Formation of a stalled early intermediate of pseudouridine synthesis monitored by real-time FRET

MARTIN HENGESBACH,¹ FELIX VOIGTS-HOFFMANN,^{1,2,4} BENJAMIN HOFMANN,^{1,2} and MARK HELM^{1,3}

¹Institute of Pharmacy and Molecular Biotechnology, Department of Chemistry, Heidelberg University, 69120 Heidelberg, Germany

²Master Program Molecular Biotechnology, Heidelberg University, 69120 Heidelberg, Germany

³Institute of Pharmacy, Johannes Gutenberg-Universität Mainz, 55128 Mainz, Germany

ABSTRACT

Pseudouridine is the most abundant of more than 100 chemically distinct natural ribonucleotide modifications. Its synthesis consists of an isomerization reaction of a uridine residue in the RNA chain and is catalyzed by pseudouridine synthases. The unusual reaction mechanism has become the object of renewed research effort, frequently involving replacement of the substrate uridines with 5-fluorouracil (f^5U). f^5U is known to be a potent inhibitor of pseudouridine synthase activity, but the effect varies among the target pseudouridine synthases. Derivatives of f^5U have previously been detected, which are thought to be either hydrolysis products of covalent enzyme-RNA adducts, or isomerization intermediates. Here we describe the interaction of pseudouridine synthase 1 (Pus1p) with f^5U -containing tRNA. The interaction described is specific to Pus1p and position 27 in the tRNA anticodon stem, but the enzyme neither forms a covalent adduct nor stalls at a previously identified reaction intermediate of f^5U . The f^5U 27 residue, as analyzed by a DNazyme-based assay using TLC and mass spectrometry, displayed physicochemical properties unaltered by the reversible interaction with Pus1p. Thus, Pus1p binds an f^5U -containing substrate, but, in contrast to other pseudouridine synthases, leaves the chemical structure of f^5U unchanged. The specific, but nonproductive, interaction demonstrated here thus constitutes an intermediate of Pus turnover, stalled by the presence of f^5U in an early state of catalysis. Observation of the interaction of Pus1p with fluorescence-labeled tRNA by a real-time readout of fluorescence anisotropy and FRET revealed significant structural distortion of f^5U -tRNA structure in the stalled intermediate state of pseudouridine catalysis.

Keywords: RNA modification; pseudouridine synthase; FRET; 5-fluorouridine; Pus inhibition; tRNA structure

INTRODUCTION

In addition to the four major nucleotides, the chemical complexity of naturally occurring RNA comprises a large variety of over 100 modified nucleotides, among which pseudouridine (Ψ) is the most frequent. Its synthesis consists of an isomerization of uridine, and is catalyzed by pseudouridine synthases (Pus), a large and ubiquitous group of enzymes that falls into six families, named after their first identified representatives: TruA, TruB, TruD, RsuA, RluA, and Pus10 (Spedaliere et al. 2004; Gurha and Gupta 2008). The mechanistic details of catalysis, which does not require any cofactors, are subject to renewed interest (Spedaliere et al. 2004; Hamilton et al. 2005, 2006), as are the structural

effects of Ψ on RNA by itself (Helm 2006). During isomerization of uridine to pseudouridine, the carbon-carbon bond of the anomeric ribose-carbon with the carbon 5 of the uracil is formed at the expense of the usual carbon-nitrogen glycosidic bond (Fig. 1A).

The mechanism is thought to involve a nucleophilic attack of the catalytic aspartate, either at the C1 of the ribose ("acyl mechanism") or—similar to the synthesis of thymidine from uracil—at the C6 of the pyrimidine ring. Following the Michael addition, the latter mechanism proceeds via a covalent intermediate referred to as "enolate." This undergoes rotation of the base and formation of the product pseudouridine (Foster et al. 2000; Spedaliere et al. 2004).

While the mechanism has so far eluded ultimate clarification, many insights have come from studies on the reaction with fluorouracil (f^5U)-containing RNA substrates (Fig. 1B). f^5U was reported to form covalent adducts with certain pseudouridine synthases, such as e.g., TruA (Huang et al. 1998) and RluA (Spedaliere and Mueller 2004; Hamilton et al. 2005, 2006), that are stable toward SDS PAGE conditions, but can be disrupted by heating to yield a hydrated product

⁴Present address: Swiss Federal Institute for Technology, Institute for Molecular Biology and Biophysics, 8093 Zürich, Switzerland.

Reprint requests to: Mark Helm, Institute of Pharmacy, Johannes Gutenberg-Universität Mainz, Staudinger Weg 5, 55128 Mainz, Germany; e-mail: mhelm@uni-mainz.de; fax: +49-6221-546430.

Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.1832510>.

FIGURE 1. Chemical structures. (A) Uracil, which is converted to pseudouridine by pseudouridine synthases (Pus). (B) 5-Fluorouracil is a potent inhibitor of some pseudouridine synthases, some of which (except for the eukaryotic Pus1p tested) form (C) 5-fluoro-6-hydroxypseudouridine (f⁵ho⁶Ψ).

(Fig. 1C). In contrast, other pseudouridine synthases are not inhibited by f⁵U-containing RNA, in which the f⁵U becomes hydrated and likely rearranged e.g., by *Escherichia coli* TruB (Spedalieri and Mueller 2004). Several crystallographic attempts have been made to trap covalent adducts, presumably between the conserved catalytic aspartate and the substrate. However, no covalent bond between nucleotide and the catalytic aspartate was observed in cocrystal structures of f⁵U-containing RNAs and *E. coli* TruB (Hoang and Ferré-D'Amaré 2001), *Thermotoga maritima* TruB (Pan et al. 2003; Phannachet and Huang 2004), *E. coli* RluA (Hoang et al. 2006), and *Pyrococcus furiosus* Cbf5 (Duan et al. 2009; Liang et al. 2009) and the base was modeled as a hydrated, rearranged product of 5-fluoro-6-hydroxy-pseudouridine (f⁵ho⁶Ψ) (Fig. 1C). In the case of RluA and Cbf5, this product may result from the X-ray sensitivity of a presumed covalent adduct, but was not unambiguously identified in the latter case.

Intriguingly, pseudouridine synthases share a structurally conserved catalytic domain, and therefore small differences in the active site geometry were suggested to account for the disparate behavior toward f⁵U-containing RNAs (Spedalieri et al. 2004). However, the possibility was left open that, despite their sequence homologies, structural resemblances in the active center, and similarities in the catalytically active amino acids, pseudouridine synthases from different families may employ diverging catalytic strategies for the synthesis of pseudouridine (Spedalieri et al. 2004).

Pseudouridine synthase 1 (Pus1), a member of the TruA family, is a multisite and multisubstrate specific enzyme, which catalyzes pseudouridine formation at several positions of mammalian cytosolic and mitochondrial tRNAs, as well as in snRNA from various species (Arлуison et al. 1998; Motorin et al. 1998; Arлуison et al. 1999; Chen and Patton 1999;

Massenet et al. 1999; Chen and Patton 2000; Hellmuth et al. 2000; Patton and Padgett 2005; Patton et al. 2005; Behm-Ansmant et al. 2006; Zhao et al. 2007; Sibert et al. 2008). These include positions in the anticodon stem of several tRNAs at positions 27 and 28. It has been shown that U27 is modified first, and depending on the substrate, U28 is modified less efficiently, apparently in an independent and slower second step (Motorin et al. 1998; Helm and Attardi 2004).

f⁵U has been used frequently in studies on cytotoxicity, and besides its inhibitory effect on thymidylate synthesis was shown to inhibit pseudouridylation (Zhao and Yu 2007; Gustavsson and Ronne 2008; Hoskins and Butler 2008). These effects can possibly be attributed to three main mechanisms, affecting either the enzyme or the RNA structure. First, formation of a covalent adduct between f⁵U and the pseudouridine synthase renders the enzyme inactive, resulting in a decreased level of the respective activity available for cellular processes. Second, f⁵U may be noncovalently bound by pseudouridine synthases, but not processed, thus blocking the enzyme for other substrates. As a third possibility, the reaction may result in the presence of a modified base that does not exhibit the desired structural effects of the pseudouridine modification in the target RNA, therefore leading to a nonfunctional RNA.

