Detection of pseudouridine and other modifications in tRNA by cyanoethylation and MALDI mass spectrometry

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ABSTRACT

Mass spectrometry plays a central role in the characterisation of modified nucleotides, but pseudouridine is a mass-silent post-transcriptional modification and hence not detectable by direct mass spectrometric analysis. We show by the use of matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry that pseudouridines in tRNA can be specifically cyanoethylated by acrylonitrile without affecting the uridines. The tRNA was cyanoethylated and then subjected to digestion with either RNase A or RNase T1. Cyanoethylated digestion fragments were identified by mass spectrometric comparison of untreated and acrylonitrile-treated samples, where the addition of one acrylonitrile resulted in a mass increment of 53.0 Da. The exact modified nucleotide could be identified by tandem mass spectrometry on the cyanoethylated digestion fragment. The methodology was used to identify additional one 4-thiouridine and one pseudouridine in tRNA^TyrII from *Escherichia coli*. Furthermore, we observed that RNase A is highly tolerant towards nucleotide modifications, only being inhibited by 2′-O-methylation, whereas RNase T1 cleavage is affected by most nucleotide modifications.

INTRODUCTION

Many cellular RNAs become heavily modified after synthesis of the primary transcript. These modifications may be additions or deletions at the terminals, excision or addition of internal sequences, intermolecular splicing and specific modifications at the single nucleotide level. The latter type of modification is generally termed post-transcriptional modification. The post-transcriptional modifications are primarily encountered in stable RNAs such as transfer RNA (tRNA) and ribosomal RNA (rRNA). The precise molecular role of post-transcriptional modification is unclear, but there are numerous examples of modifications being crucial for function of the translational apparatus (1–3). Microorganisms also use

post-transcriptional modification of their rRNAs as a means to acquire antibiotic resistance (4,5).

The most frequently occurring post-transcriptional modification, pseudouridine, is an isomer of its precursor uridine (Fig. 1A). In pseudouridine, the C5 of the uracil nucleobase is coupled via a C–C bond to the 1′-position of a ribose, whereas the coupling goes from N1 in uracil to the 1′-position of ribose in normal uridine. Formation of pseudouridine confers this nucleobase with noticeable new features. First, the pseudo-uridinylation makes N1 of uracil available as a hydrogen bond donor, which can participate in novel molecular interactions (6). Secondly, the C5–C1′ bond between uracil and ribose in pseudouridine confers this nucleotide with less rotational freedom in a tRNA context than the normal N1–C1′ bond in uridine (7), and pseudouridine consequently stabilises local RNA secondary structure (8,9). Thirdly, Ψ's N1 is a particularly reactive atom for certain chemical modifications (see below). The function of pseudouridines on a molecular level is not known, but several studies on eukaryotes and prokaryotes show that deletion of pseudouridine-synthesising genes has a negative impact on cell growth and protein synthesis rate (10,11).

Mass spectrometry plays a central role in the localisation and identification of post-transcriptional modifications (12,13). However, pseudouridine is mass-neutral compared with its uridine precursor, and consequently its mass spectrometric identification is not straightforward. Currently, the most frequently used Ψ-detection method involves RNA modification with *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium) ethylcarbodiimide *p*-tosylate (CMC), a carbodiimide reaction that modifies guanosine- and uridine-like nucleotides (14). An alkaline treatment (pH 10.4) will remove all CMC groups except those that are coupled to the N3 position in pseudouridine (15). The CMC-modified RNA is used as template for the DNA polymerase, reverse transcriptase. The reverse transcriptase terminates its polymerisation when it encounters a CMC-modified ψ, and the resulting termination product(s) are size/sequence determined by gel electrophoresis. This method is widely used with a significant success, but it has a couple of limitations. In addition to CMC-modified ψ, other naturally occurring nucleotide modifications inhibit the reverse transcriptase, thus heavily modified RNAs, such as tRNA, are not readily amenable to ψ detection by CMC-modification/reverse transcriptase

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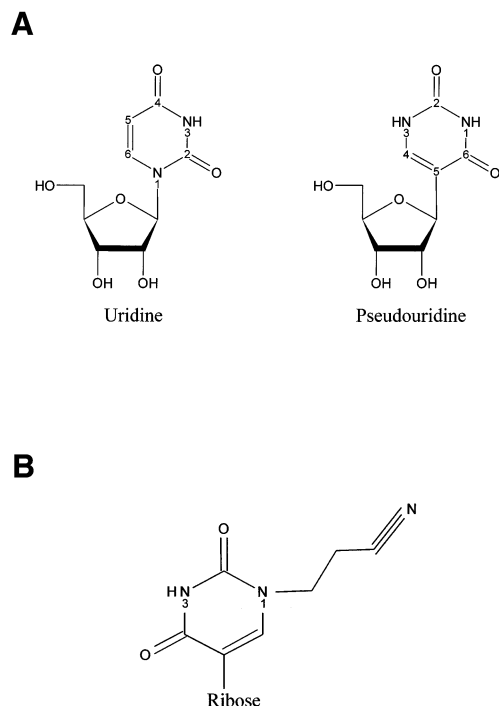*To whom correspondence should be addressed. Tel: +45 6550 2414; Fax: +45 6593 2781; Email: f.kir@bmb.sdu.dk

