Assay of both activities of the bifunctional tRNA-modifying enzyme MnmC reveals a kinetic basis for selective full modification of cmnm\(^5\)s\(^2\)U to mnm\(^5\)s\(^2\)U

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ABSTRACT

Transfer RNA (tRNA) contains a number of complex ‘hypermodified’ nucleosides that are essential for a number of genetic processes. Intermediate forms of these nucleosides are rarely found in tRNA despite the fact that modification is not generally a complete process. We propose that the modification machinery is tuned into an efficient ‘assembly line’ that performs the modification steps at similar, or sequentially increasing, rates to avoid build-up of possibly deleterious intermediates. To investigate this concept, we measured steady-state kinetics for the final two steps of the biosynthesis of the mnm\(^5\)s\(^2\)U nucleoside in Escherichia coli tRNAGlu, which are both catalysed by the bifunctional MnmC enzyme. High-performance liquid chromatography-based assays using selectively under-modified tRNA substrates gave a \(K_m\) value of 600 nM and \(k_{cat}\) 0.34 s\(^{-1}\) for the first step, and \(K_m\) 70 nM and \(k_{cat}\) 0.31 s\(^{-1}\) for the second step. These values show that the second reaction occurs faster than the first reaction, or at a similar rate at very high substrate concentrations. This result indicates that the enzyme is kinetically tuned to produce fully modified mnm\(^5\)s\(^2\)U while avoiding build-up of the nm\(^5\)s\(^2\)U intermediate. The assay method developed here represents a general approach for the comparative analysis of tRNA-modifying enzymes.

INTRODUCTION

tRNA molecules are heavily modified with many non-canonical nucleosides, which perform a number of biological functions including structural stabilization (1) and optimization of codon binding (2). These modifications range from simple methylations to complex ‘hypermodified’ residues involving multistep biosyntheses. The hypermodified nucleosides are particularly interesting, as the cellular machinery allocates a relatively large amount of energy to the biosyntheses of these modifications, indicating a high importance to cellular processes. These nucleosides are most often located in or adjacent to the anticodon (3), and are involved in key translation processes anticodon–stem loop stabilization (2), codon binding and wobble base pairing (4,5). Recent studies on the quantification of modified nucleosides show that tRNA is not always fully modified, instead containing a significant number of unmodified positions [6,7 and unpublished data]. However, partially modified precursors of hypermodified nucleosides are generally not observed, with the exception of those present in tRNAs that always contain the partially modified form (3,8–10). Together, these results suggest that biosynthetic pathways are organized to give complete hypermodified nucleosides, avoiding partially modified intermediate nucleosides. Biosynthetic tuning of this type would require a coordinated ‘assembly line’ process in which sequential modifications are performed at similar, or increasing, rates in order to efficiently construct the final products without a build-up of intermediates. We propose that this process is likely to be predominantly controlled by tuning of the activities and abundances of the tRNA-modifying proteins. Alternatively, biosynthetic tuning could involve selective degradation of partially modified tRNAs (11) or control of enzyme compartmentalization (12) in eukaryotes.

The 5-methylaminomethyl(-2-thio)uridine (mnm\(^5\)s\(^2\)U) residue, which is present in position 34 of the anticodon of Escherichia coli tRNAs Glu, Lys, Arg and, probably, Gln (3), is particularly interesting due to its involvement in wobble base pairing (4,5,13,14) and its complex
four-step biosynthetic pathway (Scheme 1). The s² group is inserted by the MnmA enzyme (15,16), while the mnm₅ group is independently formed in a three-step sequence. Initially, (s²)U is converted to 5-carboxymethylaminomethyl(-2-thio)uridine (cmnm⁵(s²)U) by a two-enzyme complex involving addition of glycine to a reactive intermediate (17–19). The cmnm⁵(s²)U base is then converted to mnm⁵s²U in two steps by the bifunctional enzyme MnmC (20–22), which initially carries out a flavin adenine dinucleotide (FAD)-mediated demodification to 5-aminomethyl(-2-thio)uridine (nm₅(s²)U), followed by methylation to mnm⁵(s²)U using S-adenosylmethionine (SAM) as cofactor. To test our proposal that the modification steps are coordinated by tuning of the kinetics and abundance of biosynthesis enzymes, we investigated the activities of the two modification steps performed by MnmC. As these two reaction steps are both performed by a single enzyme, differences in enzyme abundance for the individual steps are excluded. Therefore, control of enzyme kinetics would be necessary for our proposed assembly-line type process. To our knowledge, the partial modification nm₅(s²)U has not been reported in normal cellular tRNA, indicating that this intermediate is avoided by the biosynthetic machinery. The presence or absence of under-modified cmnm⁵(s²)U is not clear due to the natural occurrence of this nucleoside at certain tRNA positions.

