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Solution NMR analyses of the anticodon arms of proteinogenic and non-proteinogenic tRNA^{Gly}

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Abstract

Although the fate of most tRNA molecules in the cell is aminoacylation and delivery to the ribosome, some tRNAs are destined to fulfill other functional roles. In addition to their central role in translation, tRNA molecules participate in processes such as regulation of gene expression, bacterial cell wall biosynthesis, viral replication, antibiotic biosynthesis, and suppression of alternative splicing. In bacteria, glycyl-tRNA molecules with anticodon sequences GCC and UCC exhibit multiple extra-translational functions including transcriptional regulation and cell wall biosynthesis. We have determined the high-resolution structures of three glycyl-tRNA anticodon arms with anticodon sequences GCC and UCC. Two of the tRNA molecules are proteinogenic (tRNA^{Gly,GCC} and tRNA^{Gly,UCC}) and the third is non-proteinogenic (np-tRNA^{Gly,UCC}) and participates in cell wall biosynthesis. The UV-monitored thermal melting curves show that the anticodon arm of tRNA^{Gly,UCC} with a loop-closing C-A⁺ base pair melts at a 10 °C lower temperature than those of tRNA^{Gly,GCC} or np-tRNA^{Gly,UCC}. U-A and C-G pairs close the loops of the later two molecules and enhance stem stability. Mg²⁺ stabilizes the tRNA^{Gly,UCC} anticodon arm and lessens the T_m differential. The structures of the three tRNA^{Gly} anticodon arms exhibit small differences between one another, but none of them form the classical U-turn motif. The anticodon loop of tRNA^{Gly,GCC} becomes more dynamic and disordered in the presence of multivalent cations, whereas metal ion coordination in the anticodon loops of tRNA^{Gly,UCC} and np-tRNA^{Gly,UCC} establishes conformational homogeneity. The conformational similarity of the molecules is greater than their functional differences might suggest. Because aminoacylation of the full-length tRNA molecules is accomplished by one tRNA synthetase, the similar structural context of the loop may facilitate efficient recognition of each of the anticodon sequences.

Keywords

Heteronuclear NMR spectroscopy; U-turn motif; *Staphylococcus aureus*; T box riboswitch; peptidoglycan synthesis

Introduction

The functional importance of tRNA extends beyond its core cellular role in translation and relies both on its capacity to deliver amino acids and on its structure. One of these extra-translational roles involves the T box mechanism of transcription attenuation in Gram

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Accession Numbers

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Supporting information

A table listing the chemical shifts of the three RNA sequences is available free of charge via the Internet at <http://pubs.acs.org>.

positive bacteria. In this regulatory mechanism, tRNA molecules directly mediate the expression of several genes whose products are involved in amino acid metabolism in order to maintain a balanced pool of aminoacyl-tRNAs (aa-tRNAs) ^{1,2}. tRNA molecules also play a key role in the synthesis of the peptidoglycan cell wall in bacteria ^{3,4}. The short peptides that cross link the glycan moieties of the cell wall matrix are synthesized in a non-ribosome catalyzed peptidyltransferase reaction that uses aa-tRNA molecules as substrates. tRNA molecules function to prime the reverse transcription reaction of retroviruses and retrovirus-like elements. The primer initiation role of tRNA^{Lys,3} is vital for replication of the RNA genome of human immunodeficiency virus 1 (HIV-1) ^{5,6} and tRNA^{Pro} serves a similar function in murine leukemia virus (MuLV) replication ⁷. Interestingly, the primer binding sites (PBS) of members of the murine retrovirus-like VL30 family also contain segments complementary to the 3' end of tRNA^{Gly} that can functionally replace the native prolyl PBS and sustain tRNA^{Gly}-dependent MuLV growth ^{7,8}. Other tRNA-dependent mechanisms in the cell include phospholipid modification of bacterial membranes in response to environmental changes ^{9,10}, antibiotic synthesis in species of *Streptomyces* ^{11,12}, and the suppression of alternative splicing of pre-mRNA in the nucleus by tRNA^{iMet} variants ^{13,14}.

