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NCO⁻, a key fragment upon dissociative electron attachment and electron transfer to pyrimidine bases: site selectivity for a slow decay process

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Abstract

We report gas phase studies on NCO^- fragment formation from the nucleobases thymine and uracil and their N-site methylated derivatives upon dissociative electron attachment (DEA) and through electron transfer in potassium collisions. For comparison, the NCO^- production in metastable decay of the nucleobases after deprotonation in matrix assisted laser desorption/ionization (MALDI) is also reported. We show that the delayed fragmentation of the dehydrogenated closed-shell anion into NCO^- upon DEA proceeds few microseconds after the electron attachment process, indicating a rather slow unimolecular decomposition. Utilizing partially methylated thymine we demonstrate that the remarkable site-selectivity of the initial hydrogen loss as a function of the electron energy is preserved in the prompt as well as the metastable NCO^- formation in DEA. Site selectivity in the NCO^- yield is also pronounced after deprotonation in MALDI, though distinctly different from that observed in DEA. This is discussed in terms of the different electronic states subjected to metastable decay in these experiments. In potassium collisions with 1- and 3-methylthymine and 1- and 3-methyluracil, the dominant fragment is the NCO^- ion and the branching ratios as a function of the collision energy show evidence of extraordinary site-selectivity in the reactions yielding its formation.

Introduction

Electron driven reactions are of interest in many areas of science and technology, including low-temperature plasmas, the interstellar and planetary atmospheres and dense interstellar clouds as well as biological processes and medicine, just to mention a few. Pioneering studies by Sanche and co-workers[1, 2] have shown that low-energy electrons (< 15 eV) can efficiently damage DNA by inducing single- and double-strand breaks. These alterations are initiated by dissociative electron attachment (DEA) processes with the initial capture of an electron leading to a temporary negative ion (TNI), which may then decompose by spontaneous ejection of the electron (autodetachment) or by dissociation into neutral and anionic fragments. Since these landmark studies on the resonant formation of DNA strand breaks by 3-15 eV electrons, several experimental and theoretical DEA studies with segments and building blocks of DNA and RNA, in the gas phase and deposited as thin films, have been reported.[3-10] Furthermore, previously site selective bond cleavage upon DEA to biologically relevant molecules was demonstrated for the nucleobases, thymine (T) and uracil (U)[11-13], as well as for smaller molecules such as methanol and some simple organic acids.[14] These experiments demonstrated that precise control of chemical reactions in DEA can be achieved by just tuning the initial electron energy. Electron induced dissociation was shown to be controllable not only for gas phase molecules but also by scanning tunneling electrons in molecules deposited on a surface.[15-17]

Meanwhile, such site selective bond cleavage upon low-energy electron attachment in the gas phase has also been observed for a number of other biomolecular systems like thymidine[18], ribose[19, 20], clusters of a few base pairs of DNA embedded in He droplets[21], amino acids[22-25] and peptides[26]. In the majority of these studies, the discussion has been focused on single bond cleavage reactions leading to the formation of the dehydrogenated closed-shell anion

[M – H][−] [11, 13, 18, 19, 22-26] and the complementary reaction leading to H[−]. [12, 14] The loss of a hydrogen atom is fast compared to ring cleavage and the excision of heavier fragments and hence, this reaction can compete efficiently with autodetachment (see also a recent study on time-resolved dynamics of the TNI for uracil [27]). In the case of the pyrimidine bases, hydrogen abstraction is observed at electron energies below 1 eV through site selective loss of the H atom from the N1 position, while electrons with incident energies between 1 and 3 eV are required to induce hydrogen loss from the N3 position. In contrast, H[−] formation is observed at higher electron energies between 5 and 12 eV and with a much lower cross section than the H neutral loss. It was further shown that other product anions are also formed upon DEA to the nucleobases thymine (T) and uracil (U) in this energy range, with NCO[−] being the most abundant. [28] In this context, it is worth mentioning that the neutral precursor NCO is a pseudo halogen with electron affinity that exceeds those of halogen atoms (~3.6 eV). In addition to the experimental efforts, extensive theoretical studies have also been performed in order to better understand the mechanism of DEA in nucleobases. These includes calculations of potential energy surfaces as a function of the appropriate internal coordinates by Li *et al.* [29] and Takayanagi *et al.* [30] using density functional theory (DFT) and more recently by González-Ramírez *et al.* using CASSCF/CASPT2 methodology. [31] Furthermore, Burrow *et al.* [32] have studied valence and dipole-bound anion formation, while Winstead and McKoy [33, 34] and Gianturco *et al.* [35] have performed theoretical calculations on the LEE interaction with uracil, revealing resonant features in the elastic scattering cross section (formed via shape resonances). More recently, also resonant features in inelastic scattering from thymine [36] and uracil [37] have been reported. These resonances represent excited states from electron capture with simultaneous valence shell excitation (so-called core excited resonances). Nevertheless, for nucleobases the assignment of DEA reso-

nances at higher electron energies to either higher lying shape resonances[35] or core excited resonances[38] is still debatable.