In the first two cases, the effect is an inhibition of the enzyme that exceeds the extent expected from stoichiometric considerations. This is in good agreement with the work of (Zhao and Yu 2007), and is in line with their finding that there is no alteration of the behavior of f⁵U on the TLC systems used. In the presented work, the inhibitory effect of f⁵U on Pus1p was used in order to make an enzyme-bound state of the substrate tRNA amenable to analysis by FRET.

We demonstrate here the detection of a nonproductive interaction between f⁵U-containing tRNA and protein via measurement of fluorescence anisotropy and intramolecular FRET. We show that recombinant Pus1 proteins from mouse and from *Saccharomyces cerevisiae* specifically bind to human mitochondrial tRNA^{Leu(CUN)} containing f⁵U at position 27, thereby distorting parts of the tRNA structure. Unlike the previously characterized bacterial eponym of the TruA family, and the TruB and RluA pseudouridine synthases, formation of a covalent adduct with f⁵U-containing tRNA was not detected. The nucleotide itself was found to be chemically unaltered by the interaction with Pus1p. Fluorescence data obtained from the interaction with mitochondrial tRNA^{Lys} suggest that this constitutes an idiosyncratic feature typical of the interaction of Pus1p with position 27 of various tRNAs.

RESULTS

Synthesis of tRNAs that report structural changes by FRET

Substrate tRNA constructs used in this study contain two fluorophores, namely the dyes Cy3 and Cy5, which form

a well-characterized FRET-pair. FRET stands for fluorescence resonance energy transfer, and describes a distance-dependent transfer of excitation energy from the so-called “green” or donor dye to the “red” acceptor dye, which is then emitted at the fluorescence wavelength of the red dye (Lilley and Wilson 2000; Coban et al. 2006). Because of the many factors affecting FRET-based calculation of absolute distances, the technique is widely used to assess relative changes in interdyer distances.

Since such changes are best measured in the most dynamic range near the Förster radius R_0 , corresponding to the distance at 50% energy transfer between a given dye pair, the attachment sites for Cy3 and Cy5 to human mitochondrial tRNA^{Leu(CUN)} were chosen based on the X-ray structure of yeast tRNA^{Phe}, such that the interdyer distance would be around 53 Å, which is the R_0 of the Cy3–Cy5 pair (Coban et al. 2006). Hence, Cy3 was attached via a spacer to the 5-carbon of desoxyuracil at nucleotide 4, and Cy5 was attached to nucleotide 31 in the same way (details in Materials and Methods).

One-pot reactions of four-way splint ligations on the nanomole scale lead to isolated yields of 10%–15% (Kurschat et al. 2005; Hengesbach et al. 2008a). The communication of both dyes by FRET, as well as their anisotropy was assessed by bulk fluorescence spectroscopy. From the fluorescence spectra, FRET efficiencies (E_{FRET}) of ~ 0.75 for all tRNA^{Leu(CUN)} constructs were calculated as detailed in Material and Methods. This indicates that in the absence of modifying proteins, the constructs adopt a similar structure.

Pseudouridine synthase 1 binds tRNA constructs with U27, C27, f⁵U27, and f⁵U28 and distorts the f⁵U27 substrate

U27 and f⁵U27 derivatives of tRNA^{Leu(CUN)} (shown in Fig. 2) were incubated in the presence of different molar excesses of recombinant Pus1p, and E_{FRET} values were calculated from full spectra recorded at the time points indicated in Figure 3A. While E_{FRET} of U27-tRNA^{Leu(CUN)} remained essentially invariant, E_{FRET} of f⁵U27-tRNA^{Leu(CUN)} showed a strong decay during the first 10–20 min in the

FIGURE 2. Schematic of the tRNA^{Leu(CUN)} construct. The bases placed in positions 27 and 28 are indicated with their names used throughout the text.

FIGURE 3. Fluorescence spectroscopy measurements of tRNA constructs during incubation with Pus1p. (A) FRET efficiency for f⁵U27, but not for U27 construct decreases in the presence of Pus1p. (B) At the same time, the fluorescence anisotropy increases to a similar extent. (C) The FRET decrease depends on the amount of Pus1p used (different molar excesses indicated as “X”).

presence of a 20-fold excess of Pus1p, indicating the formation of a stable complex featuring an apparent structural distortion. The complexed f⁵U27 derivative subsequently remained stable in this low-FRET state over 40 min. This stabilization and other evidence outlined below argue against the effect stemming from degradation. Upon incubation, the anisotropy of acceptor fluorescence for both constructs showed a significant increase (Fig. 3B), which was also observed for a C27 and an f⁵U28 derivative (discussed below). For U27 and f⁵U27, a titration experiment with increasing

concentrations of Pus1 was conducted, which showed that the change in E_{FRET} correlated with the concentration of applied protein (Fig. 3C), as was the case for the increase in anisotropy for both constructs. The FRET decrease reached its half-maximum at a fourfold excess of Pus1p.

A number of control experiments were performed to verify that the tRNA's decrease in E_{FRET} was indeed specific to the interaction with Pus1p. Integrity of the Cy5 acceptor dye population was verified by comparison of direct excitation spectra taken before and after the incubation. Aliquots drawn before and after the incubation were submitted to denaturing PAGE. The gels were scanned with a fluorescence imaging system, which allows the detection of fmol quantities of dye-labeled RNA. Thus, the assessed degradation during incubation was consistently below 2%, excluding any relevant effect on the E_{FRET} measurement. Of note, repurified constructs showed FRET efficiencies comparable to unincubated ones.

Significant changes in FRET efficiency or fluorescence anisotropy were observed neither in protein storage buffer alone, nor in the presence of bovine serum albumin. For comparison, E_{FRET} kinetics of tRNA^{Leu(CUN)} were investigated upon incubation with a noncognate tRNA modification enzyme. In experiments with recombinant yeast m¹G9-tRNA-methyltransferase Trm10p (Jackman et al. 2003), the E_{FRET} of f⁵U27-tRNA^{Leu(CUN)} did not significantly change (Fig. 4). Importantly, the decrease of FRET efficiency observed upon incubation of f⁵U27-tRNA^{Leu(CUN)} with homologous Pus1p from mouse was similar to that observed for the yeast enzyme. These data establish that the observed time-dependent decrease of E_{FRET} in f⁵U27-tRNA is due to specific interaction of this tRNA with Pus1p.

Further investigations into the specificity of this interesting interaction were conducted using derivatives of tRNA^{Leu(CUN)} featuring f⁵U28 or a C27, respectively. The

latter constitutes a negative mutant, where the primary target residue of Pus1p, i.e., the U27 has been altered such that catalytic turnover at position 27 would be rendered impossible. Of note, a certain degree of structural distortion induced by the mutation itself is to be expected. The former derivative, f⁵U28, was investigated to probe for a potential interaction of Pus1p with its secondary target site 28. Both derivatives display a weak response in E_{FRET} (Fig. 5) similar to U27, indicating that the apparent structural distortion is observable only with f⁵U at position 27.

