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Structural and functional roles of the N1- and N3-protons of Ψ at tRNA's position 39

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ABSTRACT

Pseudouridine at position 39 (Ψ_{39}) of tRNA's anticodon stem and loop domain (ASL) is highly conserved. To determine the physicochemical contributions of Ψ_{39} to the ASL and to relate these properties to tRNA function in translation, we synthesized the unmodified yeast tRNA^{Phe} ASL and ASLs with various derivatives of U_{39} and Ψ_{39}. Ψ_{39} increased the thermal stability of the ASL (ΔT_m = 1.3 ± 0.5°C), but did not significantly affect ribosomal binding (K_d = 229 ± 29 nM) compared to that of the unmodified ASL (K_d = 197 ± 58 nM). The ASL-Ψ_{39} P-site fingerprint on the 30S ribosomal subunit was similar to that of the unmodified ASL. The stability, ribosome binding and fingerprint of the ASL with $m^1\Psi_{39}$ were comparable to that of the ASL with Ψ_{39}. Thus, the contribution of Ψ_{39} to ASL stability is not related to N1-H hydrogen bonding, but probably is due to the nucleoside's ability to improve base stacking compared to U. In contrast, substitutions of $m^3\Psi_{39}$, the isosteric m^3U_{39} and $m^1m^3\Psi_{39}$ destabilized the ASL by disrupting the A_{31}–U_{39} base pair in the stem, as confirmed by NMR. N3-methylations of both U and Ψ dramatically decreased ribosomal binding (K_d = 1060 ± 189 to 1283 ± 258 nM). Thus, canonical base pairing of Ψ_{39} to A_{31} through N3-H is important to structure, stability and ribosome binding, whereas the increased stability and the N1-proton afforded by modification of U_{39} to Ψ_{39} may have biological roles other than tRNA's binding to the ribosomal P-site.

INTRODUCTION

Transfer RNA plays a critical role in gene expression. In addition to tRNA's well-documented and essential biological function in translation, the number and variety of structural features and modified nucleosides within this relatively small RNA are also quite remarkable. Non-canonical base pairs, base triples and other structural motifs, such as the 'U-turn', were first described in yeast tRNA^{Phe} (1). Whereas some of the nucleoside modifications of tRNA are specific to particular tRNA species, such as wybutosine in tRNA^{Phe}, others are ubiquitous and invariant, such as pseudouridine, Ψ, at position 55 of tRNA's TΨC stem and loop domain.

The modification of U to Ψ occurs frequently and site-specifically in most RNAs (2–4), including tRNA (5). Ψ commonly occurs at positions 38, 39 and 40 in tRNA's anticodon stem and loop domain, ASL (Fig. 1), the most extensively modified domain in tRNA (6). A gene bank analysis (5) revealed that dT is found at position 39 at a disproportionate 39% in procaryotic and 44% in eucaryotic tRNA genes. Even more striking is that 95% of the uridines at position 39 in the transcripts of these tRNA genes are converted to pseudouridine (5). However, the physicochemical properties of Ψ at position 39 that contribute to tRNA structure and function are still not understood.

The modified nucleosides of the ASL of eucaryotic tRNA can either be subtly (7–9) or dramatically (8,10) involved in control of translation at the ribosome. Contributions to translation, in general, and ribosome binding, in particular, by modified nucleosides within the ASL, but outside of the anticodon such as pseudouridine at position 39, would support the 'extended anticodon' hypothesis (11). In yeast, the gene *DEG1* codes for the pseudouridine synthase that converts uridine to pseudouridine at positions 38 and 39 in tRNA (12). Yeast cells lacking this pseudouridine synthase activity display slow growth (12). Although this relationship has not been well characterized in eucaryotes, a similar, better understood relationship is seen in procaryotes. Pseudouridine in tRNA's ASL domain is involved in the translational control of gene expression via attenuation in bacteria. The *truA* genes in *Escherichia coli* and *Salmonella typhimurium* code for the modification enzyme, tRNA pseudouridine synthase I, which converts uridine residues to pseudouridine at positions 38, 39 and 40 in the ASL (13). A mutation in the *truA* gene results in tRNA positions 38, 39 and 40 being hypomodified (14); these mutants have disrupted transcription attenuation mechanisms resulting in reduced growth rate and reduced polypeptide chain elongation rates (15–17). Although eucaryotic cells do not regulate translation by attenuation, the absence of Ψ_{39} may affect the rate of mRNA decoding on the ribosome for all organisms (12).

Pseudouridine-39 may modulate gene expression by affecting tRNA's interaction with the ribosome. tRNA's interaction with

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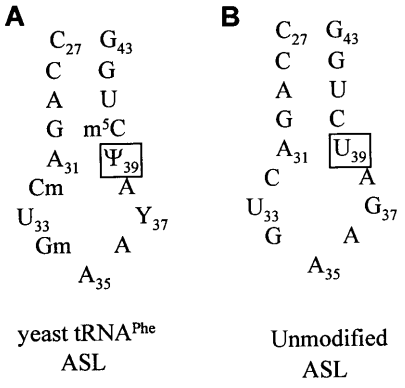

Figure 1. Primary sequence and secondary structure of ASLs. (**A**) Yeast tRNA^{Phe} ASL with six naturally occurring modifications. Ψ_{39} is boxed. (**B**) Unmodified ASL used in our study. Boxed U_{39} shows the position of all substitutions, Ψ_{39}, $m^1\Psi_{39}$, $m^3\Psi_{39}$, $m^1m^3\Psi_{39}$, m^3U_{39} and m^5U_{39}.