A

Uridine   Pseudouridine

B

Ribose

Figure 1. (**A**) Chemical structure of uridine and pseudouridine with numbering of selected atoms in the pyrimidine rings. (**B**) The proposed reaction product between ψ and acrylonitrile.

termination. Dihydrouridine and 4-thiouridine are also modified by the reported CMC procedure in ways that confer reverse transcriptase termination (14). Furthermore, the 3′-end of an RNA molecule cannot be investigated because a primer annealing site is required.

The CMC modification of ψ may also be combined with mass spectrometric identification of the modified nucleotides as reported recently (16). RNase T1 digests of CMC-modified tRNA were analysed by matrix assisted laser desorption/ionisation (MALDI) mass spectrometry in order to locate digestion fragments harbouring a CMC group. We have chosen a single-reaction chemistry to modify the ψs based on selective cyanoethylation of its uniquely accessible N1 with acrylonitrile (Fig. 1B). Specific cyanoethylation of ψ-monophosphate was reported about 30 years ago (17), and this chemistry has subsequently been applied to modification of ψs in tRNA (18,19). Normal uridine is only ~5% cyanoethylated under conditions where ψ is completely modified (19). Weakly alkaline conditions lead to the formation of the reactive nucleobase anion with either N1 or N3 being deprotonated (20,21), followed by a nucleophilic Michael-type addition to acrylonitrile. The ratio of N1 to N3 deprotonation is approximately 4:1 (20), which partly explains the selectivity for N1 cyanoethylation. However, the ratio of N1 to N3 cyanoethylation is much higher than 4:1, suggesting that N1 is inherently more reactive than N3 perhaps due to steric hindrance from the two carbonyl oxygens flanking the N3 position. We chose to pursue this chemistry because it appeared experimentally simple and could be performed under

conditions that, a priori, were not expected to degrade the RNA under investigation. We report here optimised conditions for selective ψ-modification in tRNA, and the subsequent MALDI- and tandem-mass spectrometric analysis of RNase-digested tRNA.

MATERIALS AND METHODS

Cyanoethylation

tRNA, obtained from Sigma (phenylalanine specific from brewers yeast and tyrosine specific II from *Escherichia coli*), was subjected to an ethanol/ammonium acetate precipitation in order to remove alkali ions. Optimal cyanoethylation conditions were determined by reacting tRNA with acrylonitrile (Aldrich) as follows. (i) 2 μl of 1 μg/μl tRNA plus 30 μl of 41% ethanol/1.1 M triethylammoniumacetate (pH 8.6) and 4 μl acrylonitrile (20). (ii) 18 μg of lyophilised tRNA dissolved in 10 μl of 2.7 M *N,N*-dimethyl formamide/0.5 M sodium phosphate (pH 8.8)/2.3 M acrylonitrile (18). (iii) 8 μg of lyophilised tRNA dissolved in 50 μl of 6 M urea/10 mM sodium phosphate (pH 8.8) and pre-heated to 75°C for 2 min followed by addition of acrylonitrile to 2.2 M. Various incubation times between 1 and 48 h and temperatures between 20 and 70°C were tested. The optimal conditions were found to be method (i) with incubation at 70°C for 2 h. After cyanoethylation, the reaction mixture was lyophilised, redissolved in water and the tRNA recovered by precipitation with 2 M ammonium-acetate and 2.5 vol ethanol.

RNase digestion and mass spectrometry

tRNA (1–2 pmol) was digested in 1–2 μl 50 mM 3-hydroxypicolinic acid (3-HPA). RNase A (Sigma-Aldrich) digestions were performed for 2–4 h at 37°C with 0.1 μg of enzyme. RNase T1 digestions were carried out for 2–16 h at 37°C with 20 U of enzyme (United States Biochemicals); short digestion times resulted in predominantly 2′-3′ cyclic phosphates whereas longer time gave a gradual conversion to 'linear' phosphates (18.01 Da heavier).

