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^1H , ^{15}N chemical shift assignments of the imino groups of yeast tRNA^{Phe}: influence of the post-transcriptional modifications

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Abstract

Transfer RNAs (tRNAs) are heavily decorated with post-transcriptional modifications during their biosynthesis. To fulfil their functions within cells, tRNAs undergo a tightly controlled biogenesis process leading to the formation of mature tRNAs. In particular, the introduction of post-transcriptional modifications in tRNAs is controlled and influenced by multiple factors. In turn, tRNA biological functions are often modulated by their modifications. Although modifications play essential roles in tRNA biology, methods to directly detect their introduction during tRNA maturation are rare and do not easily provide information on the temporality of modification events. To obtain information on the tRNA maturation process, we have developed a methodology, using NMR as a tool to monitor tRNA maturation in a non-disruptive and continuous fashion in cellular extracts. Here we report the ^1H , ^{15}N chemical shift assignments of imino groups in three forms of the yeast tRNA^{Phe} differing in their modification content. These assignments are a prerequisite for the time-resolved NMR monitoring of yeast tRNA^{Phe} maturation in yeast extracts.

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Key words: tRNA, imino groups, RNA resonance assignment, post-transcriptional modifications

1. Biological context

In all domains of life, the synthesis and maturation of RNAs involve post-transcriptional chemical modifications of their nucleotides at specific sites. In general, these chemical modifications have an impact on the RNA base-pairing capacities, on

the RNA recognition possibilities by proteins, and on the physico-chemical properties of the RNA itself (Li and Mason 2014; Motorin and Helm 2011; Nachtergaele and He 2017), and are thereby linked to a multitude of biological functions (Marbaniang and Vogel 2016; El Yacoubi et al. 2012; Jackman and Alfonzo 2013). Among the different RNA families, transfer RNAs (tRNAs) display not only the highest variety of chemical modifications (>90 different modifications identified), but also the highest density of modification per transcript (~8–25% of

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modified nucleotides in tRNAs of various organisms) (Boccaletto et al. 2018). tRNAs are essential components of the cellular protein synthesis machineries, but also serve additional functions as broad as amino acid delivery to membrane lipids, synthesis of peptidoglycan and antibiotics (Banerjee et al. 2010). In addition, under stress conditions, some tRNAs are cleaved creating molecules acting in signalling or as regulators of gene expression (Sobala and Hutvagner 2011). To ensure these broad varieties of function within cells, tRNAs undergo a tightly controlled biogenesis process leading to the formation of mature and functional tRNAs. In particular, the introduction of post-transcriptional modifications in tRNAs is controlled and influenced by multiple factors (Barraud and Tisné 2019). Importantly, all tRNA functions within cells are to various extents affected by modifications. For instance, roles of modifications in and around the anticodon in decoding are well-documented (El Yacoubi et al. 2012; Agris et al. 2007). In addition, oxidative or heat stress can result in changes in tRNA modifications which, in turn, results in translational reprogramming (Chan et al. 2012; Endres et al. 2015). And finally, some modifications control cleavages producing tRNA fragments (Schaefer et al. 2010; Oberbauer and Schaefer 2018; Lyons et al. 2018).

All aspects of tRNA biology are controlled and modulated by modifications, but methods to directly detect their introduction during tRNA biosynthesis are rare and do not easily provide information on the temporality of modification events. We have developed a methodology, using NMR as a tool to monitor tRNA maturation in a non-disruptive and continuous fashion (Barraud et al. 2019). Briefly, introducing isotope-labelled RNAs into unlabelled cell extracts containing the cellular enzymatic activities combined with the use of isotope-editing in NMR experiments enables the detection of the sole RNA of interest within the complex cell extract environment. The non-disruptive nature of NMR provides the means to directly monitor RNA modification events in a continuous and time-resolved fashion, by measuring successive NMR experiments on a single sample incubated directly in the NMR spectrometer.

Here we describe the production and purification of three forms of the yeast tRNA^{Phe} differing in their modification content, and we report the

¹H,¹⁵N chemical shift assignments of their imino groups (Figure 1). The first sample corresponds to a yeast tRNA^{Phe} produced by *in vitro* transcription, which presents no post-transcriptional modifications. The second sample corresponds to a yeast tRNA^{Phe} purified from yeast that contains all 14 modifications found in this tRNA. The third sample corresponds to a recombinant yeast tRNA^{Phe} produced in an *E. coli* strain overproducing this tRNA. The modification pattern of this sample resembles that of the fully modified yeast tRNA^{Phe}, but overall exhibits fewer modifications. The three sets of tRNA^{Phe} chemical shifts have been obtained from 2D (¹H,¹⁵N)-BEST-TROSY, 3D NOESY-(¹H,¹⁵N)-HSQC and 2D NOESYs experiments. The resonance assignments and especially the comparison of the imino groups chemical shifts of the three samples revealed the NMR signature of individual post-transcriptional modifications and laid the foundation for the time-resolved NMR monitoring of yeast tRNA^{Phe} maturation in yeast extracts (Barraud et al. 2019).

