

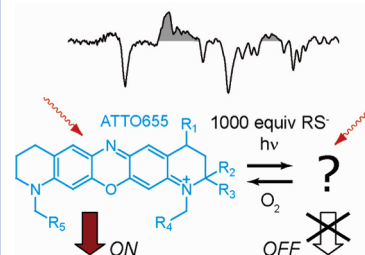
Identification of the Product of Photoswitching of an Oxazine Fluorophore Using Fourier Transform Infrared Difference Spectroscopy

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ABSTRACT The oxazine fluorophore ATTO655 is routinely applied in super-resolution fluorescence microscopy due to its reversible photoswitching between a fluorescent and a nonfluorescent state by light *in vitro* and in living cells. The molecular basis of the oxazine switching has been unclear. Here, the photoreaction of ATTO655 in the presence of a 1000-fold excess of the reductant 2-mercaptoethanol was studied using Fourier transform infrared (FT-IR) difference spectroscopy and quantum chemical calculations. The high sensitivity and selectivity of this approach allowed us to identify the chemical structure of the product of photoswitching as the fully reduced and protonated form of the oxazine. This finding opens the door for both future synthetic as well as analytical work.

SECTION Kinetics, Spectroscopy



The light-induced transition of organic fluorophores between optical states with discernible spectral properties is a key element of super-resolution fluorescence microscopy methods that employ stochastic photoswitching and single-molecule localization.^{1–5} Suitable fluorophores for these methods have to exhibit a bright fluorescent on state and a nonfluorescent off state that can be populated upon irradiation with light of appropriate wavelength. Because the majority of fluorophores have to reside in the off state at any time of the experiment, the formation of very stable off states with lifetimes considerably longer than the lifetime of the on state is mandatory to achieve optimal results.⁶ Recently, it has been demonstrated that a broad selection of conventional organic fluorophores belonging to the class of rhodamines and oxazines exhibit light-induced reversible photoswitching in the presence of millimolar concentrations of reducing thiols and oxygen, accompanied by the formation of stable dark states with lifetimes exceeding seconds even in aqueous environment.⁷ Despite their routine application in super-resolution spectroscopy, the chemical changes in most of the dyes upon photoswitching have not yet been identified. Here, we study the photoreduction of an oxazine dye, ATTO655, by infrared difference spectroscopy.

The organic fluorophore ATTO655 can undergo a reversible transition from a colored to a colorless form in a reductive environment,⁴ i.e., in the presence of reducing thiols. ATTO655 is particularly interesting as it also exhibits reversible photoswitching properties within living cells, exploiting the cellular redox system glutathione/oxygen, which recently made live-cell super-resolution imaging with organic fluorophores possible.^{7,8}

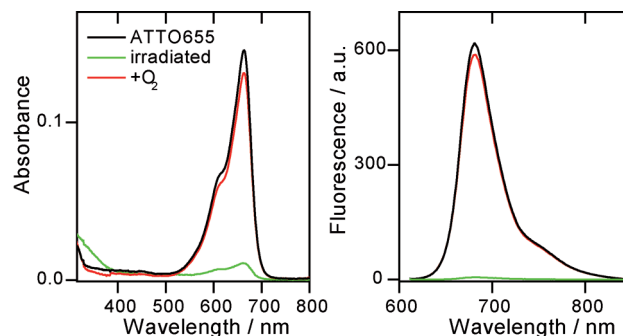


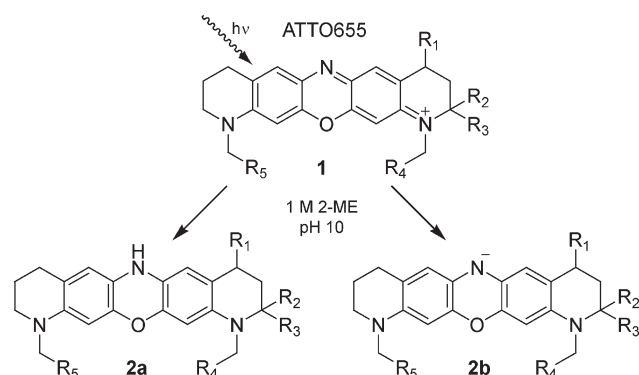
Figure 1. Absorption and emission spectra of ATTO655 in aqueous solution in the presence of 1 M 2-ME before and after irradiation with 658 nm, and after purging with oxygen. The experiment was performed at pH 9 to facilitate photoreduction by the thiolate anion.