Details on the MALDI mass spectrometry have been published previously (22). Briefly, an RNase digest was directly mixed with 0.7 μl of a 0.5 M 3-HPA matrix in 50% acetonitrile and ammonium-loaded ion exchange beads on the target plate; it was then allowed to air dry. The spectra were recorded in positive ion mode on either Perseptive STR or Bruker Reflex IV MALDI reflector Time-of-Flight (TOF) mass spectrometers. All MALDI-TOF spectra were smoothed using the 'm/z' free software (Proteometrics Inc.).

The MALDI tandem mass spectrometry data were obtained on a MicroMass MALDI Q-TOF Ultima mass spectrometer. The sample preparations were made with 0.5 μl of a 0.5 M 3-HPA matrix and 1 μl of RNase digest. All spectra were recorded in positive ion mode. MALDI ions were generated at a backing pirani pressure reading of 2.5×10^{-1} mBar N_2 to provide collisional cooling, and extracted with 50 V into the instrument. The window for ion selection was between 2 and 4 m/z values in the quadrupole during MS/MS experiment. Argon was used as collision gas at an indicated manifold pressure of 3×10^{-5} mBar in the hexapole, and the collision energy (E_{lab}) was 10 eV for MS experiments and ranged from 50 to 80 eV for MS/MS experiments. An acceleration potential
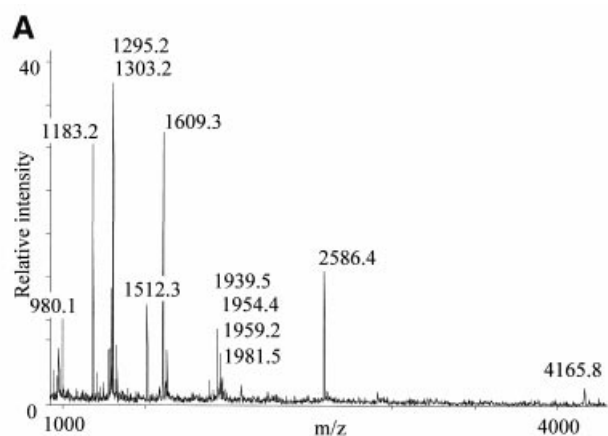
of 9.1 kV was used to introduce the ions into the orthogonal reflector TOF mass analyser. All MALDI Q-TOF spectra have been smoothed using the manufacturer supplied MassLynx software.

RESULTS

Derivatisation and mass spectrometric analysis

Because ψ is a mass-neutral post-transcriptional modification, derivatisation of this nucleotide is necessary before mass spectrometric detection. Specific cyanoethylation of N1 in ψ without concomitant modification of the uridine precursor has been known for numerous years, both with pure nucleotides (17) and in a tRNA context (18,19). The chemical reaction can be performed under mildly alkaline conditions (pH 8.5–9.0) (20), which should not significantly affect the alkali-labile phosphodiester backbone of RNA.

tRNA has previously been subjected to cyanoethylation, but the degree to which different ψs in the same molecule are modified is highly dependent on the reaction conditions. This is probably a result of the three-dimensional structure of the tRNA (18). Using yeast tRNA[Phe] as substrate, we tested ethanol/triethylamine (20), dimethylformamide/sodium phosphate (18) and urea/sodium phosphate buffers at different reaction times and temperatures to determine the best conditions for efficient but specific cyanoethylation of both ψs in the tRNA. By far the most efficient cyanoethylation was observed with the ethanol/triethylamine buffer (20), and we found a relatively short reaction time at an elevated temperature (2–4 h at 70°C) to yield the overall best results. To evaluate the cyanoethylation reactions, the tRNA was digested to completion with either RNase T1 or RNase A, and direct MALDI mass spectrometry of the digests was performed (22). Addition of a single cyanoethyl group will result in a mass increment of 53.0 Da (monoisotopic mass) compared with the unmodified digestion fragment. Figure 2 compares unmodified and cyanoethylated tRNA[Phe] that have both been subjected to RNase T1 digestion, and the accompanying table gives the assignment of the observed signals. Note that only tri-nucleotides or larger can be reliably mass analysed, because the signal from smaller digestion products is generally obscured by signals from matrix, buffers, etc. There are some interesting features already on the RNase T1 digestion of the chemically unmodified tRNA[Phe] (Fig. 2A). The fragment pattern tells us that RNase T1 cleaves efficiently after m2G, because the 1954.3 m/z (Th) species 5′-AUUUA[m2G]p-3′ is readily produced (see figures for nucleotide abbreviations). m2₂G is not equally well recognised by the enzyme as manifested by the simultaneous presence of the 1981.2 Th species 5′-C[m2₂G]CCAGp-3′ and the product of m2₂G recognition, 5′-CCAGp-3′, at 1303.2 Th. Complete resistance to enzyme cleavage was observed after the modified guanosine nucleotides, m7G, Gm and wybutosine. The signal at 1183.2 Th corresponds to the 3′-terminal RNase T1 digestion fragment without the distal acceptor adenosine (5′-CACC-3′); a minor signal at 1512.30 Th corresponds to the intact species 5′-CACCA-3′. We believe this degradation is present in the supplied tRNA[Phe], because we have not observed a similar phenomenon with any other RNase T1-digested RNA (including the tRNA[TyrII] described in the