Kinetic assay of complex tRNA-modifying enzymes is generally problematic due to the difficulty of obtaining defined under-modified tRNA substrates and the measurement of reaction progress. Previous cmnm⁵(s²)U → mnm⁵(s²)U assays have used either total tRNA from cells lacking MnmC (20–22) or substantially under-modified tRNA from in vitro transcription (18). The most accurate assay results to date indicate that the first step is rate limiting (20), but could only measure the methylation reaction and did not have the defined substrates necessary for full kinetic analysis. In order to obtain such substrates for reaction with MnmC, we overexpressed tRNA⁶Glu in an E. coli expression strain lacking MnmC, then isolated the selectively undermodified cmnm⁵(s²)U-containing tRNA species using anion-exchange high-performance liquid chromatography (HPLC). The second substrate (containing nm₅(s²)U) was formed by reaction of the cmnm⁵(s²)U-containing tRNA with MnmC in the absence of the SAM cofactor. Reaction of each substrate with MnmC was assayed using anion-exchange HPLC to monitor reaction progress. To further assess the importance of the MnmC-mediated modifications, we additionally measured growth curves for an MnmC knockout E. coli strain and its corresponding wild-type strain.

**MATERIALS AND METHODS**

**Preparation of recombinant MnmC protein**

His-tagged MnmC was cloned and expressed as previously reported (21) in E. coli BL21 cells, and purified as follows. All steps were performed at 0–4°C. Harvested cells were suspended in buffer A (50 mM Tris–HCl, pH 8.0, 200 mM KCl, 10 mM MgCl₂, 3 mM β-mercaptoethanol, 10% glycerol) with the addition of lysozyme (0.2 mg/ml), then lysed using a French press. The lysate was cleared by centrifugation (24000g for 30 min) and filtration (0.45 μM) and was applied to a HiScreen IMAC FF column (GE Healthcare) charged with Ni²⁺, using an AKTApurifier system (GE Healthcare). The column was washed with
buffer A supplemented with 5 mM imidazole then MmmC was eluted with buffer A containing 0.5 M imidazole. The protein was purified and exchanged into buffer B (buffer A lacking KCl) using an Amicon Ultra 30 000 MWCO centrifugal filter (Millipore) then applied to a 1 ml Mono Q column (GE Healthcare) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of buffer C (buffer A containing 500 mM KCl), concentrated then applied to a Superdex 200 GL10/300 size-exclusion column and eluted with buffer C. The purified protein was concentrated to 10 mg/ml in buffer D (buffer A containing only 50 mM KCl) and stored at −80°C. A yield of ∼10 mg of purified protein was obtained from 41 of expression culture. Protein concentration was calculated by Bradford assay.