Next, a different tRNA was investigated in order to gauge, whether the spectroscopic observations with f⁵U27 constitute an isolated case particular to human mitochondrial tRNA^{Leu(CUN)}, or a more general feature of Pus1p. Mitochondrial tRNA^{Lys} has been extensively characterized in our lab, including interaction studies with Pus1p. tRNA^{Lys} requires either the post-transcriptional modification m¹A9 or certain stabilizing mutations to achieve cloverleaf folding. In the absence of either, it adopts an extended hairpin structure (Helm et al. 1998), which prevents modification by Pus1p in a manner comparable to that of tRNA^{Leu(CUN)} (Helm and Attardi 2004; Voigts-Hoffmann et al. 2007). Hence, we here employ a tRNA^{Lys} construct containing a cloverleaf-stabilizing G50–C64 base pair exchange (Helm et al. 1998), in combination with a respective construct featuring the f⁵U27 residue. Remarkably, the behavior of this pair corresponds to that of the U27–f⁵U27 pair in the tRNA^{Leu(UUR)} context: while the U27 derivative shows a clear response in polarization, but little change in E_{FRET} , the f⁵U27 derivative shows an equally strong response in polarization in addition to a strong and lasting decrease in E_{FRET} in the presence of Pus1p (Fig. 5).

Fluorophore-labeled tRNA^{Leu(CUN)} constructs are substrates for pseudouridine synthase 1

While the substrate properties of fluorophore-labeled tRNA^{Lys} constructs have previously been established (Voigts-Hoffmann et al. 2007), those of fluorophore-labeled tRNA^{Leu(CUN)} remained to be demonstrated. Thus, tRNA was recovered after spectral characterization and, after repurification by denaturing PAGE, the nucleotide at position 27 was analyzed by a site-specific post-labeling assay (Hengesbach et al. 2008b). As depicted in Figure 6, tRNA was annealed to a 10-23 DNase designed to cleave between nucleotides A26 and U27, and the mixture was then submitted to temperature cycling in a PCR machine until quantitative cleavage was achieved. Because of the site- and sequence-specificity of the DNase mediated cleavage, this assay does not allow analysis of C27 or of the residue 28 in any of the constructs. For constructs containing U27 or f⁵U27, free 5'-hydroxyl groups were quantitatively phosphorylated using γ -[³²P]-ATP and polynucleotide kinase. The reaction mixture was then submitted to DNase treatment to degrade the DNase. The 3'-fragment of the tRNA, now carrying

FIGURE 4. FRET efficiency responds to specific protein binding. Changes in FRET efficiency are shown for FRET-modified tRNA (black) and the f⁵U-containing derivative (hatched) in the presence and absence of various proteins. The decrease in FRET efficiency is restricted to the eukaryotic Pus1p homologs tested, and is specific to the f⁵U27 tRNA construct.

FIGURE 5. The concurrent recording of fluorescence anisotropy and FRET efficiency allows for assignment of structural distortion of the tRNA (20 nM) during binding to the modifying enzyme. Anisotropy and FRET readings are plotted before addition of Pus1p ($c_{\text{final}} = 400$ nM) protein (indicated by “RNA only” on the x-axis), after 90 min incubation with Pus1p, and after addition of an excess of unlabeled tRNA substrate ($c_{\text{final}} = 8$ μ M).

a 5'-[32 P]-phosphate-label on nucleotide 27, was isolated by denaturing PAGE and digested to mononucleotides with nuclease P1. Analysis of this mixture by thin-layer chromatography (TLC), as shown in Figure 6B, revealed that uridine at position 27 is indeed converted to pseudouridine in good yield. For tRNA^{Leu(CUN)} this applied to both U27 (“wild-type”: 38% modification efficiency) and U27-f⁵U28 (53% modification efficiency) constructs. The tRNA^{Lys} construct was modified to an extent of 34%. Previous investigations indicate, that modification of U28 is negligible for the U27–U28 constructs under these conditions (Helm and Attardi 2004).

Pus1p does not turn over f⁵U27-containing tRNA substrates

Analysis of tRNAs containing f⁵U27 showed no detectable chemical alteration by TLC in any of altogether three different solvent systems, two of which

are shown in Figure 6B. This strongly suggests that the chemical nature of the f⁵U27 residue remains unaffected by incubation with Pus1p. In addition, f⁵U28-containing tRNAs were efficiently modified at position 27, showing that the mere presence of f⁵U does not disturb Pus1p binding or the enzymatic reaction. It is also clear that mechanistic conclusions drawn from the presented data concerning f⁵U at the primary Pus1p target site 27 cannot automatically be applied to tRNAs containing f⁵U at the secondary target site 28.

In order to further exclude alternative reaction products that may have escaped characterization of the f⁵U27–Pus1p interaction by TLC, a 24mer RNA fragment ranging from U23 to A47 was excised using a tandem DNazyme. This fragment spans the Cy5 dye attachment site, as well as the modification site. The fragment was purified by PAGE, eluted, and ethanol precipitated. After desalting with a ZipTip containing an RP-18 resin, the purified RNA was subjected to mass spectrometry analysis by MALDI. This included mixtures of (1) treated and untreated tRNA, as well as (2) U27, f⁵U27, and f⁵U28 constructs. Comparing untreated and treated U27 and f⁵U27

constructs, it could be shown that the apparent mass of the oligonucleotide is not altered upon incubation by Pus1p, i.e., there was no formation of a hydrated or defluorinated

FIGURE 6. Assessing the chemical nature of the target base by two cooperative DNazyme-based methods. (A) Scheme of the FRET-labeled tRNA construct, which is cleaved by either of two DNazymes. (B) Cleavage by DNazyme I proceeds adjacent to nucleotide 27, and allows labeling and TLC analysis of the nucleotide 27, the 5'-terminal nucleotide of the downstream fragment. (C) Double cleavage by tandem DNazyme II produces a fragment that is subjected to MALDI mass spectrometry.

product; this also holds true for the f⁵U28 construct (Fig. 6C).

Pus1p forms a stable, but not a covalent adduct with f⁵U-containing substrate tRNAs

Aliquots of reaction mixtures containing one- or twofold excess of Pus1p over tRNA were loaded onto nondenaturing polyacrylamide gels without prior heating. As can be seen in Figure 7, all constructs tested showed formation of a complex with shifted mobility on the nondenaturing gel, but f⁵U27-containing tRNAs did so to a greater extent. So far, these findings are in keeping with the known behavior of Pus–RNA interactions. Because several other pseudouridine synthases have been reported to form covalent adducts or highly stable noncovalent complexes with RNA substrates containing f⁵U, the same reaction mixtures were investigated by different denaturing PAGE systems with and without prior heating. Aliquots of f⁵U27–tRNAs in reaction mixtures with Pus1p produce a single band on urea PAGE (Fig. 7B) and SDS-PAGE (data not shown). There is thus no evidence for a covalent complex between the tRNA and Pus1p. As a control, yeast tRNA^{Phe} carrying f⁵U at position 55 showed complex formation resistant to urea PAGE when incubated with *Thermotoga maritima* TruB (data not shown). When Pus1p-treated f⁵U–tRNA was repurified on denaturing urea PAGE and refolded, an E_{FRET} very close to the original value was restored.