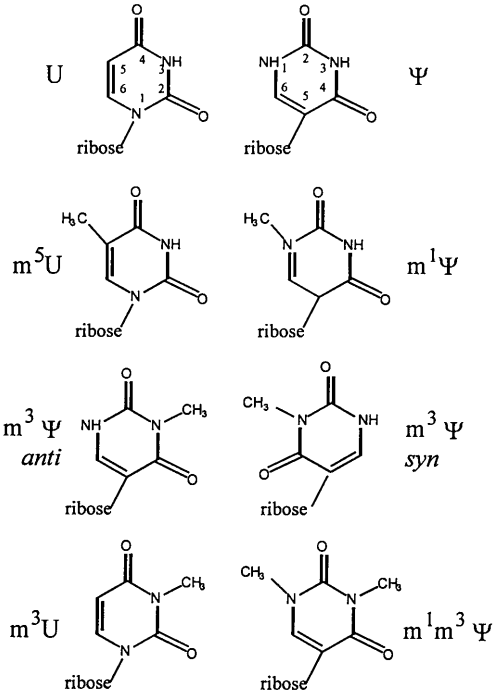

Figure 2. Structures of nucleoside substitutions. Structures of uridine and pseudouridine derivatives substituted in the ASL at position 39. Both the *syn* and *anti* conformations of $m^3\Psi_{39}$ are shown.

the ribosome could be affected by pseudouridine's ability to stabilize RNA conformation (18). The pyrimidine ring of Ψ_{39} forms a canonical Watson–Crick base pair by hydrogen bonding to A_{31}. A_{31}–Ψ_{39} interactions include the Ψ C2 carbonyl and N3 imino proton (Fig. 2); an interaction presumed important to tRNA's ASL structure and function, though not thoroughly explored. However, Ψ also has the potential to hydrogen bond by donation of the N1 imino proton. The additional hydrogen bonding could be responsible for the added stability of RNA not afforded by uridine (19). Alternatively, the stability that Ψ_{39} affords to tRNA may come from its ability to stack more effectively than uridine (20,21). The increased stability of the Ψ_{39}-modified tRNA may contribute directly or indirectly to the tRNA's interaction with the ribosome during translation. To better understand the contributions of the Ψ_{39} N1 and N3 position protons to the structure and function of tRNA, we incorporated Ψ and methylated derivatives of U and Ψ at position 39 of the heptadecamer ASL of yeast tRNA^{Phe}. Both structure and function of the modified ASLs were investigated and compared to that of the unmodified ASL.

MATERIALS AND METHODS

Synthesis and purification of modified and unmodified ASLs

Unmodified and modified heptadecamer RNAs were synthesized with the base sequence corresponding to the unmodified yeast tRNA^{Phe} ASL (Fig. 1). Synthesis was accomplished with standard RNA phosphoramidite chemistry (22) on an Applied Biosystems Model 394 automated synthesizer. The modified nucleoside phosphoramidites, pseudouridine (Ψ), N1-methylpseudouridine ($m^1\Psi$), N3-methylpseudouridine ($m^3\Psi$), and N1,N3-dimethylpseudouridine ($m^1m^3\Psi$) (Fig. 2), were prepared as previously described (23). Standard ribonucleoside phosphoramidites (A, U, C, G) and the phosphoramidites of 5-methyluridine, m^5U, and N3-methyluridine, m^3U (Fig. 2) were purchased (Glen Research, Sterling, VA and ChemGenes, Waltham, MA, respectively). ASLs were purified by ion exchange HPLC chromatography (23). Successful incorporation of the modified nucleosides was verified by quantitative nucleoside composition analysis (24).

tRNA and 30S ribosomal subunits

Yeast tRNA^{Phe} was purchased from Sigma Chemical Co. (St Louis, MO). Ribosomal 30S subunits were prepared from *E.coli* and activated according to Ericson *et al*. (25).

Determination of ASL thermal stability

ASL samples were dissolved in a buffer (10 mM sodium phosphate, pH 7.2, 100 mM NaCl, 0.1 mM EDTA). Thermal denaturations, performed in triplicate, were monitored by UV absorbance (260 nm) using a Cary 3 spectrophotometer with 10 and 2 mm path-length cells. Data points were averaged over 20 s and collected three times per minute. Denaturations and renaturations were conducted over a temperature range of 5–90°C with a ramp rate of 1°C/min. Data from denaturations and renaturations were treated similarly. No hysteresis was observed. The denaturations were concentration-independent indicating unimolecular transitions within the concentration range of 2.0–50.0 μM. The ASL-m^5U_{39} exhibited a low temperature transition as well as the high temperature transition (Fig. 3). However, the first derivative of the denaturation profile indicated that in fact a two-state transition was occurring at the defined higher melting temperature and was distinct from that at the lower temperature. Thermodynamic parameters were calculated with a Van't Hoff analysis of the data as described by Marky and Breslauer (26) using Origin software. Because monophasic denaturations

