Comprehensive Characterization of tRNA by Intact Mass Analysis

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INTRODUCTION

Transfer ribonucleic acid (tRNA) produced from the DNA gene by the multiprotein enzyme complex RNA polymerase III, (poll III) and progress through a series of maturation steps resulting in a RNA having an average mass of 25k Da and ranging in length of ~70-100 nucleotides. Of all classes of RNA, the tRNA contain the greatest density of post-transcriptional modification, ranging from simple methylations to hyper modifications having multi-enzymatic biosynthetic pathways. Structurally, tRNA contain four regions, the anticodon which base pairs with the codon of the mRNA during translation, the D-Loop, the TYC loop and the acceptor step which contains the 3' sequence CCA. The terminal adenosine carries the amino acid specific to the tRNA anticodon and is placed onto the tRNA by the tRNA's cognate protein complex, the aminoacyl tRNA synthetase (aaRS).

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) of enzymatic digests has historically been the gold standard to studying tRNA and their modification. Improvements in ion pair reversed –phase (IP-RP) chromatography, as well as mass spectrometric sensitivity and mass accuracy, now enable routine analysis of RNA oligonucleotide ranges up to 200mers. Here, using UHPLC-HRAM-MS we show the ability to fully characterize a tRNA through deconvolution of an intact mass isotopic peak envelope. Using the commercial standard $tRNA^{PHE}$ from *S. cerevisiae* we identify both known isodecoders[1], a base substitution in the acceptor stem, and show they exist primarily truncated to a CpCp 3' terminal. We further are able to identify a possible stable intermediate in the Wybutosine biosynthetic pathway[2], identify a 3' terminal phosphorylated adenosine as well as identify possible intermediates in acceptor stem maturation with monoisotopic mass errors ≤ 5ppm. Confirmation of identities was verified through traditional mass spectrometric nucleoside and mass mapping experiments.

MATERIALS AND METHODS

Liquid Chromatography: Intact and oligonucleotide separations were accomplished by reversed-phase liquid chromatography using a Thermo Scientific™ DNAPac™ RP, 4 µm, 2.1 mm X 100 mm column on a Thermo Scientific™ Vanquish™ Horizon Quaternary UHPLC system using Ion-Pairing chromatography. Nucleoside separations were accomplished by reversed-phase liquid chromatography using a Thermo Scientific™ Accucore™ C18+, 1.5 µm, 2.1 mm X 100 mm column on a Thermo Scientific™ Vanquish™ Flex UHPLC system using ammonium acetate buffers.

Elution gradient for intact mass starts at 10% B (from 0 to 0.5 min.), 40% B at 7.0 min., 90% B at 7.1 held for 0.5 min., returning to 10% B at 7.51 min. at a flow rate of 400 µL min^{-1}. The column temperature was set at 80 °C.

Elution gradient for oligonucleotide mass mapping starts at 0% B (from 0 to 1 min.), 30% B at 16 min., 90% B at 16.1 min. held for 1 min. then returning to 0% B at 17,1 min.

Elution gradient for nucleoside analysis starts at 0% B (from 0 to 3 min.), 25% B at 10 min., 99% B at 14 min. held for 1 min. then returning to 0% B at 15.1 min.

Mass Spectrometry: HRAM analyses were performed on an Thermo Scientific™ Orbitrap™ Ascend Tribrid™ mass spectrometer interfaced with a heated electrospray (H-ESI) source in negative polarity. Full scan data was acquired in Intact Protein mode at low pressure using a resolution of 240,000 at 400 m/z, mass range 800-3000 m/z, automatic gain control (AGC) 7.5e4, and max injection time (IT) 100 ms. The other instrumental conditions were quadrupole isolation of 1.2 m/z; radio frequency (RF) 35%; sheath gas, auxiliary gas, and sweep gas of 50, 10 and 1 arbitrary units, respectively; ion transfer tube temperature of 350 °C; vaporizer temperature of 320 °C; and spray voltage of 3500 V. Data was acquired using Thermo Scientific™ Xcalibur™ 4.5 and analyzed with Thermo Scientific™ Freestyle™ 1.8 and Thermo Scientific™ BioPharma Finder™ 5.1.