The transition between the two states is highly reversible and can be repeated over hundreds of cycles.^{4,7} ATTO655 can easily be photoreduced by thiols such as 2-mercaptoethanol (2-ME) in aqueous solution (Figure 1). The colorless reduced state does not show any absorption in the visible range of the electromagnetic spectrum and is nonfluorescent. By addition of oxygen, however, the colored and fluorescent state of ATTO655 is quantitatively recovered. Identification of the photoproduct by most

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Scheme 1. Two Possible Products, **2a** and **2b**, Generated by Light-Induced Two-Electron Reduction of ATTO655 **1** in the Presence of 2-ME^a



^a Molecular structure of ATTO655 adopted from Sigma-Aldrich.

analytical tools is hampered by the presence of a > 1000-fold excess of 2-ME over fluorophore. Therefore, we applied light-induced Fourier transform infrared (FT-IR) difference spectroscopy, which is capable of discriminating very small structural changes against a large constant background, even in aqueous solution. This approach is therefore commonly employed to resolve reaction mechanisms in proteins.^{9–12}

Photoreaction of ATTO655 with 2-ME and generation of the off state might proceed via one- or two-electron reduction. However, formation of a stable fluorophore radical anion under the applied experimental conditions (1 M thiol, alkaline pH) cannot be observed as corroborated by the complete loss of absorption in the visible part of the electromagnetic spectrum. The formation of radical anions results in an absorption peak at around 400 nm.^{13,14} Accordingly, the formation of a fully reduced state, i.e., the so-called leuco-form of the fluorophore, following transfer of two electrons with or without subsequent addition of a proton is most likely (Scheme 1).

FT-IR spectra were recorded in attenuated total reflection (ATR) to have access to the sample compartment. The absorption spectrum of ATTO655 was obtained by subtraction of a spectrum of ATTO655 in 1 M 2-ME minus a reference spectrum of 1 M 2-ME (Figure 2a). To tentatively assign the band pattern, we performed theoretical calculations using density functional theory on a simplified model compound **1** with $R_1-R_5 = H$. The theoretical line spectrum was broadened with Lorentzians (Figure 2b). A very good agreement with the experimental absorption spectrum was achieved, especially in the spectral region > 1340 cm^{-1} , besides a systematic shift of the calculated frequencies to lower wavenumbers. Contributions from the substituents R_1-R_5 need to be considered (see Supporting Information). Strong and broad bands are recorded from these vibrational modes, such as the asymmetric and symmetric stretch of the carboxylate group of R_4 at 1549 cm^{-1} and $\sim 1470 \text{ cm}^{-1}$, respectively,¹⁵ and the asymmetric and symmetric stretch of the sulfonate group of R_1 at around 1200 cm^{-1} and at 1067 cm^{-1} , respectively.¹⁶ One reason for the high accuracy of the vacuum calculations is the weak interaction of the ATTO655 ring system with the aqueous medium, which is reflected in its poor solubility.

