shown).

To characterize the efficiency of photoconversion, we imaged single Cy5 molecules attached to a surface under similar buffer conditions (Figure 1A–C insets). An oxygen scavenger system was added to reduce the effect of photobleaching (see the SI). The sample was illuminated with red laser light, which generated fluorescence from Cy5 and converted nearly 100% of the molecules into the dark state. Subsequent UV illumination led to nearly 100% recovery of the molecules back to the fluorescent state, indicating high photoconversion efficiencies in both directions. Similar photoswitching behavior was observed in the presence of different primary thiols, including β -mercaptoethylamine (MEA) and L-cysteine methyl ester (L-Cys-ME), and with different cyanine dyes (Cy5-diethyl, Cy5.5, Cy7, and Alexa 647) (Figure S2).

Next we used single-molecule imaging to measure the switching kinetics as a function of thiol concentration and solution pH, both of which affect the concentration of deprotonated thiol in solution. At a constant pH, the rate constant for switching to the dark state

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Scheme 1. Kinetic Pathway for Dark State Formation



(k_{off}) initially increases linearly and then saturates at high concentrations of βME (Figure 1D), indicating a transition from first-order to zeroth-order kinetics. We therefore propose a kinetic scheme in which Cy5 reversibly forms an encounter complex (EC) with the thiol anion (RS^-) before a light-driven chemical reaction generates a nonfluorescent thiol–dye adduct (Scheme 1). In this scheme, K_d is the dissociation constant for the encounter complex, σ is the absorption cross-section of Cy5, I is the excitation intensity, k_f is the de-excitation rate constant for the excited dye (Cy5^*), and k_0 is the rate constant for formation of the thiol adduct. Fitting the plot in Figure 1D to this kinetic scheme allowed us to deduce the normalized fraction of deprotonated thiol (F_{RS^-}/K_d) and the quantum yield for dark state formation, $k_0/(k_0 + k_f)$ (see the SI). The F_{RS^-}/K_d values obtained at different pH values (Figure 1E) were further used to extract the $\text{p}K_a$ (9.74 ± 0.05) of βME , which agrees with the $\text{p}K_a$ measured directly by titration of a solution of βME with NaOH (9.68 ± 0.01), supporting the proposed pathway.

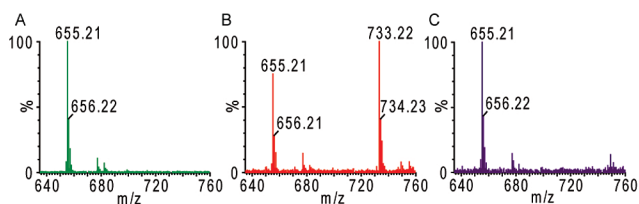
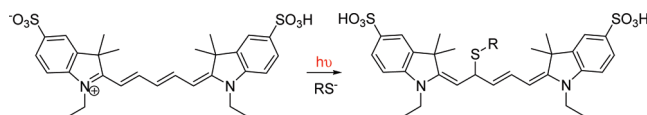


Figure 2. ESI-LC/MS measurements of the dark state of Cy5-COOH in the presence of βME . The panels show the cyanine dye–thiol mixture (A) before and (B) after red laser illumination as well as (C) after reactivation by UV excitation. The peak that appears for $[\text{M} - 2\text{H}]^-$ at m/z 655.21 corresponds to $[\text{Cy5-COO}]^-$. After red illumination, a new peak appears for the singly charged species $[\text{M} - \text{H}]^-$ at m/z 733.22. This new peak disappears after recovery by UV illumination. Because of substantial spontaneous recovery to the fluorescent state during sample preparation and measurement, the original dye peak at m/z 655.21 is not completely diminished in panel B.

Finally, we characterized the photoreaction product using high-resolution electrospray ionization liquid chromatography/mass spectrometry (ESI-LC/MS). We measured the negative-ion mass spectrum before and after photoconversion of Cy5-COOH in the presence of βME (Figure 2A–C). The spectrum before illumination reveals a major peak for $[\text{M} - 2\text{H}]^-$ at m/z 655.21 (Figure 2A), corresponding to the mass of a singly charged $[\text{Cy5-COO}]^-$. After red illumination, a new peak emerged for $[\text{M} - \text{H}]^-$ at m/z 733.22 (Figure 2B). The difference in mass between the two species is identical to that of βME (78 Da). Upon exposure of the sample to UV illumination to reactivate the dye, the new peak was eliminated (Figure 2C), indicating that it corresponds to the dark-state product. When the dark-state Cy5- βME adducts were further analyzed by tandem mass spectrometry (MS/MS), the resulting fragmentation patterns were consistent with addition of the thiol to the polymethine bridge (Figures S3–S7). Charge conservation considerations further argue against the first or middle bridge carbons as attachment sites, leaving the second carbon on the polymethine bridge as the most plausible position for thiol addition (Scheme 2), although we cannot formally exclude other potential attachment sites (see the SI for a complete analysis of the thiol attachment regiochemistry). A dark-state product was also observed upon red illumination when a different thiol (L-Cys-ME or MEA) was substituted in place of βME . In these cases, the measured mass differences between the products

Scheme 2. Proposed Structural Model of the Dark State



and reactants were again identical to the masses of the thiols (Figure S8 and Table S1). Similar results were also observed with different cyanine dyes, including Cy5-diethyl and Cy7-COOH (Table S1).

The absorption spectra, kinetics, and mass spectrometry analyses described above suggest that the dark state of the fluorophore is formed by addition of a thiol to the polymethine bridge of the cyanine dye, disrupting the fully conjugated π -electron cloud (Scheme 2). This proposed scheme is also consistent with the observation that Cy3, which has a shorter polymethine bridge but otherwise the same molecular structure as Cy5 and Cy7, is unable to switch to the dark state, whereas Cy5.5, which contains additional ring substituents but has the same polymethine bridge as in Cy5, can photoswitch,⁸ suggesting that the photoreaction is more sensitive to the structure of the polymethine bridge than to the aromatic rings.

It has been suggested that thiyl radicals can react with alkenes or other conjugated systems to form adducts.¹⁰ It is thus possible that the reaction shown in Scheme 2 occurs through radical intermediates, in which electron transfer from the thiol anion to the cyanine precedes covalent bond formation. Consistent with this hypothesis, the rate of switching to the dark state was substantially reduced in the presence of a radical quencher, isoascorbate (Figure S9).

In summary, we have used single-molecule imaging and mass spectrometry to investigate the photoswitching mechanism of cyanine dyes. These analyses show that the photoconversion into the dark state is a pH- and thiol-concentration-dependent process and that the dark-state product formed is a cyanine–thiol adduct.

Acknowledgment. We thank A. Ting, X. S. Xie, A. Tyler, A. Myers, and R. Yu for helpful discussions and M. Bruchez for his gift of the Cy5-diethyl. This work was funded in part by the National Institutes of Health (GM 086214 to X.Z.). D.R.L., R.Y.T., and X.Z. are Howard Hughes Medical Institute Investigators.

Supporting Information Available: Experimental procedures and Figures S1–S9, which describe additional kinetic and MS characterizations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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JA904588G