For isolated cases, there have been theoretical predictions of complex, concerted one step fragmentation after electron attachment, with even four-bonds breaking quasi simultaneously.[39] As discussed below in detail, however, NCO^- formation from the nucleobase anions is a complex unimolecular decomposition process, which is expected to occur on longer time scales and in more than one step. For few polyatomic molecules like valine or nitrobenzene a delayed (metastable) fragmentation extending to several microseconds after the attachment process has been reported recently.[23, 40-43] In the context of the metastable decomposition of biomolecular anions, a number of decay studies have also been reported on deprotonated biologically relevant anions formed in matrix assisted laser desorption/ionization (MALDI). These include studies on the metastable decay of the deprotonated nucleobases, nucleosides and the ribophosphate[44-47] as well as the sugars ribose[47] and fructose.[47,48] In these studies the focus resides on the role of the deprotonation site in determining the fragmentation path as reviewed in a recent colloquium[44]. It has been found that for thymine, NCO^- is the most significant fragmentation channel, which strongly depends on the deprotonation site.

Elementary ionization processes other than direct electron attachment and deprotonation are also active in biological systems and recently, several studies have been performed in order to understand how electron transfer in atom-molecule collisions can dictate the fragmentation pattern of biological components.[49] Thereby also remarkable site and bond selectivity have been shown, i.e., by tuning the collision energy, a selectivity for H^- abstraction from the N1 site vs the N3 site in the pyrimidine bases, as well as clear selectivity for the N-H vs the C-H bonds[49] were achieved. Furthermore, in recent potassium-uracil collision studies[50], we have shown that for

intermediate energies (above 30 eV in the lab frame), the most intense fragment anion formed is NCO^- , whereas, for DEA studies in the low energy region, (< 4 eV), the loss of an hydrogen atom from the parent anion, i.e., $[\text{U-H}]^-$ formation, is the most prominent.[51]

A prerequisite in the formation of NCO^- from a pyrimidine base, irrespective if the initial ion formation process is electron attachment, electron transfer, or MALDI, is the loss of at least one hydrogen atom from a nitrogen site and a two-fold cleavage of the ring. This reaction sequence represents a complex unimolecular decomposition process, which occurs on a much longer time scale than simple bond cleavage. In the present study we report the NCO^- formation from thymine, uracil and their N-methylated counterparts upon DEA and collisions with potassium atoms, as well as in the unimolecular decomposition of $[\text{M-H}]^-$ after hydrogen abstraction in DEA and deprotonation in MALDI. To get insight into the fragmentation pathways and to elucidate site selective bond cleavage processes, 1-methylthymine (1-meT), 3-methylthymine (3-meT), 1-methyluracil (1-meU) and 3-methyluracil (3-meU) were used to clearly distinguish between different sites.

Experimental

The present work has been carried out using three different crossed beam apparatus, two at the Institute for Ion Physics and Applied Physics in Innsbruck and one at the Atomic and Molecular Collisions Laboratory in Lisbon as well as a MALDI instrument at the Science Institute of the University of Iceland. In both Innsbruck experiments, a molecular effusive beam of thymine and the partially methylated analogous was formed by evaporation of their pristine powder (purchased from Sigma Aldrich or synthesized as described in[44]) from an electrically heated oven kept in vacuum. The effusive beam of molecules was crossed with an electron beam from a

standard Nier type ion source[26] in one of the instruments and from a hemispherical monochromator in the other.[12] In the latter setup the electron current was about 30 nA with a resolution of about 120 meV full width at half maximum (FWHM). In that setup, anions formed were mass analyzed with a quadrupole mass spectrometer and detected with a channeltron type secondary electron multiplier. In the setup with the Nier type ion source the electron current was about 10 μ A with a resolution of about 1 eV (FWHM) and the anions formed were accelerated by an 8 kV potential into a sector field mass spectrometer. After passing the first field-free-region (ff1) the anions were analyzed by their momentum in a magnetic sector before entering a second field-free-region (ff2). After ff2, the anions were analyzed by their kinetic energy in an electric sector. The mass and energy analyzed anions were detected with a channeltron type secondary electron multiplier. This instrument was utilized to probe the stability of anions on the μ s time scale. A metastable decay of a precursor anion with mass m_p in the ff1 is determined by variation of the acceleration voltage U . A metastable decay product in the ff1 (with mass m_f) will keep its velocity; however, the kinetic energy is different from that of the parent anion. Thus a decay product with m_f will only reach the detector if the high voltage is increased to $U_f = U \cdot m_p/m_f$. Decay reactions in the second field free region ff2 between the magnetic and electric sector are probed using the mass analyzed ion kinetic energy (MIKE) scan method.[43] The following relationship between the ratio of the precursor mass m_p and the resulting fragment ion mass m_f and the ratio of the corresponding electric sector field voltages allows the unambiguous assignment of the mass of the fragment ion:

