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# Evolutionarily conserved proteins MnmE and GidA catalyze the formation of two methyluridine derivatives at tRNA wobble positions

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## ABSTRACT

**The wobble uridine of certain bacterial and mitochondrial tRNAs is modified, at position 5, through an unknown reaction pathway that utilizes the evolutionarily conserved MnmE and GidA proteins. The resulting modification (a methyluridine derivative) plays a critical role in decoding NNG/A codons and reading frame maintenance during mRNA translation. The lack of this tRNA modification produces a pleiotropic phenotype in bacteria and has been associated with mitochondrial encephalomyopathies in humans. In this work, we use *in vitro* and *in vivo* approaches to characterize the enzymatic pathway controlled by the *Escherichia coli* MnmE•GidA complex. Surprisingly, this complex catalyzes two different GTP- and FAD-dependent reactions, which produce 5-aminomethyluridine and 5-carboxymethylaminomethyluridine using ammonium and glycine, respectively, as substrates. In both reactions, methylene-tetrahydrofolate is the most probable source to form the C5-methylene moiety, whereas NADH is dispensable *in vitro* unless FAD levels are limiting. Our results allow us to reformulate the bacterial MnmE•GidA dependent pathway and propose a novel mechanism for the modification reactions performed by the MnmE and GidA family proteins.**

## INTRODUCTION

Transfer RNAs (tRNAs) are by far the most heavily and diversely modified of all cellular RNAs (1). Modifications are introduced posttranscriptionally by specific enzymes and are critical for the fine-tuning of tRNA functions. Many of these modifications frequently appear in the anticodon wobble position (position 34) and are pivotal in the mRNA decoding process by stabilizing correct codon–anticodon interactions (1,2). Modifications at wobble uridines are classified into two groups according to their chemical structures and decoding properties: 5-hydroxyuridine derivatives ($xo^5U$) with an oxygen atom directly bonded to the C5 atom of the uracil base, and 5-methyluridine derivatives ($xm^5U$) with a methylene carbon directly bonded to the C5 atom. The last ones are mostly found in tRNAs that decode two-family box triplets ending in A or G, and include 2-thiouridine derivatives ($xm^5s^2U$) and 2′-*O*-methyluridine derivatives ($xm^5Um$). Modified nucleosides of the $xm^5s^2U$ type restrict the wobble capacity of uridine because the thiolation at position 2 of U34 stabilize the C3′-endo puckering conformation of the ribose, which facilitates the base paring with purines (A or G) and prevents misreading of codons ending in U or C (3). The $xm^5$ modification seems to be critical for decoding NNG/A codons by stabilizing U•G pairing at the wobble position as well as improving reading of the NNA codons (3–9). The lack of these modifications produces translational frameshifting and a pleiotropic phenotype in bacteria (1), whereas it has been associated with mitochondrial encephalomyopathies in humans (10) and mitochondrial dysfunction in a human cell line (11). Interestingly, some proteins involved in the biosynthesis of the $xm^5(s^2)U$ type nucleosides are evolutionarily conserved from bacteria to humans, so that the use of *Escherichia coli* to ascertain the biochemical and functional roles of such proteins may guide the study of their eukaryotic counterparts (11).

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In *E. coli*, proteins MnmE and GidA form an α2β2 heterotetrameric complex that controls the formation of 5-carboxymethylaminomethyluridine ($cmnm^5U$) in the wobble position of $tRNA^{Lys}_{mnm5s2UUU}$, $tRNA^{Glu}_{mnm5s2UUC}$, $tRNA^{Gln}_{cmnm5s2UUG}$, $tRNA^{Leu}_{cmnm5UmAA}$, $tRNA^{Arg}_{mnm5UCU}$ and $tRNA^{Gly}_{mnm5UCC}$ (Figure 1) (12–14). In certain tRNAs ($tRNA^{Lys}_{mnm5s2UUU}$, $tRNA^{Glu}_{mnm5s2UUC}$ and $tRNA^{Arg}_{mnm5UCU}$), the $cmnm^5$ group may be demodified to 5-aminomethyl ($nm^5$) and subsequently methylated in an *S*-adenosyl-L-methionine (AdoMet) dependent step to produce methylaminomethyl ($mnm^5$). Both reactions are carried out by the same enzyme called MnmC (13,15,16). Protein MnmA, together with the cysteine desulfurase IscS and sulfur transfer mediators TusA-E, is required for the thiolation at position 2 of the uridine that occurs in $tRNA^{Lys}_{mnm5s2UUU}$, $tRNA^{Glu}_{mnm5s2UUC}$, $tRNA^{Gln}_{cmnm5s2UUG}$ (17,18). Modifications at the 2- and 5-positions occur independently of each other. Meanwhile, the function of MnmA and MnmC in tRNA modification is rather well understood, the precise role of proteins MnmE and GidA remains unknown and it is unclear how many steps precede the formation of the $cmnm^5$ group (Figure 1).

Results obtained *in vivo* have shown that both the MnmE GTPase activity as well as the GidA FAD-binding activity is necessary for tRNA modification (14,19–21). Biochemical and structural studies indicated that MnmE binds 5′-formyl-tetrahydrofolate, which was proposed to be the one-carbon group donor in the modification reaction (22). Data obtained from mutants where different steps of the tetrahydrofolate (THF) pathway were interrupted indicated that the C5-methylene moiety of $mnm^5U$ or $cmnm^5U$ is derived from this pathway, although apparently not from 5-formyl-THF or methyl-THF (T. Suzuki and T. Suzuki, 22nd tRNA workshop, Sweden, 2007). Therefore, the one-carbon group donor remains uncertain. In contrast, glycine was reported to be directly incorporated into the cmnm-group of $cmnm^5U$ *in vivo* (T. Suzuki and T. Suzuki, 22nd tRNA workshop, Sweden, 2007). Curiously, taurine (τ) is incorporated into human mitochondrial $tRNA^{Lys}$ and $tRNA^{Leu(UUR)}$ in place of cmnm (23). This led to the hypothesis that proteins of the MnmE and GidA families jointly catalyze the formation of an unknown intermediate in the modification pathway of the wobble uridine, whereas the subsequent activity of a taurine or glycine transferase would be responsible for construction of the $\tau m^5$ group in humans, or the $cmnm^5$ group in yeast and bacteria (14,23,24; Figure 1). However, there is no evidence for this independent, second step. For example, no bacterial or yeast mutants have been isolated where the putative intermediate produced by the MnmE and GidA proteins accumulates. Therefore, the possibility that the complex formed by these proteins drives all the reaction steps in the $cmnm^5/\tau m^5$-side chain synthesis cannot be ruled out.

In this work, we use both *in vitro* and *in vivo* approaches to characterize the MnmE•GidA-dependent pathway. Our results indicate that the MnmE•GidA complex catalyzes two different tRNA modification reactions by which the nm and cmnm groups are incorporated into position 5 of the wobble uridine, without participation of any more proteins.

## MATERIALS AND METHODS

### Bacterial strains, plasmids, growth conditions and protein techniques

*Escherichia coli* strains and plasmids used in this study are listed in Supplementary Table S1. Deletion of the *mnmC* and *folE* genes was performed by targeted homologous recombination as described (25) using primers shown in Supplementary Table S2. Deletion of the target gene was confirmed by PCR. The N-terminal FLAG-tagged *mnmC* gene was amplified by PCR from genomic DNA of MC1000 *E. coli* strain using the specific primers FLAG-MnmC(F) (GACTATAAAGACGACGACGACAAAAACACTACTCCATACAACC, FLAG sequence underlined) and FLAG-MnmC(R) (TTACCCCGCCTTAACCGCTTTACCCTTCAAC), and cloned into pBAD TOPO TA under the $P_{araC}$ promoter, producing pIC1253. LBT (Luria–Bertani broth containing 40 mg/ml thymine) and LAT (LBT containing 20 g of Difco agar per litre) were used for routine cultures and plating of *E. coli*. When required, antibiotics were added at the following concentrations: 100 μg/ml of ampicillin, 12.5 μg/ml of tetracycline, and 25 μg/ml of kanamycin. Cell growth was monitored by measuring the optical density (OD) of the cultures at 600 nm. Recombinant proteins were purified according to standard procedures. Unless otherwise specified, protein MnmE and FLAG-GidA was purified from a *gidA::*Tn*10* mutant (strain IC5923) and *mnmE::kan* mutant (strain IC5924), respectively.

### MnmE•GidA heterotetramer formation

To obtain the MnmE•GidA heterotetramer, 10 μM final concentration of the MnmE and FLAG-GidA proteins were mixed in 40 μl of the following buffer: 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 10 mM magnesium acetate, 5 mM DTT, 5 mM $MgCl_2$ and 3% glycerol. The mix was incubated at room temperature (20–22°C) for ∼2 h.

### Isothermal titration calorimetry assay

Binding affinity of THF derivatives to MnmE was determined by isothermal titration calorimetry (ITC) in a MicroCal VP-ITC isothermal titration calorimeter (MicroCal, Northampton, MA, USA). Calorimetric experiments consisted of the titration of a 20–30 μM MnmE solution with a 300–450 μM solution of the THF derivative at 25°C in 50 mM Tris–HCl pH 7.5, 100 mM KCl, 5 mM $MgCl_2$ and 2 mM β-mercaptoethanol. To obtain the $K_d$, the data were fitted using software developed at the BIFI and implemented in Origin 7.0 (OriginLab).