2. Methods and experiments

2.1. Sample preparation

Unmodified yeast tRNA^{Phe} was prepared by standard *in vitro* transcription with an in-house produced T7 RNA polymerase containing a mutation (Pro266Leu) for improved efficiency (Guillerez et al. 2005). Transcription mix contained unlabelled NTPs (Jena Bioscience) for unlabelled samples or ¹⁵N-labelled UTP and GTP (Eurisotop) and unlabelled ATP and CTP (Jena Bioscience) for ¹⁵N-[U/G]-labelled samples. The DNA template (5' TGGTGC GAATTCTGTGGATCGAACACAGGACCTCCAGATCTTCAGTCTGGCGCTCTCCAACTGAGCTAAATCCGCTATAGTGAGTCGTATTA 3') and T7 promotor primer (5' TAATACGACTCACTATAG 3') were purchased from Eurogentec. Transcription mix contains Tris-HCl pH 8.0 20 mM, spermidine 0.5 mM, DTT 2.5 mM, Triton X-100 at 0.005 % (v/v), 200 nM of an equimolar mixture of the DNA template and T7 promotor primer, each of the 4 NTPs at 5 mM, MgCl₂ 34–38 mM, GMP 4 mM and 35 µg/mL of in-house produced T7 RNA polymerase. The transcription reaction is stopped after 3–4 h of incubation at 37°C by adding EDTA at 100 mM. The tRNA transcript is purified by ion exchange chro-

matography (MonoQ, GE Healthcare) under native conditions at 4°C with a flow-rate of 3 mL/min. Eluent A contains Na-phosphate pH 6.5 25 mM, NaCl 50 mM and MgSO₄ 5 mM; eluent B contains Na-phosphate pH 6.5 25 mM, NaCl 1 M; a gradient of 20–80 % B is performed over 25 min for RNA elution. Fractions containing the purified RNA were determined with urea/acrylamide (7 M/14%) gels migrated in TBE buffer. The purified RNA is then dialyzed extensively against Na-phosphate pH 6.5 1 mM, and refolded by heating at 95°C for 5 min and cooling down slowly at room temperature. Buffer was added to place the tRNA^{Phe} in the NMR buffer (Na-phosphate pH 6.5 10 mM, MgCl₂ 10 mM), and the sample was concentrated to ~1.5–2.0 mM using Amicon 10,000 MWCO (Millipore).

The partially modified yeast tRNA^{Phe} sample produced in and purified from *E. coli* was prepared by the overexpression of a yeast tRNA^{Phe} gene in *E. coli* using the pBSTNAV vector following similar procedures used for other tRNAs (Meinzel et al. 1988; Tisné et al. 2000; Barraud et al. 2008; Dégut et al. 2016; Dégut et al. 2019; Bou-Nader et al. 2019). Briefly, the gene coding yeast tRNA^{Phe} was cloned in the pBSTNAV vector (Addgene ID 45801) between the EcoRI and PstI restriction sites (pBSTtRNA^{Phe}). In pBSTNAV, the strong natural *E. coli* constitutive promoter *lpp* present upstream of the tRNA gene allows production of high levels of recombinant transcripts. Yeast tRNA^{Phe} was expressed in LB medium for unlabelled sample preparation or ¹⁵N-labelled Spectra-9 medium (Eurisotop) for ¹⁵N-labelled sample preparation. *E. coli* JM101TR are transformed with the pBSTtRNA^{Phe} plasmid and grown overnight at 37°C. After standard procedures of phenol extraction of soluble RNAs, yeast tRNA^{Phe} was purified by ion exchange chromatography under native conditions. First, total tRNAs are isolated by gel filtration on a HiLoad 26/600 Superdex 75 prepgrade chromatography column (GE Healthcare) equilibrated in 25 mM Na-phosphate pH 6.5. The overexpressed yeast tRNA^{Phe} was then separated from other tRNAs by an anion exchange step (MonoQ column) equilibrated in 25 mM Na-phosphate pH 6.5. tRNAs were eluted using a 450–700 mM NaCl gradient in the same buffer. The yeast tRNA^{Phe} was further purified by a hydrophobic interaction step (Phenyl Superose column) equilibrated in 25 mM Na-phosphate pH 6.5, 1.7 M ammonium sulfate. Yeast tRNA^{Phe} was eluted using a 1.7–

1.0 M ammonium sulfate reverse gradient. Fractions containing the yeast tRNA^{Phe} were identified with a gel-shift assay on a native PAGE with a DNA oligonucleotide complementary to the T-arm, anticodon stem-loop and D-arm of the overexpressed yeast tRNA^{Phe} (5' TCGAACACAGGACCTCCAGATCTTCAGTCTGGCGCTCT 3') (Dégut et al. 2016). The identified fractions were pooled and dialyzed against the NMR buffer (Na-phosphate pH 6.5 10 mM, MgCl₂ 10 mM). The sample was concentrated to ~0.5–1.0 mM using Amicon 10,000 MWCO (Millipore).