Glycyl-tRNA (tRNA^{Gly}) is one of the tRNA families with multiple extra-translational roles. In many Gram positive bacteria, expression of the gene encoding glycyl-tRNA synthetase is regulated by the T box riboswitch mechanism and tRNA^{Gly} with anticodon GCC (tRNA^{Gly,GCC}) serves as a sensor molecule ¹⁵ (Figure 1A). This transcription attenuation mechanism is sensitive to the ratio of charged tRNA to uncharged tRNA in the cell. The 5' untranslated region of the mRNA sequence-specifically binds tRNA molecules and forms one of two alternative hairpin secondary structures, the terminator hairpin and the antiterminator hairpin, depending upon the charge state of the bound tRNA. T box riboswitch selection of the appropriate tRNA molecule for regulation of the downstream gene occurs through pairing of the Specifier codon nucleotides with the nucleotides of the tRNA anticodon. Additional base pairing involving the universally conserved U₃₃ of tRNA also has been proposed ¹⁶. In many bacteria including *Bacilli* and *Staphylococcus* species, the glycyl T box riboswitch is specified by the codon 5'-GGC-3' which is complementary to the tRNA^{Gly} isoacceptor tRNA^{Gly,GCC}, but the Specifier codon 5'-GGA-3' also is represented and would be predicted to bind the isoacceptor tRNA^{Gly,UCC} ¹⁷ (Figure 1B). Binding of the non-cognate glycyl-tRNA isoacceptor tRNA^{Gly,UCC} to the 5'-GGC-3' Specifier sequence cannot be excluded and may contribute to regulation of the *glyQS* operon.

Peptidoglycan cell wall biosynthesis in bacteria involves a non-ribosomal peptidyltransferase mechanism that utilizes aminoacylated tRNA molecules as substrates for the peptide polymerization reaction. The peptidyltransferase enzymes FemABX catalyze the formation of short homopolymers that cross-link the glycan moieties and increase rigidity of the cell wall ^{18,19}. A glycyl-tRNA, first identified and sequenced in *Staphylococcus* species, participates in cell wall synthesis but is not involved in ribosome-catalyzed protein synthesis and is designated a non-proteinogenic glycyl-tRNA (np-tRNA^{Gly}) ^{3,4,20}. These np-tRNA^{Gly} molecules bear the anticodon sequence 5'-UCC-3' and are charged by glycyl-tRNA synthetase. The np-tRNA^{Gly} has a cytidine at position 37 (Figure 1C) rather than the purine found in proteinogenic glycyl-tRNA molecules and the U₃₄ base is not modified as it is in the proteinogenic tRNA^{Gly,UCC} of many bacteria ²¹ (Figure 1). The *Staphylococcal* np-tRNA^{Gly} molecules contain A₄₉-U₆₅ and A₅₁-U₆₃ base pairs at the base of the T-arm rather than the G₄₉-U₆₅ and G₅₁-C₆₃ base pairs found in most proteinogenic tRNA molecules. In *Thermus thermophilus*, substitution of the A₅₁-U₆₃ base pair for G₅₁-C₆₃ results in the loss of a direct contact between E390 of EF-Tu and the amino group of G₅₁, that substantially weakens affinity for elongation factor Tu (EF-Tu) ²²⁻²⁴. This weakened affinity for EF-Tu

limits participation of np-tRNA^{Gly} in ribosomal protein synthesis and presumably ensures a stable pool of proteinogenic tRNA^{Gly} for translation during cell wall biosynthesis^{20, 24}.