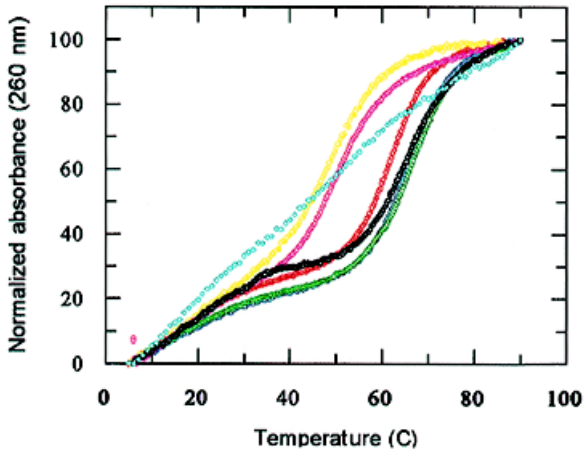

Figure 3. Thermal denaturations of ASLs. ASLs unmodified (red), Ψ_{39} (blue), $m^1\Psi_{39}$ (green), $m^3\Psi_{39}$ (yellow), $m^1m^3\Psi_{39}$ (pink), m^3U_{39} (cyan) and m^5U_{39} (black) were denatured from 5 to 90°C and renatured at a rate of 1°C/min. The UV absorbance at 260 nm was monitored. Curves are representative of multiple reiterations for the seven ASLs. The melting curve for ASL-$m^1\Psi_{39}$ (green) is coincident with that of ASL-Ψ_{39} (blue).

were observed, including the higher thermal transition for ASL-m^5U_{39}, a two-state transition was assumed.

Filter binding assay

Yeast $tRNA^{Phe}$ and ASLs were bound to 30S ribosomal subunits as previously described (8,10,27). Briefly, 10 pmol of 30S ribosomal subunits and 10 µg of poly(U) were incubated at 37°C for 20 min with increasing amounts of ^{32}P-labeled tRNA or ASL (0–50 pmol) in 40 µl of CMN buffer (80 mM potassium cacodylate, pH 7.2, 20 mM $MgCl_2$, 100 mM NH_4Cl, 3 mM β-mercaptoethanol). The reaction was then incubated on ice for 20 min, passed through nitrocellulose filters (0.45 µm) and washed twice with ice-cold CMN buffer (100 µl). The filters were air-dried and counted in a scintillation counter. K_d and standard deviation were determined using non-linear regression analysis.

Chemical probing and primer extension

Chemical probing of 16S rRNA P-site nucleosides with kethoxal and dimethyl sulfate followed by primer extension was accomplished as described by Moazed and Noller (28) except that chemical modifications were conducted at 20°C for 30 min in 40 µl of CMN buffer (8,27). Small ribosomal subunits were activated by incubating at 37°C for 30 min and then programmed with poly(U) and incubated with 50 pmol of $tRNA^{Phe}$ or ASLs.

NMR analysis

For analysis by NMR, the samples were dialyzed into phosphate buffer (10 mM phosphate, pH 6, 0.1 mM deuterated EDTA, no added NaCl). One-dimensional spectra and NOESY (τ_m = 150 ms) spectra were collected at 4°C with the samples in ^1H_2O and employed WATERGATE solvent signal suppression. Samples were exchanged into D_2O by evaporating the solution under dry N_2 in the NMR tube and re-dissolving the RNA in 99.96% D_2O and repeating the procedure. The imino proton signals were assigned from the NOESY spectra and by comparison to the spectra of closely related, assigned molecules.

RESULTS

Design of ASLs

The native yeast $tRNA^{Phe}$ ASL has five naturally occurring modifications: 2'-O-methyl C and G, Cm_{32}, Gm_{34}; wybutosine, Y_{37}; Ψ_{39}; and 5-methylcytidine, m^5C_{40}. In order to probe the structure–function relationships of the N1 and N3 protons of Ψ_{39}, the unmodified ASL and six ASLs with position 39 substitutions (Fig. 1B) were synthesized by automated chemical synthesis. The ASL sequences were based on that of the unmodified yeast $tRNA^{Phe}$ (Fig. 1A) and included introduction of the naturally occurring Ψ_{39}, the methylated pseudouridines, $m^1\Psi_{39}$, $m^3\Psi_{39}$ and $m^1m^3\Psi_{39}$, and the methylated uridines, m^3U_{39} and m^5U_{39} (Fig. 2). ASLs with $m^1\Psi_{39}$, $m^3\Psi_{39}$ and $m^1m^3\Psi_{39}$ were synthesized to probe the contribution of the N1 and N3 imino protons. The N3 position is expected to donate a proton to the canonical base pair with A_{31}. Isosteric substitutions of the methylated pseudouridines were incorporated as controls to the addition of methyl groups. The modification m^3U_{39} is isosteric with $m^3\Psi_{39}$, and m^5U_{39} is isosteric with $m^1\Psi_{39}$. By incorporating the N1 and N3 methylated Ψs at position 39, the importance of the N1 and N3 protons to the ASL's thermal stability, structure and ribosome binding were assessed.

