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The intrinsic photophysics of gaseous ethidium ions

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ABSTRACT

Ethidium is a cationic dye with fluorescence that is enhanced ~9-fold upon binding DNA. In order to better understand how the local environment modulates the behavior of this dye, we measured the photophysical properties of gaseous ethidium ions, using a quadrupole ion trap mass spectrometer that has been modified for fluorescence spectroscopy. The photodissociation maximum of gaseous ethidium measured through action spectroscopy is 485 nm and the emission maximum is 548 nm. The Stokes shift (2370 cm^{-1}) of gaseous ethidium is marginally larger than that of ethidium in non-polar solvents, and significantly less than that in polar solvents. Time-resolved fluorescence measurements of gaseous ethidium ions show two components with lifetimes of 21.4 ± 1.5 and 5.1 ± 0.7 ns, which suggest the presence of multiple conformations in the gas phase. Both lifetimes are significantly longer than that of aqueous ethidium, while the longer of the two lifetimes is remarkably similar to that of ethidium in complex with double-stranded DNA in solution. In line with this, the estimated quantum yield of gaseous ethidium is ~30% lower than that of ethidium in complex with DNA in solution, and ~10-fold higher than that of aqueous ethidium. These benchmark results provide a reference from which to better understand the factors that modulate the fluorescence of phenanthridine-based dyes by the local environment.

1. Introduction

Ethidium bromide (Scheme 1) is a classic DNA stain whose fluorescence is enhanced ~9-fold upon binding to double-stranded DNA (dsDNA) [1]. The enhancement of ethidium fluorescence is thought to arise due to an intercalative mode of binding, where the ethidium molecule lies stacked between hydrogen-bonding base pairs. Intercalation reduces the non-radiative deactivation rate constant, evident from the significant lengthening of the fluorescence lifetime of the dsDNA-ethidium complex ($\tau = 22.5$ ns) relative to aqueous ethidium ($\tau = 1.8$ ns) [2]. While the dsDNA-ethidium complex has been much-studied in solution [3–10], the mechanism of the fluorescence enhancement upon DNA binding is still not well understood [11–14]. An understanding of ethidium's *intrinsic* photophysical properties, which can be attained by removing solvent interactions and performing measurements in the gas phase, will provide a baseline from which to better understand the mechanism of its fluorescence enhancement upon binding DNA and aid the design of more efficient turn-on fluorophores.

The underlying photophysics of ethidium bromide in solution and its fluorescence enhancement upon binding to dsDNA have been extensively investigated [11–18], although no consensus has been reached [11–14,18]. In an important early contribution, Olmstead and Kearns concluded that the quenching of ethidium in polar solvents results from excited-state proton transfer from the exocyclic $-NH_2$ groups (which become more acidic in the excited state) of ethidium to solvent [11]. The significance of this quenching mechanism was supported by the short fluorescence lifetime in water (~1.8 ns) in comparison to that measured in ethanol, dimethyl sulfoxide (DMSO), and glycerol (5.0–6.0 ns), suggesting a substantially larger non-radiative deactivation rate in water. Furthermore, the fluorescence lifetime of ethidium in deuterated water (D_2O; 6.5 ns) is dramatically longer than that in H_2O. Olmsted and Kearns construed that when ethidium is intercalated in dsDNA, it is shielded from the solvent and this proton transfer is inhibited, reducing the non-radiative deactivation rate constant and resulting in a longer fluorescence lifetime and higher quantum yield [11]. Pal et al. [12] provided additional evidence in support of an excited-state proton transfer quenching mechanism upon noting the decreased fluorescence intensity and shorter lifetime of ethidium in acetone compared to that in acetonitrile, which is a less effective hydrogen-bond acceptor than acetone. Furthermore, analysis of acetonitrile/water mixtures provided evidence of a dynamic quenching of ethidium by water [12]. However, Phukan and Mitra [14] recently investigated the absorption and fluorescence of ethidium in a wider variety of solvents and found no correlation between fluorescence quantum yield (φ) and hydrogen bonding accepting ability of the solvent. For

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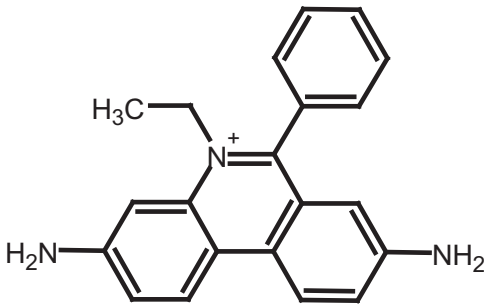

Scheme 1. The structure of ethidium.

example, the quantum yield of ethidium bromide in 1,4-dioxane (0.0061) is significantly lower than that in water (0.022), despite 1,4-dioxane's being a worse hydrogen bond acceptor. The researchers also found no correlation between solvatochromism of ethidium and solvent polarity. However, they did note that Stokes shifts in non-polar solvents are significantly smaller (2051–2273 cm^{-1}) than those found in polar solvents (2743–4139 cm^{-1}).

Luedtke et al. explored the importance of the exocyclic amines implicated in quenching via excited-state proton transfer by evaluating the fluorescence of a series of ethidium derivatives featuring substitutions of these groups [18]. In several cases, an enhancement of the derivatives' quantum yield relative to ethidium was observed. For example, substituting both $—NH_2$ groups on ethidium with urea functionalities resulted in a 57-fold enhancement in the quantum yield for the bis-urea derivative relative to ethidium. However, some derivatives with N-based functional groups that are not acidic, such as the derivative with two dimethylamino ($—N(CH_3)_2$) groups, had *reduced* quantum yields relative to ethidium, indicating the importance of deactivation pathway(s) other than excited-state proton transfer. In particular, the authors suggested that the electron donating ability of the exocyclic amino groups plays an important role in non-radiative decay.