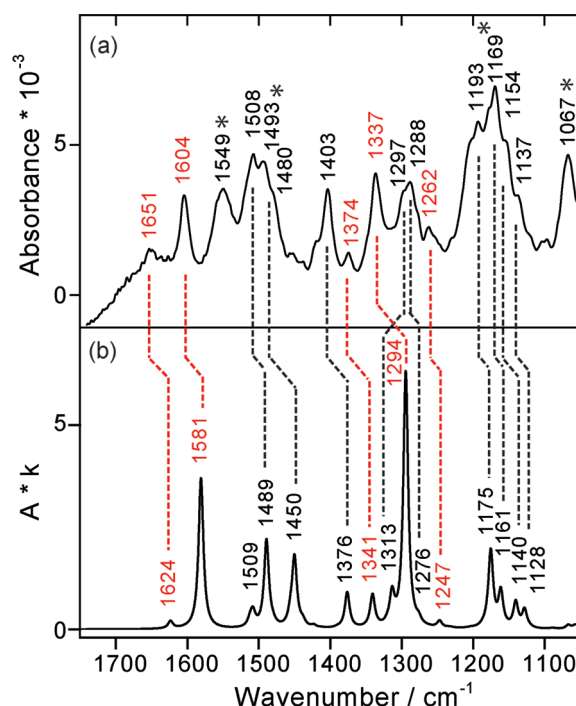


Figure 2. Measured (a) and calculated (b) infrared spectrum of ATTO655. Contributions from the substituents R_1-R_5 were not included in the calculations and are marked with an asterisk in the experimental spectrum. Bands considered being without contributions from the substituents are highlighted in red. The calculated spectrum was scaled by an arbitrary factor k .

To ensure a fast conversion of the sample by light, the pH was set to 10. The solution compartment was closed, and the sample was irradiated with 20 mW cm^{-2} at 658 nm for 1 min. The light-minus-dark difference spectrum shows negative signals of the vibrational modes of the educt and positive signals of those of the photoproduct and serves as a fingerprint of the reaction (Figure 3a). Negative signals of the educt are in agreement with those of the IR absorption spectrum of ATTO655 (see Supporting Information). The product is characterized by fewer signals than ATTO655, showing a strong band at 1490 cm^{-1} and weak bands at around 1468, 1452, 1442, 1239, and 1223 cm^{-1} . The broad rise of the baseline at > 1600 cm^{-1} is attributed to a fluctuation of the water background. The loss of bands from the educt including the high frequency mode at 1602 cm^{-1} is indicative of a reduction of the oxazine ring. After two illuminations, the sample went colorless and was stable for hours. Admission of air to the sample compartment led to recoloring of the solution.

To identify the product, theoretical calculations were performed on a variety of possible structures. To enable the direct visual comparison with the difference experiment, the difference of the theoretical spectra of the products minus that of **1** was calculated (Figure 3b,c). A good agreement of the experimental difference spectrum with the calculated spectrum of a conversion to the fully reduced state **2a** is evident, including the frequency of the most prominent band at 1482 cm^{-1} . In the comparison, it needs to be considered that the calculations were not performed in aqueous solution but in vacuum