Thermodynamic contributions of substitutions

Thermal stabilities of Ψ and methylated Ψ substituted ASLs were determined from UV-monitored thermal denaturations and compared to those of the unmodified and isosterically modified ASLs (Fig. 3). ASL-Ψ_{39} exhibited a melting temperature, T_m, of 65.1°C which was 1.3°C higher than that of the unmodified ASL (T_m = 63.8°C) (Table 1). With the substitution of Ψ for U, all three thermodynamic parameters decreased. ΔG_{37} decreased by 0.3 kcal/mol, ΔH by 1 kcal/mol and ΔS by 5 cal/mol. The N1 proton of Ψ is not involved in hydrogen bonding with A_{31} across the stem of the ASL. However, the N1 of Ψ_{39} may provide an additional locus for hydrogen bonding which is not available with the unmodified uridine (19). Therefore, blocking the hydrogen bonding ability of the N1 position of Ψ by methylation could provide insight into this position's contribution to ASL stability. The thermal stability of the ASL-$m^1\Psi_{39}$ was compared with that of ASL-Ψ_{39}. ASL-$m^1\Psi_{39}$ exhibited a 2.3°C increase in T_m (67.4°C) as compared to that of the ASL-Ψ_{39} (65.1°C) and a decrease in thermodynamic parameters (Table 1). As a control for the effect of methylating the N1 position of Ψ, the thermal stability of the ASL-m^5U_{39}, which is isosteric to ASL-$m^1\Psi_{39}$, was determined. Incorporation of m^5U_{39} slightly stabilized the ASL as evidenced by an increased T_m (0.3°C) as compared to that of the unmodified ASL. The T_m of the ASL-$m^1\Psi_{39}$ was 3.3°C higher than that of the isosteric ASL-m^5U_{39}, as compared to the 1.3°C difference between ASL-Ψ_{39} and the unmodified ASL (U_{39}). Together, these data suggest that the increased stability of the ASL-Ψ_{39}, compared to the unmodified ASL, was not due to the N1 position of Ψ providing an additional locus for hydrogen bonding. Even after the N1 position was methylated and no longer able to hydrogen

Table 1. Thermodynamic parameters for ASLs

ASL	Contribution	T_m (°C)	ΔH (kcal/mol)	ΔS (cal/mol)	ΔG_{37} (kcal/mol)
Unmodified	Unmodified	63.8 ± 0.5	–54.0 ± 2.0	–161.0 ± 8.0	–4.3 ± 0.1
Ψ_{39}[a]	Natural modification	65.1 ± 0.4	–55.0 ± 2.0	–166.0 ± 7.0	–4.6 ± 0.2
$m^1\Psi_{39}$[a]	Blocks N1 hydrogen bond	67.4 ± 0.2	–57.8 ± 0.5	–170.0 ± 1.0	–5.2 ± 0.1
m^5U_{39}	Isosteric to $m^1\Psi_{39}$	64.1 ± 0.4	–54.0 ± 2.0	–159.0 ± 5.0	–4.3 ± 0.1
$m^3\Psi_{39}$[a]	Blocks N3 hydrogen bond to A_{31}	50.6 ± 0.4	–42.0 ± 2.0	–131.0 ± 7.0	–1.8 ± 0.1
m^3U_{39}	Isosteric to $m^3\Psi_{39}$	N/D[b]	N/D	N/D	N/D
$m^1m^3\Psi_{39}$	Blocks N1 and N3 hydrogen bond	51.6 ± 0.2	–48.2 ± 0.8	–156.0 ± 3.0	–2.2 ± 0.1

Values are averages ± standard deviations derived from multiple iterations of denaturation and renaturation. ΔH and ΔS values were calculated with a van't Hoff analysis. ΔG values were calculated at 37°C.

[a]Thermodynamic values previously presented (36).

[b]N/D, could not be determined. Because the ASL-m^3U_{39} started to denature at 5°C, neither a two-state approximation nor a first derivative could be applied to the data.

bond, the ASL containing Ψ was still more stable than the uridine-containing ASL.