m/z	Position	Sequence
980.2	D16:G18	DDGp.
1183.2	C72:C75	CACC.
1295.2	T54:G57	TΨCGp.
1303.2	C27:G30	CCAGp.
1512.3	C72:A76	CACCA.
1609.2	C11:G15	CUCAGp.
1939.3	A66:G71	AAUUCGp.
1954.3	A5:[m2G]10	AUUUA[m2G]p.
1959.3	[m7G]46:G51	[m7G]UC[m5C]UGp.
1981.3	C25:G30	C[m2₂G]CCAGp.
2586.4	[m1A]58:G65	[m1A]UCCACAGp.
4165.7	A31:G42	A[Cm]U[Gm]AA[yW]AΨ[m5C]UGp.

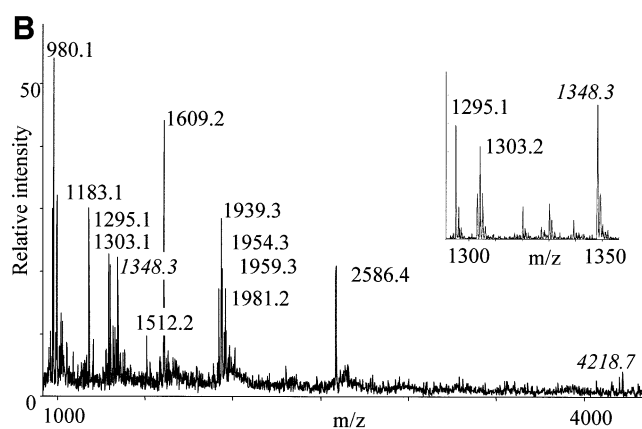| Modifications	
Gm:	2′-O-methylguanosine.
Cm:	2′-O-methylcytidine.
m1A:	1-methyladenosine.
m5C:	5-methylcytidine.
m7G:	7-methylguanosine.
D:	dihydrouridine.
m2G :	N2-methylguanosine.
Ψ:	pseudouridine.
m2₂G :	N2,N2-dimethylguanosine.
yW:	wybutosine.
T:	Ribothymidine.
p:	terminal phosphate.

Figure 2. MALDI-TOF mass spectra of yeast tRNA[Phe] digested with RNase T1 before (**A**) and after (**B**) reaction with acrylonitrile. Peaks labelled with italics contain ψs and have increased by 53.0 Da upon cyanoethylation. The insert is a zoom on the region containing the TΨCGp fragment. The table lists the expected RNase T1 fragments based on sequence and modification data present in the tRNA database (27).

following section). When comparing the spectra in Figure 2A and B, it is evident that the two fragments, which each harbour one ψ (at 1295.2 and 4165.8 Th, respectively), give rise to unambiguous signals ~53.0 Th higher upon cyanoethylation (labels in italics). The cyanoethylation is not complete as can be seen by the remaining 1295.2 Th species in the
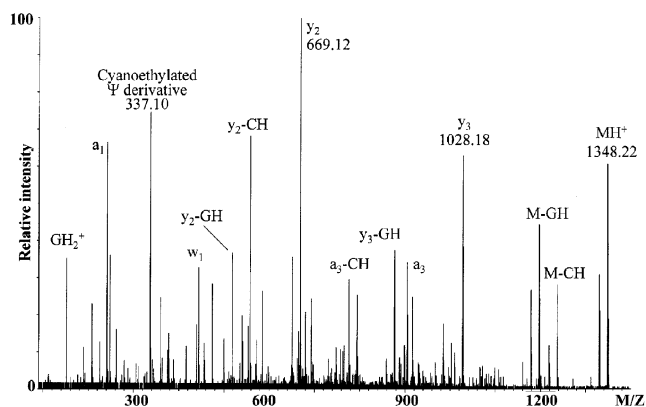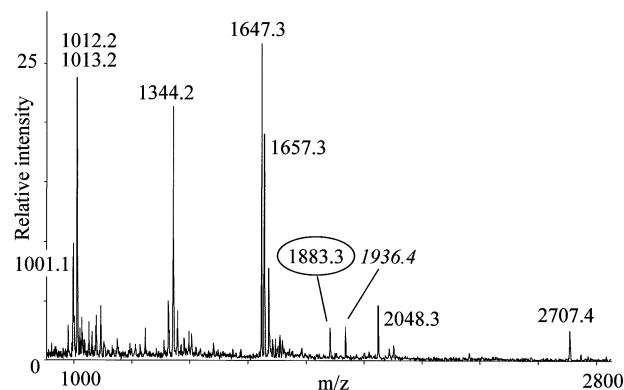
Figure 3. MALDI Q-TOF tandem mass spectrum of the 1348.22 Th singly protonated species, 5′-TØCGp-3′ + 53.03 Th, obtained through RNase T1 digestion of cyanoethylated *E.coli* tRNA^{TyrII}. The prominent fragment ions are assigned, and m/z-values used to locate the site of cyanoethylation are given.