$$\frac{E}{E_0} = \frac{m_f}{m_p} \quad (2)$$

In the Lisbon laboratory, the negative ion time-of-flight (ToF) mass spectra were obtained by neutral potassium atom collisions with 1- and 3-methylthymine and 1- and 3-methyluracil in a crossed beam experimental set-up described elsewhere.[53] Briefly, an effusive molecular beam crosses a primary beam of neutral potassium (K) atoms. In this setup K^+ ions produced in a potassium ion source were accelerated to 30-100 eV, before passing through an oven where they resonantly charge exchange with neutral potassium to produce a beam of hyperthermal atoms. Residual ions from the primary beam were removed by electrostatic deflecting plates outside the oven. The intensity of the neutral potassium beam was monitored using a Langmuir-Taylor ionization detector, before and after the ToF mass spectra collection, with typical values on the order of tens of picoampere. The effusive beam of methylated uracil or thymine was introduced into a 1 mm diameter capillary where it was crossed with the neutral hyperthermal potassium beam between two parallel plates with 1.2 cm separation. The anions produced were extracted by a 250 Vcm⁻¹ pulsed electrostatic field. The K^+ beam was pulsed with 2 μ s width and an adjusted frequency depending on the collision energy, typically 222 kHz for 100 eV. The typical base pressure in the collision chamber was 8×10^{-6} Pa and the working pressure did not rise significantly upon heating the powder samples. Mass spectra were obtained by subtracting the background measurements (without heated sample) from sample measurements. The mass calibration was carried out on the basis of the well-known anionic species formed after potassium collisions with the nitromethane molecule.[53] The solid samples were purchased from Sigma-Aldrich with a minimum purity of $\geq 99\%$ or synthesized as described in ref.[44]. They were used as delivered and evaporated at temperatures between 393K and 413K depending on the sample. The heating temperature was controlled using a PID unit. In order to test for any thermal decomposition, the spectra were recorded at different temperatures. No differences were observed in relative peak

intensities as a function of the temperatures. The extraction region and the ToF system were heated to approximately 393K throughout the measurements in order to prevent any sample deposition and thence charge accumulation on the electrodes.

Metastable decay of the deprotonated thymine and thymine derivatives was measured in a negative ion, post source decay (PSD) mode with a UV MALDI mass spectrometer (MALDI-MS) (Reflex IV, Bruker Daltonics, Bremen, Germany). The instrument and its operation have been described in detail elsewhere.[54] In brief, ions were generated in the MALDI process with an N2-laser at 337 nm. The repetition rate was 10 Hz and the power was kept about 20% above the detection threshold for the corresponding parent ions. Negative ions were extracted in pulsed delayed extraction mode with a delay time of 200 ns and accelerated into a reflectron time-of-flight (ToF) mass spectrometer. The parent ions were selected and gated into the field free region of the mass spectrometer for PSD spectra recording. The mass gate was set at ± 5 Da in all experiments. The total acceleration voltage into the field free linear region was 25 kV resulting in about 6-9 μ s flight time. The observed metastable decay occurs within this time window. After the linear flight the ions were reflected using a grid-less ion mirror and detected with a double micro-channel plate detector. The reflectron voltage was stepped down in 7 segments to assure for collection of all fragments. Individual segments are the sum of 500 individual shots, which were recorded by using the fragmentation analyses and structural ToF method FAST, within the instrumental control software FlexControl®. The alignment of individual segments and the mass calibration of the spectra were carried out with the FlexAnalyses® software also provided by the instrument manufacturer.

The sample molecules thymine and 1-methylthymine were acquired from Sigma-aldrich, Germany as high purity samples (> 98%) and used without further purification. The thymine derivative 3-methylthymine was prepared in-house from thymine. The preparation and purification was performed as described by Breeger *et al.*[55] and the structure was verified by H-NMR. Samples were prepared for measurements by co-spotting 0.5 μ L of a 1 mg/mL aqueous solution of the matrix bisbenzimidazole hydrochloride; C₂₅H₂₄N₆O•3HCl ($\geq$ 98%, Sigma-Aldrich, Germany) and 0.5 μ L of a 10 mg/mL solution of the nucleobase in methanol on a stainless steel sample carrier.

Results and discussion

The most abundant fragment anion upon DEA to isolated thymine in the electron energy range above $\sim$ 4 eV is NCO⁻. This is also the most abundant fragment ion in metastable decay of deprotonated thymine and the other pyrimidine bases such as uracil and cytosine when these are formed in MALDI[44, 46], as well as in potassium-uracil and -thymine collisions in the energy range from 30-100 eV.[50] Since NCO⁻ is formed efficiently upon attachment of a free electron, the dominant role of the NCO⁻ formation in potassium collision may seem obvious. However, in potassium collisions this channel is even enhanced compared to DEA since K⁺ can efficiently delay autodetachment in the collision complex due to considerable coulomb interaction. Therefore, the energy can be redistributed through the different degrees of freedom yielding a rather statistical dissociation in detriment of direct dissociation.[50]

