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Conformation Effects of Base Modification on the Anticodon Stem-loop of *Bacillus subtilis* tRNA^{Tyr}

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Abstract

tRNA molecules contain 93 chemically unique nucleotide base modifications that expand the chemical and biophysical diversity of RNA and contribute to the overall fitness of the cell. Nucleotide modifications of tRNA confer fidelity and efficiency to translation and are important in tRNA-dependent RNA-mediated regulatory processes. The three-dimensional structure of the anticodon is crucial to tRNA-mRNA specificity and the diverse modifications of nucleotide bases in the anticodon region modulate this specificity. We have determined the solution structures and thermodynamic properties of *B. subtilis* tRNA^{Tyr} anticodon arms containing the natural base modifications N⁶-dimethylallyl adenine (i⁶A₃₇) and pseudouridine (ψ₃₉). UV melting and differential scanning calorimetry indicate the modifications stabilize the stem and may enhance base stacking in the loop. The i⁶A₃₇ modification disrupts the hydrogen bond network of the unmodified anticodon loop including a C₃₂-A₃₈⁺ base pair and an A₃₇-U₃₃ base-base interaction. Although the i⁶A₃₇ modification increases the dynamic nature of the loop nucleotides, metal ion coordination re-establishes conformational homogeneity. Interestingly, the i⁶A₃₇ modification and Mg²⁺ are sufficient to promote the U-turn fold of the anticodon loop of *E. coli* tRNA^{Phe}, but these elements do not result in this signature feature of the anticodon loop in tRNA^{Tyr}.

Introduction

Posttranscriptional modification of RNA nucleotide bases occurs in all cells and is an important step in the maturation of these molecules^{1, 2, 3, 4}. In addition to their roles in translation^{5, 6, 7, 8, 9, 10}, base modifications have direct roles in other cellular processes that include RNA splicing^{11, 12}, viral replication¹³, and antibiotic resistance¹⁴. The diversity of base modifications is equally broad, ranging in complexity from a simple isomerization, such as uridine to pseudouridine, to elaborate modifications, such as queuosine or the Q base. The chemical properties of many base-modified nucleotides are well-characterized and the thermodynamic effects of several modified nucleotides have been examined^{4, 15, 16}.

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Atomic coordinates for the refined structures have been deposited with the Protein Data Bank under accession codes 2kzp, 2kzo, 2lbq, 2lbr. Chemical shifts have been deposited at BMRB under accession codes 17517, 17520, 17572, and 17573.

Accession Numbers

Coordinates have been deposited in the Protein Data Bank under accession numbers PDB ID: 2lac, 2la9, 2lbq, 2lbr. Chemical shifts have been deposited in the Biomolecular Magnetic Resonance Bank under accession numbers BMRB ID: 17517, 17520, 17572, and 17573.

However, the structural and dynamic effects of modified bases on RNA are less extensively described and the number of high-resolution structure investigations that examine the interplay of multiple modifications within the context of a single RNA molecule is even fewer^{13, 17, 18, 19, 20, 21}.

tRNA molecules contain a large and diverse pool of modified nucleotide bases with the greatest variety concentrated in the anticodon arm²². The anticodon arm of tRNA^{Tyr} reflects this diversity and contains modified nucleotide bases at three positions (Figure 1). The nucleotide at position 37, adjacent to the 3' nucleotide of the anticodon, is one of the most extensively modified residues in the anticodon arm²³. The modified base, i⁶A₃₇, or variants that include ms²i⁶A₃₇ and io⁶A₃₇, often follows anticodon sequences terminated by an adenine^{24, 25, 26}. i⁶A₃₇ and ms²i⁶A₃₇ are two of the most common modified forms of A₃₇ in bacterial tRNA, occurring in 10 of 46 tRNA species in *Escherichia coli*. The modification enzyme dimethylallyl (Δ^2 -isopentenyl) diphosphate:tRNA transferase (MiaA) catalyzes the addition of the dimethylallyl unit from dimethylallyl diphosphate (DMAPP) to the exocyclic amino nitrogen (N6) of A₃₇, to form i⁶A₃₇. The loss of this modification in *E. coli* and *S. typhimurium* causes defects in translation, including reduced rates of polypeptide chain elongation^{5, 6, 7}, decreased efficiency of translation⁷, increased codon context sensitivity and decreased function of suppressor tRNA species^{9, 10, 27}, increased translation misread error rates for the first codon position⁸ leading to incorrect amino acid incorporation, and decreased^{9, 10} translation misreading error rates at the third codon position leading to reduced wobble function. The most common tRNA modification, pseudouridine (ψ), is present in the anticodon arms of nearly all cellular tRNA molecules at residue 38, 39, or 40 and is interspersed at other positions throughout tRNA²⁵. Pseudouridine, an isomer of uridine containing a C1'-C5 glycosidic bond, is incorporated at positions 38-40 by the bacterial enzyme TruA. Disruption of *truA* results in reduced polypeptide elongation rates and reduced translational fidelity¹. These TruA-associated defects in Gram-negative bacteria lead to reduced growth rates¹ and cause loss of virulence in *Pseudomonas aeruginosa*²⁸. The bulky queuine (Q) base introduces three additional hydrogen bond donors and is found at the wobble position (34) of tRNAs that read codons having U at the second position and C or U at the third position. The stability of the Q-U wobble pair is less than that of Q-C but greater than that of the G-U wobble pair²⁹. In *E. coli*, queuosine confers fitness³⁰ and is essential for virulence in *Salmonella flexniri*. In the specific case of tRNA^{Tyr}, the absence of queuosine leads to amber suppressor activity³¹.

The pseudouridine and the i⁶A₃₇ modifications contribute to the biophysical and structural properties of the tRNA anticodon arm. The ms²i⁶A₃₇ modification stabilizes the codon-anticodon helix²⁴ and the i⁶A modification can alter the structure in the anticodon loop³². The loop nucleotides of the anticodon arm of unmodified *E. coli* tRNA^{Phe} adopt a tri-loop conformation in solution³², but conversion of A₃₇ to i⁶A₃₇ disrupts the tri-loop and facilitates formation of the U-turn motif³³. A similar effect is observed for the anticodon arm of human tRNA^{Lys},³ that also has a tri-loop that is disrupted by the modification threonyl-6-adenine at position 37¹⁷. ψ_{39} of tRNA increases thermodynamic stability of the stem of the anticodon arm and improves base stacking^{15, 19, 34}, but ψ_{39} modification does not significantly change the overall structure of the anticodon arm of *Escherichia coli* tRNA^{Phe} or human tRNA^{Lys},^{315, 20}.

In addition to its role in translation, tRNA has a central role in the T box riboswitch mechanism of transcriptional regulation in Gram positive bacteria. The T box riboswitch is located in the 5' untranslated (leader) regions of tRNA synthetase genes and genes involved in amino acid metabolism^{35, 36}. The transcription of these genes is attenuated when the ratio of charged to uncharged tRNA in the cell is high^{35, 37}. The T box riboswitch contains two mutually exclusive stem-loop secondary structures, the terminator and antiterminator

hairpins, responsible for pre-mature transcription termination and transcription read-through, respectively. Each T box riboswitch can bind a cognate tRNA that is related to the function of the gene downstream of the riboswitch. Read-through of the transcription termination site occurs when uncharged cognate tRNA binds and stabilizes the antiterminator hairpin by pairing of nucleotides in the 3' end of the tRNA with a seven-nucleotide bulge in the antiterminator helix³⁸. The Specifier Loop domain of the T box riboswitch contains the Specifier sequence, three nucleotides that are complementary to the anticodon nucleotides of the cognate tRNA, that is the primary specificity determinant of the T box riboswitch-tRNA interaction³⁹. The roles of tRNA base modifications in T box riboswitch function have yet to be examined, but similar to the tRNA-mRNA interaction on the ribosome, the modifications are expected to have a positive contribution to the T box riboswitch function through the anticodon-Specifier sequence interaction.

One step in our examination of the role of tRNA modification in T box riboswitch function is the determination of the biophysical effects of i^6A_{37} and ψ_{39} modifications within the anticodon arm of *Bacillus subtilis* tRNA^{Tyr} (ASL^{Tyr}). We have used heteronuclear NMR spectroscopy to determine the solution conformations of the unmodified, ψ_{39} , i^6A_{37} , and the ψ_{39} , i^6A_{37} double modified ASL^{Tyr}. The loop regions of the unmodified and $[\psi_{39}]$ -ASL^{Tyr} molecules are well ordered and contain protonated $C_{32}-A^+_{38}$ base pairs. Introduction of the i^6A_{37} modification causes loss of the $C_{32}-A^+_{38}$ base pairs and increases intermediate time-scale dynamics within the loop. The addition of cobalt hexamine to i^6A_{37} ASL^{Tyr} restores structural integrity of the loop but does not stabilize a conformation similar to the canonical U-turn motif of the tRNA anticodon arm. In the absence of divalent cations, differential scanning calorimetry and UV melting data indicate that the pseudouridine modification increases the thermal stability of the stem. Interestingly, the i^6A_{37} residue by itself stabilizes the stem but has a small destabilizing effect in the context of the ψ_{39} modification.

RESULTS

Effects of modification on RNA thermal stability

The thermal stabilities of the ASL^{Tyr} RNA hairpins (Figure 1) were investigated using UV-monitored thermal melting experiments and differential scanning calorimetry (DSC) experiments. The normalized UV thermal denaturation curves indicate that the hairpins melt in two stages (Figure 2B). The lower temperature (<50 °C) transitions presumably correspond to the destacking of the loop nucleotides. At pH 6.3 the $[\psi_{39}]$ -ASL^{Tyr} and the unmodified ASL hairpins have a protonated C-A⁺ base pair, which contributes to the stability of the molecules by forming a six (6) base pair stem instead of the five (5) base pair stem observed in the $[i^6A_{37}]$ -ASL^{Tyr} molecule. The increased absorbance of $[i^6A_{37}]$ -ASL^{Tyr} relative to ASL^{Tyr} and $[\psi_{39}]$ -ASL^{Tyr} below 70 °C suggests that the dimethylallyl modification decreases base stacking in the modified hairpins. The percent hyperchromicity observed with melting is greatest for ASL^{Tyr} and $[\psi_{39}]$ -ASL^{Tyr} (10%-11%) and is least for the dimethylallyl-modified RNAs (7% and 5% for $[i^6A_{37}]$ -ASL^{Tyr} and $[i^6A_{37}, \psi_{39}]$ -ASL^{Tyr}, respectively). The enhanced thermal stability of the stems of the $[i^6A_{37}]$ -modified RNA hairpins is consistent with the stabilization conferred by the dimethylallyl modification in the context of the *E.coli* ASL^{Trp}¹⁶, but stands in contrast to the destabilizing effect observed for the *E. coli* ASL^{Phe}³².

The DSC curves (Figure 2A) indicate that the RNA hairpins undergo a single-step melting process. The unmodified RNA hairpin appears to contain a slight heat capacity change near 50 °C that is not observed in the other three molecules, but is small relative to the change that occurs in the multi-step melting of a tetraloop receptor homodimer complex⁴⁰. This small change could reflect melting of the $C_{32}-A^+_{38}$ base pair that is present only in the unmodified RNA molecule (described below). The pseudouridine modification increased the

T_m of the RNA hairpin by 5.7 °C, indicating that ψ_{39} improves the stability of the stem. This finding is consistent with other studies that show pseudouridine improves the stability of a duplex when located at or near the end of the stem^{11, 13, 15}. The enthalpy (ΔH) of melting for the ψ_{39} ASL^{Tyr} molecule is 5.24 ± 0.06 kcal/mol/bp, which suggests additional hydrogen bonding not present in the unmodified molecule ($\Delta H = 4.17 \pm 1.20$ kcal/mol/bp). The dimethylallyl modification also confers stability to the ASL^{Tyr} stem, but not as much as the pseudouridine modification (Figure 2A). i^6A_{37} increases the T_m of the RNA hairpin by 3.7 °C and the ΔH by 1.39 kcal/mol/bp. The addition of ψ_{39} and i^6A_{37} increases the T_m by 5.0 °C and the ΔH by 0.40 kcal/mol/bp, indicating that the i^6A_{37} modification interferes slightly with the stabilizing effects of the pseudouridine modification.