MmmC knockout from an E. coli expression strain

The MmmC gene was removed from T7 express E. coli (NEB) by replacement with a chloramphenicol resistance cassette. The cassette was made by polymerase chain reaction (PCR) amplification of the cmr gene from the pDEST17 plasmid (Invitrogen) using Phusion polymerase (Finnzymes) and the following primers, each incorporating 50 bases of the MmmC gene: 5'-TGAACACTACTCATACAACCTGCAACCATTAATGGCTAGGGTGTCGGCCGCGATTTAGGGCACCCC and 5'-TACCCCCGCTTTAACCAGCTTTACCTAACACATTCCGACACTATAACC GTTACGCCCCGCCCTTTAACCTTCAAC TGCCACT. Chemically competent T7 express cells were transformed with the sPIM6 plasmid (23), kindly supplied by Donald L. Court, then made electrocompetent and transformed with the pSIM6 plasmid (23), kindly supplied by Donald L. Court and the following primers, each incorporating 50 bases of the MmmC gene: 5'-TGAACACTACTCATACAACCTGCAACCATTAATGGCTAGGGTGTCGGCCGCGATTTAGGGCACCCC and 5'-TACCCCCGCTTTAACCAGCTTTACCTAACACATTCCGACACTATAACC GTTACGCCCCGCCCTTTAACCTTCAAC TGCCACT. Chemically competent T7 express cells were transformed with the sPIM6 plasmid (23), kindly supplied by Donald L. Court, then made electrocompetent and transformed with the PCR-amplified Cam cassette. After pregrowth in LB medium then selection on Cam plates, transformed cells were grown in LB medium supplemented with carbenicillin (100 μg/ml) and stored at −80°C.

Cloning, expression and purification of under-modified tRNA<sub>Glu</sub>

The tRNA<sub>Glu</sub> gene (corresponding to RNA sequence GUCCCCUUGUCUAGGCGCACAAGCCGCGGCU UUCACGGGCGUAAACAGGGGUUCGAUUCCTCU AGGGGACGC) was amplified by PCR from NEB5a E. coli (NEB) using Phusion polymerase and the following primers to give an EcoRI cleavage site and the T7 promoter 5' of the tRNA sequence, and BsaI and HindIII cleavage sites 3' of the tRNA: 5'-CTGAACCATATGAAACACTACTCCATAACAC CTGCC, 5'-CTGAACAGATCTTTACCCCGCTTTAACC CGGTTTTA CCCCCTAACC. The cells were made electrocompetent then stored at −80°C in 10% glycerol. For assay of the FAD-dependent MmmC activity, the following reaction mixture was used: 0.4 μM MmmC, 0.5 μM Tris (400 mM, pH 7.5), 1 mM MgCl₂, 0.5 mM FAD, 30 μM adenosine 5'-triphosphate (ATP), 1 mM DTT, 100 μg/ml bovine serum albumin (BSA), and pH 7.5. The reaction was initiated by the addition of enzyme and allowed to proceed for 1 h at 30°C.

Characterization of tRNA

The purified tRNA was analysed by digestion with RNases A or T1, followed by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) (24). For identification of pseudouridine bases, tRNA (2 μM) and acrylonitrile (1.8 M) were initially dissolved in TEA-AcOH buffer (0.98 M), ethanol (36%) at a total volume of 3 μl. The mixture was incubated at 70°C for 1 h, then lyophilized, redissolved in water and exchanged into pure water using a centrifugal filter.

RNase digests were performed by incubation of tRNA (0.2 μg) with RNase A (0.1 μg, Fermentas) or RNase T1 (1 U, Ambion) in 3 mg/ml 3-HPA, 1% acetonitrile at a total volume of 3 μl at 37°C for 2 h (for RNase A) or 7 h (for RNase T1). The hydrolysed tRNA was desalted three times using a 0.025 M drop dialysis filter (Millipore) then 0.5 μl of the solution was mixed with 0.5 μl of matrix (0.6 M 3-HPA, 32 mM picolinic acid, 18 mM diaminonitrile citrate, 10% acetonitrile) on a MALDI sample plate and allowed to dry. MALDI-TOF MS was performed using a Bruker Autoflex II in negative mode with 19 kV and 16 kV ionization source voltages, 8.55 kV lens voltage, 20 kV reflector voltage and 200 ns pulsed ion extraction. Masses were externally calibrated using oligonucleotides of known sequence, or internally calibrated using invariant tRNA fragments from the sample (e.g. for accurate analysis of the anticodon–stem loop fragment).

Assay of MmmC

For assay of the FAD-dependent MmmC activity, the following reaction mixture was used: 0.4 μM MmmC, 0.5 μM Tris (400 mM, pH 7.5), 1 mM MgCl₂, 0.5 mM FAD, 30 μM adenosine 5'-triphosphate (ATP), 1 mM DTT, 100 μg/ml bovine serum albumin (BSA), and pH 7.5. The reaction was initiated by the addition of enzyme and allowed to proceed for 1 h at 30°C.

