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The origin of the dual fluorescence of protonated ellipticine in water

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ABSTRACT

Absorption and fluorescence spectroscopic measurements, as well as isothermal calorimetric titrations showed that the biexponential decay kinetics of protonated ellipticine (EH^+) fluorescence in water originates from dimerization. Due to the high equilibrium constant for the association of two EH^+ and the intense fluorescence of the dimer, deviation from the exponential emission intensity decay commences below micromolar concentrations. Dimerization must be taken into account when EH^+ concentration is determined by spectrophotometry, and when EH^+ binding to substrates are studied. The molar absorption coefficients of the monomer and dimer were determined.

1. Introduction

Ellipticine (E), a natural pyridocarbazole type alkaloid, has attracted considerable attention due to its anticancer [1] and antimalarial [2] activity. To gain a deeper understanding of the factors controlling the excited state relaxation processes, the fluorescent behavior of E was examined in organic solvents of a wide range of polarities and hydrogen bonding capabilities [3]. Self-assembling peptides was found to stabilize this alkaloid in water [4,5]. The native fluorescence of E and its protonated form (EH^+) was exploited to monitor their uptake and intracellular distribution [6,7]. The markedly different fluorescence features of E and EH^+ enabled the detection of the extent of protonation in different intracellular compartments [7] and the proton movement in mitochondria [8]. Time-resolved fluorescence measurements showed the substantial variation of the fluorescence lifetime upon binding to various subcellular constituents [9]. Biexponential decay of EH^+ fluorescence was always observed in water, but the individual emitting components could not be identified, and the variation of the average lifetime was only discussed, which has no physical meaning [10,11].

The main goal of the present studies was to unravel why EH^+ fluorescence does not follow exponential decay kinetics in aqueous solution. This knowledge is inevitably necessary for the correct interpretation of the time-resolved spectroscopic data in the presence of all types of additives, and contributes to the deeper understanding of the photophysical behavior in various microenvironments. We intend to reveal whether interaction between two EH^+ molecules occurs. Dimerization may significantly influence the biological activity, binding characteristics, and photoinitiated processes of EH^+. Therefore, it is of fundamental importance to examine the interaction between these alkaloid cations. Self-association can cause deviation from the Lambert–Beer law leading to systematic error in the spectrophotometrically measured EH^+ concentrations, which propagates to the equilibrium constants derived for EH^+ binding to various substrates. To the best of our knowledge, the dimerization has never been taken into account in the determination of the molar absorption coefficient and binding constants of EH^+. The formula of the investigated compound is presented in Scheme 1.

2. Experimental

Ellipticine (≥99% by HPLC, Fluka) was used as received. Slightly more than stoichiometric amount of concentrated HCl aqueous solution was added to ellipticine in ethanol. The solvent and the excess of HCl were evaporated under a flow of nitrogen. EH^+Cl^- salt prepared thereby was dissolved in 10^{-4} M HCl aqueous solution. The UV–visible absorption spectra were measured on an Agilent Technologies Cary60 spectrophotometer. Corrected fluorescence spectra were recorded on a Jobin-Yvon Fluoromax-4 photon counting spectrofluorometer. Fluorescence decays were collected with time-correlated single photon counting technique using the previously described instrument [12]. The results of spectrophotometric and fluorescence titrations were analyzed with homemade programs written in MATLAB 7.9. Isothermal titration calorimetry was

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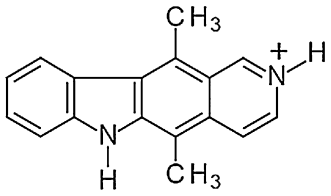

Scheme 1. Chemical structure of protonated ellipticine (EH^+).

carried out with a VP-ITC (GE Healthcare) instrument at 298 K using degassed solutions and stirring at 300 rpm. The results were analyzed using Microcal ORIGIN software. The first data point was always removed. The titrations were repeated at least three times.

3. Results and discussion

3.1. Spectrophotometric studies

A pK_a value of 7.4 ± 0.1 has been reported for EH^+ [13]. To ensure complete protonation, experiments were performed at pH 4. Figure 1 demonstrates that the absorption spectrum strongly depends on the total EH^+ concentration ($[EH^+]_T$). The apparent molar absorption coefficient (ε) was calculated on the basis of the Lambert–Beer equation. The intense band with a maximum at 301 nm shifts to 295 nm and exhibits hypochromicity, whereas the batochromic displacement in the long-wavelength domain is accompanied by intensity diminution when $[EH^+]_T$ is raised. These marked spectral changes suggest solute association. The concentration dependence of ε was analyzed assuming dimerization without formation of larger aggregates. The fraction (α) of $[EH^+]_T$ present as monomer is given by the following relationship:

$$\alpha = \frac{-1 + \sqrt{1 + 8K_D[EH^+]_T}}{4K_D[EH^+]_T} \quad (1)$$

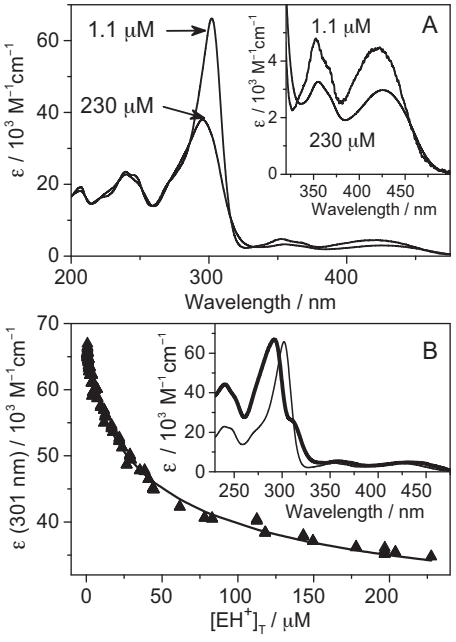

Figure 1. (A) Absorption spectra of 1.1 and 230 μM EH^+ in 10^{-4} M HCl aqueous solution. Inset displays the zoomed view of the long-wavelength range. (B) Variation of the apparent molar absorption coefficient at 301 nm with total EH^+ concentration at pH 4. The line presents the result of the nonlinear least-squares fit. Inset shows the calculated spectra for monomer (thin line) and dimer (thick line).

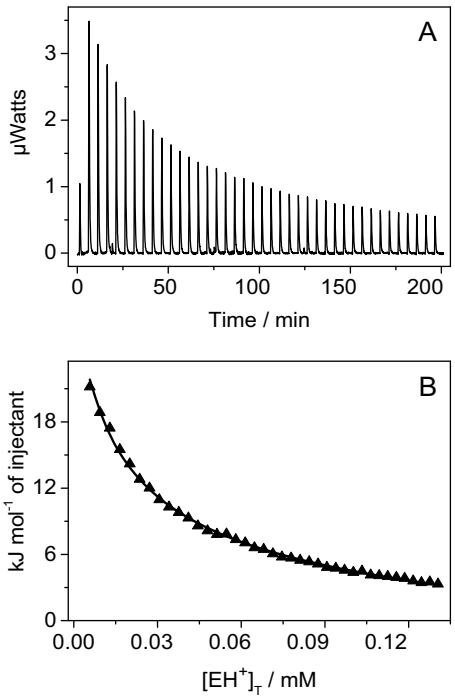

Figure 2. (A) Heat uptake upon addition of 747 μM EH^+ solution into water in increments of 7 μl at pH 4. (B) Integrated heat absorbed after injection as a function of total EH^+ concentration. The line represents the fitted function.

where $K_D = [(EH^+)_2]/[EH^+]^2$ is the equilibrium constant of dimer $(EH^+)_2$ formation. The measured apparent molar absorption coefficient (ε) is related to the molar absorption coefficients for the monomer (ε_M) and dimer (ε_D) as follows

$$\varepsilon = \varepsilon_M \alpha + \frac{\varepsilon_D}{2}(1 - \alpha) \quad (2)$$

The global analysis of the concentration dependence of the absorption spectra using Eqs. (1) and (2) provided $K_D = (1.5 \pm 0.2) \times 10^4\ M^{-1}$, and the spectra displayed as an inset in Figure 1B. The molar absorption coefficients at 301 nm were $\varepsilon_M = 66\,300 \pm 1000\ M^{-1}\ cm^{-1}$ and $\varepsilon_D = 38\,600 \pm 2000\ M^{-1}\ cm^{-1}$ for the monomer and dimer, respectively. The excellent match between the measured and computed data implies that only two EH^+ ions are associated, negligible amount of larger aggregates is produced in the $0 < [EH^+]_T \leq 230$ μM concentration range.

3.2. Thermodynamics of dimerization

To determine the thermodynamic parameters of association, isothermal calorimetric titrations (ITC) were performed at 298 K. About 81% of the solute is dimerized in 747 μM EH^+ solution. When such a concentrated solution was gradually added to water keeping pH 4 constant, the enthalpograms displayed in Figure 2 were obtained. Substantial heat is absorbed after each addition indicating that the dimer dissociation is an endothermic process. As the amount of EH^+ grows in the calorimeter cell, the extent of $(EH^+)_2$ dissociation progressively diminishes after successive injections leading to the lessening of the absorbed heat. The dependence of the integrated heat signals on the total alkaloid concentration was analyzed on the basis of the simple $2EH^+ \rightleftharpoons (EH^+)_2$ equilibrium using the association constant (K_D) and the enthalpy change (ΔH_D) of dimerization as fitting parameters. From these quantities, standard free energy (ΔG_D) and entropy changes (ΔS_D) of $(EH^+)_2$ formation were calculated using the equation

$$\Delta G_D = -RT \ln K_D = \Delta H_D - T\Delta S_D \quad (3)$$