Resonance assignments of ASL^{Tyr} molecules

The oligomeric states of the RNA molecules were examined to confirm formation of the hairpin secondary structure. The monomeric nature of the RNA is supported by moderate line widths (9-14 Hz 1H) and the near identical cross peak patterns in NH spectra at low (20 μM) and high (1.4 mM) RNA concentrations. The RNAs also run as single bands on native PAGE, consistent with a single form. The non-exchangeable 1H and ^{13}C resonances of ASL^{Tyr} and $[i^6A_{37}]$ -ASL^{Tyr} (Figure 1) were assigned using standard heteronuclear techniques^{41, 42}. The $[i^6A_{37}]$ -ASL^{Tyr} sample required 2.0 mM $Co(NH_3)_6^{3+}$ to achieve a conformationally homogeneous state and to reduce exchange broadening. For both molecules, most base and ribose 1H and ^{13}C correlations are resolved (Figures 3 and S1). The A_{38} C2 resonance of unmodified ASL^{Tyr} is slightly broadened but has a chemical shift of 149.2 ppm indicative of protonation (Figure 3)⁴³. For $[i^6A_{37}]$ -ASL^{Tyr}, the A_{38} C2 resonance shifts downfield to 153.6 ppm, indicative of the non-protonated form of adenine. In the absence of $Co(NH_3)_6^{3+}$, the A_{38} C2 resonance is very broad (≈ 130 Hz) with a chemical shift centered at 151.8 ppm. Also for $[i^6A_{37}]$ -ASL^{Tyr}, the A_{36} C8H8 resonance is absent from the ^{13}C - 1H HSQC spectrum in the absence of metal ions. All the ribose spin systems, except for the incompletely labeled 5'-terminal nucleotide, were identified using 2D or 3D HCCH-COSY and HCCH-TOCSY experiments. Three of the four adenine residues in ASL^{Tyr} yield intra-base H8-H2 correlations in the HCCH-TOCSY spectrum. The A_{38} H2-H8 correlation is not observed. Intra-residue base-to-sugar correlations were identified using 2D H(C)N experiments to obtain correlations H6-N1, H8-N9, and H1'-N1/N9. All pyrimidine correlations and all (ASL^{Tyr}) or six of eight ($[i^6A_{37}]$ -ASL^{Tyr}) purine correlations were identified in these spectra. The A_{36} and i^6A_{37} correlations are not observed due to chemical exchange.

Sequential assignments for the non-exchangeable resonances were made using 2D NOESY and 3D ^{13}C -edited NOESY experiments to identify sequential H6/8-H1' NOE connectivities. The sequential base-1' NOE connectivities are continuous through all 17 nucleotides in the 180 ms NOESY spectrum (Figure 4), but the connectivity between nucleotides U_{33} and G_{34} is weak. The H6/8-H2' and H6/8-H6/8 inter-residue connectivities also are continuous in the 180 ms spectrum except for the U_{35} H6 to A_{36} H8 step. The base-2' inter-residue connectivities from U_{33} to A_{37} are weak, consistent with the non-C3'-endo ribose ring conformations of these residues (described below).

The NH and NH₂ resonances of ASL^{Tyr} and $[i^6A_{37}]$ -ASL^{Tyr} were assigned using 1H - 1H NOESY and HNCCH experiments. Only the G_{28} - G_{30} NH resonances are sufficiently intense to yield NOE connectivities between neighboring base pairs. The G_{27} NH resonance of the terminal G-C base pair does not yield cross peaks in the NOESY spectrum. A broad guanine NH resonance, which has a chemical shift that corresponds to a non-base paired guanine (≈ 10.7 ppm), was assigned to G_{34} . The U_{39} NH resonance of ASL^{Tyr} is broadened by chemical exchange and is only observed in the 1D spectrum. The G_{34} and U_{39} NH resonances are further broadened by the i^6A_{37} modification and do not intensify with lower

temperature. The cytidine and adenine NH₂ resonances were assigned via scalar correlations using HSQC and HNCCH experiments. The upfield chemical shifts (7.20 and 6.69 ppm) of the C₃₂ H4 resonances are consistent with the lack of intra-molecular hydrogen bonding. A₃₁ of ASL^{Tyr} is the only adenine residue from either molecule that has two resolved NH₂ ¹H resonances. The dimethylallyl modification of A₃₇ causes the N6 resonance to shift downfield from 80.0 ppm to 92.3 ppm. This shift is similar to the shift observed upon dimethylallyl modification of A₃₇ in *E. coli* tRNA^{Phe32, 33}.

The [ψ₃₉]-ASL^{Tyr} and [i⁶A₃₇, ψ₃₉]-ASL^{Tyr} molecules were not isotopically enriched and so resonances were assigned using 2D NOESY and natural abundance ¹³C-¹H HSQC experiments (Figures 3 and 4). Many of the spectral features of these molecules parallel those of the ASL^{Tyr} and [i⁶A₃₇]-ASL^{Tyr} molecules described above. The most substantial spectral difference between ASL^{Tyr} and [ψ₃₉]-ASL^{Tyr} are the upfield shifts of the C6, H6, and H1' resonances that correspond to ψ₃₉. The A₃₈ is not stably protonated in [ψ₃₉]-ASL^{Tyr} (Figure S1), and deprotonates upon modification to [i⁶A₃₇, ψ₃₉]-ASL^{Tyr} (Figure 3), analogous to the deprotonation caused by introduction of the i⁶A₃₇ modification alone. The NOE cross peaks for [ψ₃₉]-ASL^{Tyr} are continuous in the base-1' region of long mixing time NOESY spectra, but the G₃₄ H8-U₃₃ H1' cross peak is weak. This sequential NOE peak is missing from all [i⁶A₃₇, ψ₃₉]-ASL^{Tyr} NOESY spectra (Figure 4). The presence or absence of a non-sequential U₃₅ H6-U₃₃ H1' peak could not be determined due to spectral overlap. The addition of Co(NH₃)₆³⁺ also was used to achieve conformational homogeneity with [i⁶A₃₇, ψ₃₉]-ASL^{Tyr}. It is notable that the cross peak patterns displayed by [i⁶A₃₇, ψ₃₉]-ASL^{Tyr} and [i⁶A₃₇]-ASL^{Tyr} molecules with Co(NH₃)₆³⁺ are very similar (Figure 4). The H1 imino resonance of ψ₃₉ is present in the ¹⁵N-¹H HSQC spectrum at 10.6 ppm and is equally sharp in both of the ψ-containing molecules. The presence of this resonance is consistent with the participation of the ψ₃₉ H1 in a water-mediated hydrogen bond with the phosphate backbone ⁴⁴.

For all molecules, the inter-nucleotide phosphate ³¹P resonances are clustered between -3.54 and -4.40 ppm and were assigned using HCP or ³¹P-¹H hetero-TOCSY-NOESY spectra ⁴⁵. The sequential P-H6/8 and P-H1' correlations are continuous throughout the [ψ₃₉]-ASL^{Tyr} (Figure 5). The P-H3' correlations and several P-H4' and P-H5'/H5' correlations are present in ³¹P-¹H HetCor spectra and provide independent confirmation of the ³¹P assignments. Notably, the ³¹P resonances remain tightly grouped indicating that the i⁶A₃₇ and ψ₃₉ modifications have little effect on the phosphate backbone conformation (Figure 5). The most significant effect of the modifications occurs at the ³¹P resonance of the phosphate 5' to ψ₃₉. This resonance shifts 0.1 ppm upfield with the introduction of pseudouridine and is likely caused by the water-mediated hydrogen bond of the *pro-R* phosphoryl oxygen with the ψ₃₉ H1 imino.

The ¹H resonances of the dimethylallyl group were assigned using NOESY spectra. The ¹H chemical shifts of the dimethylallyl groups, CH 5.05 ppm, CH₂ 4.04 ppm and 3.87 ppm, CH₃ 1.53 ppm and 1.51 ppm, are nearly the same for [i⁶A₃₇]-ASL^{Tyr} and [i⁶A₃₇, ψ₃₉]-ASL^{Tyr}. However, the CH and CH₂ resonances are shifted upfield to 4.96, 3.63, and 3.52, respectively, when Co(NH₃)₆³⁺ is absent from the samples. The protons of the dimethylallyl group have NOE cross peaks to several base and 1' protons and are discussed below. A complete list of resonance assignments, including non-protonated positions of ASL^{Tyr} and [i⁶A₃₇]-ASL^{Tyr}, is given in Table S1.

Effects of metal ions on the RNA hairpins

Metal ions, most commonly Mg²⁺, contribute to several aspects of RNA structure and function including RNA folding, tertiary structure stabilization, and catalysis. Mg²⁺ ions are crucial for maintaining the correct tertiary fold of tRNA and their binding sites within the

molecule have been identified by multiple methods^{4, 46}. In structure studies, Mg^{2+} and $\text{Co}(\text{NH}_3)_6^{3+}$ ions have been observed proximal to the loop-helix junction in the anticodon stem-loop of tRNA^{33, 47}.

Therefore, the four ASL molecules were tested for their ability to bind Mg^{2+} and to examine possible Mg^{2+} -induced conformational effects. For unmodified ASL^{Tyr} and $[\psi_{39}]$ -ASL^{Tyr}, Mg^{2+} at concentrations between 5 and 10 mM slightly weaken the intensity of the U_{39} N3H ^{15}N - ^1H cross peak and cause the ^1H resonance to shift upfield 0.2 ppm. The G_{28} - G_{30} NH resonances have small changes in chemical shift and intensity and the G_{34} resonances become extremely broadened. The ψ_{39} N1H resonance is broadened, but not shifted. The G_{28} - G_{30} chemical shift effects are consistent with binding of divalent metal ions to triplets of G-C base pairs⁴⁸. The non-exchangeable resonances also exhibit spectral changes. Most notably, the A_{38} C2 of unmodified ASL^{Tyr} shifts downfield ≈ 4.0 ppm, indicating deprotonation of the A_{38} base and loss of the C_{32} - A_{38}^+ base pair. In $[\psi_{39}]$ -ASL^{Tyr}, the A_{38} H2C2 correlation appears and has a chemical shift indicative of the neutral base. The base 6 and 8 resonances of nucleotides U_{33} - A_{38} exhibit chemical shift changes and significant broadening with the base 8 resonances of A_{36} and A_{38} broadened beyond detection. The spectra could not be improved by increasing the Mg^{2+} concentration.

The effects of metal ions on the $[\text{i}^6\text{A}_{37}]$ - and $[\text{i}^6\text{A}_{37}, \psi_{39}]$ -ASL^{Tyr} molecules are somewhat more variable. The imino ^1H resonances of the base paired G_{27} - G_{30} of both molecules broaden slightly with Mg^{2+} addition with the U_{39}/ψ_{39} imino resonances exhibiting more substantial broadening, presumably caused by increased solvent exchange. The imino ^1H resonances of U_{33} , G_{34} , and U_{35} , already broad, exhibit small changes with Mg^{2+} . These effects on the imino resonances are independent of temperature from -3°C to 15°C . The non-exchangeable base and $1'$ resonances are more sensitive to Mg^{2+} . In the absence of metal ions, the stem (G_{27} - A_{31} and U/ψ_{39} - C_{43}) resonances appear similar to the corresponding resonances of unmodified and $[\psi_{39}]$ -ASL^{Tyr} molecules, but resonances from the loop nucleotides are broadened by chemical exchange. The $[\text{i}^6\text{A}_{37}]$ -ASL^{Tyr} exhibits the poorest spectral quality (absence of A_{37} C8 resonance and severe broadening of the A_{38} C8, A_{38} C2, and U_{39} C1' resonances), but shows modest improvement with addition of up to 5 mM Mg^{2+} . As the Mg^{2+} concentration was increased beyond 10 mM, the spectral quality again began to degrade. Although Mg^{2+} leads to sharpening of some loop nucleotide resonances, the general broadening of most other resonances that accompanied Mg^{2+} addition prevented complete resonance assignment. The $[\text{i}^6\text{A}_{37}, \psi_{39}]$ -ASL^{Tyr} spectrum also exhibits the effects of chemical exchange, but the effects differ slightly from those presented by $[\text{i}^6\text{A}_{37}]$ -ASL^{Tyr}. Addition of 4 mM Mg^{2+} yielded only modest spectral improvement for loop resonances, but caused the base resonances of U_{32} , U_{33} , and A_{37} to be broadened beyond detection. Addition of Mg^{2+} to $[\text{i}^6\text{A}_{37}, \psi_{39}]$ -ASL^{Tyr} also leads to exchange broadening of the ψ_{39} N3H resonance.

