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Unexpected linear ion trap collision-induced dissociation and Fourier transform ion cyclotron resonance infrared multi-photon dissociation fragmentation of a hydrated C-glycoside of 5-fluorouridine formed by the action of the pseudouridine synthases RluA and TruB

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Abstract

As part of the investigation of the pseudouridine synthases, 5-fluorouridine in RNA was employed as a mechanistic probe. The hydrated, rearranged product of 5-fluorouridine was isolated as part of a dinucleotide and found to undergo unusual fragmentation during mass spectrometry, with the facile loss of HNCO from the product pyrimidine ring favored over phosphodiester bond rupture. Although the loss of HNCO from uridine and pseudouridine is well established, the pericyclic process leading to their fragmentation cannot operate with the saturated pyrimidine ring in the product of 5-fluorouridine. Based on the MSⁿ results and calculations reported here, a new mechanism relying on the peculiar disposition of the functional groups of the product pyrimidine ring is proposed to account for the unusually facile fragmentation.

The isomerization of uridine to pseudouridine (Ψ) is the most common post-transcriptional modification of RNA and is carried out by the pseudouridine synthase (ΨS) superfamily of enzymes.^[1,2] This internal transglycosylation occurs by breaking the C1'–N1 glycosidic bond of uridine, rotation of the free nucleobase, and reattachment to form a C1'–C5 bond (Fig. 1). To elucidate the chemical mechanism, various ΨS have been incubated with RNA in which the reactive uridine has been replaced by 5-fluorouridine (F⁵U).^[3–7] The F⁵U is isomerized to the C-glycoside and hydrated at C6;^[5,8] additionally, C2' of F⁵U suffers epimerization so that two products are formed (Fig. 2).^[9] Because MS cannot distinguish these isomeric products and the site of difference is left intact by the unusual fragmentation described below, the products of F⁵U will be abbreviated simply as F⁵U*.^a

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SUPPORTING INFORMATION

Supporting information can be found in the online version of this article.

The F⁵U* is isolated by digesting the modified RNA with S1 nuclease and alkaline phosphatase followed by preparatory scale reversed-phase high-performance liquid chromatography (HPLC); F⁵U* is isolated as the 5'-component of a dinucleotide with the nucleoside (C in the TruB substrate, U in the RluA substrate) following F⁵U, which was confirmed by mass spectrometry (MS) and nuclear magnetic resonance (NMR).^[7] Typical fragmentations of canonical nucleotides involve depyrimidination, depurination, or phosphodiester cleavage,^[10] but discrete fragmentations of the F⁵U* nucleobase account for most of the ions detected (Fig. 3(a)). Especially interesting is the apparently unprecedented loss of a neutral HNCO (43 Da) *before* phosphodiester cleavage. Although the loss of neutral HNCO from pyrimidine rings is common^[11,12] and was reported in the original MS characterization of uridine and pseudouridine,^[13–18] they lose HCNO in a pericyclic mechanism (retro-Diels-Alder reaction) that cannot operate in the case of F⁵U*. Tandem mass spectrometry experiments complemented with quantum chemical calculations were employed to investigate this unusually facile loss of HCNO from a nucleobase and the mechanism giving rise to it.

EXPERIMENTAL

General

RluA and TruB were overexpressed and purified from *E. coli* as described previously.^[19–21] The anti-codon stem-loop (ASL) from *E. coli* tRNA^{Phe} and the T-arm stem-loop (TSL) from *S. cerevisiae* containing 5-fluorouridine (F⁵U) in place of the reactive uridine ([F⁵U]ASL, GGGGAF⁵UUGAAAUCCCC; [F⁵U]TSL, CUGUGUF⁵U CGAUCCACAG) were purchased from Dharmacon, Inc. (Lafayette, CO, USA) and deprotected according to the manufacturer's instructions. All experiments were performed with water (18.2 MΩ) from a Milli-Q Synthesis system (Millipore, Billerica, MA, USA).

