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Separating and probing tautomers of protonated nucleobases using differential mobility spectrometry

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ABSTRACT

The protonated nucleobases $(C+H)^+$, $(T+H)^+$, $(U+H)^+$, $(A+H)^+$, and $(G+H)^+$ are investigated in a combined experimental and computational study using differential mobility spectrometry (DMS), mass spectrometry, and electronic structure calculations. DMS is used to isolate individual tautomeric forms for each protonated nucleobase prior to characterization with HDX or CID. The population distributions of each protonated nucleobase formed by electrospray ionization (ESI) are dominated by a single tautomeric form, as is predicted by our calculations. However, all nucleobases present additional tautomers upon ESI, with these minor contributions to the ensemble populations attributed to additional higher energy metastable species. In addition to the tautomer-derived species, additional ion signals in the DMS data are attributed to larger nucleobase-containing clusters, which fragment post-DMS to yield bare ion and fragment ion signals that are consistent with those expected for the bare protonated nucleobases. Contributions from larger clustered species are instead distinguished by monitoring DMS ion signal as declustering potential voltages are ramped.

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1. Introduction

Owing to the importance of DNA and RNA in encoding and expressing genetic information, and the central role that nucleobases play in establishing the structure and functionality of nucleic acid sequences, a great deal of experimental [1–4] and theoretical [5–8] effort has gone into determining the structures and properties of cytosine (C), guanine (G), adenine (A), thymine (T), and uracil (U). Of importance are the sites of protonation and the tautomeric forms that the nucleobases exhibit, since these variations are thought to impact mutagenic processes (*e.g.*, point mutation during nucleic acid replication) [9] and the stabilization of triplex structures [10]. Mass spectrometry and quantum chemical calculations have been employed to great success in determining nucleobase properties such as gas-phase acidity and basicity [11–17]. However, it has been shown previously that several different tautomers are likely to exist simultaneously in a given nucleobase ensemble [18,19]. For example, Salpin et al. used infrared multiple photon dissociation (IRMPD) spectroscopy to demonstrate the presence of at least two tautomeric forms of $(C+H)^+$, $(T+H)^+$, and $(U+H)^+$ in ion populations generated by ESI [20]. Comparison of the experimental IRMPD spectra with IR spectra that were calculated at the B3LYP/6–31 ++ G(d,p) level of theory indicated that the three protonated nucleobases existed predominantly as enolic tautomers, with a small sub-population of oxo tautomers. Subsequent work by Bakker et al. showed that the vibrational spectra of monohydrated protonated uracil, $(U+H)^+{\bullet}OH_2$, and cytosine, $(C+H)^+{\bullet}OH_2$, were also consistent with the presence of two tautomeric species arising from the production of two protonated forms of the associated nucleobases via ESI [21,22]. This suggests that the molecular properties of protonated nucleobases as determined by mass spectrometry are likely to correspond to an ensemble average for the various tautomeric structures that are present under the experimental conditions employed during measurement. It is therefore desirable to separate the tautomeric species prior to mass spectrometric or spectroscopic interrogation.

Various forms of ion mobility spectrometry have been employed to separate tautomers prior to MS analysis [23–25]. For example, the Attygalle laboratory recently reported on the characteriza-

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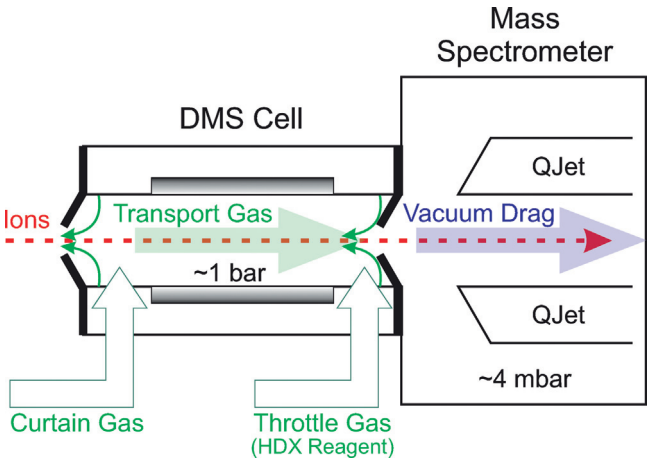

Fig. 1. The dispersion plot obtained for $(C+H)^+$ (m/z 112) with a DMS cell containing (**A**) a pure N_2 environment, and a N_2 environment seeded with 1.5% (mole ratio) (**B**) methanol vapor, and (**C**) isopropyl alcohol vapor. Error bars are 2σ obtained from Gaussian fits to the ionogram peaks. (**D**) The ionogram recorded for the m/z 112 peak in a pure N_2 environment with SV = 3500 V (highlighted green in **A**). (**Inset**) The three lowest energy tautomers of $(C+H)^+$ as calculated at the CCSD(T)/6–311++G(d,p)//B3LYP/6–311++G(d,p) level of theory. Energies are reported as standard Gibbs' energies in kJ mol^{-1}.