ASL-m^3U_{39} denatured continuously from 5°C (Fig. 3). Thus, the data could not be analyzed for T_m or other thermodynamic parameters. Disruption of the canonical A_{31}–U_{39} base pair may have destabilized the ASL stem to the extent that it denatured readily. Denaturations of ASL-$m^3\Psi_{39}$ and ASL-$m^1m^3\Psi_{39}$ proceeded as with the other ASLs exhibiting a single major thermal transition, albeit at much reduced temperatures (Fig. 3). The N3 proton of Ψ is expected to be involved in a hydrogen bond with A_{31} at the base of the stem in the ASL. The T_m of ASL-$m^3\Psi_{39}$ was 13.2°C below that of the unmodified ASL and 14.5°C below that of ASL-Ψ_{39} with correspondingly significant increases in all three thermodynamic parameters (Table 1). With introduction of the doubly methylated $m^1m^3\Psi_{39}$, ΔG_{37} decreased 0.4 kcal/mol relative to that of ASL-$m^3\Psi_{39}$; T_m increased by 1°C. Thus, introduction of both $m^3\Psi_{39}$ and $m^1m^3\Psi_{39}$ destabilized the ASL consistent with the drastic destabilization caused by m^3U. These results are strongly suggestive of the glycosidic bond of $m^3\Psi_{39}$ not reorienting to a *syn* conformation to allow the N1-H to hydrogen bond with A_{31} (Fig. 2). However, denaturation of ASLs with $m^3\Psi_{39}$ and $m^1m^3\Psi_{39}$ exhibited well-defined, two-state high temperature transitions whereas the denaturation of ASL-m^3U exhibited a continuous increase in optical density from 5°C. Thus, introduction of the N3- or N1,N3-methylated Ψs, though incapable of forming a canonical base pair, stabilized the ASL compared to that of m^3U.

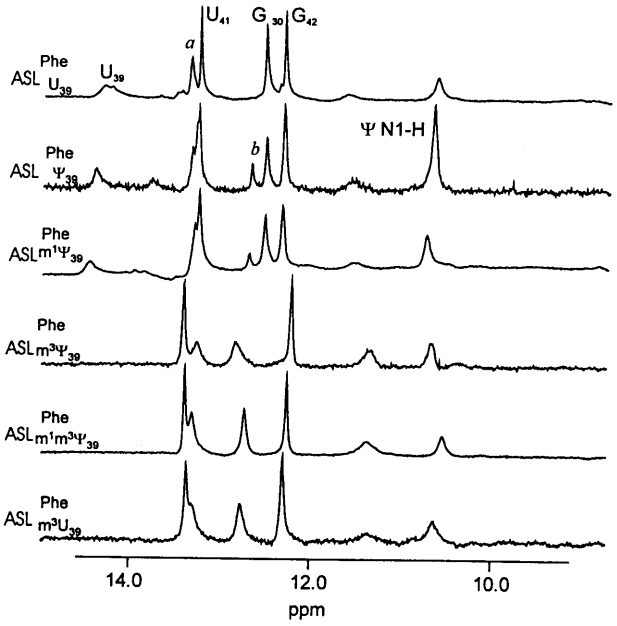

Figure 4. Imino proton NMR spectra of the ASLs. Spectra were collected from ASL samples dissolved in 10 mM phosphate buffer, pH 6, buffer at 4°C. WATERGATE was used for solvent suppression as described in Materials in Methods. *a*, resonance is either the imino of G_{43} or a loop nucleoside. *b*, additional imino proton peak observed in ASL-Ψ_{39} and ASL-$m^1\Psi_{39}$.

NMR analysis

The structures of variously modified ASLs and unmodified ASL were studied at 4°C by NMR spectroscopy in order to determine the extent of base pairing in each ASL stem and whether $m^3\Psi_{39}$ was reorienting to the *syn* conformation. The region of the spectra at which the imino proton signals are observed is presented in Figure 4 for six of the ASLs. Assignments for the non-terminal, base-paired stem imino protons are presented in the spectrum of the unmodified ASL. The sharp peak (labeled *a* in Fig. 4) downfield and adjacent to U_{41} has not been definitively assigned but is probably the imino proton of G_{43}. The signals upfield of the G_{30} resonance are imino resonances from positions not involved in canonical base pairing. All of the ASLs appear to have a hydrogen-bonded stem as judged by the U_{41}, G_{30} and G_{42} imino resonances. The N3 imino protons of Ψ_{39} and $m^1\Psi_{39}$ resonate at nearly the same position as the N3 imino proton of the U_{39} (14.4–14.5 p.p.m.). Both ASL-Ψ_{39} and ASL-$m^1\Psi_{39}$ exhibited an additional imino proton peak (labeled *b* in Fig. 4) ~0.1 p.p.m. downfield of the G_{30} imino resonance. We believe this peak occurs from an additional base-pairing interaction in the loop that is present in the ASL-Ψ_{39} and ASL-$m^1\Psi_{39}$, but not in the unmodified ASL (21). The N1-H of Ψ_{39} is likely coordinating a structured water molecule and the

Table 2. Chemical protection of 16S rRNA P-site nucleotides by yeast $tRNA^{Phe}$ or various ASLs

tRNA	16S nucleoside						
	A1339	G1338	G530	A532	A794	C795	G926
$tRNA^{Phe}$	++	+++	+++	+++	+++	+++	+++
ASL-Unmodified	++	+++	+++	+++	+++	+++	+++
ASL-Ψ_{39}	+	+++	+++	++	+++	+++	+++
ASL-$m^1\Psi_{39}$	++	+++	+++	+++	+++	+++	+++
ASL-$m^3\Psi_{39}$	+	++	++	+++	++	++	++
ASL-$m^1m^3\Psi_{39}$	+	++	++	+++	++	++	++
ASL-m^3U_{39}	+	++	++	+++	++	++	++
ASL-m^5U_{39}	++	+++	+++	+++	+++	+++	+++