Figure 1 shows the three possible pathways leading to NCO⁻ formation, from [M - H]⁻, formed after hydrogen abstraction upon electron attachment. Also given are the threshold energies, E_{th}, for each reaction step calculated at the B2PLYP/ma-TZVP level of theory [56,57]. In our calculations for reaction path c and d the energy minimization of the open chain neutral moiety sponta-

neously finds its way to a local minimum four-membered ring structure. For clarity the open chain geometries are also shown, though these are not necessary intermediates or transition states on these reaction paths. In addition to the threshold energies, also the height of the reaction barrier, E_{TS} associated with the opening of this four-membered ring to form the lowest energy open chain structure, is shown. This barrier is calculated at the B3LYP/6-311G(d,p) [58,59] level of theory using the nudged elastic band method [60] and the single point energy of the transition state geometry is then calculated at the B2PLYP/ma-TZVP level of theory. For further detail on the calculations, please see Online Resource 1. In good agreement with the energy dependency of the observed hydrogen abstraction [32] we find the thermochemical threshold for loss of the a H atom from the N1 and N3 position, to be 0.89 and 1.38 eV respectively. The reaction enthalpy for the NCO^- formation through electron attachment to the neutral thymine is equal to the sum of the threshold energies for reaction path b) and e), i.e., 3.47 eV. The threshold energy for the formation of the local minima four-membered ring on the other hand is 3.87 eV, independent of the route, and the opening of the four-membered ring is associated with a barrier of 0.35 eV.

Figure 1. Reaction scheme showing the three different possible reaction pathways leading to NCO^- formation in thymine upon electron capture, along with the threshold energies for individual reaction steps calculated at the B2PLYP/ma-TZVP level of theory.[56,57] a) H loss from N1; b) H loss from N3; c), d) and e) pathways leading to NCO^- formation. For reaction path c and d, energy minimization of the neutral moiety leads to a local minimum four-membered ring structure. For clarity the open chain geometries along these reaction paths are also shown, though these are not necessarily intermediates or transition states on these paths. The energy barrier, E_{TS} , calculated with the nudged elastic band method [60] for the opening of this four-membered ring structure to form the lowest energy open chain structure is also given.

1) NCO^- formation upon DEA to thymine and 1- and 3-methylthymine

Figure 2a shows the anion efficiency curve of NCO^- formed upon free electron attachment to the pyrimidine base thymine (T). The anion yield exhibits a rich spectrum composed of a number of

overlapping resonances. The corresponding resonant features are located at about 2.3, 4.4, 6.1, 6.8, 8.1 and 9.7 eV. The ion yield shown represents the “prompt” ion yield, i.e. the decay of the TNI has occurred in the ion source and thus on a time scale $< \sim 1 \mu\text{s}$. The thermochemical threshold calculated for the formation of NCO^- through the sequential decay mechanism shown in Figure 1 matches very well with the experimental onset of the second resonance at 4.4 eV. From the vibrational frequencies calculated here we derive an internal energy of about 0.4 eV for the neutral precursor, i.e the first weak resonance observed at 2.3 eV (onset ~ 1.5 eV) must be formed by another reaction. The prompt NCO^- ion yields for 1-meT and 3-meT, shown in Figure 2b and c, respectively, exhibit similarities to the anion efficiency curves of NCO^- from T, i.e., a series of resonances up to about 12 eV. Remarkably, though the resonance energies seem to be very similar for the three molecules, the relative yield through these resonances varies strongly between the three molecules, e.g., the most abundant resonance in T at about 6.8 eV becomes less pronounced relative to the yield at higher energies when going from $\text{T} \rightarrow \text{1-meT} \rightarrow \text{3-meT}$.

Figure 2. Prompt anion yields for NCO^- formed upon DEA to a) thymine (T), b) thymine methylated at N1 (1-meT), and c) thymine methylated at N3 (3-meT), measured with the electron monochromator.

For the pyrimidine bases the most abundant DEA fragment is the dehydrogenated closed-shell anion (here for thymine referred to as $[\text{T} - \text{H}]^-$). It is formed via a series of narrow features at electron energies below 3 eV, which are assigned to Vibrational Feshbach resonances (VFr's).[32,

61] Figure 3a shows the measurement of $[T - H]^-$ with the sector field instrument where VFr's are not resolved due to the lower energy resolution compared to the electron monochromator used in the previous study.[31] Measurements with partially deuterated molecules showed further, that in the $[T - H]^-$ formation through DEA, exclusively hydrogen atoms from the nitrogen sites are removed.[11] In the case of 1-methylthymine, hydrogen can only be removed from the N3 position and the $[1\text{-meT} - H]^-$ anion efficiency curve showed a smooth asymmetric resonance with a threshold at about 1.4 eV.[32] Thus the VFr's result only in H loss from the N1 site while the broad resonance at 1.6 eV leads to loss of hydrogen from the N3 position and is ascribed to a resonance involving a mixing of the π_2^* and σ_2^* virtual orbitals of the neutral precursor.[32] Similarly, the relative intensity of resonances in the NCO^- ion yield changes through the N1 and N3 methylation (see Figure 2). The most abundant resonance in T at about 6.8 eV becomes much less pronounced relative to the resonances at 4.4, 6.1 and 9.7 eV when going from $T \rightarrow 1\text{-meT}$. In the case of 3-meT the resonance at 6.8 eV is almost suppressed. The resonance at 8.1 eV disappears upon both N1 and N3 methylation, while the resonances at about 6.8 and 9.7 eV seemingly remain unaffected. The low signal/noise ratio of the measurements for the methylated sample prevents to derive the tendency for the weak resonance at 2.3 eV.