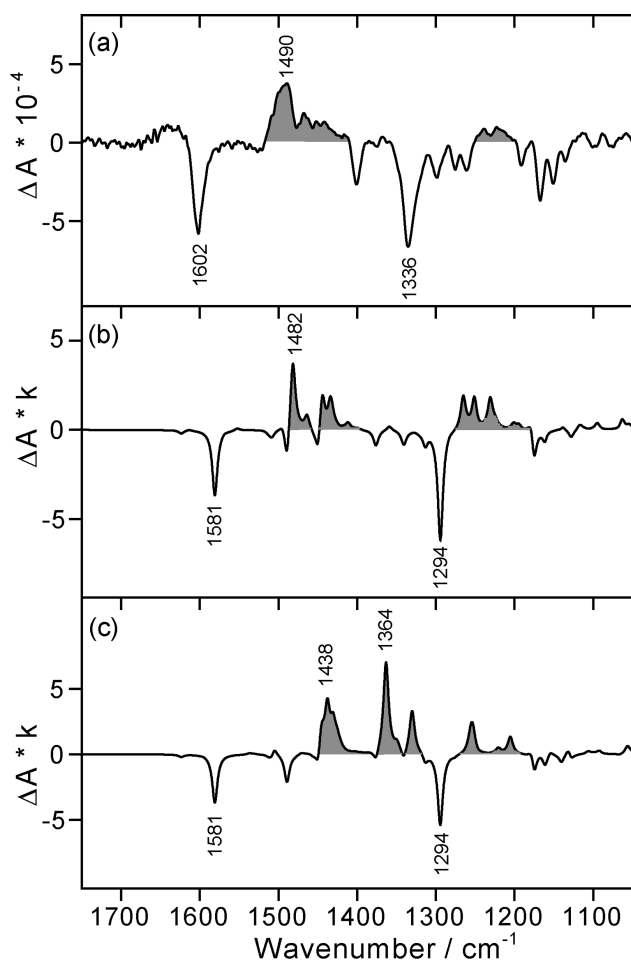


Figure 3. (a) Light-minus-dark FT-IR difference spectrum of ATTO655 after illumination with 658 nm. Calculated difference spectra of the reaction to photoproduct **2a** (b) and **2b** (c). Both calculated spectra were scaled by the same arbitrary factor k .

and that the substituents R_1 – R_5 were not included. Furthermore, the intensities cannot be as precisely determined as the frequencies. The scaling factor for subtraction of the two theoretical spectra was accordingly chosen to obtain a maximal similarity. The high level of agreement for the reaction to **2a** becomes visible, however, in the comparison to the unprotonated compound **2b**, which shows a completely different positive vibrational pattern with strong transitions at 1438 and 1364 cm^{-1} . The latter band is the decisive difference between compounds **2a** and **2b**. The molecular origin of this band is a strongly coupled vibration of the ring system of **2b** with contributions from most of the bonds (see displacement vectors in the Supporting Information). On the other hand, protonation of **2b** should lead to a N–H bending vibration with a marker band characteristic for compound **2a**. However, this vibration couples to several other modes, which prevents an isolation. Calculations accordingly predict a weak effect of deuteration on the vibrational pattern including a small downshift of the major band at 1482 cm^{-1} by only 4 cm^{-1} by the decoupling (data not shown). As a further

control, the difference spectrum to the radical species was calculated, which shows strong differences to the experiment as well (see Supporting Information). To summarize, the agreement of the vibrational difference spectrum with the calculations led to the assignment of **2a** as the product of photoswitching of ATTO655. It is proposed that the reduction might proceed via two nucleophilic attacks by the sulfide with intermediate formation of a covalent adduct as observed in another N-heterocycle, the flavin.¹⁷

In conclusion, we have identified the nature of the photo-switched state of the ATTO655 fluorophore in aqueous solution using FT-IR difference spectroscopy in combination with theoretical calculations. This finding is of great importance, as ATTO655 turned out to be a very promising photoswitchable probe for localization-based super-resolution imaging. It can be reversibly photoswitched by one irradiation wavelength only, can be operated in the presence of oxygen, in low concentration of reducing agents, and even inside a living cell.^{4,7,8} This is significantly different from other organic fluorophores that can be reversibly photoswitched, such as carbocyanines, which require the removal of oxygen¹⁸ and which were reported to follow a different switching mechanism at the ensemble level.¹⁹

The perspective of this work lies in the understanding of the nature of the dark state of ATTO655, which now opens the route to extend the palette of photoswitchable fluorophores with similar photoswitching properties and orthogonal spectral properties, thus paving the way for multicolor super-resolution imaging even in living cells. Furthermore, organic fluorophores, which can be operated as photoswitchable units, might complement the existing palette of conjugates of photochromic units and fluorophores, which have demonstrated excellent switching properties and fatigue resistance.²⁰ At the same time, we have introduced an approach to study molecular changes of reversibly photoswitchable organic fluorophores with a high level of sensitivity and specificity. We believe that this approach will be useful for further studies of this kind.

SUPPORTING INFORMATION AVAILABLE A detailed description of the experimental procedures and the calculations, the chemical structures of ATTO655 and its possible radical photoproduct, the calculated frequencies of the normal modes of **1** and possible products, a comparison of band positions, and atomic displacement vectors for the major modes of **1**, **2a**, and **2b**. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

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