Unlabelled mature yeast tRNA^{Phe} was purchased from Sigma-Aldrich, resuspended in and dialyzed against the NMR buffer (Na-phosphate pH 6.5 10 mM, MgCl₂ 10 mM) and concentrated to 0.8 mM using Amicon 10,000 MWCO (Millipore).

2.2. NMR spectroscopy

All NMR spectra of yeast tRNA^{Phe} were measured at 38°C on Bruker AVIII-HD 600 MHz and AVIII-HD 700 MHz spectrometers (equipped with TCI 5-mm cryoprobes) with 5-mm Shigemi tubes. Imino resonances of the tRNA^{Phe} samples were assigned using 2D jump-and-return-echo (¹H,¹H)-NOESY (Plateau and Gueron 1982; Sklenar and Bax 1987), 3D NOESY-(¹H,¹⁵N)-HSQC (Talluri and Wagner 1996), 2D (¹H,¹⁵N)-BEST-TROSY (Farjon et al. 2009) and standard 2D (¹H,¹⁵N)-HSQC experiments measured in the NMR buffer supplemented with D₂O 5% (v/v). For the unmodified yeast tRNA^{Phe}, three 2D jump-and-return-echo (¹H,¹H)-NOESYs were measured at a sample concentration of 1.8 mM, with mixing times of 60 ms, 100 ms and 150 ms. A 3D NOESY-(¹H,¹⁵N)-HSQC was also measured on this sample with a mixing time of 200 ms. For the partially modified yeast tRNA^{Phe} sample, two 2D jump-and-return-echo (¹H,¹H)-NOESYs were measured at a sample concentration of 1.0 mM, with mixing times of 120 ms and 150 ms. For the unlabelled mature yeast tRNA^{Phe}, a single 2D jump-and-return-echo (¹H,¹H)-NOESY was measured at a sample concentration of 0.8 mM with a mixing time of 120 ms. A 2D (¹H,¹⁵N)-BEST-TROSY spectrum was also measured at natural abundance on this sample with 128 increments in the indirect dimension and an experimental time of 2 days and 20 hours. The data were processed using TOPSPIN 3.5 (Bruker) and analysed with Sparky (<http://www.cgl.ucsf.edu/home/sparky/>).

2.3. Assignments and data deposition

High quality 2D ($^1\text{H}, ^1\text{H}$)-NOESY spectra have been obtained from all three samples of yeast tRNA^{Phe}, which enabled imino groups resonance assignments for the base-paired imino groups protected from exchange with the solvent (Figure 1). With the information on the ^{15}N chemical shifts, the 2D ($^1\text{H}, ^{15}\text{N}$)-BEST-TROSY experiments provided the means to distinguish iminos of Us and U-derived modified nucleotides (*i.e.* T54 and Ψ 55), from the ones of Gs and G-derived modified nucleotides (*i.e.* m⁷G46 and m²G10). In addition, the previously reported ^1H resonance assignment of the methyl groups of the mature yeast tRNA^{Phe} contributed to provide starting points for the assignment procedure (Agris et al. 1986). The imino resonance assignment in the acceptor and T-arm helices was performed using two starting points. First, the strong imino-imino cross peak in the 2D NOESY directly gave the assignment of the iminos of the unique G4-U69 wobble base-pair. These iminos then connect to iminos of G3 and U68, which provide the means to pursue the acceptor stem assignment (Figure 2; assignment pathway in black). Second, in the partially modified and in the mature yeast tRNA^{Phe}, the upfield methyl resonance around 1.0 ppm, which corresponds to the methyl group of T54 (m⁵U54), connects with the two iminos of Ψ 55 and with the iminos of T54 and G53. This enables to proceed with the assignment in the T-arm (Figure 2; assignment pathway in green). In addition, the Ψ 55-H3 imino connects with G18, which give the assignment of this upfield shifted imino (Figure 2; assignment in pink). The imino resonance assignment of the D-arm and of the tRNA tertiary interactions was performed using two starting points. First, in the partially modified and in the mature yeast tRNA^{Phe}, the downfield methyl resonance around 3.8 ppm, which corresponds to the methyl group of m⁷G46, connects with the imino of m⁷G46, which connects with iminos of G15, U8 and G22, the latter further connecting with the one of U12. Second, in the mature yeast tRNA^{Phe}, the methyl resonance around 2.8 ppm, which corresponds to the methyl group of m²G10, connects with the iminos of m²G10 and G24, which provide the means to complete the D-arm assignment (Figure 2; assignment pathway in red). The assignment of the anticodon arm is then easily performed through imino-imino correlations among the remaining im-