Figure 3. Panel a) Prompt ion yields for $[T-H]^-$ (dotted line), NCO^- (solid line) and H^- (dash-dotted line) formed upon DEA to thymine. Panel b) Metastable anion yields for the decomposition of $[T-H]^-$ from thymine (T) into NCO^- in the first (solid) and second (dashed line) field free region of the double-sector field mass spectrometer. Panel c) Same decay channel like for middle panel for 1-methylthymine (1-meT) and 3-methylthymine (3-meT). All anion yields shown were measured with the sector field instrument.

Assuming a partly sequential decay mechanism for the NCO^- formation, the dehydrogenated parent anion $[T-H]^-$ is the most probable intermediate (see Figure 1). Figure 3a) compares the ion yield of the two prompt anions $[T-H]^-$ and NCO^- formed upon DEA to T. The relative abun-

dance of these two anions depends strongly on the initial electron energy. At electron energies $< \sim 4$ eV, $[T - H]^-$ is more abundant than NCO^- . At higher electron energies, however, the internal energy of $[T - H]^-$ becomes sufficiently large that decomposition into NCO^- , and other low-mass anions with much weaker abundance (not discussed here) dominates. Hence, at higher incident energies, NCO^- can be formed through prompt dissociation of the TNI or through further metastable decay of $[T - H]^-$. Polyatomic molecules can accommodate a substantial amount of internal energy in their vibrational degrees of freedom and vibrational predissociation leads often to a delayed decomposition of these ions extending to the microsecond regime. In the case discussed here the metastable decomposition of $[T - H]^-$ into low-mass fragments including NCO^- can be observed mass spectrometrically. Indeed, in the present experiment we obtain direct evidence for metastable decays into NCO^- using the scan techniques introduced in the experimental section. Figure 3b) and 3c) show the electron energy dependence of the NCO^- formation through metastable decay of $[T - H]^-$, $[1\text{-meT} - H]^-$ and $[3\text{-meT} - H]^-$ formed through DEA. In the first and second field free region of the mass spectrometer the NCO^- yield shows 3 peaks located at 3.5, 5.5 and 8.8 eV. For the presently set acceleration voltage of 8 kV the time that elapsed from the formation of $[M - H]^-$ and the ion entering and exiting the two field free regions are 1 and 10 μs for the first field free region and 19 and 31.6 μs for the second field free region, respectively. The broad high-energy peak between about 8 and 11 eV is weaker for the formation of NCO^- at later times, which reflects a reduced stability of the precursor ion $[T - H]^-$ at higher electron energies, i.e. faster fragmentation.

Remarkably, however, for 1-meT the metastable NCO^- yield from $[M - H]^-$ only shows a contribution in the low energy range which, within the accuracy of the measurement, we ascribe to the metastable decay peak observed here at 3.5 eV for T. In addition, this metastable yield coincides

well with the high energy part of the $[M - H]^-$ yield from 1-meT through initial occupation of the antibonding π_2^*/σ_2^* molecular orbitals (MO).[32] In 3-meT, on the other hand, the metastable decay of $[M - H]^-$ to form NCO^- is confined to the two resonances at higher electron energies observed in the metastable decay of $[T - H]^-$. The resonance close to 5.5 eV coincides with a resonance observed in the $[M - H]^-$ yield from T (see Fig. 3a). The resonant metastable yields observed for 1-meT and 3-meT (Fig. 3c) show clearly that both the N1 and N3 dehydrogenation sites in the thymine anion contribute to the formation of NCO^- in the metastable decay of $[M - H]^-$ formed through DEA. Furthermore, it is also clear from Figure 3 and the thermochemical thresholds in Figure 1, that the site selectivity of the energy dependency of the NCO^- formation after dehydrogenation from N1 and N3 respectively, is not due to thermochemical restrictions of the respective processes. On the contrary, this site selectivity in the metastable NCO^- formation is clearly the result of the different nature of the resonances leading to the initial hydrogen abstraction. The respective contribution from both sites can thus be tuned by the initial electron energy. One may also correlate the three metastable peaks observed in Fig. 3b) with the prompt NCO^- ion yield shown for T in Figure 2a). Assuming that the prompt peaks are shifted slightly by few hundred meV upwards compared to the corresponding metastable peaks (due to a higher excess energy responsible for the prompt decay), we assign the prompt resonances at 4.4, 6.1 and 9.7 eV to the electronic states decaying also in the metastable time regime.

2) NCO^- formation upon deprotonation of thymine, 1- and 3-methylthymine in MALDI

Figure 4 compares the metastable decay spectra of $[T - H]^-$, $[1-meT - H]^-$ and $[3-meT - H]^-$ formed through deprotonation in MALDI. The figure shows that NCO^- formation in MALDI only proceeds from T and 1-meT, but is not observed from 3-meT.

Figure 4. MALDI post source decay spectra of the metastable decay of deprotonated (a) thymine (T), (b) 1-methylthymine (1-meT) and (c) 3-methylthymine (3-meT). While NCO^- is observed from the native thymine and from 1-meT, no such fragmentation is observed from 3-meT. Adapted from ref [46].