We have previously observed that $\text{Co}(\text{NH}_3)_6^{3+}$ was more effective than Mg^{2+} at stabilizing the structure of the *E. coli* tRNA^{Phe} anticodon loop³³. Thus, $\text{Co}(\text{NH}_3)_6^{3+}$ was tested for its ability to minimize the effects of chemical exchange among the loop nucleotide resonances of $[\text{i}^6\text{A}_{37}]$ -ASL^{Tyr} and $[\text{i}^6\text{A}_{37}, \psi_{39}]$ -ASL^{Tyr} without deteriorating the overall spectral quality. The addition of $\text{Co}(\text{NH}_3)_6^{3+}$ (2.0 mM) produced conformationally homogeneous samples with excellent spectral properties. All base and ribose $1'$ resonances are present and only the A_{37} C8 and A_{38} C2 resonances are moderately broad in $[\text{i}^6\text{A}_{37}]$ -ASL^{Tyr} (Figure S1). NOESY spectra (mixing time of 320 ms) were acquired in 90% $^1\text{H}_2\text{O}$ to locate the position of the $\text{Co}(\text{NH}_3)_6^{3+}$ ligands. The guanine NH and cytidine NH_2 resonances of the stem as well as H8 and H6 resonances have cross peaks with the $\text{Co}(\text{NH}_3)_6^{3+}$ protons. These peaks are consistent with coordination of $\text{Co}(\text{NH}_3)_6^{3+}$ in the major groove⁴⁹. Although the spectral data defining the stem location of $\text{Co}(\text{NH}_3)_6^{3+}$ are very good, the cobalt hexamine

complex cannot be uniquely positioned in the loop region and can only be restricted to the major groove. Very weak NOE cross peaks are observed between $\text{Co}(\text{NH}_3)_6^{3+}$ and the base protons of C_{32} , U_{33} , and A_{38} and a weak cross peak involving the CH_3 protons of the dimethylallyl modification. No evidence for coordination involving the loop nucleotide base atoms is reflected by the chemical shifts of the non-protonated ^{15}N resonances. Therefore, coordination of the $\text{Co}(\text{NH}_3)_6^{3+}$ may involve bridging between phosphate groups across the major groove.

Structure Calculations

The structures of the ASL^{Tyr} molecules were calculated using a restrained molecular dynamics routine starting from 50 sets of coordinates with randomized backbone dihedral angles. The calculations used a total of 294-300 distance constraints and 60-70 dihedral angle constraints (Table 1) to produce 8 converged structures for each molecule (Figure 6). Structures were classified as converged if they were consistent with the NMR data and maintained correct stereochemistry. The converged structures have an average of 7 distance constraint violations between 0.1 and 0.3 Å, most of these involving the loop region. All converged structures have no constraints violated by more than 0.3 Å. When the structures are arranged in order of increasing overall energy, the converged structures form a plateau with similarly low overall energies and constraint violation energies. The root mean square deviations (RMSDs) of the heavy atoms between the individual structures and the minimized mean structures are 0.36 ± 0.15 Å, 0.38 ± 0.05 Å, 0.55 ± 0.17 Å, and 1.09 ± 0.18 Å for ASL^{Tyr} , $[\psi_{39}]\text{-ASL}^{\text{Tyr}}$, $[\text{i}^6\text{A}_{37}]\text{-ASL}^{\text{Tyr}}$, and $[\text{i}^6\text{A}_{37}, \psi_{39}]\text{-ASL}^{\text{Tyr}}$, respectively. The global fold of ASL^{Tyr} is a hairpin composed of a six base pair stem and a three to five nucleotide loop (Figure 6A). The overall fold of $[\text{i}^6\text{A}_{37}]\text{-ASL}^{\text{Tyr}}$ (a five base pair stem with a five to seven nucleotide loop) is similar to ASL^{Tyr} , but the loss of some inter-residue NOEs among loop nucleotides and the loss of secondary structure proximal to the loop caused by the modification lead to a somewhat less precisely defined loop conformation (0.55 ± 0.17 Å rmsd compared to 0.36 ± 0.15 Å rmsd, respectively, for the heavy atoms). The ψ_{39} modification does not alter the global fold of the anticodon arms, but destabilizes the $\text{C}_{32}\text{-A}_{38}$ base pair in ASL^{Tyr} .

Structures of the ASL^{Tyr} and $[\psi_{39}]\text{-ASL}^{\text{Tyr}}$ loop regions

The loops of unmodified ASL^{Tyr} and $[\psi_{39}]\text{-ASL}^{\text{Tyr}}$ are similar and are composed of nucleotides $\text{U}_{33}\text{-A}_{37}$. The stems of both molecules are closed by the $\text{C}_{32}\text{-A}^+_{38}$ base pairs (Figure 7A), although exchange broadening of the A_{38} C2 resonance indicates weakening of the 38-32 base pair with the introduction of ψ_{39} . In $[\psi_{39}]\text{-ASL}^{\text{Tyr}}$, the base of G_{34} stacks against the base of U_{33} and is turned slightly away from the helix axis and towards the minor groove (Figure 7B). The base of A_{36} stacks against the base of A_{37} and is displaced toward the major groove. In unmodified- ASL^{Tyr} , the base of G_{34} is parallel to the plane of the U_{33} base but does not stack against it and is turned out into the minor groove (Figure 7A). The base of A_{36} stacks against the base of A_{37} as in $[\psi_{39}]\text{-ASL}^{\text{Tyr}}$ and also is displaced towards the major groove. The positions of the bases in the two molecules are in agreement with the observed H6-H8 and H5-H8 NOEs. The position of A_{36} is restricted by several NOEs involving its H2 resonance, including interactions with the H1' resonances of A_{36} and A_{37} , the H5'/5'' of U_{35} , and the H2' of A_{37} . These NOEs position the base of A_{36} beneath the major groove edge of the A_{37} base (Figure 7A). The position of the U_{35} base in both molecules is more variable, but tends to partition to the major groove side of the helix and oriented parallel with the helix axis (Figure 7A). Although the U_{35} base is perpendicular to the neighboring bases, the ribose moiety of U_{35} also is rotated so that the configuration about the glycosidic bonds of all residues is *anti*. About 25% of the time, the base of U_{35} is positioned on the minor groove side of the helix. NOEs involving the U_{35} base do not exclude the later conformation, nor are NOE cross peaks predicted for that conformation.

that are not observed in the NMR spectra. The structures of unmodified and ψ_{39} -modified molecules suggest a cross-strand interaction between the A₃₇ N6 and the U₃₃ O2 atoms. For the [¹³C, ¹⁵N]-labeled ASL^{Tyr}, the chemical shifts of A₃₇ N6 (80.1 ppm) and U₃₃ C2 (153.6 ppm, Figure 8A) lay between the chemical shift values observed for corresponding intra-molecular hydrogen bonded and non-hydrogen bonded atoms. The chemical shift range for adenine N6 in ASL^{Tyr} is 78.8 ppm (A₃₆) to 82.2 ppm (A₃₁). Intra-molecular hydrogen bonding of uridine O2 leads to a downfield shift of the C2 resonance with values $\approx$ 154.5 ppm for a G-U base pair, $\approx$ 153.5 ppm for a U-U base pair, and $\approx$ 152.5 ppm for an A-U base pair, and $\approx$ 152.5 ppm for a non-intra-molecular hydrogen bonded uridine base. These chemical shifts offer support for an A₃₇-U₃₃ hydrogen bond interaction of moderate strength.

The sugar-phosphate backbone conformations of the loop nucleotides are surprisingly uniform (Figure 6). The majority of the nucleotides maintain the C3'-*endo* ring pucker, but the G₃₄, U₃₅, and A₃₆ ribose groups have observable H1'-H2' couplings and ribose ¹³C chemical shifts (C1' and C4') that indicate neither pure C2'- or C3'-*endo* ring pucker conformations. In addition, the uniformly small (<5 Hz) P-C2' coupling constants for the loop residues (unmodified ASL^{Tyr}) place the ϵ torsion angles in the *trans* conformation characteristic of RNA. The phosphate backbone torsion angles β , γ , and ϵ , of residues G₃₄-A₃₆ were not constrained for the calculations, but most have values within the range common to RNA helices with most deviations from standard values centered around residue U₃₅. The ³¹P chemical shifts for all inter-residue phosphorus atoms are tightly clustered between -3.2 and -4.8 ppm (Figure 5, Table S1), indicating that the α and ζ torsion angles throughout the loop and helix adopt a *gauche* conformation. (The *trans* conformation of the α or ζ torsion angle causes a 2-3 ppm downfield shift of the corresponding ³¹P resonance).

Structures of the [⁶A₃₇]-ASL^{Tyr} and [⁶A₃₇, ψ_{39}]-ASL^{Tyr} loop regions

The loop conformations of the [⁶A₃₇]-ASL^{Tyr} and [⁶A₃₇, ψ_{39}]-ASL^{Tyr} molecules are similar and are dominated by the effects of the dimethylallyl group (Figure 7). In both molecules, the helical base stack is continuous along the 3' side of the loop from A₃₆ through C₄₃ and along the 5' strand from G₂₇ through U₃₃. The H6-H6 and H6-H5 NOEs between residues C₃₂ and U₃₃ are weak and result in a greater than normal inter-base separation. The base of G₃₄ is not stacked beneath U₃₃, but is displaced away from the helix axis on the major groove side of the loop. The relative positions of G₃₄ and U₃₃ are consistent with the absence of an H6-H8 NOE and the very weak ([⁶A₃₇]-ASL^{Tyr}) or absent ([⁶A₃₇, ψ_{39}]-ASL^{Tyr}) H8-H1' NOE. It is also worth noting that no non-sequential U₃₃H1' to U₃₅ H6 NOE could be identified for either molecule. As with ASL^{Tyr} and [ψ_{39}]-ASL^{Tyr}, the U₃₅ base resides on the major groove side of the helix. However, the orientation of this base ranges from perpendicular to the helix axis to nearly parallel with the helix axis.

In both ⁶A₃₇-modified molecules, the base plane of residue 37 is between C₃₂ and U₃₃, and the dimethylallyl group protrudes into the major groove (Figure 7). In [⁶A₃₇]-ASL^{Tyr}, the methyl groups point above the plane of the A₃₇ base in 75% of structures. The NOEs predicted by this orientation are consistent with those observed in the NMR spectra. In the structures of [⁶A₃₇, ψ_{39}]-ASL^{Tyr}, the methyl groups lie below the plane of the A₃₇ base as often as they lie above the base plane. The former orientation does not lead to a unique set of NOE cross peaks due to the sparseness of base and ribose protons that are near the dimethylallyl group and so excursions of the methyl groups below the base plane cannot be excluded by the experimental data. However, neither molecule displays evidence of multiple conformations or dynamics for the dimethylallyl protons or loop nucleotide resonances. The conformation of the dimethylallyl group is restricted by NOEs to C₃₂, U₃₃, G₃₄, A₃₇, and A₃₈. The CH₃ groups produce strong NOE cross peaks with the U₃₃ H4' and A₃₈ H2 and both resonances of the CH₂ have strong cross peaks with the A₃₈ H2. The CH₃ proton

resonances have medium cross peaks with the C₃₂ H1' and H5 and A₃₇ H2 resonances and very weak cross peaks with the U₃₂ and G₃₄ H1', G₃₄ H8, and A₃₆ H2 resonances.

Although the i⁶A₃₇-modified molecules share several structural features with the unmodified and ψ ₃₉-modified molecules, the ribose ring puckers differ significantly. All residues of the i⁶A₃₇-modified molecules have the 3'-endo sugar pucker except G₃₄ which displays a mixed 2'-endo/3'-endo conformation. This ribose conformation may favor the major groove residency of the U₃₅ base. The torsion angles of the phosphate backbone are largely within the range common for RNA helices. The greatest variability centers on the phosphate backbone angles α , β , and ϵ in the i⁶A₃₇-modified molecules. In [i⁶A₃₇]-ASL^{Tyr}, α and ϵ of G₃₄ are *trans* and *gauche*, respectively, and α , β , and ϵ of U₃₅ are not typical of A-form helical geometry. In [i⁶A₃₇, ψ ₃₉]-ASL^{Tyr}, β of U₃₃ approaches the *trans* configuration whereas α and ϵ of G₃₄ and α and γ of U₃₅ are outside the standard A-form values.