### *In vitro* transcription of *E. coli* $tRNA^{Lys}$

Unmodified *E. coli* $tRNA^{Lys}$ was prepared by *in vitro* transcription of BstNI-digested plasmid (pUC19-$tRNA^{Lys}$, 26)

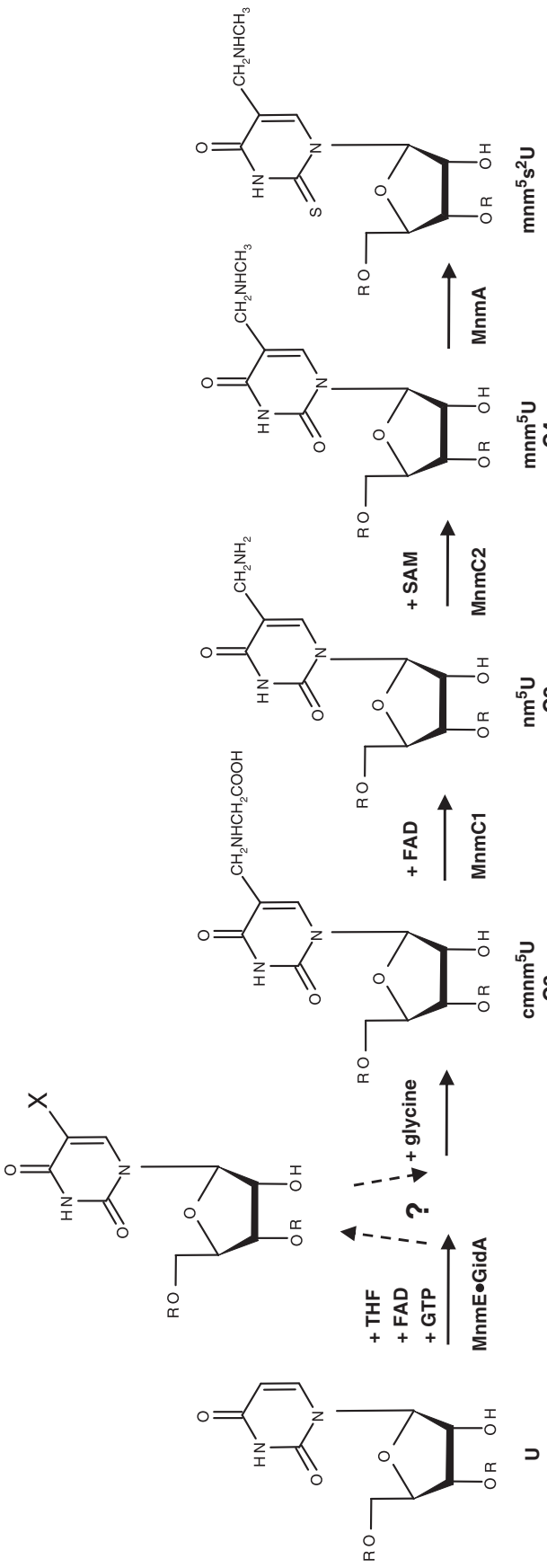


**Figure 1.** Model prior to the present study for the U34 modification pathway in $tRNA^{Lys}$ and $tRNA^{Glu}$. The first stage in the modification of U34 at position 5 is mediated by the MnmE•GidA complex (14,24), which catalyzes the production of a still unknown intermediate group (X) using GTP, FAD and a THF derivative. Glycine is subsequently incorporated by an unidentified transferase to build $cmnm^5U$. Finally, $cmnm^5U$ is first demodified to $nm^5U$ and subsequently methylated in an AdoMet-dependent step to $mnm^5U$ by the bifunctional enzyme MnmC (13,15,16). U34 may undergo thiolation at position 2 ($s^2$) mediated by MnmA (17,18). Modifications in the 2- and 5-positions occur independently of each other. Therefore, 2-thiouridine derivatives are present in tRNA obtained from *mnmE*, *gidA* and *mnmC* mutants. In this work, tRNA was completely digested to nucleosides by nuclease P1 and alkaline phosphatase before HPLC analysis. This allowed us to follow accumulation of nucleosides $s^2U$, $nm^5s^2U$, $cmnm^5s^2U$ and $mnm^5s^2U$ in modification assays where *in vivo* synthesized tRNA was used as substrate. Alternatively, tRNA was digested with nuclease P1, but not alkaline phosphatase, before TLC analysis, producing 5′-monophosphate nucleosides. C2, C3 and C4 are the designations given to 5′-monophosphate nucleosides identified in our TLC experiments ($pnm^5U$, $pcmnm^5U$ and $pmnm^5U$), where *in vitro* transcribed tRNA was mostly used as substrate.

using the Riboprobe T7-transcription kit (PROMEGA). Each reaction was performed in a final volume of 50 µl containing 40 mM Tris–HCl buffer, pH 7.9, 10 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 50 U of recombinant RNasin ribonuclease inhibitor, 2.5 mM each ribonucleotide (or 2.5 mM each rATP, rGTP and rCTP, 0.1 mM rUTP and 125 µCi [α-$^{32}$P]-rUTP [20 µCi/µl, 800 mCi/mmol], when radiolabeled tRNA was required), 2 µg of BstNI-digested plasmid, 50 U of T7-RNA polymerase and RNase-free water. The mix was incubated under mild agitation for 3 h at 37°C. At the end of the incubation period, DNA template was digested by addition of a few units of RNase-free DNase Q1 followed by an extra incubation at 37°C for 5 min. Finally, 100 µl of 0.3 M sodium acetate were added to the tube, and the radiolabeled tRNA was extracted with an equal volume (150 µl) of water-saturated phenol–chloroform–isoamyl alcohol (25:24:1), ethanol-precipitated and dried. The transcribed $tRNA^{Lys}$ was resuspended in RNase-free water and the removal of unincorporated nucleotides was performed using Micro Bio-Spin 6 Chromatography Columns (Sephadex G25 column, BioRad).

### Isolation of bulk tRNA from *E. coli*

Bacterial strains were grown in LBT broth at 37°C to about $5 \times 10^8$ cells/ml. The cells were lysed, and total RNA was prepared (27), dissolved in R200 buffer (100 mM Tris–$H_3PO_4$, pH 6.3, 15% ethanol, 200 mM KCl), and applied to a Nucleobond column (AX500) equilibrated with the same buffer. The column was washed with 6 ml of R200 and 2 ml of R650 buffer (same as R200 buffer except that the KCl concentration was 650 mM). tRNA was eluted with 7 ml of buffer R650 and precipitated with 0.7 volume of cold isopropanol, washed twice with 70% ethanol, dried, dissolved in water and quantified in a NanoDrop spectrophotometer. Note that LB broth is deficient in selenium; therefore, MnmE/GidA-specific tRNAs isolated from strains grown in this medium mostly carry sulphur at position 2.

### Determination of the MnmE•GidA enzymatic activity

To perform the modification *in vitro* of labelled $tRNA^{Lys}$ by the *E. coli* MnmE•GidA complex, the following mix was made, unless otherwise specified, in 100 µl final volume: 30 000 c.p.m. (counts per minute) of radioactive $tRNA^{Lys}$ in Buffer A (100 mM Tris–HCl pH 8, 100 mM Ammonium acetate, 5 mM $MgCl_2$, 5% Glycerol, 5 mM DTT, 0.5 mM FAD, 0.5 mM NADH, 0.5 mM NADPH, 2 mM GTP, 1 mM methylene-THF and 10 µg BSA). Glycine, alanine and serine were used at 2 mM. All cofactor solutions were freshly prepared just before use and protected from light. The enzymatic reaction was started by addition of the purified enzymes (2 µM final concentration, unless otherwise specified). A blank without enzymes was always included in each series of experiments. The reaction mixture was incubated at 37°C for 40 min with mild agitation. Then, the radiolabeled tRNA was phenol extracted, ethanol precipitated and digested with nuclease P1. The resulting nucleotides (1 µl portions) were analyzed by bidimensional thin layer chromatography (2D-TLC) on cellulose plates using isobutyric acid/concentrated ammonia/water (66:1:33 [v:v:v]) as the first dimension solvent (solvent A, 4 h), and 100 mM sodium phosphate buffer, pH 6.8/$(NH_4)_2SO_4$/n-propanol (100/60/2, v/w/v) (solvent B, 4 h) or isopropanol/concentrated HCl/water (68/18/14, v/v/v) (solvent C, 6 h 30 min) as the second dimension solvent.

To perform the modification *in vitro* of total tRNA by MnmE•GidA, we used the same procedure as above with the following modifications: the reaction mix was prepared in 200 µl of final volume and contained 40 µg of total tRNA purified from strain IC5653 (an *mnmE gidA* double mutant). At the end of the reaction, tRNA was phenol extracted, ethanol precipitated, degraded to nucleotides with nuclease P1 and, finally, treated with bacterial alkaline phosphatase. The resulting hydrolysate was analyzed by HPLC using a Develosil C30 column (250 × 4.6 mm; Phenomenex Ltd). The chromatographic conditions for gradient elution were essentially as described earlier (28). Given that total tRNA was extracted from a strain where the 2-thiouridylase MnmA protein is active, absorbance could be monitored at 314 nm to maximize the detection of thiolated nucleosides.

### Determination of the MnmC enzymatic activity

The tRNA previously modified by MnmE•GidA complex was phenolysed, ethanol precipitated and incubated with 2 µM FLAG-MnmC protein in 200 µl (total volume) of MnmC buffer {50 mM Tris–Cl (pH 8.0), 50 mM Ammonium acetate, 40 µM [*methyl*-$^{14}$C]AdoMet (60 mCi/mmole) or 0.5 mM cold SAM, 0.5 mM FAD, 5% glycerol}. After 40 min of incubation at 37°C, the tRNA was recovered by phenolization and ethanol precipitation, and digested by nuclease P1 (and by *E. coli* alkaline phosphatase when appropriated). The resulting products were analyzed by 2D-TLC on a cellulose plate (or by reverse-phase HPLC) as described earlier.

## RESULTS

### Study of the MnmE•GidA modification activity using *in vitro* synthesized $tRNA^{Lys}$ and TLC analysis

To determine whether the recombinant MnmE•GidA complex displays tRNA modification activity, we developed an *in vitro* modification assay where the independent contribution of the MnmE•GidA complex and its putative substrates and cofactors could be assessed. Basically, the reaction mix contained recombinant proteins, $^{32}$P-labeled $tRNA^{Lys}$, GTP, FAD and NADH/NADPH. In addition, we included methylene-THF as the first-choice carbon donor because its affinity for MnmE, while similar to that of the remaining THF derivatives ($K_d \sim 1$ µM) as determined by ITC, displays a slightly more favourable enthalpy, suggestive of a better interaction (Table 1). After incubation of the reaction mix, the tRNA was hydrolyzed by nuclease P1 and analyzed by 2D-TLC. Figure 2 shows that a major spot corresponding to nucleotide pU was obtained in the absence of any

**Table 1.** Affinity of MnmE to THF derivatives as determined by ITC[a]

| THF derivative | $K_d$ (μM) | $\Delta G$ (*K*cal/mol) | $\Delta H$ (*K*cal/mol) | $-T\ \Delta S$ (*K*cal/mol) |
|---|---|---|---|---|
| (6S/6R) 5-formyl-THF | 0.20 | −9.1 | −1.7 | −7.4 |
| (6S) 5-formyl-THF | 0.26 | −9.0 | −5.8 | −3.2 |
| (6R) 10-formyl-THF | 1.5 | −7.9 | −5.9 | −2.0 |
| (6S) 5-methyl-THF | 1.1 | −8.1 | −5.3 | −2.8 |
| (6R) 5,10-methylene-THF | 0.31 | −8.9 | −6.8 | −2.1 |
| (6S) THF | 0.34 | −8.8 | −6.0 | −2.8 |

[a]Data in this table indicate that all derivatives were bound by MnmE in a favorable and spontaneous way ($K_d$ and ΔG values were similar), although the binding for 10-formyl-THF and methyl-THF was the less favorable. Assuming that either there is no protonation or this is equal for all ligands, binding of methylene-THF exhibits a slightly more favorable enthalpy than the remainder.