Sample preparation

Samples of product dinucleotides were generated as described in detail elsewhere.^[7] Briefly, RluA (200 μM) and [F⁵U]RNA (150 μM) were incubated at 37 °C for 3 h in 50 mM HEPES buffer (1.6 mL), pH 7.5, containing ammonium chloride (100 mM), dithiothreitol (5 mM), and EDTA (1 mM). The resulting stoichiometric protein-RNA adduct was purified by ion-exchange chromatography and then disrupted by heating. Precipitated RluA was pelleted by centrifugation, and the free RNA (in the supernatant) was digested and dephosphorylated by the addition of S1 nuclease and calf intestine alkaline phosphatase. The resulting dinucleotide products were purified by isocratic reversed-phase HPLC over a preparatory C₁₈ column. Product-bearing fractions were combined, taken to dryness *in vacuo*, and redissolved. A small aliquot (1.2 μL) was diluted (to ~10 μM) into methanol (50%; Optima* LC/MS grade, Fisher Scientific, Pittsburgh, PA, USA) for MS analysis. The dinucleotide products from the action of TruB (10 μM) on [F⁵U]TSL (100 μM) were prepared similarly except for the omission of the ion-exchange chromatography and heat disruption since TruB and [F⁵U]TSL do not form a stable adduct.^[5]

^aIt is possible that the reported fragmentation pattern arises from markedly different contributions from each isomeric product. However, there is no evidence for the differential fragmentation of the identical pyrimidine ring of F⁵U* in the two products, and such differential fragmentation would not strongly affect the thrust of the results and conclusions.

Mass spectrometry experiments

All mass spectrometry (MS) and tandem MS (MS^n) experiments were performed on a Thermo Finnigan (Bremen, Germany) linear trap quadrupole (LTQ) Fourier transform (FT) mass spectrometer equipped with a Triversa NanoMate (Advion, Ithaca, NY, USA) direct infusion nano-electrospray ionization (nESI) ion source in the negative ion mode. For collision-induced dissociation (CID) MS^n ($n = 2-6$) experiments, the ions of interest were isolated in the LTQ using an isolation window of 1 m/z and subjected to on-resonance CID using helium as the collision gas (it also functions as a bath gas inside the ion trap) using a variable normalized collision energy (NCE) (10–15%) at a constant CID collision time (30 ms) or at a variable CID time (13–50 ms) at a constant NCE (13%), with both methods utilizing a collision q -value of 0.25. For infrared multi-photon dissociation (IRMPD) pseudo- MS^n experiments, the $[M-H]^-$ precursor anions of interest were isolated in the LTQ using an isolation window of 7 m/z , transferred to the ICR cell and subjected to continuous-wave CO_2 laser IR light (4 W (20%), $943.40 \pm 0.33 \text{ cm}^{-1}$) of variable irradiation times (10–550 ms, after a 20 ms delay after ion trapping) prior to FT-ICR excitation and detection.

Density functional theory (DFT) calculations

Geometric optimization, single point energy, and vibrational frequency calculations on selected ionic and neutral fragment structures were carried out *in vacuo* using Gaussian 09 IBM64-G09RevA.02 on 8 IBM PowerPC 4.7 GHz processors running AIX 6.1 at the B3LYP/6-311+G(d,p)//AM1 level of theory using facilities at the Center for Research Computing of the University of Louisville.

RESULTS AND DISCUSSION

Linear ion trap CID fragmentation tandem mass spectrometry

During the initial FT-ICR MS accurate mass characterization of the dinucleotide products, fragmentation was observed without deliberate activation of the precursor anion with collision gas, also known as in-source fragmentation (Fig. 3(a)). After performing ion source and transfer optics tuning with the LTQ for the $[M-H]^-$ precursor anions (585 m/z), ~5% residual fragmentation was still observed that indicated the saturated and hydrated F^5U^* was an excellent target for an informative MS^n analysis. A typical linear ion trap CID MS/MS spectrum of the 585 m/z (Fig. 3(b)) shows the product of HNCO loss (542 m/z) more prominently than the expected predominant species from phosphodiester bond cleavage (341 m/z).

Breakdown curves for the 585 m/z anions (Fig. 4) clearly show that the loss of HNCO (542 m/z) is more favorable than the usually preferred cleavage of the phosphodiester bond (341 m/z) under different CID conditions. Using the standard CID MS/MS setting (CID time = 30 ms and $q = 0.25$) causes an abrupt onset of the fragmentation at 12% NCE, and by 14% NCE all precursor anions have been fragmented (Fig. 4(a)). Breakdown curves obtained at 13% NCE with varied CID times (Fig. 4(b)) were smoother, and the normalized ion intensity of the ions at 542 m/z leveled off around 60% as opposed to around 70% with varied NCE. For both processes, the onsets are fairly similar, which suggests the transition state energies for both processes are also similar.