Glycine is a member of a four-codon box family, a set of four codons that designate the same amino acid and whose first two nucleotides are the same. In bacteria, these boxes are read by up to three different tRNA isoacceptors. Species of *Bacillus* and *Staphylococcus* use two tRNA^{Gly} isoacceptors, with the anticodon sequences 5'-U^{*}CC-3' and 5'-GCC-3' where U^{*} is a modified uridine (Figure 1). Modifications on U₃₄ can lead to opposite functional effects, enhancement of the ability of U to wobble or restriction of wobbling and enhancement of discrimination²⁵. In the case of lysine, which occupies a mixed-codon box, U₃₄ is modified to 5-methylaminomethyl-2-thiouridine (mnm⁵s²U, Figure 1) and pairing is restricted to A and G. The U₃₄ modification uridine 5-oxoacetic acid (cmo⁵U, Figure 1) allows a single tRNA isoacceptor to decode at least three valine codons in bacteria^{21, 26}. However, modification of U₃₄ is not always needed for enhanced wobbling. In *Mycoplasma mycoides* and in mitochondria and chloroplast, one tRNA isoacceptor with anticodon sequence 5'-UCC-3' reads all four glycine codons with equal efficiency²⁷⁻²⁹. Notably, 5'-GGA-3' and 5'-GGU-3' combined represent ~95% of glycine codons used in *M. mycoides* whereas these codons are used ~75% of the time in *S. aureus* or *B. subtilis*.

tRNA molecules interact with a variety of proteins and other RNA molecules in the cell to fulfill a multitude of functional roles. To examine possible contributions of structural diversity for glycine anticodon stem-loop sequences, the anticodon arms of tRNAs from *S. aureus* that participate in three cellular functions, (translation, regulation, and cell wall assembly) were selected (Figure 1). The tRNA^{Gly,GCC} participates in transcriptional regulation and translation but contains no base modifications. The tRNA^{Gly,UCC} participates in translation and in many organisms contains a U₃₄ base modification, but in some organisms, such as *M. mycoides*, is not modified. The nucleotide sequences of these anticodon loops are the same in *B. subtilis* and *M. mycoides*. The np- tRNA^{Gly,UCC} participates in cell wall biosynthesis and does not contain base modifications. These selected glycyl-tRNA isoacceptors also allow examination of the possible structural influences of base type at positions 34 and 37. We have determined the structures of these anticodon arms (Figure 1). Although the participation of the np-tRNA^{Gly,UCC} in translation is likely to be restricted by low affinity for EF-Tu, the pyrimidine-37 residue of np-tRNA^{Gly,UCC} and other np-tRNAs may limit contributions of these molecules to other anticodon-dependent processes. The structures of the tRNA^{Gly} anticodon arms differ from one another, but none of them form the classical U-turn motif seen in some tRNA anticodon arms. All of these RNA molecules form stems with at least five base pairs. The anticodon loop of tRNA^{Gly,GCC} becomes more dynamic and disordered in the presence of multivalent cations, whereas the anticodon loops of tRNA^{Gly,UCC} and np-tRNA^{Gly,UCC} become more structurally ordered by these ions. Although the U-turn is integral to ribosomal codon-anticodon pairing, it is not known if this motif is required for T box regulation or cell wall biosynthesis. A more dynamic loop structure may better accommodate the different functional roles of tRNA^{Gly}.

Materials and Methods

All enzymes were purchased from Sigma-Aldrich, except for enzymes T7 RNA polymerase, which was prepared as described³⁰. 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-Aldrich,

phosphoenolpyruvate (potassium salt) was purchased from Bachem, and 99% [^{15}N]-ammonium sulfate and 99% [$^{13}\text{C}_6$]-glucose were purchased from Isotec.