Protections are estimates from band intensities from duplicate experiments. +++, highly protected; +, slightly protected (29)

imino proton exchange is slowed enough to be detectable (21). Although there is a peak at the same location in the spectra of the other ASLs, the peak is much more pronounced in the ASL-Ψ_{39} spectrum (Fig. 4). This peak is not apparent in the spectrum of ASL-$m^3\Psi_{39}$ probably because of instability in the base caused by methylation. Hypothetically, the $m^3\Psi$ of ASL-$m^3\Psi_{39}$ could base pair to A_{31} by assuming a *syn* conformation. The N1 imino proton would be donated to form a hydrogen bond (Fig. 2). However, this does not appear to be the case, as judged by the similarity of the ASL-$m^1m^3\Psi_{39}$ and ASL-$m^3\Psi_{39}$ imino proton spectra and the thermal instability of these ASLs (Table 1).

ASLs binding to 30S ribosomal subunits

The binding of unmodified and modified ASLs to 30S ribosomal subunits was determined by a filter-binding assay. Of the seven ASLs tested, the unmodified ASL (K_d = 197 ± 58 nM), ASL-Ψ_{39} (K_d = 229 ± 29 nM) and ASL-$m^1\Psi_{39}$ (K_d = 181 ± 7 nM) all bound to poly(U) programmed 30S ribosomal subunits with similar affinities (Fig. 5). The binding of these three ASLs was comparable to that of $tRNA^{Phe}$ (K_d = 118 ± 48 nM) and to that previously reported for unmodified $tRNA^{Phe}$ ASL (K_d = 130 ± 40 nM) and for native yeast $tRNA^{Phe}$ (K_d = 100 ± 20 nM) (8). Unmodified ASL, ASL-Ψ_{39} and ASL-$m^1\Psi_{39}$ all maintain position 39 base-pairing ability with A_{31} and, as expected, do not perturb ribosomal binding. ASL-m^5U_{39} bound with a significantly lower affinity (K_d = 612 ± 230 nM) than that of the unmodified ASL even though the ASL with the m^5U_{39} substitution maintains the ability of the nucleoside to base pair with A_{31} and form the last base pair of the stem, adjacent to the loop. The reduced ribosomal binding of ASL-m^5U_{39} is likely due to assay conditions in which the ASLs are incubated with the programmed ribosomes at 37°C. At this temperature, ASL-m^5U_{39} exhibited a low temperature transition during denaturation, presumably associated with loop dynamics (Fig. 3). ASL-$m^3\Psi_{39}$, ASL-$m^1m^3\Psi_{39}$ and ASL-m^3U_{39} bound with dramatically lower affinities (K_d = 1283 ± 258, 1060 ± 189, 1161 ± 422 nM, respectively). Each of these substitutions perturbs the ability of the nucleoside at position 39 to base pair with A_{31}.

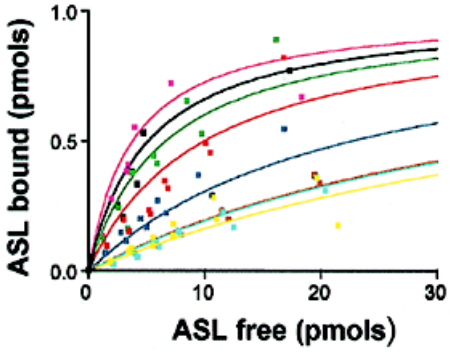

Figure 5. Binding of yeast $tRNA^{Phe}$ and variously modified ASLs to programmed 30S ribosomes. Yeast $tRNA^{Phe}$ (pink) and ASLs unmodified (green), Ψ_{39} (red), $m^1\Psi_{39}$ (black), m^5U_{39} (blue), m^3U_{39} (brown), $m^3\Psi_{39}$ (yellow) and $m^1m^3\Psi_{39}$ (turquoise) were incubated with programmed 30S ribosomes at 37°C as described in Materials and Methods. Maximum binding was normalized to $tRNA^{Phe}$ (10).

ASL protection of ribosomal RNA P-site nucleosides

Variously modified ASLs may have different affinities for the ribosome, depending on their individual associations with the ribosomal P-site. Therefore, we assayed unmodified and modified ASLs for their ability to protect seven of the known 16S rRNA P-site nucleosides (G530, A532, A794, C795, G926, G1338, A1339) (28) from chemical modification by kethoxal and dimethyl sulfate (Fig. 6). A qualitative assessment of the data, relative to that of native $tRNA^{Phe}$, was used as a measure of P-site binding (29). The unmodified ASL protected each nucleoside, as did native $tRNA^{Phe}$ (Table 2). ASL-Ψ_{39} and ASL-$m^1\Psi_{39}$ protected each of the seven 16S rRNA nucleosides to a degree similar to that of the unmodified ASL. However, ASL-$m^3\Psi_{39}$, ASL-$m^1m^3\Psi_{39}$ and ASL-m^3U_{39} afforded less protection than the ASLs capable of maintaining a Watson–Crick base pair with A_{31} (Table 2). Contrary to the filter binding assay results, ASL-m^5U_{39} protected the 16S rRNA nucleosides from chemical modification as well as the unmodified ASL