MATERIALS AND METHODS (Cont.)

Deconvolution: Deconvolution was performed using Thermo Scientific™ BioPharma Finder software 5.1, Intact Mass Analysis, with chromatogram Trace Type as TIC, and m/z Range of 800 to 3000. Source Spectra Method was Averaged Over Selected Time. Deconvolution Algorithm was Xtract with an Output Mass Range of 24000 to 26000 with a S/N Threshold of 3 and a Rel. Abundance Threshold (%) of 3. Charge Range was set from 5 to 35 with a Min. Num Detected Charge of 5 using the Nucleotide Table and Negative Charge. Under Identification, Sequence Matching Mass Tolerance was set to 10 ppm with Multiconsensus Component Merge Mass Tolerance at 10 ppm.

RNase Digestion: *Nucleoside:* An aliquot of $tRNA^{PHE}$ was heated at 95°C for five minutes and cooled in a water bath. To the cooled sample was added 1U of P1 nuclease, 0.01U RNase A, and 0.1U phosphatase and heated at 37°C for two hours. Sample was removed, taken to dryness in a speed vac, resuspended in mobile phase A and injected onto a Accucore C18+ column (2.1x100mm, 1.5um). Gradient conditions were XX.

For oligonucleotide analysis, an aliquot of $tRNA^{PHE}$ in RNase/DNase free water was made 200 mM ammonium acetate, followed by introduction of 50U RNase T1/ug RNA. Sample was placed in a heat block and allowed to digest for two hours at 40°C, taken to dryness in a speed vac, resuspended in MPA and injected onto a DNAPac column (2.1x100mm, 3um) using same mobile phase as intact analysis.

RESULTS

Intact mass measurement of $tRNA^{PHE}$ shows a charge state distribution between -9 to -26 with the most abundant charge states between -15 and -19 (**Figure 1A**). For each charge state several features are observed with a minimum and maximum relative abundance between 3 and 30 (Figure 1B). Deconvolution of the spectra from the main chromatographic peak returns several masses, the most abundant having a monoisotopic mass of 24610.450 Da. Baseline isotopic peak resolution of the -17-charge state enhances the ability of the software to accurately deconvolute the spectra (Figure 1C).

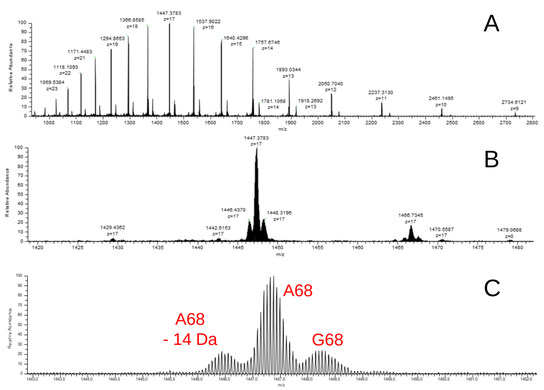

Figure 1: *Intact mass spectrum of a commercially available $tRNA^{PHE}$ standard. A: Charge state envelope. B: Zoomed image of baseline of highest abundant peak. C: Baseline isotopic resolution of the -17-charge state aids in deconvolution, and accurately identifies Both isodecoders of $tRNA^{PHE}$ as well as an A68 variant less one methylation.*

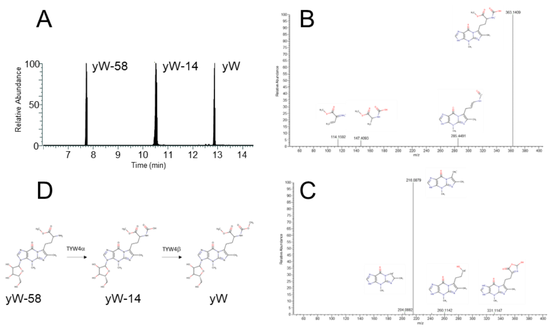

Figure 2: *Extracted ion chromatograms for Wybutosine and its two immediate precursors yW-58 and yW-14. All three were confirmed by MS/MS with Figures B and C showing the CID and HCD fragmentation of yW-14 respectively. Figure D is the predicted biosynthetic pathway, showing the yW-14 intermediate generated by the TYW4 enzyme during carboxymethylation.*