Structure of the ASL^{Tyr} stems

The conformations of the stems of the four forms of ASL^{Tyr} are very similar. The geometry of the hairpins from base pairs G₂₇-C₄₃ to C₃₂-A₃₈ is primarily A-form (Figure 6). H6/8-H6/8 and H6/8-H1',H2' NOEs from long and short mixing time NOESY spectra, respectively, are continuous (where not overlapped in [ψ ₃₉]-ASL^{Tyr}). The A₃₁-U₃₉/ ψ ₃₉ base pairs are sufficiently stable so that clear cross-strand NH-H2 NOEs can be observed, although the imino H3 resonances are weakened by solvent exchange in the presence of Co(NH₃)₆³⁺. While spectra for only the unmodified ASL^{Tyr} demonstrate the presence of a stable protonated C₃₂-A⁺₃₈ base pair, NOEs involving C₃₂ and A₃₈ and the exchange broadened A₃₈ C2 in [ψ ₃₉]-ASL^{Tyr} suggest that pseudouridylation does not fully disrupt the base pair. The torsion angles of the sugar-phosphate backbones of all molecules are within the limits of A-form geometry and are supported by chemical shift, J-coupling, and NOE data.

Comparison of the ASL structures

The anticodon loops of the ASL^{Tyr} molecules range in size from three to seven nucleotides. Surprisingly, the global conformations of the anticodon nucleotides are not dramatically altered by the ψ ₃₉ and i⁶A₃₇ modifications. The A₃₆ base is positioned on the minor groove side of the helix and stacks beneath the A₃₇ base. The U₃₅ base is positioned on the major groove side of the helix and is approximately co-planar with the base of G₃₄, which also resides on the major groove side of the helix (Figure 7). The ribose puckers of the anticodon nucleotides do change with base modification, especially U₃₅ and A₃₆ of the i⁶A₃₇-modified molecules that adopt of the C3'-endo conformation common to the A-form helix. To examine the range of loop structure effects that result from the ribose ring pucker, simulations were performed for each of the molecules while restraining the ribose rings of U₃₅ and A₃₆ to C2'-endo and C3'-endo conformations. The C2'-endo pucker results in migration of the U₃₅ base to the minor groove side of the helix in approximately 25% of the unmodified ASL^{Tyr} and [ψ ₃₉]-ASL^{Tyr} structures. The U₃₅ base was found not to occupy the minor groove in either of the i⁶A₃₇-modified molecules. Although only ASL^{Tyr} contains the well-defined C₃₂-A₃₈⁺ base pair the C₃₂ and A₃₈ bases remain largely coplanar and show minimal lateral displacement in the modified molecules. The loops appear to be stabilized primarily by stacking and hydrophobic interactions because there is no spectral evidence of hydrogen bonding among the anticodon bases, although the ASL^{Tyr} may contain an A₃₇ N6H₂-U₃₃ O2 interaction.

Although the four ASL molecules each adopt well-ordered structures, the loops do not contain the signature U-turn motif (Figure 9). In a U-turn, the sequential U₃₃ H1' to G₃₄ H8 distance is ≥ 6 Å and would lead to a break of 1'-base NOE connectivities whereas the non-

sequential U₃₃ H1' to U₃₅ H6 distance is ≈ 3.8 Å and should give rise to a moderately intense NOE. For three of four ASL^{Tyr} molecules, the sequential 1'-base NOEs are continuous, albeit weak at the U₃₃-G₃₄ step, and none contain the non-sequential U₃₃-U₃₅ interaction. Many of the NOEs predicted for a molecule containing a U-turn are a subset of the NOEs observed for the ASL^{Tyr} molecules and do not conflict with the refined structures. However, key NOEs present in the spectra of the ASL^{Tyr} molecules (U₃₃ H1' to A₃₈ H2, G₃₄ H1' to A₃₇ H2, and U₃₅ H1' to A₃₆ H2) are not compatible with the U-turn motif. These NOEs lead to a loop structure in which the phosphate backbone does not turn abruptly between U₃₃ and G₃₄, as observed in the crystalline forms of yeast and *E. coli* tRNA^{Phe} 47, 50, 51, 52 (Figure 9), but instead turns between G₃₄ and U₃₅. The turn of the phosphate backbone is maintained even when the unusual α and ζ torsion angles observed for the U-turn motif were applied during a separate set of constrained MD simulations. Another feature characteristic the U-turn motif is the *trans* conformation of backbone torsion angle α between U₃₃ and G₃₄. The ³¹P resonance corresponding to this phosphate is located in the main cluster of ³¹P peaks (Figure 5), a position not consistent with the *trans* conformation of torsion angle α . Notably, the α or ζ angles in all of the converged structures occupy the *gauche*-conformation.

Discussion

The tRNA molecules of all cells undergo base modification. However, there is limited information on how many of these modifications affect the structure and thermal stability of tRNA molecules, specifically in the anticodon stem-loop. In this study, we examined the effects of modification to positions 37 and 39, which have been shown to improve the base stacking of the 3' side of the stem and stabilize tRNA-mRNA interactions by improving intra-strand stacking within tRNA anticodon loops and inter-strand stacking between codon and anticodon bases, respectively. Here the dimethylallyl and pseudouridine modifications at positions 37 and 39 have been studied in the sequence context of the anticodon stem-loop of *B. subtilis* tRNA^{Tyr}.

Comparison with structures of other anticodon stem-loop sequences

The U-turn motif and the functional significance of this conformation of the anticodon loop for translation was first revealed by the crystal structure of fully modified yeast tRNA^{Phe} 47, 51, 52. It has recently been shown that the anticodon arm of unmodified *E. coli* tRNA^{Phe} adopts the same fold in the crystalline state⁵⁰ (Figure 9). The nearly 180° reverse of the phosphate backbone between U₃₃ and G₃₄ and concomitant stacking of the anticodon nucleotide bases, residues 34, 35, and 36, on the 3' side of the loop, primes the tRNA for pairing with the mRNA codon on the ribosome²¹. The U-turn is stabilized in these molecules by the anticodon base stack and by hydrogen bonds from the U₃₃ 2'-OH to the A₃₅ N7 and from the U₃₃ N3H to the phosphoryl oxygen 5' to A₃₆. In the crystal structure of *E. coli* tRNA^{Cys} (with the anticodon sequence 5'-GCA-3'), U₃₃ and C₃₅ form a hydrogen bond that stabilizes the U-turn. The exocyclic NH₂ group of C₃₅ donates a proton to the U₃₃ O2' while maintaining the ability of C₃₅ to form Watson-Crick base pair interactions with the mRNA⁵³. In modified *E. coli* tRNA^{Lys} ASL (with the anticodon mnm^{5s2}-UUU), the U₃₃ NH-O1P hydrogen bond is maintained, but no interaction between U₃₃ and U₃₅ was identified¹⁸.

Although the U-turn motif is prevalent in the anticodon arm of the crystal structures of tRNA molecules, in solution the *B. subtilis* unmodified and [ψ ₃₉]-, [i⁶A₃₇]-, and [ψ ₃₉, i⁶A₃₇]-ASL^{Tyr} molecules do not form this conserved structural motif. Our data indicate that the unmodified and partially modified molecules are composed of a five-base pair stem with a seven-nucleotide loop or a six-base pair stem with a five-nucleotide loop. Similar to the *B. subtilis* unmodified and [ψ ₃₉]-ASL^{Tyr}, the anticodon arms of the unmodified and ψ ₃₉-

modified human ASL^{Lys},³ the unmodified *E. coli* ASL^{Phe}, and the unmodified *E. coli* tRNA^{Val} lack a defined U-turn structure^{15, 54, 55}. However, the imino spectrum of the unmodified *E. coli* tRNA^{Val} molecule in the presence of metal ion was similar to the native *E. coli* tRNA^{Val}, indicating that a U-turn was probably present⁵⁵. Interestingly, the addition of i⁶A₃₇ and metal ion in the context of the *E. coli* ASL^{Phe} molecule resulted in the formation of a U-turn³³. Similarly, introducing N6-threonylcarbamoyl-A₃₇ (t⁶A₃₇) into the *E. coli* ASL^{Lys} in the presence of ψ_{39} at pH 7 with metal ion promotes the structure toward the U-turn conformation¹⁸. These data would suggest base modifications are critical for the formation of the archetypical U-turn motif. However, this trend does not continue with the *B. subtilis* ASL^{Tyr} as the introduction of the i⁶A₃₇ modification alone or with ψ_{39} in the presence or absence of metal ions does not lead to a U-turn (Figure 9). Similarly, the introduction of t⁶A₃₇ at pH 5.0 into the human ASL^{Lys},³ which has the same nucleotide sequence as *E. coli* ASL^{Lys}, does not result in a U-turn¹⁷. Also analogous to the [i⁶A₃₇]-ASL^{Tyr} and [i⁶A₃₇, ψ_{39}]-ASL^{Tyr} molecules, a C₃₂-A⁺₃₈ base pair is disrupted by the t⁶A₃₇ modification. Unlike the [i⁶A₃₇]-ASL^{Tyr} and [i⁶A₃₇, ψ_{39}]-ASL^{Tyr} molecules, though, the human and *E. coli* ASL^{Lys} molecules have no cross-strand NOE's from the t⁶A₃₇^{17, 18}. These results indicate that modification at residue 37 may be necessary to form the U-turn, but is not sufficient in all cases.

The structures of i⁶A₃₇-modified *B. subtilis* ASL^{Tyr} and *E. coli* ASL^{Phe} demonstrate the sensitivity of the conformational effects of the i⁶A₃₇ modification to sequence context. The nucleotide sequences of these two molecules are very similar. The *E. coli* ASL^{Phe} anticodon sequence is 5'-GAA-3' and residue 32 is a uridine. The i⁶A₃₇ modification of *E. coli* ASL^{Phe} and metal ion binding lead to a stable U-turn fold³³, but these two elements are not sufficient to drive U-turn formation in the *B. subtilis* ASL^{Tyr}. Like the C₃₂-A⁺₃₈ base pair of ASL^{Tyr}, the U₃₂-A₃₈ base pair of *E. coli* ASL^{Phe} is disrupted by the modification, and suggests the structural difference between the ASL^{Phe} and ASL^{Tyr} anticodon loops arises mainly from the base identity of nucleotide 35. The stacking energy for the purine base of ASL^{Phe} is more favorable than for the pyrimidine base of ASL^{Tyr}. Also, when paired with the adenine nucleotide of the codon, the O4 atom of U₃₅ is not positioned to hydrogen bond with the U₃₃ 2'-OH²¹, although this positional restriction may be relaxed outside the context of the ribosome. The lack of a U-turn conformation with the i⁶A₃₇-modification may offer a role for the queuosine modification at position 34 (Q₃₄) in ASL^{Tyr}. The Q₃₄ base contains a dihydroxycyclopentene ring (Figure 1) that lends three hydrogen bond donors and substantial bulk to residue 34. Although the functional groups introduced by Q₃₄ are not positioned to form additional hydrogen bonds with the Watson-Crick edge of the paired codon base, these attributes may increase the intra-loop base-sugar or base-base hydrogen bond network among the loop nucleotides. The bulkiness of the Q₃₄ base also introduces additional stacking surface for the U₃₅ base. Q₃₄ at the 5' end of the anticodon loop may then function with the i⁶A₃₇ modification at the 3' end to stabilize stacking of the anticodon bases and re-mold the architecture of the loop.

Computational methods have been used to study the conformational features of N⁶-dimethylallyl adenine. The methyl groups in the [i⁶A₃₇]-ASL^{Tyr} and [ψ_{39} , i⁶A₃₇]-ASL^{Tyr} are oriented in a downward direction (Figure 7). This position of the methyl groups was found to be a low energy configuration of the dimethylallyl group in the absence of the ms² modification⁵⁶. The addition of ms² results in an upward orientation of the methyl groups⁵⁶. This may improve base stacking along the 3' side of the loop and along with Q₃₄, promote the formation of a U-turn.

Effects of ψ_{39} and i⁶A₃₇ modification on the stability of the anticodon loop

Addition of ψ_{39} to the ASL^{Tyr} molecule did not significantly alter the structure from the unmodified molecule. Residue U₃₃ is stacked between C₃₂ and G₃₄ in both molecules and,

unlike the *E. coli* ASL^{Phe} and human ASL^{Lys},³ molecules that form compact tri-loop structures, the ASL^{Tyr} molecules form a five (5) base loops that are well ordered.