To further characterize the f⁵U27–tRNA complex with Pus1p, and to assess whether its formation was reversible, its behavior toward an excess of unlabeled, competing substrate was analyzed in our spectroscopic E_{FRET} assay. After recording FRET kinetics over 60–90 min, a 400-fold molar

excess of total tRNA from *E. coli* over FRET-labeled tRNA was added, and data acquisition was continued for another 60–90 min. Some tRNAs from *E. coli* carry a U27, and since *E. coli* does not possess modifying activities for pseudouridine formation at position 27, this position serves as a substrate for Pus1p. In contrast to samples containing no Pus1p or a U27 construct, the FRET efficiency increased for the f⁵U-containing substrates upon addition of *E. coli* tRNA. The E_{FRET} level almost recovered to the original values before addition of Pus1p (Fig. 8). At the same time, anisotropy for all constructs decreased to values before addition of Pus1p (Fig. 5). This relaxation indicates that both U27 and f⁵U27 substrates were out-competed by the excess of unlabeled substrate tRNA, and that the interaction of the f⁵U-containing substrate with Pus1p must therefore be reversible. It thus argues against formation of a covalent f⁵U substrate–Pus1p adduct of a type previously described in the literature. The reversibility of the Pus1p–tRNA interaction is further characterized by a titration of Pus1p shown in Figure 8. It is clearly apparent that the plateau level of the decreased E_{FRET} is strongly dependent on the concentration of Pus1p, while the subsequent relaxation to the original is E_{FRET} comparable.

DISCUSSION

Fluorescently labeled tRNA constructs are substrates for Pus1p

As previously shown for human mitochondrial tRNA^{Lys} (Voigts-Hoffmann et al. 2007), tRNA^{Leu(CUN)} labeled with Cy3 and Cy5 as a FRET pair is also a substrate for Pus1p. The comparable behavior of fluorescence anisotropy values in U27 and f⁵U27 constructs upon addition of Pus1p indicates similarities in the mode of binding. In addition, competition experiments with excess unlabeled substrate tRNA show that the interaction is reversible in both cases as judged from fluorescence anisotropy. Together with the data derived from analysis of pseudouridine formation, the behavior of the system analyzed can be considered resembling the natural enzymatic turnover.

FRET as a useful reporter for structural changes in tRNA

By determining changes in FRET efficiency—and thus changes in spatial distance between the attachment sites within the tRNA—structural alterations of the substrate tRNA can be assessed. FRET of untreated samples remained stable even

FIGURE 7. Gel electrophoresis of tRNA^{Leu(CUN)}–Pus1p complexes. (A) Nondenaturing gel electrophoresis of reaction mixtures containing FRET-labeled tRNA constructs and Pus1p shows a distinct band in addition to a smear, which migrates slower than the tRNA alone. The intensity of this band and the smear is highest for the f⁵U27 construct. (B) Urea PAGE of aliquots from the same reaction mixtures shows single bands.

FIGURE 8. Kinetics of FRET efficiency of tRNA^{Leu(CUN)} f⁵U27 ($c = 20$ nM) after addition of Pus1p ($c_{\text{final}} = 400$ nM) at $t = 0$. The distorting effect titrates with the excess of Pus1p used (indicated as “X”) and is in all cases reversible to yield a FRET efficiency comparable to unbound tRNA. “Blank” indicates the control measurement of tRNA in the absence of protein.

over time periods of up to 3 h, which shows that any changes in FRET are specific to the conditions tested. Whereas the f⁵U-containing construct shows a significant decrease in FRET efficiency when binding to Pus1p, the U27 construct does not. Taking the change in fluorescence anisotropy into consideration, this strongly indicates a distortion of the f⁵U substrate by Pus1p.

A two-step model of substrate binding by pseudouridine synthases was proposed (Kealey et al. 1994; Hur et al. 2006), separating the rapid binding of substrate RNA to the pseudouridine synthase from a slower flipping of the target base, aligning it in the catalytic pocket. The data presented here for the f⁵U-tRNA support this model; the quick association of Pus1p with the substrate is reflected in the fluorescence anisotropy, whereas distortion of the RNA is a slower process, which manifests in a significant decrease in FRET efficiency after ~10 min (Fig. 3). The difference of the fluorescence anisotropy and FRET kinetics during the first 15 min also shows that there is no significant contribution of the fluorophores’ orientational freedom to the FRET effect in the system analyzed here. The fact that the f⁵U27 constructs of both tRNA^{Leu(CUN)} and tRNA^{Lys} show decreasing E_{FRET} upon Pus1p binding suggests that the structural distortion is not an isolated event of one particular tRNA. It will be interesting to investigate resemblances to, e.g., the λ -shaped tRNA structure of the modification intermediate of the archaeosine tRNA-guanine transglycosylase (Ishitani et al. 2003).

Our data suggest, that the structural distortion is not observed in the U27-tRNAs, because it is related to a step of the catalytic process distinct from binding, which is therefore not synchronized in the ensemble observed here. Because the introduction of f⁵U27 stalls the catalytic

process at this step, a significant population of this intermediate accumulates, thus allowing its observation.

Characterization of base modification by two cooperative DNAzyme-based approaches

Due to the limited amounts of modified RNA available, sensitive methods are required to assess the chemical nature of the modification. In this study, two different approaches based on site-specific cleavage of the target RNA by DNAzymes are employed. The first method uses a 10-23 type DNAzyme to cleave adjacent to the base to be analyzed, making it available for post-labeling (Hengesbach et al. 2008b). This method is comparable in strategy and sensitivity to the method employing RNase H (Zhao and Yu 2004, 2007). The second strategy cleaves the target RNA using a construct consisting of two DNAzyme motifs in one sequence. This tandem DNAzyme makes a defined RNA fragment available to MALDI-TOF mass spectrometry. The productive combination of both methods allows for a thorough characterization of the modified base with high sensitivity in the picomole range.

Interaction between Pus1p and the f⁵U substrate is not productive and does not lead to formation of a covalent adduct

Determination of the physicochemical properties of f⁵U at position 27 in the tRNA showed that neither chromatographic behavior in three different systems, nor the apparent mass of the nucleotide of interest were changed upon incubation with Pus1p. The complex formed by the f⁵U27-containing substrates was shown to be more stable compared to the U27 constructs under conditions of non-denaturing gel electrophoresis. However, urea and SDS PAGE analysis, as well as experiments where the f⁵U-containing substrate was released from Pus1p by addition of excess unlabeled substrate tRNA, show that the interaction does not stall at a covalent intermediate, but at an earlier step than previously reported. This noncovalent, nonproductive binding of f⁵U substrates may be an additional mechanism contributing to the inhibitory effect of 5-fluorouracil observed in vivo on pseudouridine synthases (Gustavsson and Ronne 2008).

Specificity of the formation of a distorted intermediate

The structural distortion, as evidenced by a decrease in E_{FRET} , was observed for two homologous Pus1p enzymes, showing that this feature is not restricted to a single species and likely to be idiosyncratic for Pus1p. The fact that two different tRNAs, i.e., tRNA^{Lys} and tRNA^{Leu(CUN)} behave similarly in terms of anisotropy and E_{FRET} suggests, that the distortion may be a feature of most substrate tRNAs. It thus

appears to represent a mode of recognition by Pus1p, which is general with respect to the tRNA, but site specific for f⁵U-containing tRNAs. In contrast, our data do not yield insight into the mechanism of pseudouridine formation at position 28 of the same tRNAs.