Preparation of RNA samples

The RNA sequences for the ASL^{Gly} molecules shown in Figure 1 were synthesized *in vitro* using T7 RNA polymerase and a synthetic DNA template. The nucleotide sequence of the stem corresponds to residues 27–43 of full-length tRNA^{Gly} molecules. To facilitate transcription using T7 RNA polymerase, the first three base pairs of the stems were modified from the native sequences. 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 [^{13}C - ^{15}N]-enriched 5'-NTPs as described³¹. 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 6.2, and 2.0 mM EDTA, and dialyzed extensively against 10 mM NaCl, 10 mM potassium phosphate, pH 6.2, and 0.02 mM EDTA, using a Centricon-3 concentrator (Amicon Inc.). The samples were diluted with buffer to a volume of 0.33 ml and lyophilized to a powder. For experiments involving the non-exchangeable protons, the ASL^{Gly,GCC} and ASL^{Gly,UCC} samples were exchanged twice with 99.9% D₂O and then resuspended in 0.33 ml of 99.96% D₂O and annealed. The ASL^{Gly,UCC} sample was mixed with Co(NH₃)₆³⁺ dissolved in D₂O to a final concentration of 2.0 mM Co(NH₃)₆³⁺. After annealing, the np-ASL^{Gly,UCC} sample was dialyzed against low salt buffer containing 3.0 mM MgCl₂ in 99.9% D₂O. For experiments involving detection of the exchangeable protons, the samples were dialyzed against appropriate buffer (2.0 mM Co(NH₃)₆³⁺ or 3.0 mM MgCl₂) containing 90% H₂O/10% D₂O. The samples contained 30–100 A₂₆₀ OD units of RNA oligonucleotide in 0.33 ml ($\approx$ 0.4–1.5 mM).

NMR spectroscopy

Spectra were acquired on Varian Inova 500 MHz (^1H -[^{13}C , ^{15}N , ^{31}P] probe) and 600 MHz and 800 MHz (^1H -[^{13}C , ^{15}N] 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) ^{13}C - ^1H HSQC spectra were collected to identify ^{13}C - ^1H chemical shift correlations. Sugar spin systems were assigned using 3D HCCH-TOCSY (8 ms and 24 ms DIPSI-3 spin lock) experiments collected in D₂O. 2D HCN experiments were used to identify intra-residue base-ribose correlations. Pyrimidine C2 and C4 resonances were assigned from H6-C2 and H5-C4 correlations using 2D H(CN)C and 2D CCH-COSY experiments and a 2D H(N)CO experiment for np-ASL^{Gly,UCC} uridine NH-[C2, C4] resonances^{32–34}. 2D ^{15}N - ^1H HSQC spectra optimized for 2-bond HN couplings were collected to identify purine N7 and adenine N1 and N3 resonances. 2D ^{15}N - ^1H HSQC spectra were collected to identify ^{15}N - ^1H chemical shift correlations. Sequential assignments and distance constraints for the non-exchangeable resonances were derived at 25 °C from 2D ^1H - ^1H NOESY spectra (t_m = 90, 180, and 420 ms) and 3D ^{13}C -edited NOESY spectra (t_m = 180 and 400 ms). Assignments and distance constraints for the exchangeable resonances were derived at 12 °C from 2D NOESY spectra (t_m = 160 and 360 ms) acquired in 90% $^1\text{H}_2\text{O}$.

$^3J_{H-H}$ and $^3J_{P-H}$ coupling constants were estimated using DQF-COSY and ^{31}P - 1H experiments, respectively. $^3J_{C-P}$ coupling constants also were estimated for np-ASL^{Gly,UCC} using the CECT-HCP experiment.³⁵