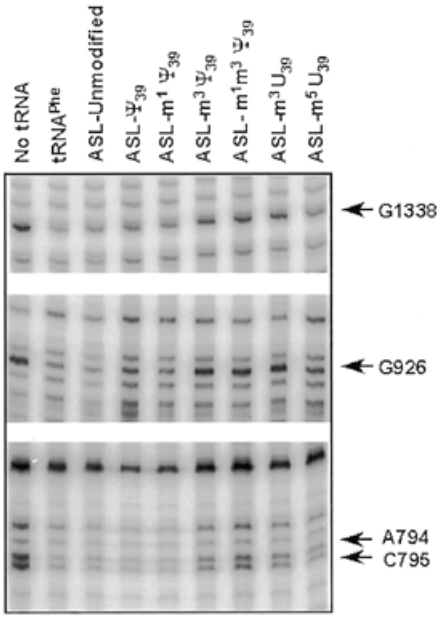

Figure 6. Chemical protection of 16S rRNA P-site nucleosides by yeast tRNA^{Phe} or various ASLs. ASLs unmodified, m$^1\Psi_{39}$, m$^3\Psi_{39}$, m^{1}m$^3\Psi_{39}$, m^{3}U$_{39}$, m^{5}U$_{39}$ and native yeast tRNA^{Phe} were incubated with programmed 30S ribosomes. Footprints are ASLs protecting 16S rRNA P-site nucleotides from kethoxal and DMS modification. Although seven 16S rRNA P-site nucleosides were probed (Table 2), the results of only G1338, G926, A794 and C795 are shown.

(Fig. 6). The chemical protection assay was conducted at 20°C, a temperature below that of the low-temperature transition observed for ASL-m^{5}U$_{39}$ (Fig. 3). In contrast, at the 37°C incubation temperature of the filter-binding assay, the ASL would have undergone low temperature transition.

DISCUSSION

Ψ at position 39 of the ASL of ~40% of all bacterial tRNAs plays an important role in the control of gene expression through attenuation. Presumably Ψ_{39} modulates the ability of these tRNAs to bind to appropriately programmed ribosomes. Ψ_{39} is even more prevalent in eucaryotic cytoplasmic tRNAs (5) and also appears to play a central role in translation (12). However, the physicochemical mechanism by which Ψ_{39}, in comparison to U$_{39}$, modulates translation in either procaryotes or eucaryotes has not been elucidated. In the *anti* conformation, Ψ is isosteric with that of its unmodified precursor U. The immediately obvious difference between the two nucleosides is the additional imino proton at the N1 position of Ψ, a proton that can be donated for hydrogen bonding. We probed the contributions of Ψ_{39} to the thermal stability, structure and function of yeast tRNA^{Phe} by assessing the importance of both of the nucleoside's imino protons at N3 as well as at N1.

We have shown that Ψ_{39} compared to U, stabilized the yeast tRNA^{Phe} ASL structure, moderately increasing the T_m by 1.3°C (Table 1). Incorporation of Ψ has been shown to increase the stability of a variety of RNA constructs, including tRNAs (30), duplex oligoribonucleotides (20,31,32) and single-stranded regions of RNAs (18,20). Recently, incorporation of Ψ into position 39 of the human tRNA^{Lys3} ASL moderately stabilized that RNA as compared to the unmodified ASL (21). Thus, both single-stranded and double-stranded regions of model RNAs, as well as the ASLs of yeast tRNA^{Phe} and human tRNA^{Lys3} were stabilized by the introduction of Ψ as the only modification. Stabilization of RNA structure had been attributed to pseudouridine being more versatile in its ability to hydrogen bond due to the N1 proton (19). However, NMR analysis of the imino protons suggested that the ASL-Ψ_{39} had similar base pairing to that of the unmodified ASL. Although the N1 hydrogen of Ψ appears to be coordinating a structured water molecule (30,21), the Ψ pyrimidine ring could add stability to the RNA by forming a more stable base stacking arrangement that would then be propagated through a helix or into an adjacent loop to stabilize stacking of the neighboring nucleosides (18).

By incorporating the methylated derivatives of Ψ, we were able to block the ability of the Ψ N1 and/or N3 positions to hydrogen bond and examine if the stabilizing effects of Ψ were still present. When the proton from the N1 position of Ψ was replaced with a methyl group in the tRNA^{Phe} ASL construct, the ASL actually exhibited an increased stability resulting in an increase of 2.0°C in T_m as compared to the ASL-Ψ_{39} (Table 1). In comparison, when the isosteric proton in uridine at position 5 was replaced with a methyl, the T_m increased by only 0.3°C compared to the unmodified ASL. These results suggest that the increased stability from incorporating Ψ_{39} resides in the nucleoside's ability to stack with a more thermodynamically favored arrangement and not from additional hydrogen bonding. Methylation of the N1 position improved pseudouridine's ability to stack in comparison to uridine. Methylation of the isosteric position 5 of cytidine and the N1 position of guanosine in the stem and loop, respectively, of a DNA analog of the yeast tRNA^{Phe} ASL also improved base stacking interactions (33).