Summary and Outlook

To our knowledge, this is the first FRET-based investigation of an RNA–protein complex involving a modification enzyme. We have shown here that FRET-labeled tRNAs provide an excellent means to address tRNA structure even in the presence of a modification enzyme. We could show by anisotropy and FRET that the interaction is specific for f⁵U27-tRNA and Pus1p, and by MALDI and TLC that it is nonproductive, thus representing a stalled and probably early intermediate of pseudouridine synthesis. It was shown that a substrate tRNA containing a 5-fluorouracil at the target base is bound to and distorted by pseudouridine synthase 1, but does not form a covalent adduct with the protein. The fact that 5-fluorouracil is not turned over by Pus1p is in contrast to studies on certain other pseudouridine synthases, found to either form covalent abortive intermediates or to produce hydrated reaction products. The present study thus leads to the conclusion that the exact type of pseudouridine synthase and especially the structural details of the active site are critically important in determining at which step f⁵U inhibits pseudouridine formation by Pus enzymes.

This makes usage of f⁵U at the target nucleotide a valuable tool to trap the tRNA–protein complex at an intermediate of pseudouridine synthesis, whose apparently unusual tRNA structure, and possible resemblances to other structural intermediates of modification processes (Ishitani et al. 2003) can then be studied in more detail. Definition of the spectral properties of this stalled intermediate, as done here, should pave the way to single-molecule observations of the tRNA structural rearrangements accompanying catalysis in real time.

MATERIALS AND METHODS

Preparation of recombinant enzymes

Plasmids encoding recombinant modification enzymes were kindly provided by Henri Grosjean (Université Paris-Sud) (*S. cerevisiae* PUS1), Eric Phizicky (University of Rochester) and Jane E. Jackman (Ohio State University) (*S. cerevisiae* TRM10), and Jeff Patton (University of South Carolina) (mouse PUS1). Induction and purification was essentially performed as previously described (Arluison et al. 1998; Motorin et al. 1998; Chen and Patton 1999; Constantinesco et al. 1999; Jackman et al. 2003), with the modifications described below. The proteins were expressed in Rosetta(DE3)-pLysS (Novagen). Cells were grown on LB medium supplemented with ampicillin (AMP, 100 µg/mL) and chloramphenicol (CAM, 30 µg/mL) or with kanamycin (KAN, 34 µg/mL) and CAM.

Preparatory cultures of 50 mL LB medium with the appropriate antibiotics were inoculated with single colonies and incubated overnight at 37°C. The main cultures (up to 1 L) were inoculated with 20–35 mL of preparatory culture, and grown at 37°C, with gentle agitation at 250 rpm. When OD₆₀₀ reached 0.6, protein expression was induced by addition of 1 mM IPTG. For Pus1p, temperature was reduced to 25°C prior to induction and the medium was supplemented with 20 µM ZnCl₂. After 4 h, cells were pelleted, washed with PBS, and stored at –80°C after shock freezing in liquid nitrogen.

Purification of *S. cerevisiae* and mouse Pus1p was performed on ice or at 4°C. The pellet was resuspended in 15 mL buffer a1 (50 mM sodium phosphate at pH 8.0, 200 mM NaCl, 1 mM DTT, and 0.5 mg/mL dodecyl maltoside) (DM, Fluka), supplemented with 0.1% TritonX-100 and two complete miniprotease inhibitor tablets and lysed by sonication. Debris was pelleted at 40,000 g, 4°C for 20 min. and the supernatant loaded onto a 1 mL Ni²⁺-chelating column (Amersham, GE Healthcare). After washing with 30 mL of buffer a1 containing 42 mM imidazole, the protein was eluted with 150 mM imidazole in buffer a1. The protein was buffer-changed into buffer b1 (Tris-HCl at pH 8.5, 10% glycerol, 1 mM DTT, 1 mM MgCl₂, and 0.5 mg/mL DM) on PD-10 columns (Amersham), loaded on a 1 mL Resource Q anion exchange column (Amersham) and eluted with a linear gradient of 0–300 mM NaCl in buffer b1 over 14 mL. Fractions with pure protein were identified by SDS-PAGE. Glycerol was added to 50% and aliquots were stored at –20°C or shock frozen in liquid nitrogen and kept at –80°C. Mouse Pus1p was eluted from the Ni²⁺-chelating column with a linear gradient of 0–300 mM imidazole in buffer a1, and the pooled fractions were dialyzed into buffer c1 (50 mM Tris-HCl at pH 7.5, 00 mM NaCl, 10 mM MgCl₂, 1 mM DTT, and 25% glycerol) overnight with one buffer change, and glycerol was added to 50% before freezing.

Purification of Trm10p from *S. cerevisiae* was carried out similarly, with buffer a2 (20 mM sodium phosphate at pH 8.0, 10% glycerol, 4 mM MgCl₂, 500 mM NaCl, 0.5 mM β-mercaptoethanol, and 1 mM adenosine), supplemented with 20 mM imidazole and protease inhibitors for lysis and 20 mM imidazole for washing. After dialysis into buffer b2 (20 mM Tris-HCl at pH 7.5, 4 mM MgCl₂, 2 mM EDTA, 55 mM NaCl, 1 mM DTT, and 10% glycerol), pooled fractions were concentrated (10 kDa MWCO concentrators, Amicon, Billerica) and glycerol was added to 50% final concentration before freezing.

SDS gel electrophoresis

SDS-PAGE for analysis of protein purity and for studies on covalent interactions between f⁵U27-tRNA and Pus1p was carried out on discontinuous mini-gels (resolving gel: 0.4 M Tris-HCl at pH 8.8, 10% acrylamide, 0.1% SDS; stacking gel: 0.125 M Tris-HCl at pH 6.8, 5% acrylamide, 0.1% SDS; electrophoresis buffer: 25 mM Tris, 250 mM glycine, and 0.1% SDS). Samples were diluted at least twofold with loading buffer (63 mM Tris-HCl at pH 6.8, 25% glycerol, 2% SDS, and bromophenolblue). For protein purity analysis, but not for studies on covalent interaction, the samples were heated to 95°C for 4 min before loading. Gels were run at 200 V constant for ~35 min, washed with ultrapure water, stained with colloidal Coomassie and destained in water to enhance contrast. After 30 min incubation in gel drying solution, they were enclosed between two cellophane sheets and dried in a convection oven (GelAir, Bio-Rad).

Denaturing and nondenaturing polyacrylamide gel electrophoresis

Two picomoles of each tRNA construct in 15 μ L H₂O were denatured for 3 min at 60°C and refolded by addition of 4 μ L 5X Pus1 buffer (0.1 M Tris-HCl at pH 8.0, 10 mM MgCl₂, 0.1 M ammonium acetate, 0.1 mM EDTA, and 0.5 mM DTT) by slow cooling to room temperature over 10 min. Two or 4 pmol of Pus1p in 1 μ L were added and incubated at 30°C for 60 min; for negative controls, only Pus1 storage buffer was added. Aliquots for nondenaturing gel electrophoresis were withdrawn and mixed with an equal volume of loading buffer containing 1X TBE and 60% glycerol. Aliquots for denaturing PAGE were withdrawn and mixed with an equal volume of loading buffer containing 1X TBE and 90% formamide. The samples were loaded onto 8% polyacrylamide gels without and with urea, respectively. Nondenaturing gels were run at 4°C, denaturing gels at room temperature until a bromophenolblue-containing marker lane reached the lower 10% of the gel. Gels were stopped and immediately scanned on a Typhoon 9400 fluorescence scanner (GE Healthcare) for Cy3 and Cy5 fluorescence.