One of the most abundant signals in the deconvolution results was identified as a demethylation of the A68 tRNA. Looking at the most probable labile methylation suggests a loss of methylation on the Wybutosine (yW) sidechain, which would result in a monoisotopic mass of 494.1761 Da. To verify if the demethylated signal in the deconvolution result was the yW-14 intermediate, extracted ion chromatogram (EIC) in the nucleoside data for the protonated mass were generated and returned a feature at ~10.6 min, between yw-58, a precursor, and yW (**Figure 2A**). Examining the collisional induced dissociation (CID) and High Energy Collision Dissociation (HCD) MS/MS filters for 495 (**Figure 2B** and **3C**) yielded fragmentation consistent with a yW-14 structure.

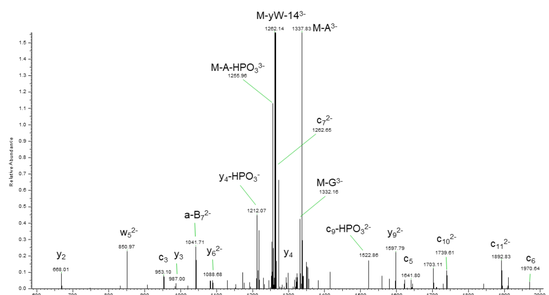

Figure 3: *Mass mapping results from the signature digestion product from $tRNA^{PHE}$ containing the yW-14 modification at position 37.*

A mass mapping experiment was then performed to verify the presence of the yW-14 modification within $tRNA^{PHE}$. The primary fragmentation channel for wybutosine containing oligonucleotides is loss of the bulky tricyclic modification. Zooming into the baseline exposes a number of low abundant fragment ions which are diagnostic for the yW-14 containing oligonucleotide and follow typical CID RNA ladder fragment ions (**Figure 3**).

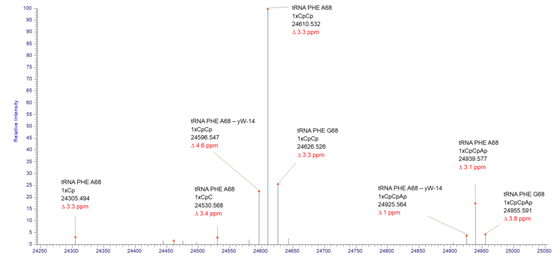

Figure 4: *BioPharma Finder™ 5.1 deconvoluted results with annotation showing the presence of three variants of $tRNA^{PHE}$ containing 3' truncations, the predominate being a CpCp terminal.*

The 3' ends of tRNA end in the CCA motif. This three-nucleotide sequence is added post-transcriptionally to the tRNA by CTP:ATP nucleotidyltransferase. Each nucleotide is joined to the tRNA sequentially from their respective triphosphate precursors. Using BioPharma Finder™ 5.1 Intact Mass Analysis we show that the tRNA exists in various forms of maturation, with the predominate species being CpCp for all three variants (**Figure 4**). Surprisingly, a 3' of CpCpAp was also identified with mass deltas between theoretical and observed at < 4 ppm. The presence of the 3' adenosine phosphate could suggest the possibility of an intermediate step in the aminoacylation pathway.

CONCLUSIONS

In these experiments we have used ion pairing reverse phase liquid chromatography coupled to accurate mass spectrometry to identify an intact tRNA through deconvolution of its negative charge state distribution. We were further able to identify the known isodecoder as well as an under modified variant. We further show that, for this sample, the predominant species exist as a single 3' truncated species CpCp with other low abundant identifications characterized as being intermediates in 3' maturation.

REFERENCES

1. Keith G, Dirheimer G. *Evidence for the existence of an expressed minor variant tRNAPhe in yeast*. Biochem Biophys Res Commun. **1987** Jan 15;142(1):183-7
2. Noma et al., *Biosynthesis of wybutosine, a hyper-modified nucleoside in eukaryotic phenylalanine tRNA.* EMBO J. **2006** May 17;25(10):2142-54

TRADEMARKS/LICENSING

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