Deactivation pathways that involve rotation of the peripheral phenyl substituent on ethidium have been proposed as an alternative mechanism of non-radiative decay [13,18,19]. Sommer et al. [19] proposed the significance of phenyl group rotation upon measurement of time-resolved fluorescence spectra of ethidium in glycerol, which shifts ~40 nm to the red on the subnanosecond timescale. Prunkl et al. [13] made a case for the contribution to quenching of a charge transfer (CT) state, accessed by twisting of the phenyl ring upon electronic excitation. This suggestion was made based on the comparison of ethidium to derivatives with differing electron-donating capabilities in place of the phenyl ring and supported by calculations. The authors proposed that upon intercalation into dsDNA, the CT state becomes less accessible, in part perhaps because the rotation of the pendant phenyl ring on ethidium is hindered, resulting in a reduction of the non-radiative decay rate constant and an enhancement of fluorescence.

In this work, the photophysical properties of gaseous ethidium are reported. These are measured making use of a modified quadrupole ion trap (QIT) mass spectrometer equipped for fluorescence spectroscopy. By performing gas-phase fluorescence measurements, any deactivation due to solvent proton transfer or collisional deactivation are eliminated. This study of ethidium's *intrinsic* behavior lays the groundwork to better understand the factors that drive the fluorescence enhancement of this fluorophore upon binding dsDNA.

2. Material and methods

2.1. Mass spectrometry and gas-phase fluorescence spectroscopy

Ethidium bromide was purchased from Sigma (Oakville, ON, Canada) at a concentration of 25 mM in H_2O and diluted to a concentration of 5 μM in 50/50 (v/v%) methanol–water. Rhodamine 575 (Rh575) was obtained from Exciton Corporation (Dayton, OH, USA) and was diluted to a concentration of 1 μM in 50/50 (v/v %) methanol–water. Ions were transferred from solution into the gas phase via electrospray ionization (ESI) operated in positive ion mode using spray voltages between 3 and 4 kV.

Gaseous ions were accumulated in the trapping region of a quadrupole ion trap (QIT, Bruker, Esquire 3000+) mass spectrometer that has been modified to enable photodissociation and fluorescence measurements of gaseous ions. This experimental set-up has been described in detail previously [20,21]. Ions are accumulated in the trapping region of the QIT at an adjustable pressure ($0.6–2.3 \times 10^{-3}$ mbar, depending on the experiment) of room temperature helium for 10–1000 ms. The desired ion (ethidium m/z 314 or rhodamine 575 m/z 415) is mass selected. Irradiation of trapped mass-selected ions is synchronized with mass spectrometry (MS)/MS events defined in the MS control software using a trigger from the mass spectrometer that opens a shutter after ion isolation. After the shutter is closed, an MS/MS spectrum is recorded upon scanning the product ions out of the trap in a mass-selective manner. For fluorescence experiments, irradiation parameters and helium pressure were adjusted such that <5% of the ethidium photodissociated, while these parameters were re-adjusted to favor photodissociation for the other experiments (see Table S1). The data shown is the sum of repeated measurements of multiple ion populations. For the fluorescence measurements, these were interspersed with measurements made without ions in the trap (see Fig. S2), which were used for background subtraction.

The light source used for photodissociation and fluorescence excitation experiments is a *Tsunami* Titanium:Sapphire laser (Ti:Sapph; Spectra-Physics, Mountain View, CA) pumped by a Nd:YVO_4 laser (Millenia 10s, Spectra-Physics, Mountain View, CA). This laser system generates pulsed tunable IR light in the wavelength range of 700–1080 nm, which is then frequency doubled. To enable irradiation of the ions in the QIT, two 1.2 mm holes have been drilled in the ring electrode such that the frequency doubled output of the Ti:Sapph intersects the trapped, gaseous ions.

Fluorescence from trapped ions is collected through a third hole (2.0 mm diameter) in the QIT ring electrode (~1 cm internal radius), which is orthogonal to the optical excitation axis. A lens inserted into the ring electrode assists in the collection of the fluorescence light, which then passes outside of the vacuum chamber through a UV-fused silica window with an anti-reflective coating. The collected fluorescence light then passes through a long-pass filter (Chroma Technology Corp., Rockingham, VT, USA) in order to reduce scattered laser light. The filtered light can then be sent to one of two detectors: a single-photon avalanche diode (SPAD) that enables measurement of time-resolved fluorescence using time-correlated single photon counting (TCSPC) techniques [21], or a spectrograph coupled to an electron-multiplying charge coupled device (EM-CCD) for the measurement of fluorescence emission spectra. For TCSPC measurements, the repetition rate of the laser was reduced from 80 MHz down to 10 MHz by passing the laser beam through a pulse picker (Conoptics, Danbury, CT, USA). The EM-CCD was operated using a 50 kHz readout rate, 4× pre-amplified gain, an electron-multiplication gain of 210, and at a temperature of 198 K. To measure accurate emission maxima, the spectrograph/EM-CCD system was externally calibrated with gaseous rhodamine 640 ($\lambda_{em}^{max} = 561$ nm) [22].