Distance and torsion angle constraints

Interproton distances were estimated 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 3.2, 4.2, 5.2, or 6.2 Å, respectively. Cross peaks observed only at mixing times 180 ms were classified as extremely weak and given 7.2 Å upper bound distance constraints to account for the possibility of spin diffusion. All distance constraints were given lower bounds of 1.8 Å. 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 medium, 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 and by the chemical shifts of non-protonated base ^{15}N and ^{13}C carbonyl resonances. 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 $^3J_{HH}$, $^3J_{HP}$, and $^3J_{CP}$ couplings³⁶. Residues with $^3J_{H1'-H2'} < 5$ Hz and C3' resonances between 70–74 ppm were constrained to C3'-*endo*. Ribose rings with $^3J_{H1'-H2'} \approx 5$ Hz and with C3' and C4' resonances between 74–76 and 84–86 ppm, respectively, were left unconstrained. The angle δ was constrained as $85^\circ \pm 30^\circ$ and $160^\circ \pm 30^\circ$ for C3'-*endo* and C2'-*endo* sugars respectively. For residues 27–33 and 37–43, γ was constrained to the *gauche*⁺ conformation ($60 \pm 20^\circ$)³⁶. γ was left unconstrained for the anticodon residues. Dihedral angle restraints for the β and ϵ torsion angles were derived from $^3J_{P-H5'}$, $^3J_{P-H5''}$, and $^3J_{P-H3'}$ couplings estimated in 2D ^{31}P - 1H HetCor spectra and $^3J_{P-C2'}/P-C4'$ couplings measured in 2D ctHSQC spin-echo difference spectra. For stem residues, β was constrained to the *trans* conformation ($180 \pm 20^\circ$) if $^3J_{P-C4'}$ was > 5 Hz. Σ was constrained to the *trans* conformation ($-150 \pm 20^\circ$) for residues with $^3J_{P-C2'} < 5$ Hz and $^3J_{P-C4'} > 5$ Hz. α and ζ were constrained to $-65 \pm 20^\circ$ for the stem residues 27–31 and 39–43. Because a down-field shifted ^{31}P resonance is associated with the *trans* conformation of α or ζ , and because no such shift is observed for any of the ^{31}P resonances in the RNA molecules, α and ζ were loosely constrained to exclude the *trans* conformation ($0 \pm 120^\circ$) for residues 32–38. Although all base 6/8-1' intra-residue NOE cross peak intensities support the *anti* configuration about the glycosidic bond, no dihedral angle constraints were used for the 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 obscured due to spectral overlap.

Structure refinement was carried out with simulated annealing and restrained molecular dynamics (rMD) calculations using Xplor-NIH v2.19³⁷. Starting coordinates for ASL^{Gly} were generated using Insight II (Accelrys, San Diego, CA) and were based on standard A-form helical geometry. 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. Eight 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 Pymol and UCSF Chimera.

Thermal stability

UV melting studies were performed using 0.5 A₂₆₀ 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 60 s and snap cooled on ice before each melting experiment. A₂₆₀ absorbance spectra from 10–92 °C were acquired (1.0 °C per minute) on a Jasco J-815 circular dichroism spectrometer equipped with a peltier heating apparatus. The melting curves were acquired in triplicate and averaged.

RESULTS

RNA thermal stability

The thermal stability of ASL^{Gly,GCC}, ASL^{Gly,UCC}, and np-ASL^{Gly,UCC} (Figure 1) were investigated using UV melting experiments to determine overall molecular stability (T_m). The normalized UV thermal denaturation curves indicate that the ASL^{Gly} molecules melt in one step (Figure 2). We have observed two-stage melting transitions of other anticodon arms (tyrosine and phenylalanine) with the lower temperature (<50 °C) transition corresponding to the destacking of the loop nucleotides^{34, 38}. In these two systems, the anticodon contains an adenine nucleotide which may facilitate stacking of the unpaired loop nucleotides. Two of the glycine molecules (ASL^{Gly,GCC} and np-ASL^{Gly,UCC}) display similar T_m values around 70 °C and the T_m of ASL^{Gly,UCC} is 10 °C lower. Both ASL^{Gly,GCC} and np-ASL^{Gly,UCC} form 6–7 Watson-Crick base pair stems whereas ASL^{Gly,UCC} forms five regular base pairs and a protonated C-A⁺ base pair at pH 6.3 (see below). Additionally, the degree of base stacking differs among the molecules. The hyperchromicity associated with melting is least for np-ASL^{Gly,UCC} (9%) and greatest for ASL^{Gly,GCC} and ASL^{Gly,UCC} (16% and 14%, respectively). Mg²⁺ raises the T_m of ASL^{Gly,UCC} to the level of np-ASL^{Gly,UCC} and has only a slight (~1 °C) destabilizing effect on np-ASL^{Gly,UCC} (Figure 2).