The 'ion tree' of the linear ion trap CID MSⁿ experiments (Supplementary Fig. S1 and Table S1, see Supporting Information) reveals the extensive and diverse fragmentation of this novel nucleoside. A detailed discussion of all fragmentation patterns and ion structures exceeds the scope of this communication, but several points demand attention. After the loss of one HNCO molecule, a second one is lost from 542 *m/z* in the CID MS³ stage to afford a species at 499 *m/z*, but this second loss of HCNO is less favorable than phosphodiester bond rupture, which affords the species at 298 *m/z*. The breakdown curves for MS³ of the anions of 542 *m/z* (Fig. 5) reveal that both cleavage of the phosphodiester bond and loss of a second HNCO occur with lower energy of fragmentation than similar processes in the anions of 585 *m/z*. In CID MS⁴, the anion at 499 *m/z* loses HF and other neutrals.

Fourier transform ion cyclotron resonance infrared multi-photon dissociation tandem mass spectrometry

The LTQ-FT offers the option of performing CID experiments and then transferring the remaining precursor ions and the fragment ions to the ICR cell for high-resolving power and high mass measurement accuracy detection. This allows for the discovery of elemental compositions of fragment ions and thus lends additional support for the fragmentation mechanism proposed on the basis of the ion trap CID MSⁿ data. After MS², however, the ion intensities for the fragment ions become very low; additionally, the 1/3 low-mass cut-off associated with ion trap CID prevents the detection of low *m/z* fragment ions.

IRMPD in the ICR cell offers the opportunity to fine-tune the fragmentation by controlling the laser energy and irradiation time. In general, IRMPD only samples the lowest energy pathways while delivering pseudo-MSⁿ data because the fragment ions themselves can absorb IR photons until they fragment to afford the next generation of ions. The FT-ICR IRMPD MS spectrum (Fig. 6) shows the loss of HNCO under these conditions still remains more favorable than cleavage of the phosphodiester bond, even at the lowest non-zero laser energy setting and the minimal irradiation time sufficient to induce and detect fragment anions. FT-ICR IRMPD MSⁿ breakdown curves (Supplementary Fig. S2, see Supporting Information) confirm the proposed anion structures deduced from the ion trap CID MSⁿ data and show that all anions eventually fragment to yield H₂PO₄⁻, and, ultimately, PO₃⁻.

Mechanisms for HNCO loss

The facile loss of neutral HNCO is interesting by itself because the established retro-Diels-Alder mechanism for the same fragmentation in uridine and pseudouridine cannot apply to F⁵U*, and, therefore, a new mechanism based on the idiosyncratic characteristics of F⁵U* must operate. The C–C glycosidic bond is shared with pseudouridine, so it is not a unique feature, but hydration both saturates the pyrimidine ring and introduces a hydroxyl group at C6. The fluoro group also dramatically changes the charge characteristics of the pyrimidine ring (Fig. 7). Saliently, C5 in F⁵U* bears a partial positive charge (+0.282) compared to a partial negative charge in uridine (−0.349) and pseudouridine (−0.202). The partial positive charge on C5 and the associated lengthening – and therefore – weakening – of the C4–C5 bond allows its easier cleavage.

A mechanism that relies on the relative positioning of the hydroxyl group and charge distribution in F^5U^* leads to the loss of H–N1, C2, and O^2 to generate a zwitterionic intermediate that can tautomerize to an aldehyde/amide or close to a β -lactam (Fig. 8). Formation of the zwitterion, **2**, is likely facilitated by a hydrogen bond between the oxyanion end and the 2'-hydroxyl group of the trailing pyrimidine, for that stabilizing interaction is seen in the energy-minimized structure of the parent dinucleotide, **1** (Supplementary Fig. S3, see Supporting Information). These three candidate structures for the ions at 542 m/z were subjected to DFT energy minimization with unrestricted bond rotation, and the resulting energies (B3LYP electronic energy + AM1 thermal enthalpy) of each fragment was summed with that of HCNO for comparison to the dinucleotide of F^5U^* , **1**. The aldehyde/amide, **3**, was calculated to be 12.5 kcal/mol higher in energy than **1**. During minimization, the zwitterion, **2**, collapsed to the β -lactam, **4**, which was calculated to be 26.5 kcal/mol higher than the dinucleotide of F^5U^* . Extrusion of O^4 , C4, and N3–H by homolysis of the C5–C4 and N3–C2 bonds was also considered and results in a diradical that collapses during minimization to afford the β -lactam. These results favor the aldehyde/amide, **3**, over the β -lactam if product formation is under thermodynamic control, but the fragmentation may well be under kinetic control so that β -lactam formation dominates.