The addition of ψ to the ASL^{Tyr} RNA hairpin increased the stability of the stem by 5.7 °C, which is similar to the effect observed in the human ASL^{Lys},³ in low salt, acidic conditions. The ΔH of melting of the [ψ_{39}]-ASL^{Tyr} hairpin obtained from DSC measurements was 1.07 kcal/mol per base pair higher than the unmodified molecule. The increase of the ΔH of melting is most likely due to the water-mediated hydrogen bond between the pseudouridine and the phosphate backbone⁴⁴. Additionally, ψ_{39} increases the stability of the 31-39 base pair¹⁵ and contributes to the overall enthalpy. Although the transition event occurring at lower temperatures (<50°C) in the UV curve is not as robust as in the unmodified ASL^{Tyr} hairpin, the [ψ_{39}]-ASL^{Tyr} UV melting curve does suggest that the [ψ_{39}]-ASL^{Tyr} has a well stacked loop. This finding is consistent with the structure of the [ψ_{39}]-ASL^{Tyr} hairpin. However, the ψ_{32} modification in the context of the *E. coli* ASL^{Phe} hairpin only increases the T_m of the molecule by 3.5 °C⁵⁴, suggesting a pseudouridine at the stem-loop junction confers greater stability than when located in the loop proper with the water-mediated hydrogen bond to the phosphate backbone intact⁵⁴.

The dimethylallyl modification of A₃₇ N6 opens up the helix proximal to the anticodon loop as evidenced by deprotonation of A₃₈ and loss of the U₃₉ H3 resonance. Broadening of base and sugar-phosphate backbone resonances of nucleotides in and near the loop indicate that i⁶A₃₇ destabilizes the structure of this region of the ASL^{Tyr} molecules and increases nucleotide mobility. The increased mobility in this region appears to be the result of steric clash between the modification and cross-strand nucleotides. The position of the dimethylallyl group in the helix leads to NOEs from dimethylallyl methyl protons to C₃₂ and U₃₃ base protons as well as disruption of the C₃₂-A⁺₃₈ base pair. While the i⁶A₃₇ modification increases the dynamics of nucleotides within the loop region, the basic fold of the modified RNA hairpin in the presence of metal ion remains largely unchanged.

Although the dimethylallyl modification of A₃₇ decreases the compact nature of the loop nucleotides, the DSC data indicate that the modification increases the stability of the hairpin stem by 3.7 °C and the ΔH by 1.39 kcal/mol. The absence of transitions at lower temperatures (< 50°C) in the UV spectra confirms that the dimethylallyl modification is disrupting base stacking in the loop. A lower temperature transition is observed in the i⁶ modified ASL^{Trp} molecule¹⁶ and the addition of the dimethylallyl group improved the stability of the hairpin compared to the unmodified form of the molecule¹⁶. Interestingly, the t⁶A₃₇ modification in the human ASL^{Lys},³ at pH 6.0 does not significantly affect the T_m or the thermal stability of the molecule compared to the unmodified molecule¹⁷. At pH 5.0, the T_m of the t⁶A₃₇ molecule was elevated $\approx$ 2 °C, but the thermal stability was comparable to the unmodified human ASL^{Lys},³¹⁷, which parallels the melting data obtained from the [i⁶A₃₇]-ASL^{Tyr} molecule. Conversely, at pH 7.2 the t⁶A₃₇ modification decreases the T_m as well as the thermal stability, as does the i⁶A modification in the *E. coli* ASL^{Phe}^{17, 33}.

Significance of the pseudouridine and dimethylallyl modifications for translation

One of the most common modifications found in tRNA molecules is the pseudouridine modification. The pseudouridine synthase TruA catalyzes the isomerization of uridines 38, 39, and 40 in the anticodon arms of bacterial tRNA to form the C₁-C₅ glycosidic bond and introduces the additional NH functional group in the base⁵⁷. When located in a helical stem, pseudouridine confers additional stability to the helix by forming a water-mediated hydrogen bond to the phosphate backbone through the N1 NH group. The addition of pseudouridine at position 39 leads to increased stability and stacking at the anticodon arm stem-loop junction^{15, 54}. Presumably, the increased stability conferred by the ψ at this location enhances translation efficiency and improves growth rate compared to tRNA

molecules lacking the ψ modification^{58, 59}. In *Saccharomyces cerevisiae*, loss of the $\psi_{38/39}$ modifications in tRNA^{Tyr}, tRNA^{Lys}, and tRNA^{Trp} impairs decoding of the amber UAG stop codon and decreases read-through efficiency during translation resulting in decreased growth rate⁵⁸. In *Pseudomonas aeruginosa*, mutation of the *truA* gene leads to cells deficient in expressing functional type III secretion genes and are unable to sustain virulence²⁸.

The importance of the dimethylallyl modification of A₃₇ for tRNA function has been well-studied in *E. coli*^{5, 6, 7, 9, 10, 27} and modification of A₃₇ to i⁶A₃₇ is necessary to form the hypermodified base, ms²i⁶A₃₇ through the activity of MiaB²³. In *B. subtilis*, tRNA^{Tyr} can exist in two forms^{60, 61, 62}, the modified i⁶A₃₇ and the hypermodified ms²i⁶A₃₇. In stationary-phase cells, the hypermodified form predominates whereas in exponential growth phase cells, the single-modified form of tRNA^{Tyr} is the dominant form⁶⁰. The hypermodified form is better able to decode the UAC codon⁶⁰ and the presence of the thiomethyl modification correlates with the onset of sporulation⁶².

In *Salmonella typhimurium*, modification of A₃₇ to 2-methylthio-N⁶-(4-hydroxyisopentenyl) adenosine (ms²i⁶A) can increase translational efficiency. Suppression of the amber codon by tRNA^{Tyr}, tRNA^{Gln}, and tRNA^{Ser} containing ms²i⁶A₃₇ exhibited decreased codon context sensitivity, particularly when flanked on the 3' side by an adenine or a cytidine⁶. Readthrough of amber codons followed by an adenine increases in the absence of the hypermodified A₃₇⁶. Interestingly, the loss of ψ_{39} in suppressor tRNA^{Tyr} and tRNA^{Gln} decreases the translational efficiency of the molecules by two- and 10-fold, respectively⁶. However, the absence of ψ_{39} does not alter the codon context sensitivity of tRNA^{Tyr} and tRNA^{Gln}⁶, indicating that the ms²i⁶A₃₇ and m²A₃₇ modifications are the principle forces in mitigating mistranslation caused by codon context but are not sufficient for maintaining translation efficiency.

Materials and Methods

All enzymes were purchased from Sigma Chemical, except for enzymes T7 RNA polymerase and MiaA, which were prepared as described^{63, 64}. Deoxyribonuclease I type II, pyruvate kinase, adenylate kinase, and nucleotide monophosphate kinase were obtained as powders, dissolved in 15% glycerol, 1 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.4, and stored at -20 °C. Guanylate kinase and nuclease P1 were obtained as solutions and stored at -20 °C. Unlabeled 5' nucleoside triphosphates (5'-NTPs) were purchased from Sigma, phosphoenolpyruvate (potassium salt) was purchased from Bachem, and 99% [¹⁵N]-ammonium sulfate and 99% [¹³C]-methanol were purchased from Cambridge Isotope Labs. γ , γ -dimethylallyl diphosphate was purchased from Sigma.

Preparation of RNA samples

The RNA sequence for *B. subtilis* ASL^{Tyr} shown in Figure 1 was synthesized *in vitro* using T7 RNA polymerase and a synthetic DNA template. The nucleotide sequence of the stem corresponds to residues G₂₇-C₄₃ of full-length *B. subtilis* ASL^{Tyr}. Unlabeled RNA molecules were prepared from 10 ml transcription reactions using 4 mM 5'-NTPs. Isotopically labeled RNA molecules were prepared from 10 ml transcription reactions using 3 mM uniformly [¹³C-¹⁵N]-enriched 5'-NTPs as described^{65, 66}. The RNA molecules were purified by passage through 20% (w/v) preparative polyacrylamide gels, electroeluted (Schleicher & Schuell), and precipitated with ethanol. The purified oligonucleotides were dissolved in 1.0 M NaCl, 20 mM potassium phosphate, pH 6.1, and 2.0 mM EDTA, and dialyzed extensively against 10 mM KCl, 10 mM potassium phosphate, pH 6.1, and 0.02 mM EDTA, using a Centricon-3 concentrator (Amicon Inc.). The samples were diluted with buffer to a volume of 300 ml and lyophilized to a powder. For experiments involving the

non-exchangeable protons, the samples were exchanged twice with 99.9% D₂O and then resuspended in 300 µl of 99.96% D₂O. For experiments involving detection of the exchangeable protons, the samples were resuspended in 300 ml of 90% H₂O / 10% D₂O. The samples contained 50-110 A₂₆₀ OD units of RNA oligonucleotide in 300 ml (≈1.2–2.5 mM). The [ψ_{39}]-ASL^{Tyr} oligonucleotide was chemically synthesized (Dharmacon, Boulder, CO), ethanol precipitated after deprotection, and dialyzed as described above.

Preparation of the A₃₇ (N6)–dimethylallyl modified ASL^{Tyr}

The dimethylallyl modification was introduced at position A₃₇ of purified ASL^{Tyr} and [ψ_{39}]-ASL^{Tyr} oligonucleotides using the enzyme MiaA dimethylallyl diphosphate:tRNA transferase (MiaA) and dimethylallyl diphosphate (DMAPP). Histidine-tagged MiaA was expressed in *E. coli* and purified using Ni²⁺ affinity resin as described⁶³. The typical modification reaction was performed using 50 µM oligonucleotide, 125 µM DMAPP, 50mM Tris-HCl pH 7.6, 10mM MgCl₂, 0.1 mg/ml BSA, 5mM β-mercapto-ethanol, and 26 µg/ml MiaA. The reactions were incubated overnight at 30 °C. Upon completion, the solution was extracted with phenol:chloroform:isomyl alcohol (25:24:1) and chloroform, and the RNA precipitated with ethanol. The reactions were monitored using 20% denaturing PAGE. The N6-modified oligonucleotides migrate slower than unmodified ASL^{Tyr} and leads to a band shift equivalent to ~1 additional nucleotide. Using base ¹³C-¹H cross peaks that shift upon modification, no unmodified material could be detected in HSQC spectra of the N6-modified samples.

NMR spectroscopy

All spectra were acquired on Varian Inova 500 MHz (¹H- [¹³C, ¹⁵N, ³¹P] probe) and 600 MHz (¹H- [¹³C, ¹⁵N] cryoprobe) spectrometers. Solvent suppression for ¹H homonuclear spectra collected in 90% H₂O was achieved using the binomial scheme. The data points were extended by 25% using linear prediction for the indirectly detected dimensions. NMR spectra were processed and analyzed using Felix 2007 (Felix NMR Inc., San Diego, CA).

Two-dimensional (2D) ¹³C-¹H HSQC spectra were collected to identify ¹³C-¹H chemical shift correlations. Sugar spin systems were assigned using 3D HCCH-TOCSY (16 ms and 24 ms DIPSI-3 spin lock) experiments collected in D₂O. A 3D HCCH-TOCSY (56 ms DIPSI-3 spin lock) was collected to establish the intra-base H2-C2-C8-H8 correlations in adenine residues. A 3D HCN experiment⁶⁷ was used to identify intra-residue base-ribose correlations.

Sequential assignments and distance constraints for the non-exchangeable resonances were derived at 25 °C from 2D ¹H-¹H NOESY spectra (τ_m = 120, 180, and 460 ms) and 3D ¹³C-edited NOESY spectra (τ_m = 180 and 400 ms). Pyrimidine C2 and C4 resonances were assigned from H6-C2 and H5-C4 correlations using 2D H(CN)C and 2D CCH-COSY experiments. 2D ¹⁵N-¹H HSQC spectra optimized for 2-bond HN couplings were collected to identify purine N7 and adenine N1 and N3 resonances. For the exchangeable resonances, 2D ¹⁵N-¹H HSQC spectra were collected to identify ¹⁵N-¹H chemical shift correlations. 2D NOESY spectra (τ_m = 160 and 360 ms) were acquired in H₂O and at 12 °C to obtain distance restraints involving exchangeable protons.