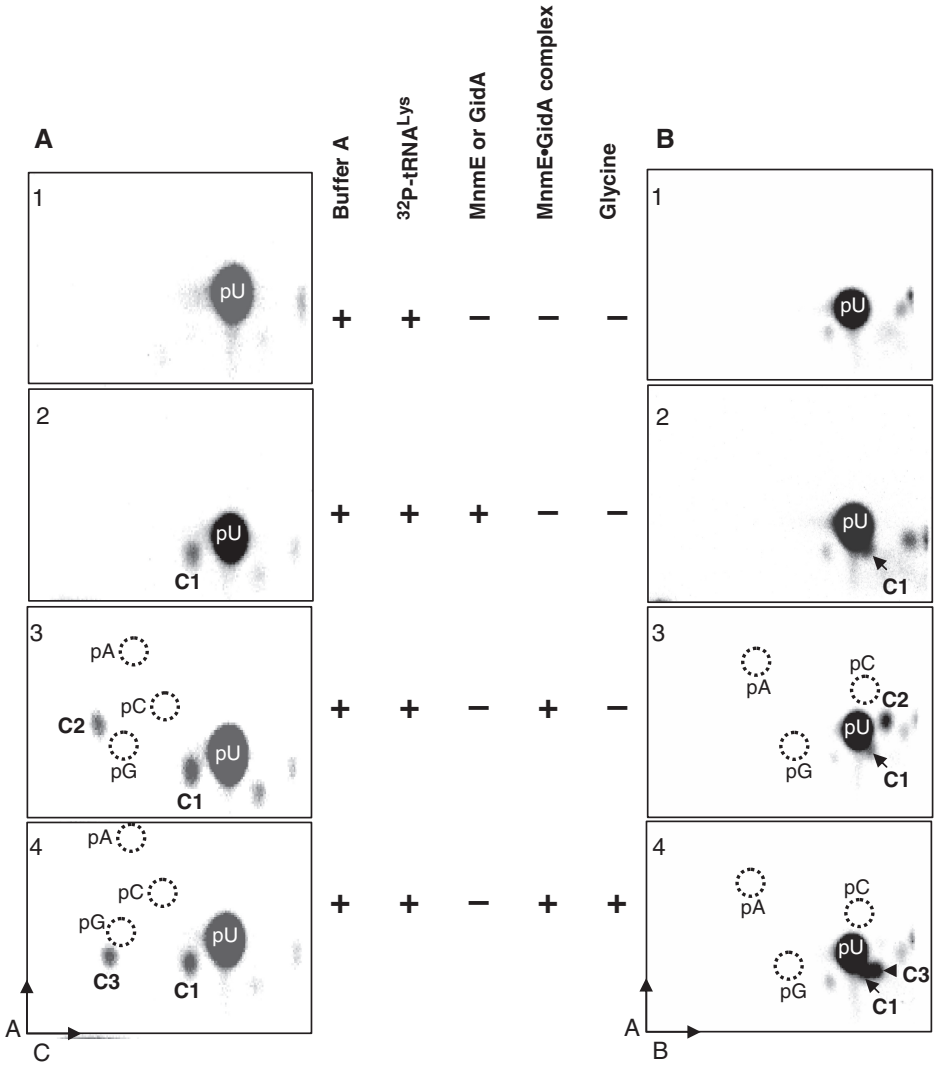


**Figure 2.** The complex MnmE•GidA catalyzes the formation of two nucleotides, C2 and C3, on *in vitro* transcribed $tRNA^{Lys}$. Autoradiograms of tRNA hydrolysates resulting from *in vitro* modification reactions performed in buffer A in the presence (+) or absence (−) of the indicated components. (**A** and **B**) Correspond to the autoradiograms of the TLC analysis in solvents A/C and A/B, respectively. Positions of modified 5' phosphate nucleotides C1, C2 and C3 are indicated. Circles in dotted lines show the migration of the canonical nucleotides used as UV markers. Identical results to those shown in panels A1, B1, A2 and B2 were obtained in the presence of glycine (data not shown). In this article, we show that C2 and C3 correspond to $nm^5U$ 5′-monophosphate ($pnm^5U$) and $cmnm^5U$ 5′-monophosphate ($pcmnm^5U$), respectively.

protein (panels A1 and B1), and that one additional compound (C1) appeared after addition of either MnmE or FLAG-GidA (Figure 2, panels A2 and B2). Interestingly, when the assay was performed in the presence of the MnmE•GidA complex, a new compound, named C2, was also detected (Figure 2, panels A3 and B3). Formation of C2, but not C1, was absolutely dependent on the presence of FAD and GTP

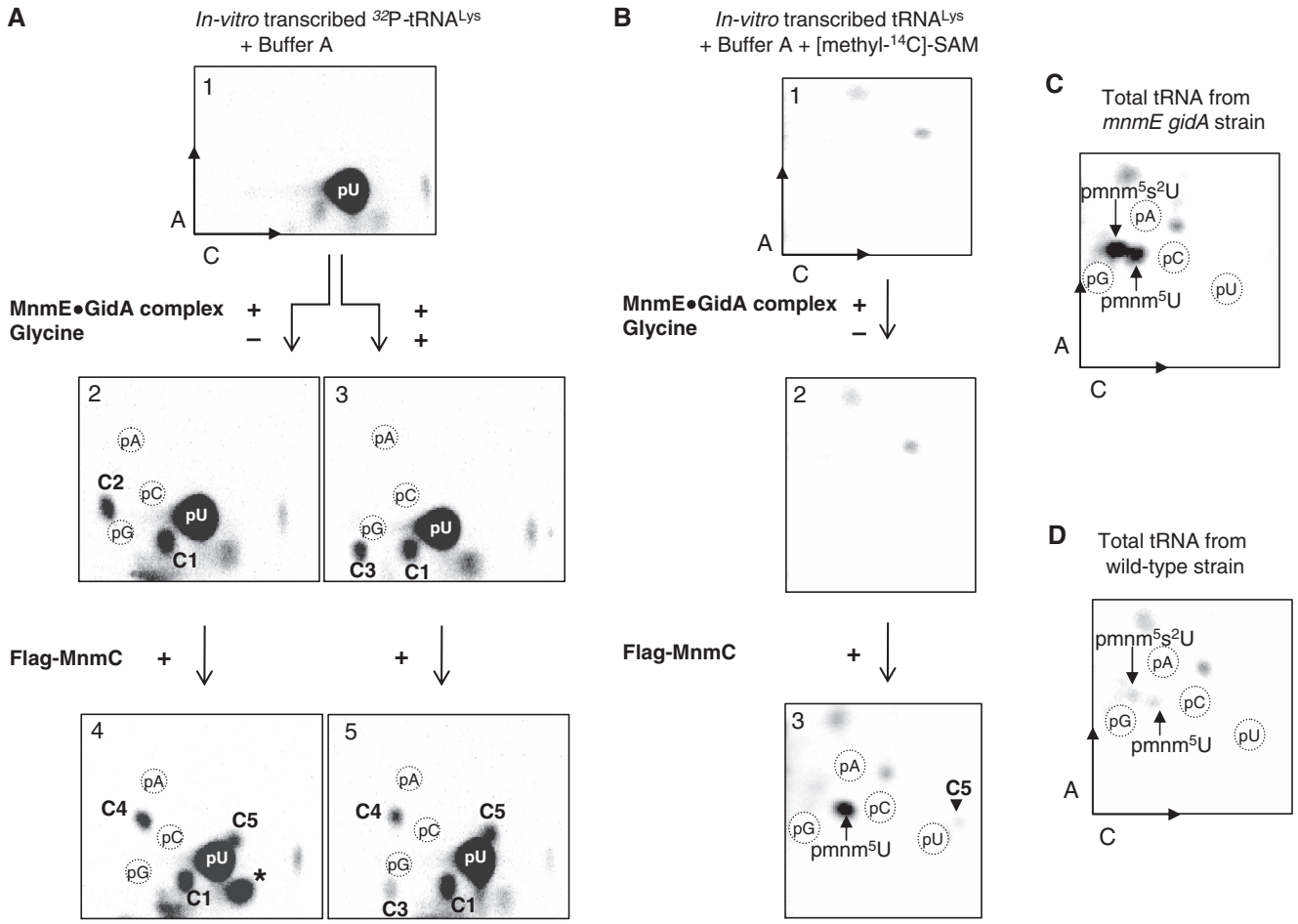


**Figure 3.** MnmC converts C2 and C3 to C4 ($mnm^5U$ 5′-monophosphate, $pmnm^5U$). 2D-TLC autoradiograms of tRNA hydrolysates resulting from *in vitro* modification reactions. Solvent system A/C was used for the TLC analysis. (**A**) [α-$^{32}$P]-UTP-labelled *in vitro* transcribed $tRNA^{Lys}$ was incubated in buffer A (**A1**), modified by the addition of MnmE•GidA to form C2 ($pnm^5U$) in the absence of glycine (**A2**), or C3 ($pcmnm^5U$) in the presence of this amino acid (**A3**). C2 and C3 were converted to compound C4 ($pmnm^5U$) by MnmC (**A4** and **A5**) (**B**) Cold *in vitro* transcribed *E. coli* $tRNA^{Lys}$ was incubated in buffer A with 40 μM [*methyl*-$^{14}$C]AdoMet (**B1**). Addition of MnmE•GidA (in the absence of glycine) produces C2 ($pnm^5U$), which is not visible in this step because both the $tRNA^{Lys}$ as well as the methyl group added to U34 are not radiolabeled (**B2**). Incubation of the resulting tRNA with MnmC, FAD and [*methyl*-$^{14}$C]AdoMet produces $pmnm^5U$ (**B3**). (**C** and **D**) Total tRNA from an *mnmE gidA* mutant or a wild-type strain, respectively, were treated as in (B), and the products resulting from the MnmC activity are shown.