In the next round of MS^n , the β -lactam can extrude HNCO, perhaps in a pericyclic process comparable to the formation of the “B” fragments during MS of β -lactam antibiotics[22]^b or by a mechanism allowed by the novel combination of β -hydroxyl and α -fluoro groups (Fig. 8). Alternatively, the β -lactam may reversibly form the zwitterion, which can fragment with the release of HNCO (Fig. 8). Based on the similar onsets of HNCO loss and phosphodiester cleavage in the energy-resolved CID plots, it is reasonable to conclude that both proceed through transition states of comparable energy, but the preponderance of fragments with HNCO loss indicates that the difference in transition state heights for the two processes is significant. Detailed assessment of these issues will require RRKM calculations to determine the exact transition states for each, which lies beyond the scope of this communication. Ion spectroscopy^[24,25] would also be a powerful tool for the elucidation of this system, for the plausible fragmentation products were subjected to DFT vibrational frequency calculations, which indicate that the candidate products would be readily differentiated by their IR spectra (Supplementary Fig. S4, see Supporting Information), specifically in the carbonyl stretching region.

CONCLUSIONS

The fragmentation of the pyrimidine ring in F^5U^* is strikingly facile, with loss of HNCO occurring in-source and more readily than phosphodiester bond cleavage. Although HNCO loss is observed with uridine and pseudouridine, the retro-Diels-Alder mechanism that operates with these unsaturated pyrimidines cannot hold for the saturated pyrimidine ring of

^bThe pericyclic fragmentation of β -lactam antibiotics to give a ketene product is established,^[23] but the process proposed here for HNCO extrusion is not. However, such pericyclic cleavage of the antibiotics would not result in a change of mass because the nitrogen of the β -lactam is doubly substituted and so generates RNCO (where R represents the remaining atoms of the antibiotic), so the proposed pericyclic cleavage pathway would only become detectable upon further fragmentation, such as those seen for the “B” ions.^[22]

F⁵U*. A plausible mechanism that relies on the unique relative disposition of functional groups in F⁵U* nicely accounts for the observed HNCO loss but must be verified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The conversion of uridine into pseudouridine (Ψ) by pseudouridine synthases (ΨS).

Figure 2.

When the pseudouridine synthases TruB and RluA are incubated with substrate RNA containing F⁵U in place of the reactive uridine, two rearranged and hydrated products (F⁵U*) form; they differ in stereochemistry at C2'.^[9] F⁵U* is isolated as part of a dinucleotide with the trailing nucleoside in the RNA because S1 nuclease cannot cut after nonplanar nucleobases.^[7]

Figure 3.

The linear ion trap mass spectra of the F^5U^* dinucleotide: (a) prior to isolation, CID or IRMPD and (b) after isolation of 585 m/z and CID fragmentation at 13% NCE for 30 ms and a q of 0.25. * peaks not present in the solvent or readily deduced by likely fragmentation.

Figure 4.

The breakdown curves for the parent dinucleotide after CID fragmentation: (a) varying collision NCE (10–15%) at constant irradiation time (30 ms) and (b) varying collision irradiation time (13–50 ms) at constant NCE (13%).

Figure 5.

The isolated fragment ion after the first HNCO loss (542 m/z) is subjected to varying CID irradiation time (10–100 ms) at constant NCE (13%).

Figure 6.
After isolation and passage into the ICR cell, the lowest non-zero laser energy still favors HNCO loss over phosphodiester cleavage.

Figure 7.

Natural bond order calculations show the different partial charges in the pyrimidine rings of (a) F^5U^* , (b) Ψ , and (c) U . For F^5U^* , R denotes the rest of the dinucleotide product (Fig. 2); for U and Ψ , R denotes ribose.

Figure 8.

The proposed mechanism for facile HNCO loss from F^5U^* , **1**. The initial HNCO loss (A) leads to the zwitterion (**2**), which can tautomerize (B) to the aldehyde/amide (**3**) or close (C) to the β -lactam (**4**). **2** can extrude a second HNCO (D) to generate the enol (**5**), as can **3** (E). **4** can fragment to afford **5**, either by a pericyclic process (F) or by a mechanism (G) made possible by the relative disposition of the hydroxyl and fluoro groups (which formally yields the zwitterionic resonance form of **5**). R denotes the rest of the dinucleotide product (Fig. 2).