Hence, initial deprotonation at the N3 site is a prerequisite for the NCO^- formation in MALDI and no further decay is observed after deprotonation at N1. This is very different from the metastable decay of T, 1-meT and 3-meT after hydrogen loss upon DEA, where NCO^- formation is observed from $[1\text{-meT} - \text{H}]^-$ as well as $[3\text{-meT} - \text{H}]^-$. Furthermore, similar to the MALDI experiments, classical dynamics simulations of the decay of the deprotonated species in their electronic ground state [44, 46] show NCO^- formation nearly exclusively after deprotonation from the N3 site. Hence, the classical dynamics simulations showed NCO^- formation in four out of ten of the trajectories simulated for thymine deprotonation at N3, but for thymine deprotonation at N1, only one out of ten simulations resulted in NCO^- formation. Similarly, nine out of ten trajectories simulated for $[1\text{-meT} - \text{H}]^-$ resulted in NCO^- formation but no fragmentation was observed for $[3\text{-meT} - \text{H}]^-$. In MALDI the deprotonated anion is formed through proton transfer under multiple

collision condition in the expanding plume of matrix and analyte molecules, and the resulting internal energy of small deprotonated molecular anions produced in this process is estimated to be around 4-5 eV.[62, 63] Consequently, these deprotonated ions are formed in their electronic ground state but with considerable internal energy. Similarly the classical dynamics simulations are based on the condition that the anions have reached the thermally equilibrated electronic ground state before dissociation occurs and considerable internal energy is added to accelerate the trajectories. In the case of thymine, and its methylated analogs the internal energy added was 8 eV (above room temperature). Hence, both in the MALDI experiment and in the classical dynamics simulations, the internal energy of the deprotonated parent ions is considerably higher than the thermochemical threshold for their metastable decay, independent of the deprotonation site (see step c, d and e in Figure 1). Nonetheless, in MALDI and in our classical dynamics simulations NCO^- is only observed after deprotonation from N3 (with the exception of one trajectory in the simulations). We note that such statistical decay model was previously applied successfully for the metastable decay of the amino acid valine.[23] In the study for valine, the decomposition process $[\text{M} - \text{H}]^- \rightarrow \text{HCOO}^- + \text{neutrals}$, was examined for $[\text{M} - \text{H}]^-$ formed through DEA and in MALDI, and it was found to be independent of the formation mechanism of $[\text{M} - \text{H}]^-$, i.e. if it was formed by deprotonation in MALDI or DEA. In the presently studied decay for thymine, a purely statistical decay model seems to be applicable only in a limited way for $[\text{M} - \text{H}]^-$ formed in the DEA process since it misses the metastable decay starting from H-loss from the N1 position. As mentioned above, in the case of hydrogen loss from the N3 position in DEA the TNI is initially formed through a single electron occupation of the low lying π_2^* orbital, mixing with the repulsive σ_2^* . The TNI then relaxes through hydrogen loss to the electronic ground state of $[\text{M} - \text{H}]^-$. The bulk of the excess energy brought in by the attaching electron and through the electron

affinity of $[M - H]^-$ (3.5 eV) is distributed within the internal degrees of freedom of the $[M - H]^-$ fragment. Correspondingly the metastable NCO^- formation is observed from the higher energy side of the resonance where sufficient internal energy is available for further fragmentation. Metastable decay from $[M - H]^-$ formed through hydrogen loss from N1, on the other hand, proceeds exclusively when the TNI leading to the precursor ion, $[M - H]^-$, is formed through a core excited resonance located close to 5.5 eV. Hence, here the TNI leading to hydrogen loss is formed in an electronically excited state with two electrons in a formerly unoccupied MO and a single vacancy in a previously occupied MO. This may also lead to the formation of $[M - H]^{-*}$, an electronically excited state of the dehydrogenated molecular ion. In that case, further fragmentation to NCO^- after hydrogen loss from N1 proceeds from an electronically excited state, which in turn explains the absence of this channel in metastable decay of the ground state precursor ions. We also note that the metastable peak at about 5.5 eV matches well with the DEA resonance for H^- formation from the N1 position (see Figure 3a). Thus we tentatively assign a common electronically excited TNI state as a precursor for the fragment anions formed at this energy.

3) NCO^- formation upon collisions of potassium atoms with 1- and 3-methyluracil and 1- and 3-methyl thymine

Figure 5 shows the negative ion ToF mass spectrum of 1-methyl uracil recorded at 30 eV (lab frame) potassium impact energies, where the most intense anion is assigned to NCO^- . Apart from NCO^- , also a number of other, lower intensity fragment anions have been observed in potassium collisions with pyrimidine bases as thymine and uracil at 30, 70 and 100 eV collision energies (see e.g. Almeida *et al* [64]). In this contribution, however, we limit our discussion to NCO^- . In order to have a conclusive set of data on the NCO^- formation, several mass spectra of the four

different methylated pyrimidine bases have been recorded in the energy range from 30 to 100 eV. These provide information on the site and bond selectivity of fragmentation pathways involved in the decomposition of the TNI of the pyrimidine bases, when these are formed in potassium collisions. Recently, we have shown that in potassium collisions with methylated pyrimidine bases, H^- formation can be completely inactivated when the N-H bond is replaced by N-CH₃. [49] Accordingly, the mechanism leading to NCO^- formation can be interpreted as a multiple step mechanism initiated by the loss of the hydrogen atom from the N1 and/or the N3 positions. In electron transfer experiments on potassium collisions with thymine (T) and uracil (U), it was proposed that the dehydrogenated parent anion $[M-H]^-$ is indeed a precursor in the formation of the fragments like NCO^- that require bond cleavages in the ring. [50] Thus the initial position of the hydrogen loss will dictate the fragmentation yield as we shall show here.