tion of tautomer populations of deprotonated hydroxybenzoic acid with travelling wave ion mobility spectrometry [26]. This work challenges the notion that ESI–MS results reflect solution phase population distributions, and demonstrates that tautomer populations can be tuned by varying ESI source conditions. We have also recently reported on the use of ion mobility to characterize tautomer populations generated *via* ESI by using differential mobility spectrometry (DMS) [27–30] to separate and probe the nitrogen- and oxygen-protonated tautomers of *para*-aminobenzoic acid. [31,32] By taking advantage of the different DMS behaviors of the two tautomers, we could examine the MS/MS fragmentation patterns and HDX behaviors of each species individually and demonstrate that each structure did, indeed, exhibit its own characteristic physicochemical properties. We also demonstrated that a great deal of care had to be taken in HDX experiments since high vapor pressures of HDX reagent can drive *in situ* tautomerization via proton-transfer relay networks established upon ion-solvent clustering [31]. Studies like these show that ESI solvent effects are not necessarily the primary criteria that determine relative tautomer (or, by extension, isomer or conformer) population distributions. Instead, a variety of post-ESI instrument conditions could potentially contribute to the observed sub-populations within a gas phase ensemble.

Here, we utilize the DMS technique to separate and study the individual tautomeric forms of protonated adenine, $(A+H)^+$, guanine, $(G+H)^+$, cytosine, $(C+H)^+$, thymine, $(T+H)^+$, and uracil, $(U+H)^+$ that are generated via ESI. The various tautomers of these molecules are studied individually by HDX and CID, and we show that the relative tautomer populations can be manipulated post-ESI and post-DMS by using the instrument declustering potential to selectively fragment high-energy, kinetically trapped tautomers prior to MS characterization.

2. Methods

2.1. Experimental details

A SelexION differential mobility spectrometer was used in conjunction with a QTRAP 5500 system (SCIEX; Concord, ON) (Fig. 1). Instrument parameters included a ESI probe voltage of 5500 V, a source temperature of 32 °C, nebulizing gas pressure of 20 psi, and auxiliary gas pressure of 0 psi. The DMS was set to a temperature of 150 °C, and nitrogen was used as both the curtain gas (20 psi) and collisionally activated dissociation gas (~9 m Torr) for all experiments. Nucleobase solids were purchased from Sigma-Aldrich and subsequently dissolved in a 50:50 mixture of ultrapure water and methanol with 0.1% formic acid to yield solutions of 10 ng/mL. Analyte solutions were pumped into the ESI source at 7 μL/min. HPLC-grade methanol, isopropanol, and deuterium oxide were also purchased from Sigma-Aldrich and used without further purification or dilution.

DMS experiments involved the stepping of the separation voltage (SV) from 0 to 4000 V in 500 V increments. At each SV, the compensation voltage (CV) was scanned from −80 V to 15 V in increments of 0.1 V to produce an ionogram. A dispersion plot [33], which plots optimal conditions for ion transmission as a function of SV and CV, was then generated. Dispersion plots enable the identification of the DMS behavior of particular ions according to known patterns [34]. These data were acquired for each nucleobase in a pure N_2 DMS environment, as well as with DMS environments that had been seeded with 1.5% (mole ratio) methanol (MeOH) and isopropanol (IPA) chemical modifiers. Hydrogen-deuterium exchange (HDX) experiments were conducted through the infusion of deuterium oxide into the throttle gas. These experiments were undertaken under two different HDX conditions. In the first implementation, the throttle gas was bubbled through D_2O to saturate the N_2 with HDX reagent. This yields maximum rates of HDX in the junction chamber between the DMS cell and the orifice of the mass spectrometer, as described in reference [31]. In the second implementation, the throttle gas sampled only the headspace above the D_2O HDX reagent vessel, resulting in a lower D_2O partial pressure and slower rates of HDX [31]. In this way, the DMS cell was used to select a specific tautomer prior to HDX, which was monitored by recording a full scan mass spectrum (Q1).

Enhanced product ion (EPI) scans were also conducted for each of the separated nucleobase tautomers. Following DMS isolation of a given tautomer, the collision energy (CE) of the Q2 ion trap was ramped from 0 V to 60 V in 0.25 V increments, while recording the complete mass spectrum at each interval. By plotting the fraction of the parent and each fragment ion present as a function of collision energy, breakdown curves were produced.

2.2. Computational details

All possible tautomeric forms of the protonated nucleobases (C, G, A, T, and U) were considered. Optimization and frequency calculations (T = 298.15 K, P = 1 atm) were performed at the B3LYP level of theory using a 6–311++G(d,p) basis set as implemented in Gaussian 09 [35]. Harmonic frequency calculations were conducted for all tautomers to estimate thermochemical corrections to the DFT electronic energies. These calculations also generated harmonic vibrational spectra for the tautomers of $(C+H)^+$, $(T+H)^+$, and $(U+H)^+$ for comparison with the experimental IRMPD spectra reported in reference [20] as a means of validating our computational methodology. Using the calculated standard Gibbs' energies, the various tautomers were sorted energetically to determine the species most likely to be present in the probed ensembles. The four lowest energies tautomers of each protonated nucleobase were then carried forward for treatment with the coupled cluster single, double, and perturbative triple excitations method (*i.e.*, CCSD(T)/6–311++G(d,p) level of theory). These improved electronic energies were combined with the DFT thermochemical corrections to produce the standard Gibbs' energies that we report in this manuscript. Calculated structures and thermodynamic data are provided in the supporting information.

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