(Supplementary Figure S1), which suggests that only C2 is related to the MnmE•GidA pathway.

Next, we analyzed the role of glycine in the *in vitro* modification reaction. Addition of this amino acid, but not alanine or serine, led to disappearance of C2 and production of a new compound, designated C3 (Figure 2, panels A4 and B4, and Supplementary Figure S2). Formation of C3 was also dependent on inclusion of both GTP and FAD in the reaction mix (Supplementary Figure S2). According to reference maps (29), C3 corresponds to $cmnm^5U$ 5′-monophosphate ($pcmnm^5U$), but C2 cannot be assigned.

### MnmC converts nucleotides C2 and C3 ($pcmnm^5U$) to $mnm^5U$ 5′-monophosphate ($pmnm^5U$)

MnmC is a bifunctional enzyme whose C-terminal domain catalyzes demodification of $cmnm^5(s^2)U$ to $nm^5(s^2)U$, whereas its N-terminal domain independently methylates $nm^5(s^2)U$ to form the final product $mnm^5(s^2)U$ (16; Figure 1). Therefore, considering that $cmnm^5U$ and $nm^5U$ are substrates for MnmC, we decided to use this enzyme to investigate the nature of compounds C2 and C3 ($pcmnm^5U$) generated by the MnmE•GidA activity. $tRNA^{Lys}$, previously modified *in vitro* by MnmE•GidA (in the presence or not of glycine), was incubated with protein FLAG-MnmC, in a buffer containing FAD and SAM (Figure 3A). Subsequently, the tRNA was hydrolyzed using nuclease P1 and the resulting nucleotides were analyzed by TLC. Interestingly, C2 and most of C3 ($pcmnm^5U$) disappeared, whereas two new compounds migrating as $pmnm^5U$ (C4) and $pm^5U$ (C5), according to previous chromatographic diagrams (29), were generated (Figure 3A, compare panels 2 and 3 with 4 and 5, respectively). Synthesis of $m^5U$ could be explained if our MnmC preparation contained traces of TrmA, which catalyzes the SAM-dependent methylation of the uracil in position 54 in all *E. coli* tRNAs. Note that the

compound labeled with an asterisk in panel 4 of Figure 3A is already present in other chromatograms, with variable relative intensity. Thus, we think it is a product of either an unusual cleavage or tRNA degradation.

Conversion of C3 to pmnm$^5$U (C4) by MnmC (Figure 3A, panels 3 and 5) is in agreement with our prediction that C3 is pcmnm$^5$U. Conversion of C2 to pmnm$^5$U (C4) (Figure 3A, panels 2 and 4) suggests that C2 is pnm$^5$U since it works as a substrate for MnmC but does not exhibit the chromatographic mobility of pcmnm$^5$U. If so, this is surprising because it has never before been reported that nm$^5$U may be a direct product of the MnmE•GidA complex activity.

To further explore the capability of MnmC to work on the C2 nucleotide produced by MnmE•GidA, we proceeded as follows (Figure 3B). Five micrograms of unlabeled *in vitro* transcribed tRNA$^{Lys}$ were incubated in buffer A with MnmE•GidA and [*methyl*-$^{14}$C]AdoMet, in the absence of glycine, to form C2 (pnm$^5$U), according to our previous results (Figure 3A, panel 2). In this case, production of pnm$^5$U could not be detected (Figure 3B, panel 2) because the *in vitro* transcribed tRNA used as substrate was unlabelled, and [*methyl*-$^{14}$C]AdoMet does not work as a methyl donor in the formation of nm$^5$U by MnmE•GidA. Then, the tRNA modified by the MnmE•GidA complex was incubated with MnmC, FAD and [*methyl*-$^{14}$C]AdoMet, hydrolyzed and analyzed by TLC. As shown in Figure 3B, panel 3, a radioactive compound whose migration characteristics correspond to those of pmnm$^5$U could be detected. Moreover, 15 µg of total tRNA isolated from either an *mnmE gidA* double mutant (strain IC5653; Figure 3C) or a wild-type strain (MG1655; Figure 3D) were treated in the same way as in Figure 3B. Addition of MnmC, FAD, and $^{14}$C-radiolabeled SAM to total tRNA previously modified *in vitro* by the MnmE•GidA complex (in the absence of glycine, i.e. under conditions where only the nucleotide corresponding to C2, pnm$^5$U, is formed) produced compounds whose migration characteristics are those of pmnm$^5$U and pmnm$^5$s$^2$U (Figure 3C and D). The tRNAs isolated from the wild-type strain that are substrates for MnmE•GidA/MnmC are mostly modified, which may explain the less amount of labeled products obtained in this case (compare Figure 3C and D). Altogether these experiments support that MnmC transforms nucleotide C2 (pnm$^5$U), produced by MnmE•GidA in the absence of glycine, to pmnm$^5$U.

### Ammonium ion or glycine may be used by the MnmE•GidA complex to synthesize nm$^5$U or cmnm$^5$U, respectively

If compound C2 is pnm$^5$U, we wondered whether ammonium is the source of its amine group, given that the nucleotide is synthesized in the absence of glycine. Figure 4 shows that removal of ammonium acetate from the reaction buffer prevents formation of C2 (compare panels B and C). It is important to note that stimulation of the MnmE GTPase activity (which is essential for the modifying function of this protein; 20) requires potassium or ammonium ions (30). In the *in vitro* modification reaction, potassium ions are present, at least, 10 mM due

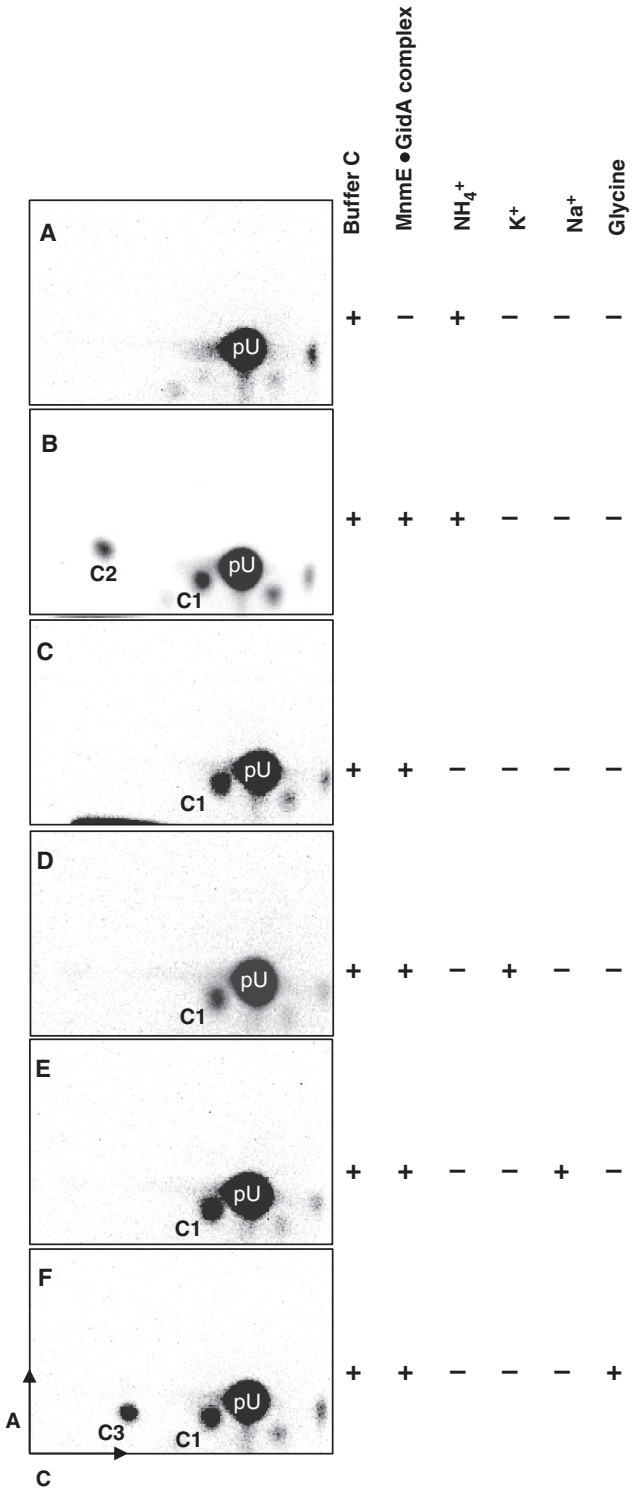


**Figure 4.** Ammonium ion is indispensable to form C2 (pnm$^5$U) but not C3 (pcmnm$^5$U). 2D-TLC autoradiograms of tRNA hydrolysates resulting from *in vitro* modification reactions performed in buffer C (same as buffer A, but without ammonium acetate) supplemented (+) or not (−) with the indicated components. Salts were added to buffer C at 100 mM, but note that potassium ions are always present at, at least, 10 mM after the addition of the MnmE•GidA complex. Solvent system A/C was used for the TLC analysis.

to the addition of the MnmE•GidA complex (whose formation is carried out in a buffer containing 50 mM KCl). This concentration might be suboptimal for efficient stimulation of the MnmE GTPase activity (31), explaining that elimination of ammonium from the reaction mix impairs the *in vitro* tRNA modification reaction. However, when ammonium acetate was substituted by potassium acetate, the modification reaction does not work either, given that C2 ($pnm^5U$) is not produced (Figure 4D). The same result was obtained when sodium acetate was the substituent (Figure 4E). Interestingly, when ammonium ions are removed but glycine is added, C3 ($pcmnm^5U$), but not C2 ($pnm^5U$), is formed (Figure 4F). Considering that the synthesis of both nucleotides requires the presence of GTP (Supplementary Figures S1 and S2), we conclude that 10 mM KCl is enough to stimulate the MnmE activity and that ammonium may be used by the MnmE•GidA complex to synthesize the $nm^5$ group. Moreover, given that C3 ($pcmnm^5U$) may be obtained when glycine, but not ammonium, is present in the buffer (Figure 4F), we propose that C2 ($pnm^5U$) is not an intermediate in the C3 ($pcmnm^5U$) formation and that synthesis of both compounds is mediated by MnmE•GidA through different and independent pathways (Figure 5). Strikingly, only compound C3 ($pcmnm^5U$) is produced when ammonium and glycine are simultaneously present in the reaction mix (Figure 2, panels A4 and B4). Therefore, we think that the glycine-dependent reaction is competitively more effective under the conditions used in these assays.