Resonance assignments of the ASL^{Gly} molecules

Due to self-complementarity, the RNA sequences used in this study can adopt either hairpin (monomer) form or a duplex form with internal loops of different sizes. Therefore, the oligomeric states of the RNA molecules were assessed using the NH spectra of each of the molecules. The hairpin forms have moderate line widths (9–14 Hz ¹H) and NH peak patterns that are independent of RNA concentration. ASL^{Gly,UCC} and np-ASL^{Gly,UCC} yield a single set of NH resonances consistent with the hairpin helix and give rise to single bands on native

PAGE gels. The NH spectrum of ASL^{Gly,GCC} also yields a single set of peaks immediately after annealing. However, additional peaks appear in the spectrum after several hours. The hairpin-duplex equilibrium was confirmed using a NOE-based ¹⁵N-filtered spectrum³⁹. At low RNA concentration (<0.4 mM) and <5 mM NaCl, the duplex constitutes <5% of the RNA population. Native PAGE analysis also shows that ASL^{Gly,GCC} forms a mixture of monomer and dimer species.

Under conditions of low salt and RNA concentration <0.4 mM, ASL^{Gly,GCC} yields good quality spectra with no evidence of duplex. However, the base ¹H and ¹³C resonances of ASL^{Gly,UCC} and np-ASL^{Gly,UCC} exhibit limited dispersion with a few resonances broadened by chemical exchange (Figure 3). To improve spectral quality, Mg²⁺ and Co(NH₃)₆³⁺ were tested for their ability to bind the RNA molecules and promote conformational homogeneity. For np-ASL^{Gly,UCC}, 3.0 mM Mg²⁺ yields quality spectra with improved resolution. For ASL^{Gly,UCC}, 2.0 mM Co(NH₃)₆³⁺ was found to improve spectral dispersion and reduce exchange broadening whereas Mg²⁺ was less effective and resulted in general resonance broadening. Therefore, the solution NMR studies for ASL^{Gly,UCC} and np-ASL^{Gly,UCC} were performed in the presence of Co(NH₃)₆³⁺ and Mg²⁺, respectively.

The non-exchangeable ¹H and ¹³C resonances of the three ASL^{Gly} molecules (Figure 1) were assigned using standard heteronuclear techniques^{40, 41}. Most of the base and ribose ¹H and ¹³C correlations are resolved for each of the molecules (Figure 3). In the absence of metal ion, the A₃₈ C2 resonance of ASL^{Gly,UCC} is slightly broadened and has a chemical shift of 148.6 ppm indicative of N1 protonation (Figure 3). With the addition of Co(NH₃)₆³⁺, the A₃₈ base deprotonates and the C2 resonance shifts downfield to 153.2 ppm. The ribose spin systems, except for the incompletely labeled 5'-terminal nucleotides, were identified using 2D or 3D HCCH-COSY and HCCH-TOCSY experiments. For ASL^{Gly,UCC} and np-ASL^{Gly,UCC}, spectral overlap even in the 3D spectrum limited unambiguous assignment of some 4' and 5' resonances. 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 purine (ASL^{Gly,GCC} and np-ASL^{Gly,UCC}) or four of six purine (ASL^{Gly,UCC}) correlations were identified in these spectra. The G₂₈ and A₃₈ correlations of ASL^{Gly,UCC} are not observed due to chemical exchange.

Sequential assignments for the non-exchangeable resonances were made using 2D NOESY and 3D ¹³C-edited NOESY experiments to identify sequential H6/8-H1' NOE connectivities. The sequential base-1' NOE connectivities are continuous through all 17 nucleotides at long mixing times (400 ms). The connectivity is continuous in the 180 ms NOESY spectrum of ASL^{Gly,GCC} (Figure 4), but at this mixing time the inter-residue NOE is weak between nucleotides U₃₃ and U₃₄ and broken between nucleotides U₃₄ and C₃₅ in spectra of ASL^{Gly,UCC} and np-ASL^{Gly,UCC}. In addition, while peak overlap near the diagonal prevented detection of many potential H6-H6 inter-residue NOEs, sequential H5-H6 crosspeaks were identified that support stacking of several pyrimidine bases in the loop regions of ASL^{Gly,UCC} and np-ASL^{Gly,UCC}.