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**RESULTS**

**Preparation of selectively under-modified tRNA\textsuperscript{Glu} substrates for MnmC**

In order to prepare selectively under-modified tRNA\textsuperscript{Glu}, we assembled a tRNA overexpression system (25) using an *E. coli* strain that lacks the MnmC gene. As the expression construct, the *E. coli* tRNA\textsuperscript{Glu} gene was cloned into a protein expression plasmid (pSGA12) containing a T7 promoter for inducible expression. An additional T7 promoter was added directly 5' to the tRNA gene in order to expedite tRNA processing (as the RNA transcripts from this promoter should begin at the first position of the tRNA and therefore not require enzymatic 5'-cleavage). To obtain an expression strain lacking MnmC, we used the recombineering method (23) to replace the MnmC gene in T7 expression cells with an antibiotic resistance cassette. The tRNA expression plasmid was then used to transform ΔMnmC cells, from which tRNA\textsuperscript{Glu} was expressed and isolated by phenol extraction (6). The major tRNA 'modivariants' (10) obtained were then separated by anion-exchange HPLC (Figure 1a and b) then partially sequenced by MALDI-MS analysis of RNase digests (Figure 2). All major peaks in the HPLC chromatogram were found to be tRNA\textsuperscript{Glu} based on this MALDI sequencing (Figure 2a–c and Supplementary Figure S1), showing that a very high level of expression was sustained by the cells. A single modivariant (tRNA 1, Figure 1a and c) was found to contain the desired cmnm\textsuperscript{5}s\textsuperscript{2}U modification (Figure 2c) as well as the other expected mass-detectable tRNA\textsuperscript{Glu} modifications (T and m\textsuperscript{2}A, Figure 2a–c). The presence of the two expected pseudouridine (Ψ) nucleotides in this tRNA was then confirmed by reaction with acrylonitrile followed by RNase/MS analysis (Supplementary Figure S2) (24). The other modivariants obtained lacked anticodon–stem loop modifications (Supplementary Figure S1), as labelled in Figure 1c.

The purified cmnm\textsuperscript{5}s\textsuperscript{2}U-containing tRNA 1 was reacted with purified recombinant MnmC (21) in the absence or presence of the SAM cofactor to give nm\textsuperscript{5}s\textsuperscript{2}U or mm\textsuperscript{5}s\textsuperscript{2}U-containing tRNA, respectively (tRNA 5 and 6, Figure 1c). These tRNA products were further purified by anion-exchange HPLC and analysed by MALDI-MS of RNase T1 digests of the tRNA products. Mass peaks corresponding to the anticodon–stem loop fragments confirmed the identity of each tRNA (Figure 2d and e). High purity of the tRNAs 1 and 5 was determined from the lack of contaminating under-modified fragments in MALDI-MS spectra, the single major HPLC peak detected for each tRNA, and the observed complete reaction with MnmC as measured by HPLC.

**Development of an HPLC-based assay for both activities of MnmC**

Anion-exchange HPLC of the cmnm\textsuperscript{5}s\textsuperscript{2}U- mm\textsuperscript{5}s\textsuperscript{2}U- and mm\textsuperscript{5}s\textsuperscript{2}U-containing tRNAs showed that each elutes at a different retention time (Figure 3a and b), allowing an HPLC-based assay to be used to measure both activities...
of the enzyme. As full separation of tRNA 5 and 6 was not obtained, a computational peak deconvolution was employed to calculate the area of each peak (Figure 3b). Preliminary studies confirmed the linear response of the areas of the product and substrate peaks, and the approximate saturating concentration of the SAM cofactor (100 mM). Subsequently, steady-state kinetic assays for each reaction step were carried out. The conditions for each assay were chosen to be similar in order to compare kinetic constants, and were based on conditions known to optimize enzyme activity (20) with the addition of MgCl₂ to stabilize tRNA (26). Rate versus substrate concentration curves are shown in Figure 3c and d. Fitting a rectangular hyperbola to these curves gives the Michaelis–Menten constants shown in Table 1. The Km for the second reaction is substantially lower (∼9-fold) than that of first reaction, and the kcat constant is not significantly different. This shows that the enzyme binds the second substrate (tRNA 5) tighter than the first (tRNA 1). This clearly will result in a higher rate of reaction for the second step, or similar rates at very high substrate concentrations where the kcat constant becomes dominant. The kcat/Km value, a general indicator of activity, is correspondingly eight times higher for the second reaction step, showing substantially higher activity at intermediate substrate concentrations.