Figure 5. Negative ion Time-of-flight mass spectrum of ions formed in collisions of potassium atoms with 1-meU at 30 eV lab frame collision energy.

In the present discussion we concentrate on the possible fragmentation pathways shown in Figure 1 and will not explore reactions initiated by hydrogen loss from the C5 and/or C6 positions,

which are energetically much less favorable (threshold energy > 2 eV).[29] However, initial hydrogen loss from the carbon positions, while requiring a more complex mechanism for the NCO^- formation, may be dominating at higher collision energies (> 100 eV).[50]

Figure 6. NCO⁻ branching ratios in collisions of potassium atoms with N-site methylated uracil and thymine molecules. The dotted lines are just to guide the eye.

We see from the possible pathways in Figure 1 that after electron transfer and formation of a TNI, the system can evolve through paths a) and/or b) with H loss from the N1 and/or the N3 position, respectively. In the case that the loss of hydrogen stems from the N3 position, NCO^- formation may further proceed along d) and/or e), whereas abstraction from the N1 position leads to a NCO^- formation through c) only. In order to evaluate the contribution of such paths, NCO^- branching ratios (i.e. $\text{OCN}^-/\sum X^-$ where the denominator corresponds to the sum of the ion yields of all fragment anions) were obtained as function of the collision energy for the four methylated pyrimidines 1- and 3-meT and 1- and 3-meU. The resulting ratios are plotted in Figure 6. In the case of 1-meU and 3-meU the relative difference between the two molecules is approximately 0.1 for all collision energies with the exception of 70 eV. Therefore, NCO^- formation from potassium collisions with 1-meU is more favorable than in the case of 3-meU. In the case of 1-meU, the decomposition process is initiated by the loss of hydrogen from the N3 position leading to the intermediate $[1\text{-meU} - \text{H}]^-$ anion formation. The reaction will then follow path d) and/or e), whereas path c) will remain blocked. In contrast, for 3-meU the only pathway that is opened is c) since methylation in the N3 position suppresses NCO^- formation from paths d) and e). As a result we observe a lower NCO^- branching ratio for 3-meU in comparison with 1-meU.

In the lower panel of Figure 6, we show the results from collisions of potassium atoms with 1-meT and 3-meT. A close inspection of this figure shows remarkably similar NCO^- branching ratios for collision energies up to 50 eV, indicating very limited site selectivity at low collision energies. For higher collision energies (>50 eV), on the other hand, a distinct site selectivity, favoring NCO^- formation from 1-meT, appears.

For collision energies below 50 eV, which correspond to collision times longer than 40 fs, the potassium cation formed after electron transfer to the target molecule may be present sufficiently long in order to stabilize the TNI through coulomb interaction within the collision complex. This ensures that pathways d) and e) are open together with pathway c). This rationale can be interpreted in terms of the spatial orientation of the molecular orbitals. The incoming electron can be trapped in the C5 methyl group position π^* orbital, with time enough to be transferred to σ^* N-CH₃ from the N3 position methyl group. This assumption seems reasonable since a similar transition has been described recently by Almeida *et al.* [64], in context to the loss of a methyl group in potassium collisions with 1-meT and 3-meU. Therefore, in this unimolecular decomposition such coupling will allow accessing the three pathways c), d) and e) in Figure 1, which in turn means similar NCO⁻ branching ratio for energies up to 50 eV. For higher collision energies (70 and 100 eV), the K⁺ stabilizing effect will not be effective enough, due to its reduced transit time, and this coupling may no longer hold. In this energy range, the relative difference between the NCO⁻ branching ratios from 1-meT and 3-meT, is thus increased to about 0.2 in favor of NCO⁻ formation from 1-meT. This effect is mostly noticeable for energies above 50 eV and these results show an unprecedented site- and bond-selectivity regarding the concerted fragmentation mechanism yielding NCO⁻ formation in DNA pyrimidine bases upon electron transfer.

Conclusion

In this work we presented negative ion formation in N-site methylated pyrimidine derivatives through DEA, MALDI, and potassium-molecule collisions experiments, with particular focus on NCO⁻ formation. This is the dominant fragment anion formed upon DEA to gas phase thymine at electron energies $\sim$ 4eV, the only fragment observed in metastable decay of deprotonated thymine

in MALDI, and, in the case of potassium-N-site methylated pyrimidine collisions, NCO^- is the most abundant fragment for collision energies above 30 eV.