### Reverse-phase HPLC analysis of total *E. coli* tRNA modified *in vitro* by the MnmE•GidA complex

To confirm the TLC data and further investigate how proteins MnmE, GidA and MnmC work, we performed the *in vitro* modification assay using total tRNA purified from a double mutant *mnmE gidA* instead of the *in vitro* transcribed $tRNA^{Lys}$. In this case, the resulting tRNA was analyzed, after digestion with nuclease P1 and alkaline phosphatase, by HPLC, using some purified nucleosides as controls (Figure 6A). Previous HPLC analysis of tRNA purified from null *mnmE* or *gidA* mutants (where protein MnmA is active) revealed accumulation of $s^2U$ and disappearance of $mnm^5s^2U$ in the corresponding chromatograms (14). In this work, we observed that $s^2U$ remained accumulated after performing the *in vitro* modification reaction in the absence of the MnmE•GidA complex (Figure 6B). However, when this complex was added to a reaction mix containing ammonium but not glycine, $s^2U$ was converted into $nm^5s^2U$ (Figure 6C). Note that ammonium was indispensable for this conversion since $s^2U$ remained accumulated in the absence of the cation (Figure 6D). Moreover, when the reaction mix contained glycine but not ammonium, $s^2U$ was partially converted into $cmnm^5s^2U$, whereas no production of $nm^5s^2U$ was observed (Figure 6E). Altogether these results support the idea that the MnmE•GidA complex catalyzes the *in vitro* biosynthesis of $nm^5s^2U$ and $cmnm^5s^2U$ in *E. coli* tRNA through two independent reactions and without participation of other proteins.

Curiously, both $cmnm^5s^2U$ and $nm^5s^2U$ were detected after the simultaneous addition of ammonium and glycine to the reaction mix (Figure 6F). This result differs from that obtained when the MnmE•GidA complex activity was analyzed using *in vitro* synthesized $tRNA^{Lys}$ and TLC. In such a case, $pcmnm^5U$ was the only product detected when both ammonium and glycine were included in the reaction (compound C3 in Figure 2, panels A4 and B4), in spite of we detected formation of $pnm^5U$ (C2) if only ammonium was present (Figure 2, panels A3 and B3). Thus, it seems that the glycine-dependent reaction is competitively more effective when the *in vitro* synthesized $tRNA^{Lys}$ is the substrate. Considering that this tRNA is completely unmodified, in contrast to the total tRNA purified from the *mnmE gidA* strain (Figure 6), it is tempting to speculate that some tRNA modification(s) outside the 5-position of U34 modulate the efficiency of the reactions mediated by the MnmE•GidA complex.

To firmly establish the identities of the nucleosides resulting from the *in vitro* MnmE•GidA activity, we again analyzed their ability to be used as substrates by the bifunctional enzyme MnmC. As expected, MnmC converted the peaks assigned to $nm^5s^2U$ and $cmnm^5s^2U$ into $mnm^5s^2U$ (Supplementary Figure S3, compare panels B and D with C and E, respectively).

To further demonstrate that $nm^5U$ is not an intermediate in the synthesis of $cmnm^5U$ mediated by MnmE•GidA, the bulk tRNA purified from an *mnmC2* mutant (where $nm^5s^2U$ accumulates) was used as substrate for an *in vitro* modification reaction mediated by MnmE•GidA in the presence of glycine (with or without ammonium) and, as expected, no conversion of $nm^5s^2U$ to $cmnm^5s^2U$ was observed (Supplementary Figure S4). In addition, we used total tRNA extracted from an *mnmE gidA* mutant as substrate in a reaction catalyzed by MnmE•GidA in the presence of ammonium (Figure 7). After phenol extraction and ethanol precipitation, the resulting tRNA, which carried $nm^5s^2U$ (Figure 7B), was mixed with MnmE•GidA and glycine (in the presence or absence of ammonium). The HPLC pattern of the tRNA remained unaltered after this second reaction (Figure 7C), supporting that $nm^5s^2U$ is not a substrate for MnmE•GidA.

Finally, we thought that if the MnmE•GidA activity is able to introduce the $nm^5$ group at the wobble uridine, the modified nucleoside should be found in the tRNA isolated from a null *mnmC* mutant. As expected, HPLC analysis of tRNA from an *mnmC::kan* strain revealed the presence of $nm^5s^2U$, in addition to $cmnm^5s^2U$, in the hydrolysates (Figure 6G). Curiously, this nucleoside was previously not identified in tRNA from an *mnmC1* mutant (13), which we now know carries a nonsense mutation in the *mnmC* gene. Here, we have carefully analyzed the tRNA extracted from mutant *mnmC1* and detected the presence of $nm^5s^2U$ (Figure 6H). Whether such a discrepancy may be due to the different growth medium used in each case is a question that remains to be explored.

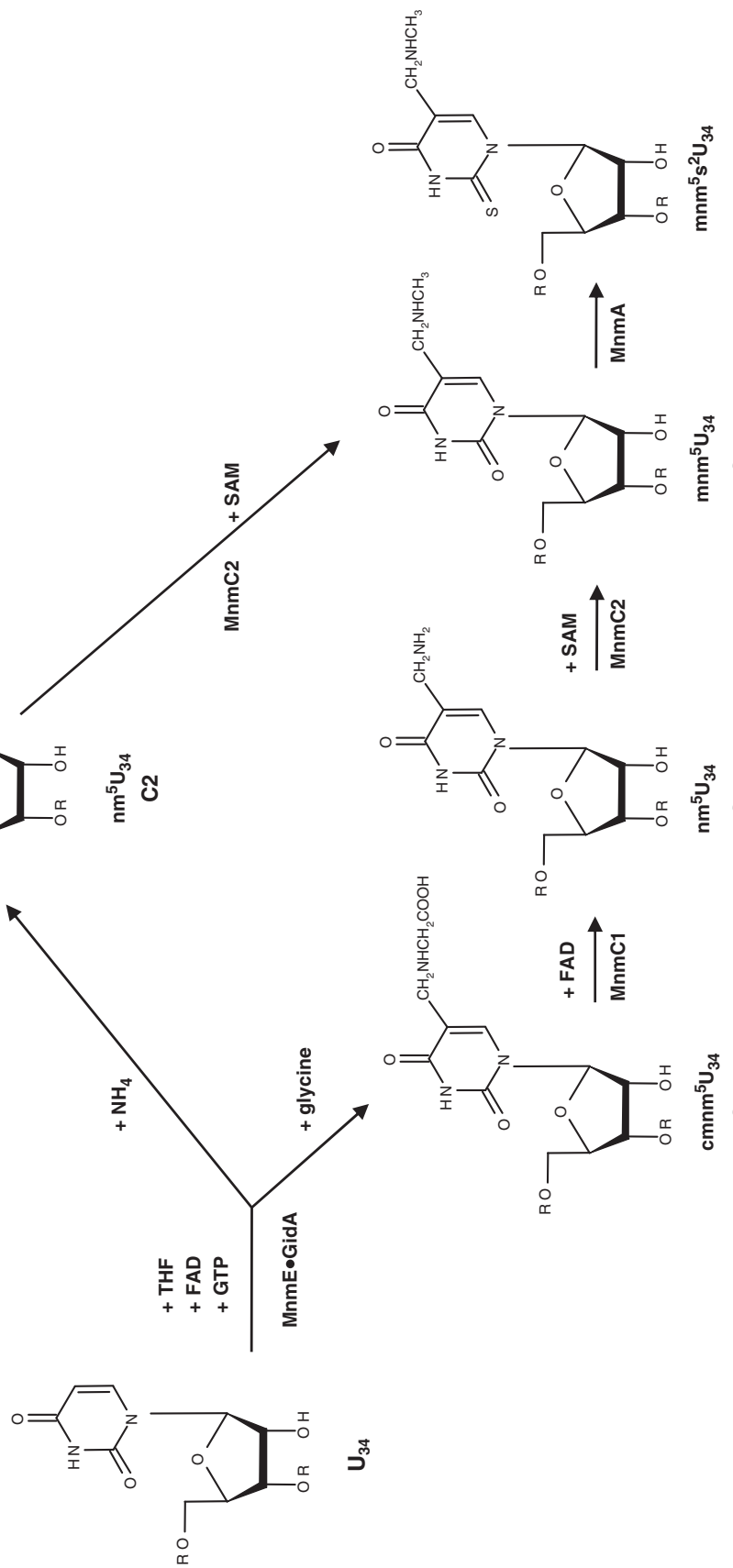


**Figure 5.** A new model of the U34 modification pathway. As in the old model (Figure 1) the modifications at the 2- and 5-positions occur independently of each other. C2, C3 and C4 are the designations given to 5′-monophosphate nucleosides identified in our TLC experiments (pnm$^5$U, pcmnm$^5$U, and pmnm$^5$U).