magnification in Figure 2B. However, the specificity of the cyanoethylation for ψ is noteworthy: not even the 1954.3 Th species 5′-AUUUA[m^{2}G]p-3′, containing three uridines, exhibits a noticeable degree of reaction with the acrylonitrile. Thus, the cyanoethylation reaction is very selective for ψ under the conditions used. Preliminary experiments indicate that cyanoethylated ψ does not inhibit the polymerising activity of reverse transcriptase (data not shown), so this derivatisation method probably cannot be used to detect ψs by primer extension approaches.

The cyanoethylation approach only locates ψs to a defined RNase digestion fragment, but does not reveal the exact modified position. We therefore performed a tandem mass spectrometry experiment (23) on the cyanoethylated 5′-TψCGp-3′ RNase T1 fragment (1348.25 Th). The mass spectrometric fragmentation was performed on a hybrid MALDI quadrupole-TOF mass spectrometer that allows selective isolation and subsequent gas-phase fragmentation by collisional activation of the ion of interest. The outcome of this experiment is shown in Figure 3, a spectrum rich in information about the structure of the analyte. Amongst others, a series of y-fragment ions [originating from cleavage of the 5′ P–O bonds of the phosphodiester groups; nomenclature according to (24)] is observed. The y-ions are prominent in the experimental set-up used (23). The difference between the parent ion (MH$^+$) and the y$_3$ ion corresponds to the expected 5′-T nucleotide (320.04 Th observed, 320,04 Th calculated). Between the y$_3$ and the y$_2$ ions is a difference of 359.06 Th, which fits precisely with the presence of a cyanoethylated ψ at nucleotide two in the selected digestion fragment. Thus, tandem mass spectrometry can be used to pinpoint the chemically modified nucleotide. The intense signal at 337.10 Th may be explained as a cyanoethylated ψ nucleotide, which has lost CH$_3$CN, a commonly observed neutral fragment loss (25). More work is required to verify this interpretation and to determine whether the 337.10 Th fragment is generally diagnostic for cyanoethylated ψ.

In our standard protocol for RNase digestion of 5S rRNA prior to mass spectrometry, we used the addition of 3-HPA in order to partly denature the secondary structure of the substrate and thereby obtain a more complete cleavage (22).
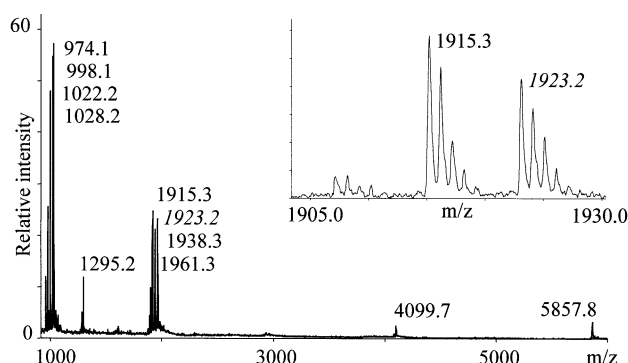

m/z	Position	Sequence
1001.2	A14:D16	AGDp.
1012.2	A9:C11	A[m^{2}G]Cp.
1013.2	G57:U59	G[m^{1}A]Up.
1344.2	G3:U6	GGAUp.
1647.2	A29:U33	AGA[Cm]Up.
1657.2	A64:U68	AGAAUp.
2048.3	G42:U47	GGAG[m^{7}G]Up.
2241.4	[Gm]34:Ψ39	[Gm]AAyWAΨp.
2707.4	G18:C25	GGGAGAGCp.

Modifications
Gm: 2′-O-methylguanosine.
Cm: 2′-O-methylcytidine.
m^{1}A: 1-methyladenosine.
m^{7}G: 7-methylguanosine.
D: dihydrouridine.
m^{2}G: N2-methylguanosine.
Ψ: pseudouridine.
yW: wybutosine.
T: Ribothymidine.
p: terminal phosphate.