³J_{H-H} and ³J_{P-H} coupling constants were estimated using DQF-COSY and ³¹P-¹H experiments, respectively. ³J_{C-P} coupling constants were estimated for ASL^{Tyr} and [⁶A₃₇]-ASL^{Tyr} using the ¹³C-¹H ct-HSQC spin-echo difference method⁶⁸.

Distance and torsion angle constraints

Interproton distance estimates were obtained from cross peak intensities in 2D NOESY and 3D ^{13}C -edited NOESY spectra. Using the covalently fixed pyrimidine H5-H6 distance (≈ 2.4 Å) and the conformationally restricted sugar H1'-H2' distance (2.8-3.0 Å) as references, peak intensities were classified as strong, medium, weak, or very weak and their corresponding proton pairs given upper bound distance constraints of 3.0, 4.0, 5.0, or 6.0 Å, respectively. Cross peaks observed only at mixing times ≥ 180 ms were classified as extremely weak and given 7.0 Å upper bound distance constraints to account for the possibility of spin diffusion. All distance constraints were given lower bounds of 0.0 Å to improve convergence of the calculations. Only the intra-residue sugar-to-sugar constraints involving H5' and H5'' resonances included in the calculations are considered conformationally restrictive. Distance constraints involving exchangeable protons were estimated from 360 ms mixing time NOESY spectra and were classified as weak, very weak, or extremely weak.

Watson-Crick base pairs were identified by observation of a significantly downfield shifted NH or NH₂ proton resonance and the observation of strong G-C NH-NH₂ or A-U H2-NH NOEs. The C₃₂-A⁺₃₈ interaction was based on the A₃₈ C2 upfield chemical shift ⁶⁹. Hydrogen bonds were introduced as distance restraints of 2.9 ± 0.3 Å between donor and acceptor heavy atoms and 2.0 ± 0.2 Å between acceptor and hydrogen atoms.

Ribose ring pucker and backbone dihedral constraints were derived from $^3J_{\text{HH}}$, $^3J_{\text{HP}}$, and $^3J_{\text{CP}}$ couplings ⁷⁰. Ribose rings with $^3J_{\text{H1}'\text{-H2}'}$ > 7 Hz and with C3' and C4' resonances between 76-80 and 85-86 ppm, respectively, were constrained to C2'-*endo*. Residues with $^3J_{\text{H1}'\text{-H2}'}$ < 5 Hz and C3' resonances between 70-74 ppm were constrained to C3'-*endo*. Residues with intermediate $^3J_{\text{H1}'\text{-H2}'}$ couplings were left unconstrained. For residues 27-33 and 37-43, γ was constrained to the *gauche*⁺ conformation ($50 \pm 40^\circ$) ⁷⁰. The NOE intensities for these residues of ASL^{Tyr} and [⁶A₃₇]-ASL^{Tyr} with resolved H4'-H5' and H4'-H5' NOE cross peaks are consistent with the *gauche*⁺ conformation. γ was left unconstrained for the anticodon residues. The conformations of the γ torsion angles of [ψ_{39}]-ASL^{Tyr} and [⁶A₃₇, ψ_{39}]-ASL^{Tyr} from G₂₇-C₃₂ and A₃₈-C₄₃ could not be confirmed as *gauche*⁺ due to spectral overlap, but the conservation of ¹H chemical shifts between the corresponding structures, NOE patterns, and ³¹P-¹H scalar couplings support the regular A-form geometry. Dihedral angle restraints for the β and ϵ torsion angles were derived from $^3J_{\text{P-H5'}}$, $^3J_{\text{P-H5'}}$, and $^3J_{\text{P-H3'}}$ couplings estimated in 2D ³¹P-¹H HetCor spectra and $^3J_{\text{P-C2'}/\text{P-C4'}}$ couplings measured in 2D ctHSQC spin-echo difference spectra. For stem residues, β was constrained to the *trans* conformation ($180 \pm 40^\circ$). β was loosely constrained to the *trans* conformation ($180 \pm 70^\circ$) for loop residues except G₃₄-A₃₇. ϵ was constrained to the *trans* conformation ($-150 \pm 50^\circ$) for residues with $^3J_{\text{P-H3'}}$ > 5 Hz or $^3J_{\text{P-C2'}}$ > 5 Hz. P-C2' couplings of ASL^{Tyr} and [⁶A₃₇]-ASL^{Tyr} indicate that the ϵ angles of G₂₇-C₃₂ and A₃₈-C₄₂ have the A-form *trans* conformation. α and ζ were constrained to $-70 \pm 30^\circ$ for the stem residues (G₂₇-C₃₂ and A₃₈-C₄₃). Because a down-field shifted ³¹P resonance is associated with the *trans* conformation of α or ζ , and because no such shift is observed for any of the ³¹P resonances in the RNA molecules, α and ζ were loosely constrained to exclude the *trans* conformation ($0 \pm 120^\circ$) for residues U₃₃-A₃₇. No dihedral angle constraints were used for the glycosidic angle χ .

Structure calculations and refinement

An initial set of structures was calculated using a shortened version of the simulated annealing protocol (described below). A list of all proton pairs in the calculated structures closer than 5.0 Å (representing expected NOEs) was compared to the list of constraints. The NOESY spectra were then re-examined for predicted NOEs absent from the constraint list.

In some cases, this allowed the unambiguous assignment of previously unidentified NOEs, but, in other cases, the predicted NOEs were unobservable due to spectral overlap or the broadening of resonances by exchange with solvent.

Structure refinement was carried out with simulated annealing and restrained molecular dynamics (rMD) calculations using Xplor-NIH v2.19⁷¹. Starting coordinates for ASL^{Tyr} were generated using Insight II (Accelrys, San Diego, CA) and were based on standard A-form helical geometry. Starting coordinates for the [ψ_{39}]-ASL^{Tyr} model were created by atom replacement within the ASL^{Tyr} model coordinate file. Starting coordinates for the [i^6A_{37}]-ASL^{Tyr} and [ψ_{39} , i^6A_{37}]-ASL^{Tyr} models were created by adding dimethylallyl groups to the A_{37} N6 atoms of the ASL^{Tyr} and [ψ_{39}]-ASL^{Tyr} models, respectively, using Insight II. Parameters for the ψ_{39} and i^6A_{37} residues were obtained from⁵⁶. The structure calculations were performed in two stages. Beginning with the energy minimized starting coordinates, 50 structures were generated using 80 ps of rMD at 1200K with hydrogen bond, NOE-derived distance and base-pairing restraints. The system then was cooled to 25 K over 12 ps of rMD. Force constants used for the calculations were increased from 2 kcal mol⁻¹ Å⁻² to 30 kcal mol⁻¹ Å⁻² for the NOE and from 2 kcal mol⁻¹ rad⁻² to 30 kcal mol⁻¹ rad⁻² for the dihedral angle constraints. Each structure was then minimized with constraints at the end of the rMD. Ten structures were selected for the final refinement. The criteria for final structure selection included lowest energies, fewest constraint violations, and fewest predicted unobserved NOEs (¹H pairs less than 3.5 Å apart, but no corresponding cross peak in the NOE spectra). A second round of rMD was performed on these structures using protocols similar to those used in the first round of structure calculation. The major difference was the starting temperature of 300 K followed by cooling to 25 K over 28 ps of rMD. Ten refined structures for each model were analyzed using Xplor-NIH and Insight II.

Thermal stability

UV melting studies were performed using 0.5 A_{260} units RNA hairpin dissolved in NMR buffer (10 mM NaCl, 10 mM potassium phosphate, pH 6.3, and 0.05 mM EDTA). The samples were heated to 90°C for 90 s and snap cooled on ice before each melting experiment. A_{260} absorbance spectra from 10-92 °C were acquired (1.0 °C per minute) on a Pharmacia Ultrospec 2000 UV-Visible spectrophotometer equipped with a peltier melting apparatus. The melting curves were acquired in triplicate and averaged.

Differential scanning calorimetry

Measurements were performed using a VP-DSC differential scanning microcalorimeter (MicroCal, LLC, Northampton, MA) at a scan rate of 90°C h⁻¹. RNA hairpin concentration was 10 μM in low salt NMR buffer (10mM Phosphate Buffer, 10 mM KCL, 0.02 mM EDTA) at pH 6.3. Before measurements, the RNA hairpins were heated to 90°C for 90 s and snap cooled on ice. Following the cooling, the sample and buffer were degassed for 15 min at room temperature. During the heating cycles a pressure of 2 atm was maintained in the cells to prevent degassing. A background scan collected with buffer in both cells was subtracted from each scan. Data were analyzed with Origin 7.0 software (MicroCal) to obtain the transition temperature (T_m), the calorimetric enthalpy of thermal unfolding (ΔH), and the van't Hoff enthalpy. The ITC measurements were performed in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DMAPP	dimethylallyl diphosphate
ASL^{Tyr}	anticodon stem-loop of <i>B. subtilis</i> tRNA ^{Tyr} , GUA
[i⁶A₃₇]-ASL^{Tyr}	A ₃₇ (N ⁶ -dimethylallyl)-modified anticodon stem-loop of <i>B. subtilis</i> tRNA ^{Tyr} , GUA
MiaA	dimethylallyl (Δ^2 -isopentenyl) diphosphate:tRNA transferase
ms²i⁶A	(2-thiomethyl, N ⁶ -dimethylallyl)-adenine
NTP	nucleoside triphosphate
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	NOE spectroscopy
DQF-COSY	double quantum filtered correlated spectroscopy
2D	two dimensional
3D	three dimensional
HetCor	heteronuclear correlation
HMQC	heteronuclear multiple quantum coherence
HSQC	heteronuclear single quantum coherence
MD	molecular dynamics
RMSD	root mean square deviation
NH	imino
NH₂	amino

References

1. Björk, GR. Biosynthesis and function of modified nucleosides. In: Söll, D.; RajBhandary, U., editors. tRNA: Structure, Biosynthesis, and Function. ASM Press; Washington, D.C.: 1995. p. 165-206.
2. Garcia, GA.; Goodenough-Lashua, DM. Mechanisms of modifying enzymes. In: Grosjean, H.; Benne, R., editors. Modification and Editing of RNA: the alteration of RNA structure and function. ASM Press; Washington, D.C.: 1998.
3. Winkler, ME. Genetics and regulation of tRNA and rRNA modification. In: Grosjean, H.; Benne, R., editors. Modification and editing of RNA: the alteration of RNA structure and function. ASM Press; Washington, D.C.: 1998.
4. Agris PF. The importance of being modified: Roles of modified nucleosides and Mg²⁺ in RNA structure and function. Prog Nucleic Acid Res Mol Biol. 1996; 53:79-129. [PubMed: 8650309]
5. Diaz I, Pedersen S, Kurland CG. Effects of *miaA* on translation and growth rates. Mol Gen Genet. 1987; 208:373-6. [PubMed: 3312947]