To further support our idea that partially hypermodified nucleosides negatively affect translation processes, we measured the growth rate of a well-defined MnmC knockout strain from the Keio collection of E. coli knockouts (Figure 4). Exponential fits to the growth curves of the ΔMnmC and wild-type strains reveal a larger growth constant, k, for the wild-type strain. As the knockout strain is expected to contain the cmmn⁵s⁵U.
Figure 2. MALDI-MS spectra of RNase digests of tRNA 1, 5 and 6. (a) RNase A digest of tRNA 1 (cmnm5s2U-tRNA\textsuperscript{Glu}). (b) RNase T1 digest of tRNA 1. (c) Expanded section of (b) showing the anticodon–stem loop fragment of tRNA 1. Extra peaks are assigned as follows. a: 3165.5, Fragment A [AAUCCCCUAGcpcp = cyclic phosphate]; b: 3182.9, Fragment B (AAUCCCCUAGcp); c: 3203.7, Fragment A – H + K and Fragment B – H + Na; d: 3221.3, Fragment B – H + K; e: 3235.6, Fragment C (CCUC\textsuperscript{cmnm5s2U}UUC\textsuperscript{m2AC}Gcp); f: 3242.4, Fragment A – 2H + 2K; g: 3258.4, Fragment B – 2H + 2K; h: 3274.4, Fragment C – H + K and Fragment D (CCUC\textsuperscript{cmnm5s2U}UUC\textsuperscript{m2AC}Gcp) – H + Na; i: 3290.9, Fragment D – H + K; j: 3296.0, Fragment B – 3H + 3K. (d) Section of a RNase T1 digest of tRNA 5 (nm5s2U-tRNA\textsuperscript{Glu}) showing the nm5s2U-containing fragment. Extra peaks are assigned as follows. a: 3165.3, Fragment A (AAUCCCCUAGcpcp); b: 3177.8, Fragment E (CCUC\textsuperscript{nm5s2U}UUC\textsuperscript{m2AC}Gcp); c: 3182.9, Fragment B (AAUCCCCUAGcp); d: 3204.2, Fragment B – H + Na; e: 3215.8, Fragment E – H + K; f: 3221.2, Fragment B – H + K; g: 3233.5, Fragment F (CCUC\textsuperscript{nm5s2U}UUC\textsuperscript{m2AC}Gcp) – H + K; h: 3243.4, Fragment B – 2H + Na + K; i: 3258.5, Fragment B – 2H + 2K; j: 3270.1, Fragment F – 2H + 2K; k: 3296.1, Fragment B – 3H + 3K; l: 3307.8, Fragment F – 3H + 3K. (e) Section of an RNase T1 digest of tRNA 6 (mnm5s2U-tRNA\textsuperscript{Glu}) showing the mnm5s2U-containing fragment. Extra peaks are assigned as follows. a: 3165.4, Fragment A (AAUCCCCUAGcpcp); b: 3182.9, Fragment B (AAUCCCCUAGcp); c: 3191.0, Fragment G (CCUC\textsuperscript{nm5s2U}UUC\textsuperscript{m2AC}Gcp); d: 3204.9, Fragment B – H + Na; e: 3221.0, Fragment B – H + K; f: 3229.3, Fragment G – H + K; g: 3246.4, Fragment H (CCUC\textsuperscript{nm5s2U}UUC\textsuperscript{m2AC}Gcp) – H + K; h: 3258.9, Fragment B – 2H + 2K; i: 3267.3, Fragment G – 2H + 2K; j: 3283.9, Fragment H – 2H + 2K; k: 3296.5, Fragment B – 3H + 3K; l: 3306.7, Fragment A – 3H + 3K; m: 3322.0, Fragment H – 3H + 3K; n: Fragment B – 4H + 4K. The RNA fragments analysed in (a) and (b) are labelled with mass and sequence on each spectrum, and coloured in red on each corresponding tRNA diagram. The data in (c)–(e) are calibrated to the constant fragment B (AAUCCCCUAGcpcp, 3182.9) to allow accurate determination of the variable anticodon fragment.
modification in place of mnm⁵, the result shows that this cmnm⁵ under-modification is detrimental to cell growth.