When the N3 proton of U$_{39}$ was replaced with a methyl, there was a dramatic and almost complete loss of conformational stability by the ASL-m^{3}U$_{39}$, which started to denature at 5°C. The nucleoside's ability to hydrogen bond with A$_{31}$ across the stem was disrupted, as revealed in the NMR spectra. However, incorporation of the isosteric, m$^3\Psi_{39}$ significantly rescued the RNA's thermal stability, though the T_m (50.6°C) was still 14.5°C below that of the ASL-Ψ_{39} (Table 1). One could postulate that m$^3\Psi_{39}$ was in the *syn* conformation allowing the N1 proton to base pair with A$_{31}$. However, no evidence of such a bond was found in the NMR spectrum of ASL-m$^3\Psi_{39}$, which was very similar to that of ASL-m^{3}U$_{39}$. Although previous NMR studies (19,30,34) have shown that Ψ does not adopt a *syn* conformation to base pair with A through the Ψ C2 carbonyl and N1 imino proton, these studies have never addressed the effect of blocking canonical base pairing of Ψ with A by methylating the Ψ N3 proton. We have shown that m$^3\Psi$ in the *anti* conformation and not able to form a Watson–Crick base pair does not rotate to the *syn* conformation to base pair with A through the Ψ C2 carbonyl and N1 imino proton. Further support for m$^3\Psi$ retaining the *anti* conformation and for Ψ contributing to stacking interactions, rather than additional hydrogen bonding, was found in the results of the thermal denaturation experiments with ASL-m^{1}m$^3\Psi_{39}$. With both the N1 and N3 protons of Ψ replaced with methyl groups there was no possibility of any base pair formation. Yet the stability of the ASL increased slightly as compared to that of m$^3\Psi_{39}$. Thus, the glycosidic bond of m$^3\Psi$ must have remained in an *anti* conformation

even though a reorientation of the glycosidic bond to a *syn* conformation would have allowed the $m^3\Psi_{39}$ to base pair with A_{31}. We can conclude from these results that the N3 proton of Ψ is involved in an important base pairing hydrogen bond with A_{31} to form the terminal base pair of the anticodon stem. More importantly, one can conclude from these observations together with those of others (18,20,21) that Ψ_{39} stabilizes the ASL though the nucleoside's ability to accommodate a more stable stacking interaction.

We have shown that Ψ_{39} increases the stability of the ASL as compared to U_{39} of the unmodified ASL. The methylated Ψs we have synthesized to determine the contributions of Ψ in the ASL of tRNA could be used to study Ψ contributions in other RNAs. An understanding of the physicochemical effects of methylated Ψs on tRNA structure and stability as a model could be transferred to interpreting the roles of the naturally occurring methylated Ψ in rRNAs (35).

The increased stability of tRNAs afforded by Ψ_{39} may be directly responsible for the modification's role in modulating translation. Ψ_{39} may enable both procaryotic and eucaryotic tRNAs to bind more effectively to the programmed ribosome. *DEG1* mutants of yeast are hypomodified at positions 38 and 39 and have a reduced growth rate (12) which could also be a result of a reduced affinity of tRNA to bind to the ribosome. If tRNA aminoacylated with the correct amino acid but lacking the Ψ_{39} modification could not effectively bind to the ribosome there would be less efficient translation and, consequently, reduced growth rates. We assayed the ability of the variously modified ASLs to bind to programmed ribosomes and compared the results to each other and to the unmodified ASL. The three ASLs in which the A_{31}–U_{39} base pair was negated and exhibited significantly decreased thermal stability (Table 1), ASL-m^3U_{39}, ASL-$m^3\Psi_{39}$ and ASL-$m^1m^3\Psi_{39}$, had significantly lower affinities for the ribosome. It should be noted that the ribosomal binding assay conditions could contribute to the decreased binding. At the assay temperature (37°C) the ASLs with methylated N3 positions have already started melting (Fig. 3). However, ASL-m^3U_{39}, ASL-$m^3\Psi_{39}$ and ASL-$m^1m^3\Psi_{39}$ also protected 16S rRNA P-site nucleosides to a lesser degree than that of the ASLs maintaining Watson–Crick base pairing with A_{31}, even though this assay was conducted at 20°C. Thus, the base pair adjacent to the anticodon loop is important to tRNA stability and ribosome binding. We found little discrimination in ribosome binding or ability to protect certain 16S rRNA P-site nucleosides from chemical modification between the unmodified, Ψ, and $m^1\Psi_{39}$ modified ASLs. Our ribosomal binding and 16S nucleotide chemical protection assays only probe the ability of our ASL constructs to bind to the 30S P-site. However, the results may be different if the ability of the ASL constructs to bind to the 30S ribosomal A-site was probed. Thus, we can conclude that either Ψ_{39} did not contribute to ribosomal binding of the ASLs or its contribution was not observed in the assays we used. We also can conclude that hydrogen bonding of the N1 position of Ψ is not vital to tRNA stability or to 30S P-site binding.

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