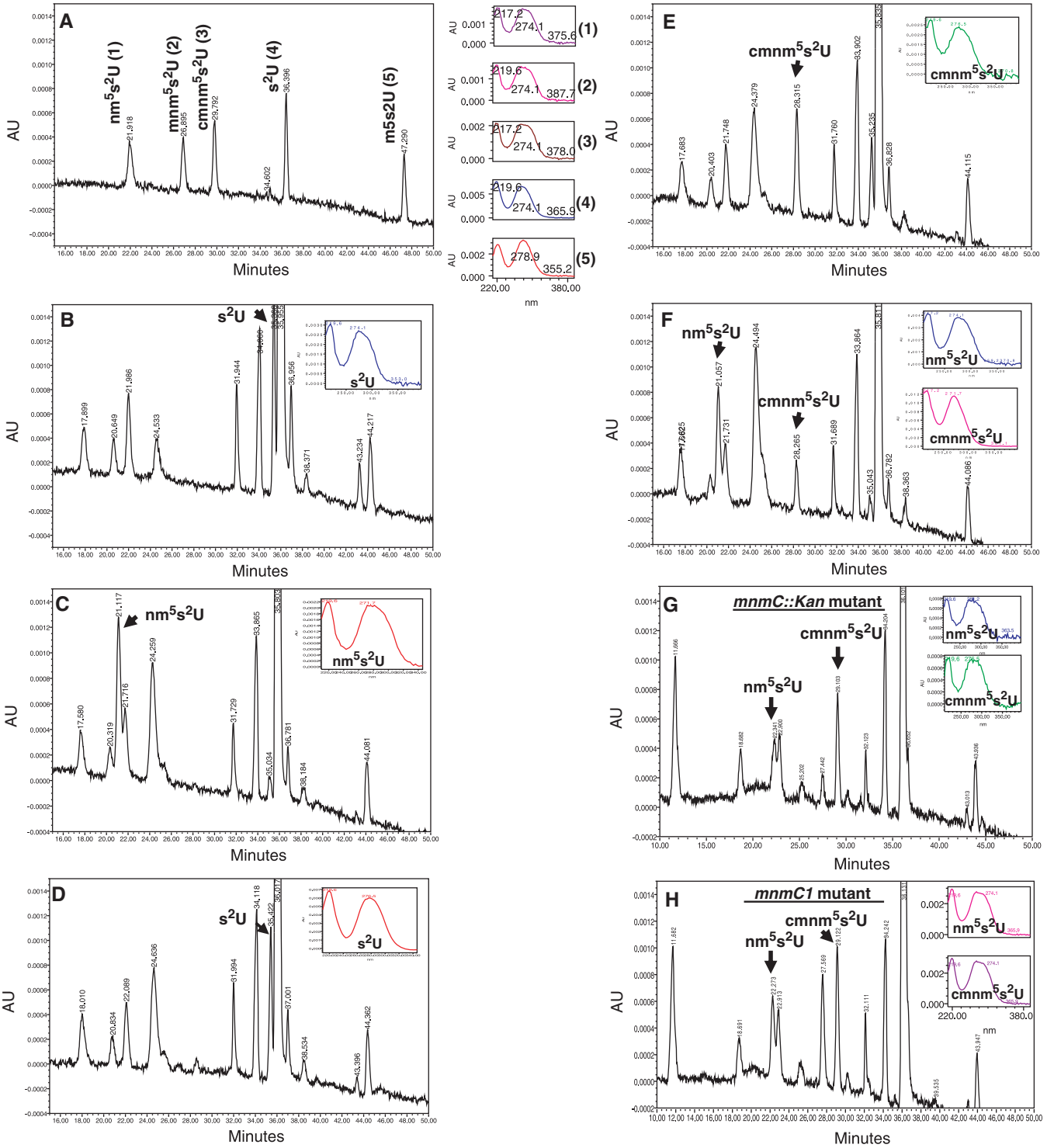


**Figure 6.** HPLC analysis of the MnmE•GidA activity. (**A**) Elution times and UV spectra of the reference ribonucleosides. (**B**) *In vitro* modification reaction performed in the absence of the MnmE•GidA complex. (**C**) As in B, but adding MnmE•GidA. (**D**) As in C except ammonium acetate was removed from buffer. (**E**) As in (D), but adding glycine. (**F**) As in (C) (i.e. in buffer A, which contains ammonium acetate), but adding glycine. (**G** and **H**) Bulk tRNA from *mnmC::kan* and *mnmC1* mutants, respectively. *AU*, absorbance units.

## Involvement of FAD and NADH in the tRNA modification reaction

Mutations affecting the FAD-binding domain of GidA impair tRNA modification *in vivo* (14, 21). Here, we have shown that FAD is essential for the *in vitro* modification reaction (Supplementary Figures S1 and S2). It was previously postulated that GidA-bound reduced FAD is required for the last step in the synthesis of the cmnm$^5$-group, and that GidA-bound FAD receives its electrons from NADH, given that GidA binds NADH

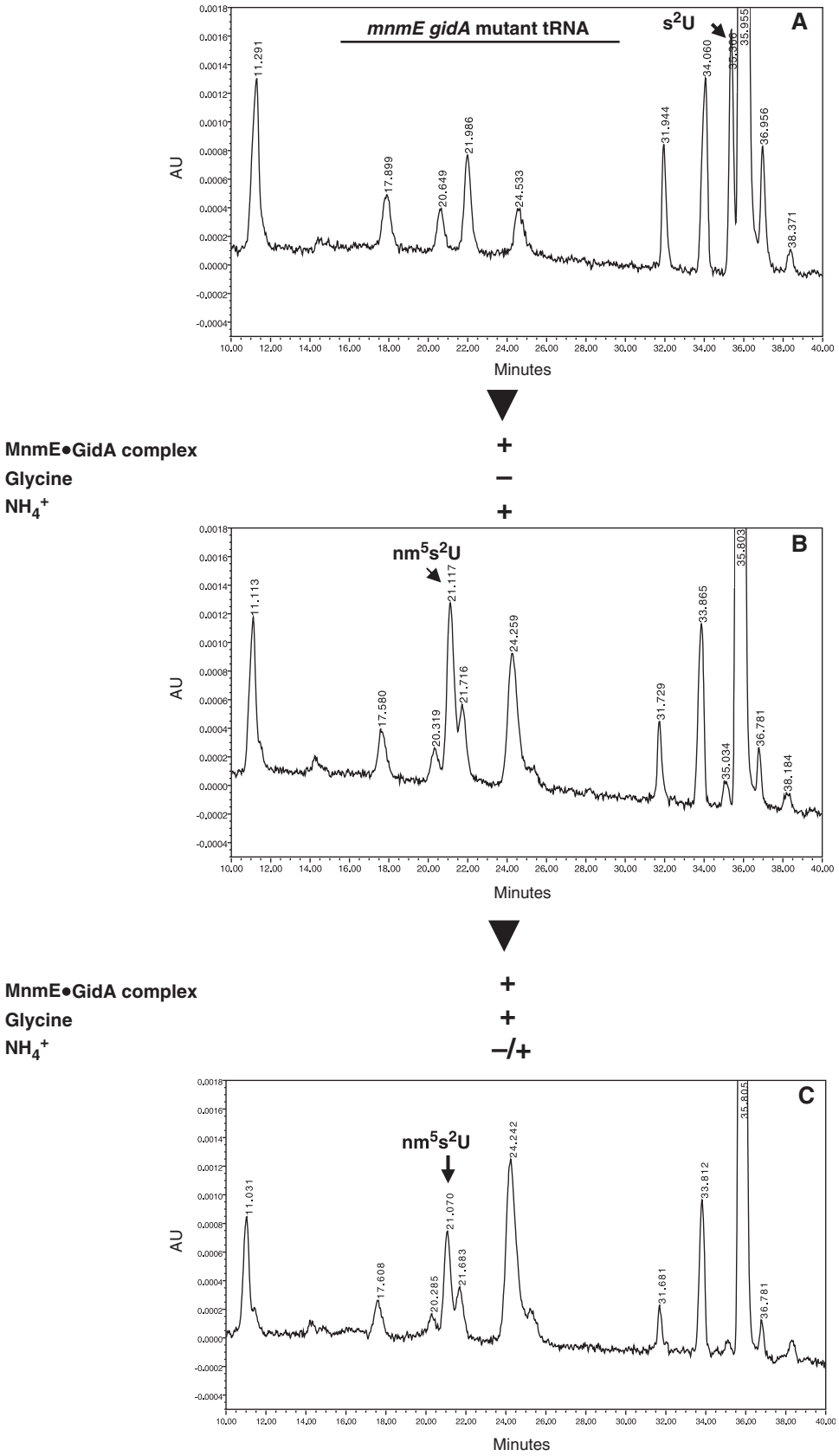


**Figure 7.** The $nm^5s^2U$ nucleoside is not an intermediate in the biosynthetic pathway of $cmnm^5s^2U$. Bulk tRNA from an *mnmE gidA* double mutant (**A**) was incubated with MnmE•GidA in buffer A to form $nm^5s^2U$ (**B**). After phenol extraction and ethanol precipitation, the resulting tRNA was incubated with MnmE•GidA and glycine in buffer A, with or without ammonium acetate (**C**).

but not NADPH (22,32). Curiously, we have found that NADH (or NADPH) does not seem to be necessary for the synthesis *in vitro* of $nm^5s^2U$ and $cmnm^5s^2U$ if FAD is present at a high concentration (1 mM) (Figure 8A and B). Note that appropriate control experiments indicate that $nm^5s^2U$ and $cmnm^5s^2U$ do not accumulate if FAD is removed, though NADH or NADPH remains in the reaction mix. Synthesis of $nm^5s^2U$ and $cmnm^5s^2U$ is drastically reduced when FAD is used at 2 μM (Figure 8C and D). However, under such conditions, the addition of NADH (but not NADPH) at 0.5 mM substantially improves the $nm^5s^2U$ and $cmnm^5s^2U$ synthesis, mainly of the last one (Figure 8C and D). These results suggest that NADH may play a critical role in tRNA modification depending on cell FAD concentrations.

Curiously, it has been reported that TrmFO, a GidA paralogue involved in the FAD-, NADPH- and methylene-THF-dependent methylation of uridine at position 54 of tRNAs, is able to catalyze the *in vitro* modification reaction in the absence of NADPH (33). Given that NADPH is thought to be required for FAD reduction, the mentioned finding was attributed to copurification of the enzyme with the oxidized and reduced forms of flavin. In this respect, it should be mentioned that we have used dialyzed proteins to perform the experiments shown in Figure 8. Considering the relatively low affinity of GidA for FAD ($K_d \sim 3$ μM; our own unpublished results) and NADH ($K_d \sim 11$ μM; 32), we find it unlikely that the dialyzed GidA protein used in our experiments carried these cofactors.