Figure 4. MALDI-TOF mass spectrum of cyanoethylated yeast tRNA^{Phe} digested with RNase A in the presence of 3-HPA. The circled peak represents the 34–39 fragment that has lost the nucleobase from the wybutosine by acidic hydrolysis. The cyanoethylated version of this fragment is labelled in italics (see text for details). The table lists the expected RNase A fragments based on data present in the tRNA database (27).

The acid was omitted in the digestions used to obtain the Figure 2 spectra because the N-glycosidic bond of post-transcriptionally modified nucleotide wybutosine is hydrolysed under mildly acidic conditions (26). Figure 4 depicts an RNase A digestion of cyanoethylated tRNA^{Phe} in the presence of 50 mM 3-HPA. The calculated value for the wybutosine-containing fragment is 2241.4 Th, but instead a fragment at 1883.3 Th appeared, reflecting a hydrolysis of the N-glycosidic bond of wybutosine. The wybutosine-containing fragment ends in a ψ, and therefore its cyanoethylated product at 1936.4 Th is also observed. The presence of the cyanoethylated RNase A digestion fragment demonstrates that RNase A can cleave after this chemically modified pyrimidine nucleotide. We also observed RNase A cleavage after thymine, m^{5}C, dihydrouridine and ψ, but not Cm with this tRNA.

Escherichia coli tRNA^{TyrII}

The tRNA^{Phe} from yeast is probably the best characterised tRNA, with all post-transcriptional modifications identified. We therefore investigated the less characterised *E.coli* tRNA^{TyrII} to see if our method could reveal additional modifications to the ones reported in the tRNA database (27) (http://www.uni-bayreuth.de/departments/biochemie/trna/. The database is now maintained by M. Sprinzl and

m/z	Position	Sequence	Modifications
974.1	C44:G46	CCGp.	Gm: 2'-O-methylguanosine.
998.1	C29:G31	CAGp.	ms²i6A: 2-methylthio-N^6-
1022.2	A59:G61	AAGp.	isopentenyladenosine.
1028.2	C16:G18	C[Gm]Gp.	s⁴U: 4-thiouridine.
1295.2	T63:G66	TΨCGp.	Ψ: pseudouridine.
1907.2	*[s4U]8:G13*	*[s4U]UCCCGp.*	Q: queuosine.
1915.3	A53:G58	ACUUCGp.	T: Ribothymidine.
1938.3	U47:G52	UCACAGp.	p: terminal phosphate.
1961.3	C19:G24	CCAAAGp.	
4099.6	A32:G43	ACUQUA[ms²i6A]	
		AΨCUGp.	
5857.8	A67:A85	AAUCCUUCCC	
		CCACCACCA.	

Figure 5. MALDI-TOF mass spectrum of *E.coli* tRNA^{TyrII} digested with RNase T1. The fragment revealing an additional 16.0 Da post-transcriptional modification is labelled with italics in the mass spectrum and the table; the insert is a zoom of the corresponding region. The table lists the expected RNase T1 fragments based on sequence and modification data present in the tRNA database (27).

K. S. Vassilenko). Figure 5 presents the outcome of an RNase T1 digestion on the unmodified tRNA. The spectrum clearly has one difference compared with the expected fragment pattern: the calculated fragment at 1907.2 Th (position 8 to 13; 5′-[s4U]UCCCGp-3′, where s4U is 4-thiouridine) is essentially replaced by a signal at 1923.2 Th (Fig. 5 insert). This mass increment of ~16.0 Da may reflect an extra oxygen atom (as a hydroxyl group) or the replacement of an oxygen atom with a sulfur atom as in s4U, but neither the exact nature nor the position of the modification could be determined from MALDI-TOF data. Digestion of the tRNA^{TyrII} with RNase A ruled out location of the 16.0 Da post-transcriptional modification on nucleotides 8 and 13, because these nucleotides occurred in RNase A digestion fragments at the predicted m/z ratios (data not shown). Thus, the detected modification must be located between positions 9 and 12 (5′-UCCC-3′ + 16.0 Da). The RNase A digestion did not reveal any further unreported modifications.