6. Ericson JU, Bjork GR. tRNA anticodons with the modified Nucleoside 2-methylthio-N6-(4-hydroxyisopentenyl)adenosine distinguish between bases-3' of the codon. *J Mol Biol.* 1991; 218:509–16. [PubMed: 2016742]
7. Hagervall TG, Ericson JU, Esberg B, Ji-nong L, Björk GR. Role of tRNA modification in translational fidelity. *Biochim Biophys Acta.* 1990; 1050:263–6. [PubMed: 2207153]
8. Harrington KM, Nazarenko IA, Dix DB, Thompson RC, Uhlenbeck OC. In vitro analysis of translational rate and accuracy with an unmodified tRNA. *Biochemistry.* 1993; 31:7617–22. [PubMed: 7688564]
9. Björnsson A, Isaksson LA. UGA codon context which spans three codons. Reversal by ms²i⁶A₃₇ in tRNA, mutation in *rpsD*(S4) or streptomycin. *J Mol Biol.* 1993; 232:1017–29. [PubMed: 8371264]
10. Bouadloun F, Srichaiyo T, Isaksson LA, Björk GR. Influence of modification next to the anticodon in tRNA on codon context sensitivity of translational suppression and accuracy. *J Bacteriol.* 1986; 166:1022–7. [PubMed: 3086285]
11. Hall KB, McLaughlin LW. Properties of a U1/mRNA 5' splice site duplex containing pseudouridine as measured by thermodynamic and NMR methods. *Biochemistry.* 1991; 30:1795–801. [PubMed: 1993194]
12. Donmez G, Hartmuth K, Luhrmann R. Modified nucleotides at the 5' end of human U2 snRNA are required for spliceosomal E-complex formation. *RNA.* 2004; 10:1925–33. [PubMed: 15525712]
13. Bilbille Y, Vendeix FAP, Guenther R, Malkiewicz A, Ariza X, Vilarrasa J, Agris PF. The structure of the human tRNA^{Lys3} anticodon bound to the HIV genome is stabilized by modified nucleosides and adjacent mismatch base pairs. *Nucleic Acids Res.* 2009; 37:3342–53. [PubMed: 19324888]
14. Persaud C, Lu Y, Vila-Sanjurjo A, Campbell JL, Finley J, O'Connor M. Mutagenesis of the modified bases, m⁵U₁₉₃₉ and ψ ₂₅₀₄, in *Escherichia coli* 23S rRNA. *Biochem Biophys Res Comm.* 2010; 392:223–7. [PubMed: 20067766]
15. Durant PC, Davis DR. Stabilization of the anticodon stem-loop of tRNA^{Lys,3} by an A⁺-C base-pair and by pseudouridine. *J Mol Biol.* 1999; 285:115–31. [PubMed: 9878393]
16. Kierzek E, Kierzek R. The thermodynamic stability of RNA duplexes and hairpins containing N6-alkyladenosines and 2-methylthio-N6-alkyladenosines. *Nucleic Acids Res.* 2003; 31:4472–80. [PubMed: 12888507]
17. Stuart JW, Gdaniec Z, Guenther R, Marszalek M, Sochacka E, Malkiewicz A, Agris PF. Functional anticodon architecture of human tRNA^{Lys3} includes disruption of intraloop hydrogen bonding by the naturally occurring amino acid modification, t⁶A. *Biochemistry.* 2000; 39:13396–404. [PubMed: 11063577]
18. Sundaram M, Durant PC, Davis DR. Hypermodified nucleosides in the anticodon of tRNA^{Lys} stabilize a canonical U-turn structure. *Biochemistry.* 2000; 39:12575–84. [PubMed: 11027137]
19. Durant PC, Bajji AC, Sundaram M, Kumar RK, Davis DR. Structural effects of hypermodified nucleosides in the *Escherichia coli* and human tRNA^{Lys} anticodon loop: the effect of nucleosides s²U, mcm⁵U, mcm⁵s²U, mnm⁵s²U, t⁶A, and ms²t⁶A. *Biochemistry.* 2005; 44:8078–89. [PubMed: 15924427]
20. Tworowska I, Nikonowicz EP. Base pairing within the ψ ₃₂, ψ ₃₉-modified anticodon arm of *Escherichia coli* tRNA^{Phe}. *J Am Chem Soc.* 2006; 128:15570–1. [PubMed: 17147349]
21. Weixlbaumer A, Murphy F. V. t. Dziargowska A, Malkiewicz A, Vendeix FA, Agris PF, Ramakrishnan V. Mechanism for expanding the decoding capacity of transfer RNAs by modification of uridines. *Nat Struct Mol Biol.* 2007; 14:498–502. [PubMed: 17496902]
22. Cantara WA, Crain PF, Rozenski J, McCloskey JA, Harris KA, Zhang X, Vendeix FA, Fabris D, Agris PF. The RNA modification database, RNAMDB: 2011 update. *Nucleic Acids Res.* 2011; 39:D195–201. [PubMed: 21071406]
23. Björk, GR. Modified nucleosides at positions 34 and 37 of tRNAs and their predicted coding capacities. In: Grosjean, H.; Benne, R., editors. *Modification and Editing of RNA*. ASM Press; Washington, D.C.: 1998. p. 577-582.
24. Motorin Y, Bec G, Tewari R, Grosjean H. Transfer RNA recognition by the *Escherichia coli* Δ^2 -isopentenyl-pyrophosphate:tRNA Δ^2 -isopentenyl transferase: dependence on the anticodon arm structure. *RNA.* 1997; 3:721–33. [PubMed: 9214656]

25. Sprinzl M, Horn C, Brown M, Ioudovitch A, Steinberg S. Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res.* 1998; 26:148–53. [PubMed: 9399820]
26. Xie W, Zhou C, Huang RH. Structure of tRNA dimethylallyltransferase: RNA modification through a channel. *J Mol Biol.* 2007; 367:872–81. [PubMed: 17292915]
27. Connolly DM, Winkler ME. Genetic and physiological relationships among the *miaA* gene, 2-methylthio-N⁶-(Δ^2 -isopentenyl)-adenosine tRNA modification, and spontaneous mutagenesis in *Escherichia coli* K-12. *J Bacteriol.* 1989; 171:3233–46. [PubMed: 2656644]
28. Ahn KS, Ha UW, Jia JH, Wu DH, Jin SG. The *truA* gene of *Pseudomonas aeruginosa* is required for the expression of type III secretory genes. *Microbiology-Sgm.* 2004; 150:539–47.
29. Meier F, Suter B, Grosjean H, Keith G, Kubli E. Queuosine modification of the wobble base in tRNA^{His} influences *in vivo* decoding properties. *EMBO J.* 1985; 4:823–7. [PubMed: 2988936]
30. Dineshkumar TK, Thanedar S, Subbulakshmi C, Varshney U. An unexpected absence of queuosine modification in the tRNAs of an *Escherichia coli* B strain. *Microbiology.* 2002; 148:3779–87. [PubMed: 12480882]
31. Kersten H. The nutrient factor queuine: biosynthesis, occurrence in transfer RNA and function. *Biofactors.* 1988; 1:27–9. [PubMed: 3076430]
32. Cabello-Villegas J, Winkler ME, Nikonowicz EP. Solution conformations of unmodified and A₃₇ N⁶-dimethylallyl modified anticodon stem-loops of *Escherichia coli* tRNA^{Phe}. *J Mol Biol.* 2002; 319:1015–34. [PubMed: 12079344]
33. Cabello-Villegas J, Tworowska I, Nikonowicz EP. Metal ion stabilization of the U-turn of the A₃₇ N⁶-dimethylallyl-modified anticodon stem-loop of *Escherichia coli* tRNA^{Phe}. *Biochemistry.* 2004; 43:55–66. [PubMed: 14705931]
34. Yarian CS, Basti MM, Cain RJ, Ansari G, Guenther RH, Sochacka E, Czerwinska G, Malkiewicz A, Agris PF. Structural and functional roles of the N1- and N3-protons of ψ at tRNA's position 39. *Nucleic Acids Res.* 1999; 27:3543–9. [PubMed: 10446245]
35. Henkin TM. Transcription termination control in bacteria. *Curr Opin Microbiol.* 2000; 3:149–53. [PubMed: 10745002]
36. Henkin TM. Riboswitch RNAs: using RNA to sense cellular metabolism. *Genes Dev.* 2008; 22:3383–90. [PubMed: 19141470]
37. Henkin TM, Yanofsky C. Regulation by transcription attenuation in bacteria: how RNA provides instructions for transcription termination/antitermination decisions. *Bioessays.* 2002; 24:700–7. [PubMed: 12210530]
38. Grundy FJ, Henkin TM. Conservation of a transcription antitermination mechanism in aminoacyl-tRNA synthetase and amino acid biosynthesis genes in gram-positive bacteria. *J Mol Biol.* 1994; 235:798–804. [PubMed: 8289305]
39. Grundy FJ, Hodil SE, Rollins SM, Henkin TM. Specificity of tRNA-mRNA interactions in *Bacillus subtilis* *tyrS* antitermination. *J Bacteriol.* 1997; 179:2587–94. [PubMed: 9098057]
40. Vander Meulen KA, Davis JH, Foster TR, Record MT Jr. Butcher SE. Thermodynamics and folding pathway of tetraloop receptor-mediated RNA helical packing. *J Mol Biol.* 2008; 384:702–17. [PubMed: 18845162]
41. Pardi A. Multidimensional heteronuclear NMR experiments for structure determination of isotopically labeled RNA. *Methods Enzymol.* 1995; 261:350–80. [PubMed: 8569503]
42. Dieckmann T, Feigon J. Assignment methodology for larger RNA oligonucleotides: application to an ATP-binding RNA aptamer. *J Biomol NMR.* 1997; 9:259–72. [PubMed: 9204555]
43. Legault P, Pardi A. *In situ* probing of adenine protonation in RNA by ¹³C NMR. *J Amer Chem Soc.* 1994; 116:8390–1.
44. Arnez JG, Steitz TA. Crystal structure of unmodified tRNA^{Gln} complexed with glutamyl tRNA synthetase and ATP suggests a possible role for pseudouridines in stabilization of RNA structure. *Biochemistry.* 1994; 33:7560–7. [PubMed: 8011621]
45. Kellogg GW, Schweitzer BI. Two- and three-dimensional ³¹P-driven NMR procedures for complete assignment of backbone resonances in oligodeoxyribonucleotides. *J Biomol NMR.* 1993; 3:577–95. [PubMed: 8219742]
46. Laing LG, Gluick TC, Draper DE. Stabilization of RNA structure by Mg²⁺ ions - specific and nonspecific effects. *J Mol Biol.* 1994; 237:577–587. [PubMed: 8158638]

47. Shi HJ, Moore PB. The crystal structure of yeast phenylalanine tRNA at 1.93 angstrom resolution: A classic structure revisited. *RNA*. 2000; 6:1091–105. [PubMed: 10943889]
48. Allain FH, Varani G. Structure of the P1 helix from group I self-splicing introns. *J Mol Biol*. 1995; 250:333–53. [PubMed: 7608979]
49. Kieft JS, Tinoco I. Solution structure of a metal-binding site in the major groove of RNA complexed with cobalt (III) hexammine. *Structure*. 1997; 5:713–21. [PubMed: 9195889]
50. Byrne RT, Konevega AL, Rodnina MV, Antson AA. The crystal structure of unmodified tRNA^{Phe} from *Escherichia coli*. *Nucleic Acids Res*. 2010; 38:4154–62. [PubMed: 20203084]
51. Robertus JD, Ladner JE, Finch JT, Rhodes D, Brown RS, Clark BFC, Klug A. Structure of yeast phenylalanine tRNA at 3 Å resolution. *Nature*. 1974; 250:546–51. [PubMed: 4602655]
52. Kim SH, Suddath FL, Quigley GJ, Mcpherso A, Sussman JL, Wang AHJ, Seeman NC, Rich A. 3-Dimensional tertiary structure of yeast phenylalanine tRNA. *Science*. 1974; 185:435–40. [PubMed: 4601792]
53. Nissen P, Thirup S, Kjeldgaard M, Nyborg J. The crystal structure of Cys-tRNA^{Cys}-EF-Tu-GDPNP reveals general and specific features in the ternary complex and in tRNA. *Structure*. 1999; 7:143–56. [PubMed: 10368282]
54. Cabello-Villegas J, Nikonowicz EP. Solution structure of ψ_{32} -modified anticodon stem-loop of *Escherichia coli* tRNA^{Phe}. *Nucleic Acids Res*. 2005; 33:6961–71. [PubMed: 16377777]
55. Vermeulen A, McCallum SA, Pardi A. Comparison of the global structure and dynamics of native and unmodified tRNA^{Val}. *Biochemistry*. 2005; 44:6024–33. [PubMed: 15835891]
56. Tewari R. Conformational preferences of modified nucleic acid bases N6-(Δ^2 -isopentenyl) adenine and 2-methylthio-N6-(Δ^2 -isopentenyl) adenine by the quantum chemical PCICO calculations. *Int J Quantum Chem*. 1988; 34:133–42.
57. Hur S, Stroud RM. How U38, 39, and 40 of many tRNAs become the targets for pseudouridylation by TruA. *Mol Cell*. 2007; 26:189–203. [PubMed: 17466622]
58. Lecointe F, Namy O, Hatin I, Simos G, Rousset JP, Grosjean H. Lack of pseudouridine 38/39 in the anticodon arm of yeast cytoplasmic tRNA decreases *in vivo* recoding efficiency. *J Biol Chem*. 2002; 277:30445–53. [PubMed: 12058040]
59. Charette M, Gray MW. Pseudouridine in RNA: What, where, how, and why. *IUBMB Life*. 2000; 49:341–51. [PubMed: 10902565]
60. Menichi B, Heyman T. Study of tyrosine tRNA modification in relation to sporulation in *Bacillus subtilis*. *J Bacteriol*. 1976; 127:268–80. [PubMed: 819420]
61. Vold BS. Post-transcriptional modifications of anticodon loop region - alterations in isoaccepting species of tRNAs during development in *Bacillus subtilis*. *J Bacteriol*. 1978; 135:124–32. [PubMed: 97263]
62. Buu A, Menichi B, Heyman T. Thiomethylation of tyrosine tRNA is associated with initiation of sporulation in *Bacillus subtilis* - effect of phosphate concentration. *J Bacteriol*. 1981; 146:819–22. [PubMed: 6163767]
63. Leung HCE, Chen YQ, Winkler ME. Regulation of substrate recognition by the MiaA tRNA prenyltransferase modification enzyme of *Escherichia coli* K-12. *J Biol Chem*. 1997; 272:13073–83. [PubMed: 9148919]
64. Davanloo P, Rosenberg AH, Dunn JJ, Studier FW. Cloning and expression of the gene for bacteriophage-T7 RNA-polymerase. *Proc Natl Acad Sci*. 1984; 81:2035–39. [PubMed: 6371808]
65. Nikonowicz EP, Sirr A, Legault P, Jucker FM, Baer LM, Pardi A. Preparation of ¹³C and ¹⁵N Labeled RNAs for heteronuclear multidimensional NMR-studies. *Nucleic Acids Res*. 1992; 20:4507–13. [PubMed: 1383927]
66. Batey RT, Inada M, Kujawinski E, Puglisi JD, Williamson JR. Preparation of isotopically labeled ribonucleotides for multidimensional NMR-spectroscopy of RNA. *Nucleic Acids Res*. 1992; 20:4515–23. [PubMed: 1383928]
67. Marino JP, Diener JL, Moore PB, Griesinger C. Multiple-quantum coherence dramatically enhances the sensitivity of CH and CH₂ correlations in uniformly C-13-labeled RNA. *J Amer Chem Soc*. 1997; 119:7361–66.
68. Legault P, Jucker FM, Pardi A. Improved measurement of ¹³C, ³¹P J coupling constants in isotopically labeled RNA. *FEBS Lett*. 1995; 362:156–60. [PubMed: 7720863]