ino resonances (Figure 2; assignment pathway in yellow). Overall, the 2D spectra provided the main source of information for imino-groups assignments, but the 3D NOESY-($^1\text{H}, ^{15}\text{N}$)-HSQC measured on the unmodified tRNA^{Phe} was in some cases helpful to unravel certain ambiguities in crowded regions. The chemical shifts determined here for the three samples of yeast tRNA^{Phe} agree well with previously reported ^1H chemical shifts (Heerschap et al. 1985).

Post-transcriptional modifications generate chemical shift variations that one can easily observe when comparing chemical shifts of the partially modified and/or mature yeast tRNA^{Phe} with the ones of the unmodified sample. Importantly, the analysis of these chemical shift variations in between the three tRNA^{Phe} samples can reveal the NMR signature of individual post-transcriptional modifications. For instance, the T54 modification has a moderate effect on the chemical shifts of the imino group of U54 ($\Delta\delta$: ^1H = -0.08 ppm; ^{15}N = -1.26 ppm; Figure 1B). Similarly, the m²G10 and m⁷G46 modifications have moderate and strong effects on the chemical shifts of the imino group of G10 ($\Delta\delta$: ^1H = -0.04 ppm; ^{15}N = -1.10 ppm; Figure 1C) and G46 ($\Delta\delta$: ^1H = +0.75 ppm; ^{15}N = +2.93 ppm; Figure 1C), respectively. More subtle differences can also be analysed in detail (Barraud et al. 2019). The source of these chemical shift variations can be identified with the aid of the 3D structure of yeast tRNA^{Phe}, which enables the precise identification of the nearest modifications to an imino group (Shi and Moore 2000). The most striking difference in the chemical shift of the tRNA^{Phe} samples certainly corresponds to the large variation in the position of the imino group of T54 in the mature yeast tRNA^{Phe} when compared with the partially modified yeast tRNA^{Phe} ($\Delta\delta$: ^1H = -1.12 ppm; ^{15}N = -1.64 ppm, Figure 1B). This large change is induced by the m¹A58 modification in the T-loop of the mature tRNA^{Phe} (Figure 1A), and is most probably very pronounced because T54 forms a non-canonical base-pair with A58, the chemical environment of which is highly perturbed by the positive charged appearing on the A58 base after methylation at position N1.

Spectra were referenced to 2,2-dimethyl-2-silapentanesulfonic acid (DSS) using an external sample of 0.5% DSS and 2 mM sucrose in H₂O/D₂O (Bruker), and indirect chemical shift referen-

cing for ^{15}N according to IUPAB (Markley et al. 1998). Chemical shifts for the unmodified, partially modified and mature yeast tRNA^{Phe} have been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>) under accession number 50095, 50096 and 50097, respectively.

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Figure 1. The ^1H , ^{15}N chemical shift assignments of the imino groups in the base-pairs of the three forms of the yeast tRNA^{Phe} differing in their modification content. **(A)** Sequence and cloverleaf representation of unmodified yeast tRNA^{Phe} produced by *in vitro* transcription (left), partially modified yeast tRNA^{Phe} produced in *E. coli* (middle), and fully modified yeast tRNA^{Phe} produced in yeast (right). Main tertiary interactions are represented with thin lines and modifications are displayed in bold. **(B)** Portion of a (^1H , ^{15}N)-BEST-TROSY spectrum and imino group assignments of Us and U-derived modified nucleotides. Signals from the unmodified yeast tRNA^{Phe} are in blue; signals from the partially modified yeast tRNA^{Phe} are in green; and signals from the fully modified yeast tRNA^{Phe} are in red. **(C)** Portion of a (^1H , ^{15}N)-BEST-TROSY spectrum and imino group assignments of Gs and G-derived modified nucleotides. Signals are displayed with the same colour-code as in panel B.

Figure 2. Portion of a 2D ($^1\text{H}, ^1\text{H}$)-NOESY summarizing the connexion enabling chemical shift assignments of the imino groups in the base-pairs of the fully modified yeast tRNA^{Phe}. Main connexions used to assign the four tRNA-arms are displayed on the 2D spectra, with the corresponding assignments on the right side of the spectrum in black for the acceptor arm and in green for the T-arm. The connexion enabling the assignment of G18 is in pink. Connexions used to assign the D-arm and the anticodon arm are shown in red and in yellow, respectively, with the corresponding assignments displayed above the spectrum.