Synthesis of tRNA derivatives

Dye-labeled derivatives of human mitochondrial tRNA^{Leu}^{CUN} were synthesized by a construction-kit approach based on splint ligation (Kurschat et al. 2005; Hengesbach et al. 2008a). Two of the fragments for each construct contained cyanine dyes, introduced site specifically by NHS-conjugation after solid phase synthesis.

For tRNA^{Leu}^{CUN} constructs, RNA oligonucleotides 5'-ACdt^{Cy3}-UUUAAAGGAUA-3', 5'-ACAGCUAUCCAUUG-3', 5'-Gdt^{Cy5}CUUAGGCCCAAAAAUUUGGUGCAACUCCAAAAUAAAGUACCA-3', and DNA template 5'-TGGTACTTTTATTTGGAGTTGCA CCAAAATTTTGGGGCCTAAGACCAATGGATAGCTGTTATCCTTTAAAAGTTATAGTGAGTCGTATTAAGCTTCGCGCG-3' were used. For the tRNA^{Lys} construct, oligos AUUAACCUUUUA, CA Cdt^{Cy3}GUAAGCUAACUUAGC and GUDt^{Cy5}AAAGAUUAGGAGAACCAACACCUCCUACAGUGACCA on the DNA template 5'-GGTGGGTCTGCTTGGTCACTGTAAAGAGGTGTTGGTTCTCTTAATCTTTAACTTAAAAGGTTAATGCTAAGTTAGCTTTACAGTGATTTTGGGTACC-3' were used. All these RNAs were obtained from IBA. At sites denoted dt, 5'-dimethoxytrityl-5-[N-(trifluoroacetylaminohexyl)-3-acrylimido]-2'-deoxyuridine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite was coupled during the synthesis instead of uridine phosphoramidites, replacing uridine in the sequence with a 2'-deoxythymidine, which carries a linear spacer of 11 atoms ending in a primary amino moiety, to which NHS derivatives of Cy3 and Cy5 dyes were conjugated post-synthetically. The respective conjugation sites are indicated as dt^{Cy3} and dt^{Cy5}, respectively. RNA pACAGCUAUCCAf⁵UG (f⁵U stands for 5-fluorouracil; p stands for 5'-phosphate) for tRNA^{Leu}^{CUN} and RNA pAf⁵UUAACCUUUUA for tRNA^{Lys} were obtained from Dharmacon.

Oligonucleotides synthesized without a 5'-monophosphate were phosphorylated by incubation for 60 min at 37°C with T4 polynucleotide kinase (0.75 u/ μ L T4-PNK, Fermentas) in buffer KL (50 mM Tris-HCl at pH 7.4, and 10 mM MgCl₂), supplemented with 5 mM DTT and 2 mM ATP. The phosphorylated RNA was used immediately in enzymatic ligation or stored at -20°C until use. Splint ligation was performed as follows: stoichiometric amounts of

the various respective RNA fragments (5–10 μ M) were hybridized to stoichiometric amounts of DNA template by heating to 75°C and slow cooling to room temperature over 10 min in buffer KL supplemented with 5 mM DTT and 2 mM ATP. T4 DNA ligase was added (2 u/ μ L) and ligation was carried out overnight at 16°C. Template DNA was removed by addition of 0.02–0.1 u/ μ L DNase I followed by 15 min incubation at 37°C. RNAs were purified by denaturing PAGE, identified by imaging Cy5 fluorescence with a Typhoon scanner (GE Healthcare), eluted from the gel, and precipitated with ethanol. Concentrations were calculated from absorption at 254 nm, as determined on a Nanodrop ND-1000 spectrometer.

Modification of tRNA with Pus1p

Before addition of protein, tRNA samples were denatured and refolded in Pus1 buffer as described above. Derivatives of tRNA carrying Cy3 and Cy5 (0.5 μ M) and U27 or f⁵U27, respectively, were incubated with 0.5 equivalents of *S. cerevisiae* Pus1p, as determined by UV absorbance and Bradford assay, respectively, for 30 min at 30°C in Pus1 buffer.

DNAzyme-based position specific modification assay

Site-specific modification analysis was achieved by sequence-directed cleavage with a 10-23 type DNAzyme I designed (sequence for tRNA^{Leu}^{CUN}: 5'-AAAATTTTGGGGCCTAAGACC AAGGCTAGCTACAACGAGGATAGCTGTTATCCTTTAA-3', sequence for tRNA^{Lys}: GGTTCTCTTAATCTTTAACTTAAAAGGTT AAGGCTAGCTACAACGAGCTAAGTTAGCTTTACAGTG from IBA) to cut between nucleotides 26 and 27, followed by 5'-labeling, nuclease P1 digestion, TLC, and autoradiography on phosphor-imager plates. Protein was removed from the modification reaction mixture by extraction with two times two volumes of water-saturated phenol and two times five volumes of water-saturated diethylether to remove contaminant phenol from the solution. After evaporation of the diethylether under ambient conditions, the RNA was precipitated with ethanol. Modified or control tRNA with ten equivalents of DNAzyme was heated to 80°C for 2 min in DNAzyme buffer (100 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 10 mM MgCl₂), cooled to 37°C at -0.3°C/min, and incubated at 37°C for 5 min in a PCR machine. This cycle was repeated 10 times, which produced complete and specific cleavage of the target tRNA between nucleotides 26 and 27. The free 5' OH on nucleotide 27 generated by DNAzyme cleavage was phosphorylated using T4 polynucleotide kinase. An equal volume of T4-PNK forward reaction buffer (Fermentas) supplemented with 0.1 mM ATP, 1 μ L [γ -³²P]-ATP (10 μ Ci/ μ L), and 0.5 u/ μ L T4-PNK was added to the cleavage reaction and incubated for 60 min at 37°C. The DNAzyme was degraded with 0.025 u/ μ L of DNase I during 15 min of incubation at 37°C to facilitate purification of the 3'-terminal tRNA fragments on a 20% denaturing polyacrylamide gel. Fragments were identified by autoradiography and fluorescence imaging, excised, eluted, and precipitated as described above, resuspended in 20 mM ammonium acetate at pH 5.3 containing 1.0 μ g/ μ L of *E. coli* total tRNA as carrier, and digested to 5' mononucleotides with nuclease P1 overnight at room temperature. Thin layer chromatography was performed on 10 or 20 cm Merck cellulose TLC plates with buffer A (70% isobutyric acid, 28.9% H₂O, 1.1% ammonia [25%] v/v/v), buffer B (70% isopropanol,

15% conc. HCl, 15% H₂O, v/v/v), and buffer C (60% w/v ammonium sulfate, 2% v/v *n*-propanol, 100 mM phosphate buffer at pH 7.4) (Keith 1995; Grosjean et al. 2007). Radiolabeled nucleotides were visualized by autoradiography on phosphorimager screens and quantified by peak volume integration with the software Imagequant 5.2 (Molecular Dynamics, GE). Retention characteristics of 5-fluorouracil mononucleotides on two-dimensional TLC were identified by chromatography of 10 µg digested *E. coli* total tRNA, mixed with 5'-³²P 5-fluorouracil mononucleotides on 10 × 10 cm² cellulose TLC plates F₂₅₄ (Merck), which contain a fluorescent dye. Radioactively labeled 5-fluorouracil mononucleotides were obtained from untreated f⁵U27 tRNA^{Leu(CUN)} as described above. Radioactivity was visualized by autoradiography, while nonradioactive nucleotides, present in much greater amounts, were identified as dark spots during UV irradiation, as they quench the fluorescence of the F₂₅₄ plates. The two images were overlaid according to two radioactive and colored markers.