69. Simorre JP, Zimmermann GR, Mueller L, Pardi A. Triple-resonance experiments for assignment of adenine base resonances in C-13/N-15-labeled RNA. *J Amer Chem Soc.* 1996; 118:5316–17.
70. Varani G, Aboulela F, Allain FHT. NMR investigation of RNA structure. *Prog Nucl Mag Reson Spec.* 1996; 29:51–127.
71. Schwieters CD, Kuszewski JJ, Tjandra N, Clore GM. The Xplor-NIH NMR molecular structure determination package. *J Mag Reson.* 2003; 160:65–73.

Figure 1.

(A) Sequence and modified nucleotide positions corresponding to the anticodon arm of *B. subtilis* tRNA^{Tyr}_{GUA}. Nucleotide numbering corresponds to the full-length tRNA molecule. ψ, pseudouridine; Q, queuosine; A*, (2-thiomethyl, N6-dimethylallyl)-adenine. (B) Unmodified form of the parent sequence used in this study. The chemical structures of 2-thiomethyl, N6-dimethylallyl-adenine (ms²i⁶A), pseudouridine, and queuosine are shown.

Figure 2.

(A) Overlay of the DSC curves of unmodified (red), [ψ₃₉]-ASL^{Tyr} (black), [i⁶A₃₇]-ASL^{Tyr} (green), and [i⁶A₃₇, ψ₃₉]-ASL^{Tyr} (blue). The raw normalized excess heat capacity relative to buffer-only solution is plotted. (B) UV melting curves of the unmodified and modified hairpins using the same color scheme as A. The unmodified and [ψ₃₉]-ASL^{Tyr} molecules appear to exhibit two melting transitions—the broad lower temperature transitions presumably correspond to destacking of the loop nucleotide bases. The apparent T_m 's based on UV melting profiles of unmodified, [ψ₃₉]-ASL^{Tyr}, [i⁶A₃₇]-ASL^{Tyr}, and [i⁶A₃₇, ψ₃₉]-ASL^{Tyr} are 70.0 °C, 75.7 °C, 73.7 °C, and 76.1 °C, respectively.

Figure 3.

Comparison of two-dimensional ^{13}C - ^1H HSQC spectra of the base C6/8 and C2 regions of (A) unmodified ASL^{Tyr}, (B) [$i^6\text{A}_{37}$, ψ_{39}]-ASL^{Tyr}, and (C) [$i^6\text{A}_{37}$, ψ_{39}]-ASL^{Tyr} saturated with 2 mM $\text{Co}(\text{NH}_3)_6^{3+}$ RNA molecules at pH 6.4. The sequence-specific resonance assignments are shown. Spectra B and C were acquired at natural abundance and therefore do not display C5 coupling to the pyrimidine C6 resonances. The chemical shift of the A_{38} C2 resonance is diagnostic of protonation and indicates the presence of a stable A_{38} - C_{32} base pair. The A_{38} C2H2 cross peak is significantly weakened by chemical exchange upon modification (B), but appears with near unit intensity in C. Addition of $\text{Co}(\text{NH}_3)_6^{3+}$ stabilizes the anticodon loop of [$i^6\text{A}_{37}$, ψ_{39}]-ASL^{Tyr} and leads to the appearance of the A_{37} C8H8 resonance that is lost to exchange broadening with modification. The *in vitro* transcription reaction of $^{13}\text{C}/^{15}\text{N}$ -labeled unmodified ASL^{Tyr} was primed with unlabeled 5'-GMP, therefore the G_1 C8H8 does not appear in A.

Figure 4.

Comparison of the base-1' proton regions and sequential connectivities in NOE spectra of the ASL^{Tyr} molecules. Two-dimensional NOESY-HSQC spectra with selective excitation of the C1' nuclei to suppress pyrimidine H5 resonances of (A) unmodified ASL^{Tyr} and (C) [⁶A₃₇]-ASL^{Tyr}. Standard two-dimensional NOESY spectra are shown for the unlabeled molecules (B) [ψ₃₉]-ASL^{Tyr} and (D) [⁶A₃₇, ψ₃₉]-ASL^{Tyr}. The correlations between U₃₃ and G₃₄ are weak in all spectra, except D where the box indicates the absence of the cross peak. The H1' resonances of ψ₃₉ are shifted upfield to 4.35 and 4.40 ppm in B and D, respectively, due to substitution of the C1'-N1 glycosidic bond (uridine) with a C1'-C5 glycosidic bond (pseudouridine).

Figure 5.

Sequential H6/8-P connectivities in the ^{31}P - ^1H HeteroTOCSY-NOESY spectrum of $[\psi_{39}]$ -ASL^{Tyr}. The connectivity is disrupted between U₃₃ and G₃₄. This inter-residue step is also weak in the NOESY spectrum (Figure 4B). All ^{31}P resonances cluster between -3.4 and -4.5 ppm, supporting the [g, g] conformation of all α , ζ torsional angles. The ^{31}P resonances of the other forms of ASL^{Tyr} have similar distributions and were assigned using the HCP experiment (unmodified ASL^{Tyr} and $[i^6\text{A}_{37}]$ -ASL^{Tyr}) or the HetCor experiment ($[i^6\text{A}_{37}, \psi_{39}]$ -ASL^{Tyr}).

Figure 6. Superposition of 8 converged structures of (A) unmodified, (B) [ψ_{39}]-ASL^{Tyr}, (C) [i^6A_{37}]-ASL^{Tyr}, and (D) [i^6A_{37} , ψ_{39}]-ASL^{Tyr} RNA hairpins. Views are into the major grooves of the anticodon loops. The RMSDs between the individual structures and the average structure are listed in Table 1. The greatest variability occurs among the anticodon bases and reflects the lower number of constraints for these residues, especially for the G₃₄ and U₃₅ bases.

Figure 7.

Stereoviews of the loop regions of structure of (A) unmodified, (B) [ψ_{39}]-ASL^{Tyr}, (C) [i^6A_{37}]-ASL^{Tyr}, and (D) [i^6A_{37} , ψ_{39}]-ASL^{Tyr} RNA hairpins. The coloring scheme as follows: the anticodon bases G₃₄, U₃₅, and A₃₆ (*blue*), C₃₂ and A₃₈ (*pink*), A₃₈ (*orange*), A₃₈ (*red*), ψ_{39} (*brown*), dimethylallyl modification of A₃₇ (*green*). The base of U₃₅ is depicted in the orientation most frequently observed among the converged structures, although orientations of the base parallel and perpendicular to the helix axis are represented in all four molecules.

Figure 8.

H6-C2 regions of H(CN)C spectra of (A) unmodified ASL^{Tyr} and (B) [i⁶A₃₇]-ASL^{Tyr} and H5-C4 regions of CCH-COSY spectra of (C) unmodified ASL^{Tyr} and (D) [i⁶A₃₇]-ASL^{Tyr}. The base C2 resonance is sensitive to the electronic state of the O2 atom and tends to display a downfield chemical shift with the formation of an intra-molecular hydrogen bond. The C2

chemical shifts of U₃₃ and U₃₅ suggest hydrogen bond participation of the O2 atoms. The structures show a hydrogen bond between U₃₃ O2 and A₃₇ N6H₂ that is also consistent with the A₃₇ N6 chemical shift. Only the intra-residue 2'-OH is suggested as a hydrogen bond partner for U₃₅ O2. Although these uridine resonances remain downfield in [i⁶A₃₇]-ASL^{Tyr}, the binding of Co(NH₃)₆³⁺ in the loop and possible coordination involving the O2 atoms introduces an alternative cause of the shifts. The weakening of the protonated A₃₈-C₃₂ base pair indicated by the A₃₈ C2 chemical shifts upon modification (Figures 3 and S1) is also reflected in the C₃₂ C4 chemical shifts.

Figure 9.

Comparison of the anticodon loop regions of (A) solution NMR structure of unmodified *E. coli* tRNA^{Phe} (PDB 1KKA), (B) X-ray crystal structure of *E. coli* tRNA^{Phe} (PDB 1IBL), (C) solution NMR structure of fully modified human tRNA^{Lys,3} (PDB 1FL8), and (D) solution NMR structure of *B. subtilis* [⁶A₃₇, ψ_{39}]-modified tRNA^{Tyr}. The U-turn fold is present in B and C and is characterized by stacking of the anticodon bases (blue) along the minor groove side of the helix and the NH-P hydrogen bond from U₃₃ (orange). Molecules A and D do not form this same fold and maintain minor groove positions for residues 34 and 35 with stacking of U₃₃ and G₃₄ bases. Fully modified *E. coli* tRNA^{Phe} contains ms² i⁶A₃₇ and ψ_{32} .

Table 1

Summary of experimental distance and dihedral angle constraints and refinement statistics for ASL^{Tyr} molecules

Constraint	ASL ^{Tyr}	[ψ 39]- ASL ^{Tyr}	[i^6 A ₃₇]- ASL ^{Tyr}	[i^6 A ₃₇ , ψ 39]- ASL ^{Tyr}
NOE distance constraints				
intraresidue ^a	110	109	110	109
interresidue	157	178	178	164
mean number per residue	15.7	16.8	16.9	16
NOE constraints by category				
very strong (1.8 - 3.0 Å)	12	8	8	9
strong (1.8 - 4.0 Å)	35	35	28	28
medium (1.8 - 5.0 Å)	114	142	147	163
weak (1.8 - 6.0 Å)	79	57	74	62
very weak (1.8 - 7.0 Å)	16	15	18	22
base pair constraints				
total	34	30	28	28
dihedral angle constraints				
ribose ring ^b	48	48	48	48
backbone	70	70	70	70
mean number per residue	6.9	6.9	6.9	6.9
Violations				
average distance constraints > 0.6 Å ^c	4.0	2.0	4.2	3.2
average dihedral constraints > 0.5° ^d	2.0	2.7	1.2	1.3
RMSD from ideal geometry^e				
Heavy Atoms (Å)	0.36 ± 0.15	0.38 ± 0.05	0.56 ± 0.17	1.09 ± 0.18

^a Only conformationally restrictive constraints are included.

^b Three torsion angles within each ribose ring were used to constrain the ring to either the C2'-endo or C3'-endo conformation as described in the text. The ring pucker of residue G34 was not constrained for the reported structures.

^c A distance violation of 0.6 Å corresponds to 5.0 kcal energy penalty.

^d A dihedral angle violation of 0.5° corresponds to 0.05 kcal energy penalty.

^e Calculated against the minimized average structure.