To obtain more information on the location of the +16 Da post-transcriptional modification, we performed a mass spectrometric fragmentation study on the m/z 1923.22 RNase T1 digestion product (Fig. 6), and again the y-fragment ions predominate. The y_5 ion corresponds to the loss of 322.04 Th, as expected from the presence of 4-thiouridine at the 5′-end of the selected ion. The y_4 ion originates from an
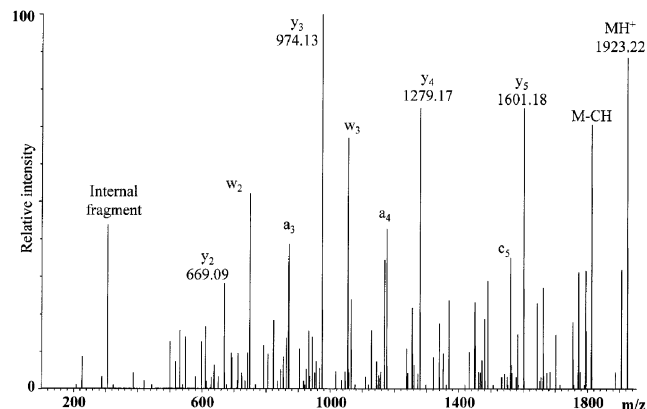

Figure 6. MALDI Q-TOF tandem mass spectrum of the 1923.22 Th singly protonated species obtained through RNase T1 digestion of *E.coli* tRNA^{TyrII}. The y-ion series is given together with the m/z values. Other prominent fragment ions are assigned.
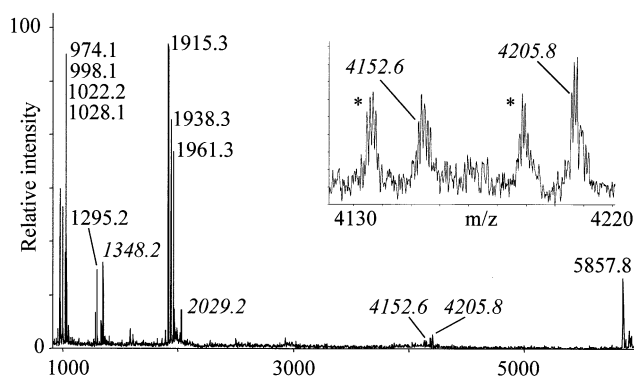

Figure 7. MALDI-TOF mass spectrum of *E.coli* tRNA^{TyrII} digested with RNase T1 after reaction with acrylonitrile. Peaks corresponding to cyanoethylated species are labelled in italics. The insert is a zoom on the region containing the putatively doubly pseudo-uridinylated fragment; the asterisk-labelled peaks are the accompanying 2′-3′ cyclic phosphate-harbouring species. See Figure 5 for a table of expected chemically unmodified fragments.

additional loss of 322.01 Th. This unveils that the +16.0 Da post-transcriptional modification is located in the second nucleotide of the selected ion (position 9 in the tRNA), because a uridine at this position would have resulted in loss of calculated 306.03 Th going from y_5 to y_4. Of the known uridine modifications in *E.coli* (28), the modified nucleotide could be 2-thiouridine, 4-thiouridine or 5-hydroxyuridine.

We then cyanoethylated the tRNA^{TyrII} to see if we could detect ψs additional to the ones in positions 40 and 64 (27). Digestion of the cyanoethylated tRNA with RNase T1 (Fig. 7) shows that the fragment 5′-TψCGp-3′ (1295.19 Th unmodified) exhibits the expected mass shift of 53.0 Da to 1348.2 Th, and that the other ψ-harbouring fragment, position 32 to 43, appears as a doublet at 4152.6 and 4205.8 Th (in addition to the two associated species ending in 2′-3′ cyclic phosphates ~18.0 Da lighter) with one and two cyanoethyl groups coupled, respectively. This suggests that an extra, hitherto unreported, ψ is present in the latter fragment. Because the position 32 to 43 fragment in the RNase T1 digest

turns up as a doublet upon cyanoethylation, the reaction of the two ψs with acrylonitrile has not run to completion. It is possible that either the queuosine or the 2-methylthio-N^6-isopentenyladenosine in the position 32–43 RNase T1 fragment had reacted with acrylonitrile, but an RNase A digestion of the cyanoethylated tRNA^{TyrII} did not support this possibility (data not shown). Sequence elucidation by tandem mass spectrometry is not feasible with this fragment, because the MALDI Q-TOF instrument only allows specific ion selection up to 4000 Th.

Figure 7 also yields additional information about the position of the 8–13 fragment (5′-[s4U]XCCCGp-3′) with the unknown +16.0 Da modification, because it becomes doubly cyanoethylated to yield a 2029.2 Th signal. Thus, the unknown nucleotide in this fragment also reacted with acrylonitrile. A priori we had expect this fragment to be singly cyanoethylated, because 4-thiouridine reacts readily with acrylonitrile forming an *S*-cyanoethyl product (21). The double cyanoethylation strongly indicates that the nucleotide at position 9 in tRNA^{TyrII} is also a 4-thiouridine: 2-thiouridine is reported not to react with acrylonitrile (21), and 5-hydroxyuridine does not have the chemical properties to perform a nucleophilic addition to acrylonitrile.