A previous free electron attachment study with thymine embedded in cold helium droplets[65] showed that $[\text{T} - \text{H}]^-$ is the only fragment anion formed when an electron attaches to thymine in the droplet. In addition, the anion efficiency curve of $[\text{T} - \text{H}]^-$ upon DEA to superfluid helium droplets doped with thymine matches well with the total anion yield obtained for DEA to gas phase thymine. This led to the conclusion that, due the highly efficient energy dissipation to the surrounding matrix, the formation of all low-mass anions is quenched in helium droplets in favor of the $[\text{T} - \text{H}]^-$ formation. [65] The present DEA results directly confirm that NCO^- is formed in a sequential decay reaction with the dehydrogenated closed shell anion as the intermediate product. Previously it was assumed that multistep bond cleavage reactions, such as the NCO^- formation studied here are less selective than single bond dissociations.[39] Here we show that the remarkable site selectivity in the H^- and $[\text{M} - \text{H}]^-$ reaction channels in the pyrimidine bases is preserved for the subsequent decay reaction into NCO^- . Furthermore, this is true even if the decay takes place several tens of microseconds after the initial electron attachment process. Comparison with MALDI results shows that the contribution to the metastable anion yield at ~ 5.5 eV in the DEA experiments (and thus also in the prompt ion yields of H^- and $[\text{M} - \text{H}]^-$) results from a core excited resonance and is thus not observable in the statistical decay of the electronic ground state in MALDI and in classical dynamics simulations. The present results show that not only the total amount of internal energy determines the extent of this metastable decay channel but also the initial electronic state of the TNI leading to the formation of the $[\text{T} - \text{H}]^-$ precursor ion.

From the electron transfer experiments in potassium collisions with N-site methylated pyrimidines, NCO^- branching ratios obtained show remarkable site- and bond-selectivity in this decay channel. In the case of N-site methylated uracil, the selectivity is verified for all collision energies. However for N-site methylated thymine the selectivity depends strongly on the collision energy. The dwell time of the potassium cation in the coulomb complex will dictate the fragmentation pathways leading to the NCO^- formation. For longer collision times some fragmentation channels are not accessible without cation stabilization, while for shorter collision times, they are blocked. In the latter situation, the selectivity of the NCO^- formation is clearly visible. To our knowledge the studies presented here show for the first time that site-selectivity in molecular fragmentation up on electron transfer can be conserved, even in complex, concerted reaction mechanisms that require multiple bond breaking.

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Reaction scheme for the thermal decomposition of 4-methyl-2,6-pyridinedione (1):

- Pathway a):** Direct thermal decomposition of 1 (4-methyl-2,6-pyridinedione) with $E_{th}^{H(LOH)} = 0.89 \text{ eV}$ to form a radical cation intermediate (N1), which then loses NCO^- with $E_{th}^{M(D)} = 2.09 \text{ eV}$ to form a 4-methyl-2,6-pyridinedione radical cation (N1 or 3).
- Pathway b):** Initial loss of NCO^- from 1 with $E_{th}^{H(LOH)} = 1.38 \text{ eV}$ to form a radical cation intermediate (N3-), which then loses NCO^- with $E_{th}^{M(D)} = 2.50 \text{ eV}$ to form a 4-methyl-2,6-pyridinedione radical cation (N1 or 3).
- Final Product:** A 4-methyl-2,6-pyridinedione radical cation (N1 or 3) with $E_{15} = 0.35 \text{ eV}$.

The energy axis is labeled **Energy**.

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure captions

Figure 1. Reaction scheme showing the three different possible reaction pathways leading to NCO^- formation in thymine upon electron capture, along with the threshold energies for individual reaction steps calculated at the B2PLYP/ma-TZVP level of theory.[56,57] a) H loss from N1; b) H loss from N3; c), d) and e) pathways leading to NCO^- formation. For reaction path c and d, energy minimization of the neutral moiety leads to a local minimum four-membered ring structure. For clarity the open chain geometries along these reaction paths are also shown, though these are not necessarily intermediates or transition states on these paths. The energy barrier, E_{TS} , calculated with the nudged elastic band method [60] for the opening of this four-membered ring structure to form the lowest energy open chain structure is also given.

Figure 2. Prompt anion yields for NCO^- formed upon DEA to a) thymine (T), b) thymine methylated at N1 (1-meT), and c) thymine methylated at N3 (3-meT), measured with the electron monochromator.

Figure 3. Panel a) Prompt ion yields for $[\text{T-H}]^-$ (dotted line), NCO^- (solid line) and H^- (dash-dotted line) formed upon DEA to thymine. Panel b) Metastable anion yields for the decomposition of $[\text{T-H}]^-$ from thymine (T) into NCO^- in the first (solid) and second (dashed line) field free region of the double-sector field mass spectrometer. Panel c) Same decay channel like for middle panel for 1-methylthymine (1-meT) and 3-methylthymine (3-meT). All anion yields shown were measured with the sector field instrument.

Figure 4. MALDI post source decay spectra of the metastable decay of deprotonated (a) thymine (T), (b) 1-methylthymine (1-meT) and (c) 3-methylthymine (3-meT). While NCO^- is observed

from the native thymine and from 1-meT, no such fragmentation is observed from 3-meT. Adapted from ref [46].

Figure 5. Negative ion Time-of-flight mass spectrum of ions formed in collisions of potassium atoms with 1-meU at 30 eV lab frame collision energy.

Figure 6. NCO^- branching ratios in collisions of potassium atoms with N-site methylated uracil and thymine molecules. The dotted lines are just to guide the eye.