### MnmE•GidA uses methylene-THF to form the C5-methylene moiety of $nm^5U$ and $cmnm^5U$

While studying the requirements for the *in vitro* synthesis of $nm^5U$ by MnmE•GidA, we observed that removal of methylene-THF from the reaction mix did not affect the accumulation of the modified nucleoside. This suggests that MnmE copurifies with the one-carbon unit donor, a behavior that has also been observed for TrmFO (34,35). To overcome this difficulty and further investigate the carbon donor used by MnmE•GidA, we first constructed a *folE::cat* mutant. *folE* encodes GTP cyclohydrolase I, which is the first enzyme of the *de novo* THF pathway (36). HPLC analysis of tRNAs purified from the *folE::cat* mutant revealed the disappearance of nucleoside $mnm^5s^2U$ and accumulation of $s^2U$, in relation to the wild-type strain (Table 2). This finding supports that THF works as the one-carbon unit donor in the MnmE•GidA modification pathway. Then, strain *folE::cat* was independently transformed with plasmids expressing proteins GST-MnmE and FLAG-GidA with the aim to purify both proteins from cells where THF is presumably very limited, since only some folate-related salvage pathway may be active (36). Finally, an *in vitro* modification assay was performed with bulk tRNA extracted from an *mnmE gidA* double mutant and proteins purified from the *folE::cat* strain. The relative accumulation of $nm^5s^2U$ and $s^2U$ observed under different concentrations of the MnmE•GidA complex and folate derivatives is shown in Table 3. Curiously, when no external source of folate is included in the reaction mix (see entry 'Proteins/No folates' in Table 3), $nm^5s^2U$ still accumulates if the MnmE•GidA complex is added to 2 μM (Table 3, column A). This suggests that the amount of protein MnmE that is added to the mix still provides a concentration of the one-carbon unit donor that is enough to lead the *in vitro* modification of tRNA. Accordingly, when the concentration of MnmE•GidA in the mix is reduced from 2 to 0.35 μM (Table 3, column B), no significant conversion of $s^2U$ to $nm^5s^2U$ is observed in the absence of exogenous folate. Moreover, if under such conditions, the folate derivative is added to 50 μM, major increases of $nm^5s^2U$ are only seen with methylene-THF, THF or methyl-THF. These results help us to discard 5-formyl-, 10-formyl- and methenyl-THF as the one-carbon unit donor. However, given that no one-carbon unit is carried by THF, it is possible that our preparation of this compound contained traces of the true one-carbon unit donor, which may be methylene-THF or methyl-THF. We assume that traces of one of them are also present in the other one, explaining that the *in vitro* reaction works with both substrates. The use of the folate derivative at 1 μM (Table 3, column C) still led to the production of $nm^5s^2U$ in the case of THF, methylene-THF and methyl-THF, with methylene-THF appearing to be slightly more effective. A further decrease in the concentration of these derivatives did not lead to conclusive results since the efficiency of the reaction was drastically reduced (data not shown), probably because the affinity of MnmE for them is in the low micromolar range (Table 1).

*glyA* and *metF* mutations block the synthesis of 5-formyl-THF and methyl-THF, respectively. Considering that the *in vivo* $^{13}C$ incorporation from glycine to $mnm^5s^2U$ takes place in *glyA* and *metF* mutants similarly to the wild-type strain, it was suggested that 5-formyl-THF and methyl-THF are not the direct substrates to form the C5-methylene moiety of $mnm^5s^2U$ (T. Suzuki and T. Suzuki, 22nd tRNA workshop, Sweden, 2007). We have verified such a proposal, finding that $mnm^5s^2U$ accumulates in *glyA* and *metF* null mutants at the wild-type level (Table 2). These results, together with those shown in Table 3, prompt us to conclude that methylene-THF is the one-carbon unit donor in the modification reaction catalyzed by MnmE•GidA. If so, data from Table 3 also suggest that there is no substrate inhibition with THF and methyl-THF since $nm^5s^2U$ accumulates even when both compounds are added at 1 mM (final concentration) and, according to our hypothesis, methylene-THF should be a minor contaminant in the respective preparations. This behaviour is different from that described for TrmFO, whose methylene-THF-dependent activity is inhibited by concentrations of THF higher than 2 μM (33). Given that the affinity of MnmE for all THF derivatives tested is similar (Table 1), we think that either the binding to methylene-THF is kinetically favored or the affinity for this derivative significantly increases when MnmE is bound to its partners.

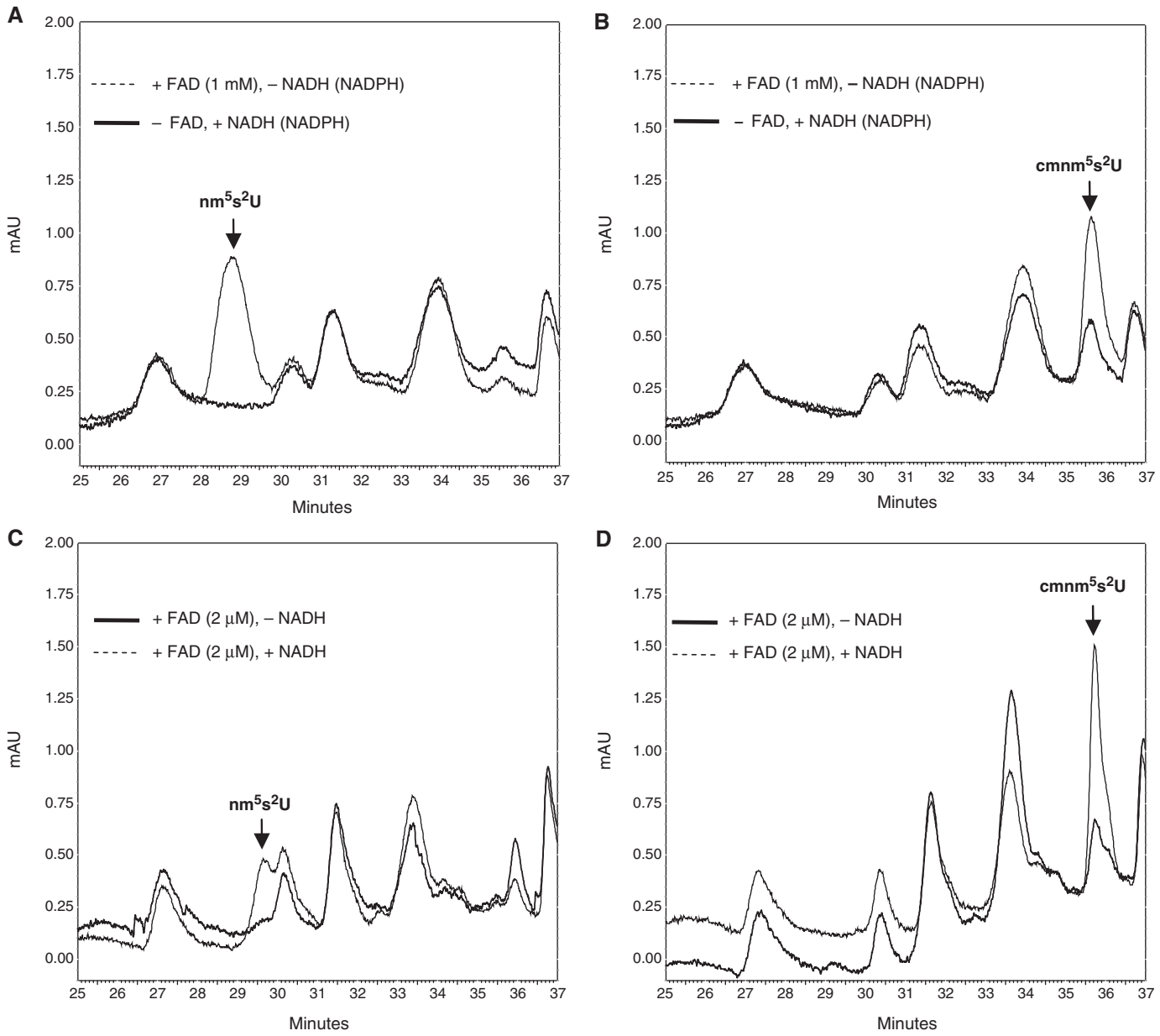


**Figure 8.** NADH is dispensable for *in vitro* tRNA modification unless FAD levels are limiting. The effect of FAD, NADH and NADPH on the $nm^5s^2U$ and $cmnm^5s^2U$ formation, in bulk tRNA purified from a double *mnmE gidA* mutant, was monitored by HPLC analysis. The MnmE and GidA proteins were previously dialysed against TBS buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl and 10 mM $MgCl_2$). (**A** and **B**) Formation of $nm^5s^2U$ (ammonium acetate, but not glycine, is present in the reaction mix) and $cmnm^5s^2U$ (glycine, but not ammonium acetate, is present in the mix), respectively, when FAD (1 mM) was added in the absence of exogenous NADH and NADPH (thin line) and when NADH or NADPH was added at 0.5 mM in the absence of FAD (bold line). (**C** and **D**) Formation of $nm^5s^2U$ and $cmnm^5s^2U$, respectively, when FAD is added to 2 μM, in the absence of NADH and NADPH (bold line), or in the presence of NADH (0.5 mM, thin line). NADPH (0.5 mM) did not improve the production of $nm^5s^2U$ and $cmnm^5s^2U$ when FAD was used at 2 μM (data not shown).

## DISCUSSION

To characterize the enzymatic pathway of $mnm^5U$ biosynthesis in *E. coli*, we have purified proteins MnmE, GidA and MnmC and checked their ability to modify tRNA *in vitro*. Our results show, for the first time, that the MnmE•GidA complex catalyses the synthesis of $nm^5U$ and $cmnm^5U$ in tRNA through two independent reactions (Figures 3A and 6), in which ammonium and glycine are the source of the amino and carboxymethylamino moiety, respectively (Figure 5). The identity of the nucleosides synthesized by the MnmE•GidA complex *in vitro* ($nm^5U$ and $cmnm^5U$) was confirmed by the fact that, in a buffer containing FAD and SAM, they were transformed to $mnm^5U$ by a recombinant MnmC protein (Figure 3 and Supplementary Figure S3). *In vivo* results also indicated that $nm^5U$ is a direct product of the MnmE•GidA activity because a peak corresponding to this nucleoside could be clearly identified in the HPLC analysis of total tRNA extracted from an *mnmC* mutant (Figure 6G and H). It should be emphasized that $nm^5U$ is a final product,

and not a stable intermediate, of the MnmE•GidA activity since it is not a substrate for this complex both *in vitro* (Figure 7 and Supplementary Figure S4) as well as *in vivo* (Figure 6G and H; note in these panels that $nm^5U$ accumulates in an *mnmC* mutant where the MnmE•GidA complex is active). Therefore, the pathway controlled by MnmE and GidA may be reformulated as shown in Figure 5 and, more schematically, 9A.

We have previously shown that the GTPase activity of MnmE and the FAD-binding ability of GidA are required for tRNA modification *in vivo* (14, 19, 20). Here, we demonstrate that the two *in vitro* reactions catalyzed by MnmE•GidA are GTP- and FAD-dependent (Supplementary Figures S1 and S2), suggesting that the synthesis of $nm^5U$ and $cmnm^5U$ share a similar mechanism.