DISCUSSION

As an extension of previous work, where we reported a MALDI mass spectrometric method to screen for post-transcriptional modifications (22), we have implemented a specific cyanoethylation of ψ, which is then amenable to mass spectrometric detection. The reaction is generally very selective for ψ versus its uridine precursor; no appreciable cyanoethylation of uridines was observed in any of the shown spectra. The degree of ψ cyanoethylation under the applied conditions is 50% or better as estimated from the signal intensities in the mass spectra (note that signal intensities are generally analyte-dependent, and quantifications are therefore not very reliable with our experimental set-up). However, if we aimed at a quantitative conversion of ψ to its cyanoethylated form, undesired cyanoethylated fragments started to appear, probably from reaction of uridine nucleotides. Yoshida and Ukita (18) noted that the degree of cyanoethylation is dependent on the sequence context of a given ψ, which we have also observed. This is likely to be due to dissimilar accessibility of the ψs in tRNA's three-dimensional structure. Thus, different RNA molecules may require different reaction times in order to attain the optimal degree of ψ modification. We have also performed the RNase digestions prior to the cyanoethylation in order to avoid effects of RNA higher order structure. The approach was, in principle, successful, but it introduced difficulties in the subsequent purification: whereas vacuum drying followed by ethanol precipitation sufficed for the intact cyanoethylated tRNA, purification of the cyanoethylated digestion fragments required a chromatographic step due to the small size of the digestion fragments.

The cyanoethylation of a given RNase digestion fragment does not tell us where the ψ is located if the fragment contains more than one uridine. This is often the case in RNase T1 generated fragments. The application of RNase A sometimes resolves the ambiguity, but not for sequence stretches consisting of many pyrimidine nucleotides. We therefore

successfully used MALDI tandem mass spectrometry as a means to pinpoint the chemically modified nucleotide. The theoretical upper limit for this approach is 4000 Th, determined by the mass spectrometric hardware, but it is, in practice, probably lower due to reduced sequence information from larger singly charged species (23). Limbach and co-workers have used HPLC isolation of CMC-modified RNase T1 digestion fragments followed by sequential exonuclease digestion and mass spectrometry to determine the position of modified ψs (16). This method should also be applicable here. An alternative approach would be to perform tandem mass spectrometry on multiply charged ions generated by electrospray ionisation.

The RNase digestion pattern is dependent on whether post-transcriptionally modified positions are refractory to RNase cleavage. The natural cleavage site for RNase T1 is on the 3′-side of guadinylates, but in the present study we also observed cleavage after N^2-methylguanine and less efficiently after N^2,N^2-dimethylguanine. Cleavage after prolonged incubation with RNase T1 has also been observed after N^1-methylguanine (S.Douthwaite, M.F.Liu and F.Kirpekar, unpublished results). In contrast, we observed complete RNase T1 inhibition by the guadinylate derivatives 2′-*O*-methyl guanine, N^7-methylguanine, queuosine and wybutosine. X-ray crystal data show that the both the N^1, N^2 and N^7 positions of guanosine are involved in the substrate recognition by the enzyme (29), but there is apparently only an absolute requirement for unmodified N^7 in the RNase T1-mediated cleavage. RNase A, which cleaves after pyrimidine nucleotides, is much less selective in its choice of substrate in that we have only observed inhibition by 2′-*O*-methylated pyrimidine nucleotides. The 2′-*O*-methyl inhibition is understandable in light of the RNase cleavage mechanism, where hydrolysis of the phosphodiester backbone proceeds over a 2′-3′ cyclic phosphate intermediate. RNase A cleavage was observed after 5-methylcytidine, thymidine, dihydrouridine, pseudouridine and 4-thiouridine, as well as the cyanoethylated derivatives of the latter two nucleotides.

The 'traditional' CMC-derivatisation affects post-transcriptionally modified nucleotides other than ψ (14), and acrylonitrile likewise reacted with 4-thiouridine [as well as inosine (21)] in the present study. This was not a problem but rather aided the identification of the additional 4-thiouridine in *E.coli* tRNA^{TyrII}. Analysing the unreacted tRNA already revealed the presence of an additional modification, and cyanoethylation gave a strong indication of the identity of the modification. In conclusion, mass spectrometry in combination with cyanoethylation as a means to analyse post-transcriptional modifications is experimentally simple and chemically gentle, and the methodology complements the CMC/alkali approach with either mass spectrometric (16) or reverse transcriptase/electrophoretic (14) detection.

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