DISCUSSION

The MnmC enzyme catalyses the final two steps of the biosynthesis of the mnm⁵(s²)U₃₄ nucleoside in tRNA. The mnm⁵ biosynthesis pathway seems to be tuned to give only the fully modified residue, in particular avoiding nm⁵ under-modification [cmnm⁵s²U has been detected in vivo (8); however, it is not clear whether this is due to under-modification or the natural presence of cmnm⁵ in certain tRNAs]. The biosynthetic machinery could be regulated in a number of ways, for example by selective degradation of partially modified tRNA (11), controlled expression [or compartmentalization, in eukaryotes (12)] of particular modifying enzymes or by optimization of enzyme activities and specificities. This study reveals that the two transformations catalysed by MnmC are regulated by enzyme kinetics, as the second substrate is bound tighter than the first. Even at relatively high substrate concentrations (~3 μM), the two reactions would be performed at similar rates, resulting in a relatively low concentration of the partially modified intermediate. Degradation of the under-modified tRNA cannot be ruled out as an additional mechanism; however, this seems unlikely given the existing kinetic control and the

Table 1. Michaelis–Menten constants for each MnmC catalysed reaction

| Reaction                        | $K_m$ (nM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (μM⁻¹ s⁻¹) |
|---------------------------------|------------|-----------------|--------------------------|
| cmnm⁵s²U → nm⁵s²U              | 600 ± 200  | 0.34 ± 0.04     | 0.56                     |
| nm⁵s²U → mnm⁵s²U              | 70 ± 40    | 0.31 ± 0.05     | 4.5                      |

Figure 3. HPLCs and Michaelis–Menten plots for each MnmC catalysed reaction. (a) Representative HPLC showing complete separation of tRNA 1 from tRNA 5. HPLC gradient: 100 mM Tris, 50 mM MgCl₂, 175 → 180 mM NaCl over 1 → 20 min. (b) Representative HPLC showing partial separation of tRNA 5 from tRNA 6, and calculated peaks for each tRNA. HPLC gradient: 100 mM Tris, 50 mM MgCl₂, 160 → 165 mM NaCl over 1 → 30 min. (c) Michaelis–Menten plot for the FAD-dependent cmnm⁵s²U → nm⁵s²U demodification. (d) Michaelis–Menten plot for the SAM-dependent nm⁵s²U → mnm⁵s²U methylation. Rates in (c) and (d) represent the amount of substrate formed in a 40 μl reaction per minute per milligram enzyme.
inefficiency associated with degradation of a highly modified tRNA.

In terms of the applicability of our in vitro study to in vivo enzyme activity, several aspects need to be considered. It is possible that the modification reactions are affected in vivo by other cellular components. Importantly, Mg\(^2+\) and NH\(_4\)\(^+\) concentrations were reported previously to affect enzyme activity. However, this is unlikely to substantially change the results reported here, as both activities of MnmC were reported to be similarly affected by changes in the concentrations of these ions (20). Intracellular SAM concentrations are reported to be similar to those used in our assay (27), so our result is likely to be relevant in this respect. Other modifying enzymes have been found to be dependent on the state of tRNA processing, such as TilS, which modifies tRNA\(^{\text{Ile}}\) at a precursor stage (28). None of the cmnm\(^5\)-containing tRNA developed in this article, future studies will be carried out to further elucidate the regulation of tRNA modification and the impact of partial hypermodification on genetic processes.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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