Fragment isolation by tandem DNAzyme cleavage

In order to further clarify the physicochemical properties of the modified base, a 24-nucleotide fragment was excised from Pus1p-treated or untreated full-length tRNA with and without f⁵U, respectively, and subjected to MALDI-TOF mass spectrometry. Modification was carried out as described above, and protein was removed by extraction with 1 vol water-saturated phenol (2X) and 2–3 vol water-saturated diethylether (3X). The tandem DNAzyme II (sequence: 5'-TTGGAGTTGCACAAAAGGCTAGCTACAACGATTTTGGGGCCTAAGACCAATGGAGGCTAGCTACAACGAAGCTGTTATCCTTTAAAAGT-3'; IBA) was designed with cleavage sites targeted between A22 and U23, and between A47 and U48. Cleavage was carried out as described above for the post-labeling assay. The DNAzyme was removed by denaturing PA gel electrophoresis. The fragment carrying the Cy5 dye was identified by Typhoon fluorescence imaging, excised from the gel, and eluted in 0.5 M ammonium acetate overnight. The RNA was ethanol precipitated, and purified by Millex RP-18 ZipTips (Millipore) with elution in acetonitrile. The fragment was subjected to MALDI-TOF mass spectrometry on a Bruker Daltonics BiFlex III (Bruker Daltonic), using an internal oligonucleotide standard (Bruker, No. 206200) with masses of 3645.4, 6117.0, and 9191.0 Da as calibration source. Expected masses were calculated using ChemDraw, including both a 5'-hydroxyl group and a 2'–3' cyclic phosphate from DNAzyme cleavage.

Fluorescence spectroscopy

In order to determine the orientational freedom of the tRNA, fluorescence anisotropy, in addition to FRET, was measured using a TECAN safire² plate reader (Tecan). For these measurements, CoStar 96-well plates (Corning) with darkened walls were used. tRNAs were refolded in 1X Pus buffer by denaturing at 60°C for 3 min and slow refolding to room temperature over >10 min. Solutions of 21 nM tRNA (2 pmol) were prepared in 95 µL, and FRET, as well as anisotropy, were measured. Five microliters of glycerol-free Pus1p solution of different concentrations (or glycerol-free storage buffer only as negative control) were added to achieve the different molar excesses described. FRET and anisotropy were measured either alternating or in an automated kinetics mode. The temperature during all measurements was 28 ± 1°C.

The measurement settings for anisotropy were: excitation wavelength 635 nm, emission wavelength 680 nm, emission bandwidth 10 nm, 50 reads per well, and 50 ms between move and flash. As only relative changes are being discussed, a G factor accounting for different orientational sensitivity differences was not determined experimentally and thus not introduced into the calculation. Anisotropy was calculated using the Tecan X-fluor software.

Measurement settings for FRET: Excitation wavelength: 530 nm with 10-nm bandwidth, emission wavelength scan from 555 to 720 nm (10 nm bandwidth) with 1-nm steps and 2-ms integration per data point; averaged over 2 reads per well. FRET efficiency was calculated from the average fluorescence intensity from five data points around the intensity maximum (Cy3: 566–570 nM, Cy5: 668–672 nM). FRET was calculated using donor and acceptor emission intensities upon donor excitation, where $E_{\text{FRET}} = I_A / (I_D + I_A)$.

For out-competing experiments, the solution containing 20 nM substrate tRNA and different concentrations of Pus1p after 90 min was adjusted to contain 8 µM total tRNA from *E. coli* (Roche) in 1X Pus buffer. FRET kinetics were measured continuously over 90 min.

ACKNOWLEDGMENTS

We thank Heiko Rudy for mass spectrometry measurements and Roman Teimer for unpublished data. We thank Henri Grosjean, Eric Phizicky, Jane E. Jackman, and Jeff Patton for providing plasmids, and Andres Jäschke for constant support. M. Hengesbach was funded by the Landesgraduiertenförderung Baden-Württemberg. M. Helm acknowledges funding by the DFG (HE 3397/3).

Received July 17, 2009; accepted December 3, 2009.

REFERENCES

- Arлуison V, Hountondji C, Robert B, Grosjean H. 1998. Transfer RNA-pseudouridine synthetase Pus1 of *Saccharomyces cerevisiae* contains one atom of zinc essential for its native conformation and tRNA recognition. *Biochemistry* **37**: 7268–7276.
- Arлуison V, Buckle M, Grosjean H. 1999. Pseudouridine synthetase Pus1 of *Saccharomyces cerevisiae*: Kinetic characterisation, tRNA structural requirement and real-time analysis of its complex with tRNA. *J Mol Biol* **289**: 491–502.
- Behm-Ansmant I, Massenet S, Immel F, Patton JR, Motorin Y, Branlant C. 2006. A previously unidentified activity of yeast and mouse RNA:pseudouridine synthases 1 (Pus1p) on tRNAs. *RNA* **12**: 1583–1593.
- Chen J, Patton JR. 1999. Cloning and characterization of a mammalian pseudouridine synthase. *RNA* **5**: 409–419.
- Chen J, Patton JR. 2000. Mouse pseudouridine synthase 1: Gene structure and alternative splicing of pre-mRNA. *Biochem J* **352**: 465–473.
- Coban O, Lamb DC, Zaychikov E, Heumann H, Nienhaus GU. 2006. Conformational heterogeneity in RNA polymerase observed by single-pair FRET microscopy. *Biophys J* **90**: 4605–4617.
- Constantinesco F, Motorin Y, Grosjean H. 1999. Characterization and enzymatic properties of tRNA(guanine 26, N², N²)-dimethyltransferase (Trm1p) from *Pyrococcus furiosus*. *J Mol Biol* **291**: 375–392.
- Duan J, Li L, Lu J, Wang W, Ye K. 2009. Structural mechanism of substrate RNA recruitment in H/ACA RNA-guided pseudouridine synthase. *Mol Cell* **34**: 427–439.