Biochemical and structural studies previously indicated that MnmE binds 5′-formyl-THF (22). Consequently, a tRNA modification mechanism based on the formyl transfer onto C5 of the wobble uridine was proposed (22). However, we have found here that MnmE exhibits a similar affinity for different THF derivatives, including 5′-formyl-THF (Table 1), which questions the role of this compound as the one-carbon unit donor in the modification reaction. We have analyzed the nucleoside composition of tRNA extracted from mutants where the THF pathway is inactivated at different stages. Our data support the idea that the C5-methylene moiety of $xm^5(s^2)U$ nucleosides is derived from the THF pathway, since $s^2U$ accumulates in a *folE* mutant, although not from 5-formyl- or methyl-THF, since the level of $mnm^5s^2$ in *glyA* and *metF* mutants is similar to that of the wild-type strain (Table 2). Moreover, we have performed *in vitro* modification assays in the presence of different THF derivatives (Table 3). Our data indicate that neither 5-formyl-, 10-formyl- or methenyl-THF is likely to be the one-carbon unit donor in the modification reaction. Altogether, these results prompt us to conclude that methylene-THF is the source of the C5 methylene moiety of $nm^5U$ and $cmnm^5U$.

We have shown that NADH is dispensable for the *in vitro* function of MnmE•GidA unless FAD levels are limiting (Figure 8). This suggests that FAD undergoes an oxidation-reduction cycle during synthesis of $nm^5U$ and $cmnm^5U$ (Figure 9B). In such a cycle, FAD receives electrons from other participant in the reaction and, subsequently, donates them to some reaction intermediate. Probably, there is a spontaneous oxidation of the reduced FAD during the *in vitro* modification that may explain, together with the low affinity of GidA for FAD ($K_d \sim 3\,\mu M$, our own unpublished results), the low efficiency of the reaction when FAD is used at 2 μM. Under these circumstances, addition of NADH would facilitate production of the reduced FAD, improving the second step of the FAD cycle associated with the reduction of some reaction intermediate. Based on these considerations, we propose a novel catalytic mechanism for the $nm^5U$ and $cmnm^5U$ modification process (Figure 9C), where a general acid of MnmE•GidA converts the methylene-THF into a reactive iminium ion (step 1), in analogy to the reaction catalyzed by thymidylate synthase (37). This allows the addition of the amine or carboxymethylamine group to the methylene group at N5 of THF (step 2). Then, FAD-bound GidA performs a dehydrogenation reaction (step 3), facilitating the nucleophilic attack by the C5 atom of U34 (step 4), which has been activated, again in analogy with the thymidilate synthase reaction, through the attack at the C6 position of U34 by a general acid of the MnmE•GidA complex. Later, $FADH_2$ serves as the reducing agent of the Schiff's base (step 7). This step is followed by the liberation of the MnmE•GidA complex and production of $cmnm^5U$ and $nm^5U$. Our model involves that the THF-binding domain of MnmE and the FAD-binding domain of GidA should be close to the C5 position of the wobble

**Table 2.** Levels of $mnm^5s^2U$ and $s^2U$ in mutants of the THF pathway

| | $mnm^5s^2U/s^4U$[a] | $s^2U/s^4U$[a] |
|---|---|---|
| BW25113 (wt) | 0.047 | 0.000 |
| *folE::cat* | 0.002 | 0.024 |
| *metF::kan* | 0.046 | 0.000 |
| *glyA::kan* | 0.038 | 0.000 |

[a]The numbers are calculated as the absorbance of $mnm^5s^2U$ or $s^2U$ relative to the absorbance of $s^4U$ at 314 nm and represent the mean values of at least two independent experiments.

**Table 3.** Synthesis of $nm^5s^2U$ in the presence of different THF derivatives

| Reaction mix[a] | A[b] | | B[b] | | C[b] | |
|---|---|---|---|---|---|---|
| | $nm^5s^2U/s^4U$ | $s^2U/s^4U$ | $nm^5s^2U/s^4U$ | $s^2U/s^4U$ | $nm^5s^2U/s^4U$ | $s^2U/s^4U$ |
| No proteins/No folates | 0.0000 | **0.0246** | 0.0000 | **0.0248** | 0.0000 | 0.0244 |
| Proteins/No folates | 0.0142 | **0.0178** | 0.0003 | **0.0271** | nd | nd |
| Proteins/methylene-THF | **0.0459** | 0.0026 | **0.0498** | 0.0069 | 0.0457 | 0 |
| Proteins/THF | **0.0486** | 0.0033 | **0.0465** | 0.0055 | 0.0357 | 0.0072 |
| Proteins/5 formyl-THF | 0.003 | **0.0264** | 0.001 | **0.0279** | nd | nd |
| Proteins/10 formyl-THF | 0.0274 | 0.0134 | 0.0033 | **0.0269** | nd | nd |
| Proteins/methyl-THF | **0.0503** | 0.0024 | **0.0422** | 0.0063 | 0.0225 | 0.0114 |
| Proteins/methenyl-THF | 0.0132 | **0.0222** | 0.0034 | **0.0314** | nd | nd |

[a]tRNA purified from an *mnmE gidA* double mutant and buffer A were used for performing the *in vitro* modification reaction in the presence of the components specified in the column. MnmE and GidA were used at a final concentration of 2 μM (A) and 0.35 μM (B and C). Folates were used at a final concentration of 1 mM (A), 50 μM (B) and 1 μM (C).
[b]The numbers are calculated as the absorbance of $nm^5s^2U$ or $s^2U$ relative to the absorbance of $s^4U$ at 314 nm. Bold numbers are used to highlight the highest values. nd, not done.

uridine of tRNA. The structural rearrangements of the MnmE•GidA heterotetramer that are coupled to the GTPase cycle of MnmE (32) and to the FAD binding of GidA (our own unpublished results) might help in the construction of the active center. Further biochemical and structural studies will be required to clarify the detailed mechanism of the MnmE•GidA-dependent reactions.

MnmE and GidA, but not MnmC, are conserved proteins from bacteria to humans. The MnmE and GidA homologues in yeast (MSS1 and MTO1) and humans (GTPBP3 and MTO1) are nucleus-encoded proteins involved in modification of mitochondrial tRNAs (38–41). The lack of MnmC determines that, in yeast, the final product of the MSS1/MTO1 pathway is $cmnm^5U$. In humans, taurine is incorporated into tRNAs in place of glycine, producing $\tau m^5U$ (23). Both nucleosides are probably synthesized in mitochondria by the MnmE and GidA homologues without the participation of other proteins, through a reaction similar to that described here. If so, the complex formed by the human homologues should be able to use taurine in place of glycine. It should be noted that GTPBP3 largely conserves the MnmE properties (11,42), which supports our proposal that the tRNA modification reaction catalyzed by the eukaryotic homologues should be basically similar to that performed by the *E. coli* proteins. The presence of $nm^5(s^2)U$ in mitochondrial tRNA has not been reported

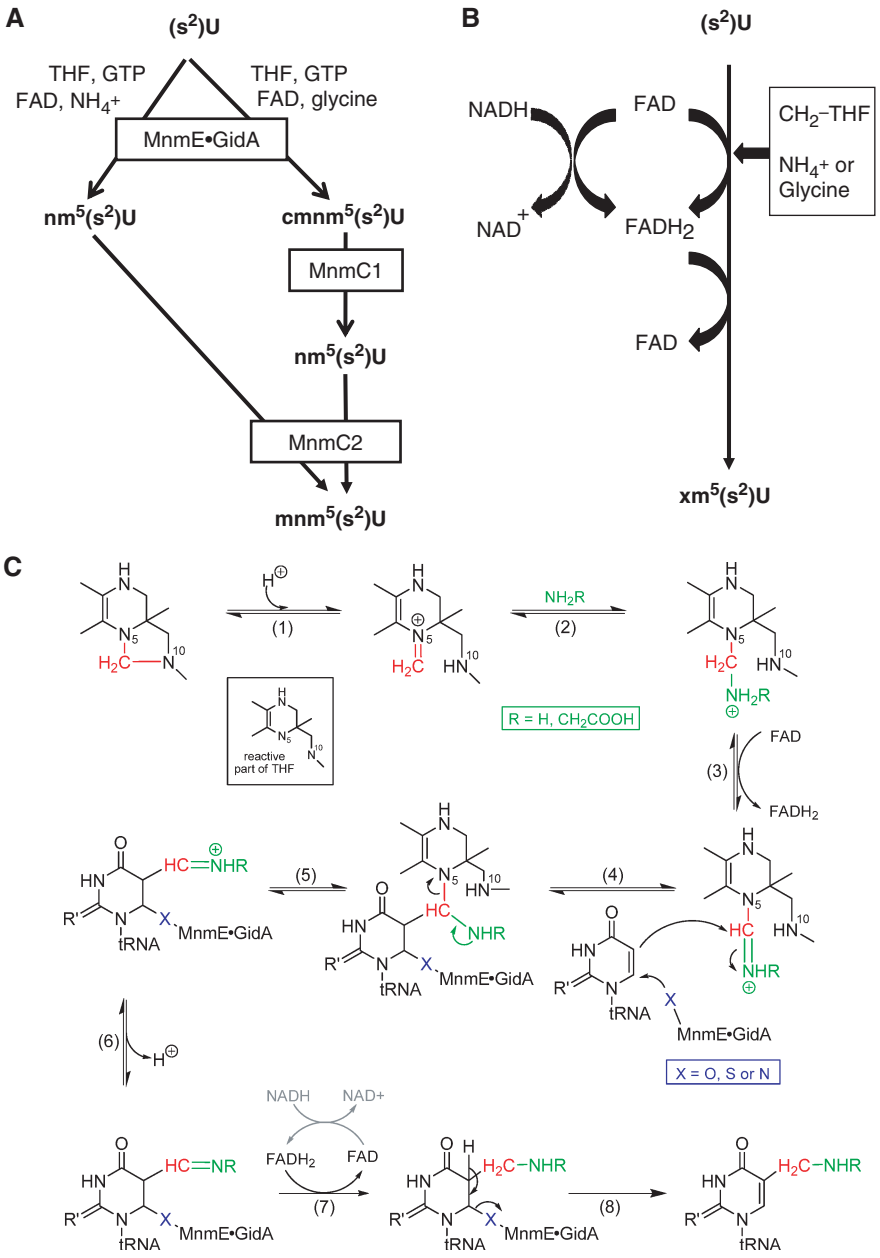


**Figure 9.** The MnmE•GidA tRNA modification pathway. (**A**) A schematic of the new formulation for the MnmE•GidA pathway. (**B**) Hypothetical FAD-dependent steps in the MnmE•GidA-mediated reaction. (**C**) The proposal chemical mechanism for the MnmE•GidA catalyzed reactions.

so far. However, in the light of our results, we think this issue should be further explored.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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*Conflict of interest statement*. None declared.

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