- Foster PG, Huang L, Santi DV, Stroud RM. 2000. The structural basis for tRNA recognition and pseudouridine formation by pseudouridine synthase I. *Nat Struct Biol* **7**: 23–27.
- Grosjean H, Droogmans L, Roovers M, Keith G. 2007. Detection of enzymatic activity of transfer RNA modification enzymes using radiolabeled tRNA substrates. *Methods Enzymol* **425**: 55–101.
- Gurha P, Gupta R. 2008. Archaeal Pus10 proteins can produce both pseudouridine 54 and 55 in tRNA. *RNA* **14**: 2521–2527.
- Gustavsson M, Ronne H. 2008. Evidence that tRNA modifying enzymes are important in vivo targets for 5-fluorouracil in yeast. *RNA* **14**: 666–674.
- Hamilton CS, Spedaliere CJ, Ginter JM, Johnston MV, Mueller EG. 2005. The roles of the essential Asp-48 and highly conserved His-43 elucidated by the pH dependence of the pseudouridine synthase TruB. *Arch Biochem Biophys* **433**: 322–334.
- Hamilton CS, Greco TM, Vizthum CA, Ginter JM, Johnston MV, Mueller EG. 2006. Mechanistic investigations of the pseudouridine synthase RluA using RNA containing 5-fluorouridine. *Biochemistry* **45**: 12029–12038.
- Hellmuth K, Grosjean H, Motorin Y, Deinert K, Hurt E, Simos G. 2000. Cloning and characterization of the *Schizosaccharomyces pombe* tRNA:pseudouridine synthase Pus1p. *Nucleic Acids Res* **28**: 4604–4610.
- Helm M. 2006. Post-transcriptional nucleotide modification and alternative folding of RNA. *Nucleic Acids Res* **34**: 721–733.
- Helm M, Attardi G. 2004. Nuclear control of cloverleaf structure of human mitochondrial tRNA(Lys). *J Mol Biol* **337**: 545–560.
- Helm M, Brule H, Degoul F, Cepanec C, Leroux JP, Giege R, Florentz C. 1998. The presence of modified nucleotides is required for cloverleaf folding of a human mitochondrial tRNA. *Nucleic Acids Res* **26**: 1636–1643.
- Hengesbach M, Kobitski A, Voigts-Hoffmann F, Frauer C, Nienhaus GU, Helm M. 2008a. RNA intramolecular dynamics by single-molecule FRET. In *Current protocols in nucleic acid chemistry*. pp. 11.12.1–11.12.22. Wiley, New York.
- Hengesbach M, Meusburger M, Lyko F, Helm M. 2008b. Use of DNazymes for site-specific analysis of ribonucleotide modifications. *RNA* **14**: 180–187.
- Hoang C, Ferré-D'Amaré AR. 2001. Cocrystal structure of a tRNA Ψ 55 pseudouridine synthase: Nucleotide flipping by an RNA-modifying enzyme. *Cell* **107**: 929–939.
- Hoang C, Chen J, Vizthum CA, Kandel JM, Hamilton CS, Mueller EG, Ferré-D'Amaré AR. 2006. Crystal structure of pseudouridine synthase RluA: Indirect sequence readout through protein-induced RNA structure. *Mol Cell* **24**: 535–545.
- Hoskins J, Butler JS. 2008. RNA-based 5-fluorouracil toxicity requires the pseudouridylation activity of Cbf5p. *Genetics* **179**: 323–330.
- Huang L, Pookanjanatavip M, Gu X, Santi DV. 1998. A conserved aspartate of tRNA pseudouridine synthase is essential for activity and a probable nucleophilic catalyst. *Biochemistry* **37**: 344–351.
- Hur S, Stroud RM, Finer-Moore J. 2006. Substrate recognition by RNA 5-methyluridine methyltransferases and pseudouridine synthases: A structural perspective. *J Biol Chem* **281**: 38969–38973.
- Ishitani R, Nureki O, Nameki N, Okada N, Nishimura S, Yokoyama S. 2003. Alternative tertiary structure of tRNA for recognition by a post-transcriptional modification enzyme. *Cell* **113**: 383–394.
- Jackman JE, Montange RK, Malik HS, Phizicky EM. 2003. Identification of the yeast gene encoding the tRNA m1G methyltransferase responsible for modification at position 9. *RNA* **9**: 574–585.
- Kealey JT, Gu X, Santi DV. 1994. Enzymatic mechanism of tRNA (m⁵U54)methyltransferase. *Biochimie* **76**: 1133–1142.
- Keith G. 1995. Mobilities of modified ribonucleotides on two-dimensional cellulose thin-layer chromatography. *Biochimie* **77**: 142–144.
- Kurschat WC, Muller J, Wombacher R, Helm M. 2005. Optimizing splinted ligation of highly structured small RNAs. *RNA* **11**: 1909–1914.
- Liang B, Zhou J, Kahen E, Terns RM, Terns MP, Li H. 2009. Structure of a functional ribonucleoprotein pseudouridine synthase bound to a substrate RNA. *Nat Struct Mol Biol* **16**: 740–746.
- Lilley DM, Wilson TJ. 2000. Fluorescence resonance energy transfer as a structural tool for nucleic acids. *Curr Opin Chem Biol* **4**: 507–517.
- Massenet S, Motorin Y, Lafontaine DL, Hurt EC, Grosjean H, Branlant C. 1999. Pseudouridine mapping in the *Saccharomyces cerevisiae* spliceosomal U small nuclear RNAs (snRNAs) reveals that pseudouridine synthase pus1p exhibits a dual substrate specificity for U2 snRNA and tRNA. *Mol Cell Biol* **19**: 2142–2154.
- Motorin Y, Keith G, Simon C, Foiret D, Simos G, Hurt E, Grosjean H. 1998. The yeast tRNA:pseudouridine synthase Pus1p displays a multisite substrate specificity. *RNA* **4**: 856–869.
- Pan H, Agarwalla S, Moustakas DT, Finer-Moore J, Stroud RM. 2003. Structure of tRNA pseudouridine synthase TruB and its RNA complex: RNA recognition through a combination of rigid docking and induced fit. *Proc Natl Acad Sci* **100**: 12648–12653.
- Patton JR, Padgett RW. 2005. Pseudouridine modification in *Caenorhabditis elegans* spliceosomal snRNAs: Unique modifications are found in regions involved in snRNA–snRNA interactions. *BMC Mol Biol* **6**. doi: 10.1186/1471-2199-6-20.
- Patton JR, Bykhovskaya Y, Mengesha E, Bertolotto C, Fischel-Ghodsian N. 2005. Mitochondrial myopathy and sideroblastic anemia (MLASA): Missense mutation in the pseudouridine synthase 1 (PUS1) gene is associated with the loss of tRNA pseudouridylation. *J Biol Chem* **280**: 19823–19828.
- Phannachet K, Huang RH. 2004. Conformational change of pseudouridine 55 synthase upon its association with RNA substrate. *Nucleic Acids Res* **32**: 1422–1429.
- Sibert BS, Fischel-Ghodsian N, Patton JR. 2008. Partial activity is seen with many substitutions of highly conserved active site residues in human Pseudouridine synthase 1. *RNA* **14**: 1895–1906.
- Spedaliere CJ, Mueller EG. 2004. Not all pseudouridine synthases are potentially inhibited by RNA containing 5-fluorouridine. *RNA* **10**: 192–199.
- Spedaliere CJ, Ginter JM, Johnston MV, Mueller EG. 2004. The pseudouridine synthases: Revisiting a mechanism that seemed settled. *J Am Chem Soc* **126**: 12758–12759.
- Voigts-Hoffmann F, Hengesbach M, Kobitski AY, van Aerschot A, Herdewijn P, Nienhaus GU, Helm M. 2007. A methyl group controls conformational equilibrium in human mitochondrial tRNA^{Lys}. *J Am Chem Soc* **129**: 13382–13383.
- Zhao X, Yu YT. 2004. Detection and quantitation of RNA base modifications. *RNA* **10**: 996–1002.
- Zhao X, Yu YT. 2007. Incorporation of 5-fluorouracil into U2 snRNA blocks pseudouridylation and pre-mRNA splicing in vivo. *Nucleic Acids Res* **35**: 550–558.
- Zhao X, Patton JR, Ghosh SK, Fischel-Ghodsian N, Shen L, Spanjaard RA. 2007. Pus3p- and Pus1p-dependent pseudouridylation of steroid receptor RNA activator controls a functional switch that regulates nuclear receptor signaling. *Mol Endocrinol* **21**: 686–699.

RNA

A PUBLICATION OF THE RNA SOCIETY

Formation of a stalled early intermediate of pseudouridine synthesis monitored by real-time FRET

Martin Hengesbach, Felix Voigts-Hoffmann, Benjamin Hofmann, et al.

RNA 2010 16: 610-620 originally published online January 27, 2010

Access the most recent version at doi:[10.1261/ma.1832510](https://doi.org/10.1261/ma.1832510)

References

This article cites 44 articles, 16 of which can be accessed free at:
<http://rnajournal.cshlp.org/content/16/3/610.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

To subscribe to *RNA* go to:
<http://rnajournal.cshlp.org/subscriptions>