The NH and NH₂ resonances were assigned using ¹H-¹H NOESY and HNCCH experiments. For all molecules, the NH resonances of the first five neighboring base pairs yield NOE connectivities between each other. For ASL^{Gly,GCC} and np-ASL^{Gly,UCC}, the NH resonance connectivities extend to a sixth base pair. The NH spectrum of ASL^{Gly,GCC} contains a broad guanine NH resonance at 10.56 ppm that was assigned to G₃₄. The U₃₃ NH resonance at 12.95 ppm of ASL^{Gly,GCC} is broad and very weak as well. The U₃₃ and U₃₄ NH resonances of ASL^{Gly,UCC} and np-ASL^{Gly,UCC} also are broad and only observed in the 1D spectra between 10.5 and 11.5 ppm. 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 of ASL^{Gly,UCC} are consistent with the lack of intra-molecular hydrogen bonding (Figure 5). In all molecules, the C₃₅ and C₃₆ NH₂ ¹H resonance pairs and the NH₂ ¹⁵N resonances are shifted upfield by ~1 ppm (¹H) and ~2 ppm (¹⁵N) relative to those of the base paired cytidine residues (Figure 5). The C₃₇ NH₂ resonances of np-ASL^{Gly,UCC} exhibit similar upfield shifts.

The inter-nucleotide phosphate ³¹P resonances are clustered between -3.54 and -4.60 ppm for all molecules and partial assignments were obtained using HCP or ³¹P-¹H hetero-TOCSY-NOESY spectra⁴². 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 of ASL^{Gly,UCC} and np-ASL^{Gly,UCC} remain tightly grouped indicating that the metal ions have little effect on the phosphate backbone conformation (Figure 3) and point to weak metal ion coordination to the phosphate backbone. A complete list of resonance assignments, including non-protonated positions, is given in Table S1.

Metal ions and the RNA hairpins

In high-resolution structure studies of tRNA, Mg²⁺ and Co(NH₃)₆³⁺ ions have been observed proximal to the loop-helix junction in the anticodon stem-loop of tRNA^{43,44}. As noted above, the multivalent ions Co(NH₃)₆³⁺ and Mg²⁺ were needed to obtain high-quality spectra for ASL^{Gly,UCC} and np-ASL^{Gly,UCC}, respectively. In the absence of multivalent ions, the A₃₈ C2 resonance at 148.6 ppm marks the presence of a C₃₂-A⁺₃₈ base pair in ASL^{Gly,UCC} (Figure 3). Both Co(NH₃)₆³⁺ and Mg²⁺ lead to loss of this base pair and deprotonation of A₃₈. Mg²⁺ reduces the conformational variability of the anticodon nucleotides at 10 mM concentration, but causes broadening of resonances throughout the spectrum and substantial spectral overlap in the NOESY. Co(NH₃)₆³⁺ (2 mM) also reduces the conformational variability of the loop nucleotides, but does not lead to excessive line broadening. The most significant chemical shift change caused by Co(NH₃)₆³⁺ is the N1 base resonance of G₂₈ (~0.3 ppm). The remaining chemical shift changes involve loop nucleotide resonances and are minor (<0.1 ppm). The resonances of np-ASL^{Gly,UCC} exhibit a similar pattern of chemical shift changes with 3 mM Mg²⁺. The addition of Co(NH₃)₆³⁺ to np-ASL^{Gly,UCC} does not produce additional resonance changes, but was used to localize ion binding. The 90% ¹H₂O NOESY spectrum supports non-specific association of Co(NH₃)₆³⁺ in the loop region. In the 90% ¹H₂O NOESY spectra, NOEs between the guanine and uridine NH and cytidine NH₂ protons of the stem with the Co(NH₃)₆³⁺ protons confirm the expected coordination of Co(NH₃)₆³⁺ at the G₂₈-U₄₂ base pair⁴⁵. Although the NOE spectral data defining the stem location of Co(NH₃)₆³⁺ are very good, the cobalt hexamine complex can only be restricted to the major groove side of the loop. Very weak NOE cross peaks are observed between Co(NH₃)₆³⁺ and the base protons of residues 32, 33, 37 and 38. However, there is evidence for coordination involving the U₃₃ O4 carbonyl atom of the base. The chemical shifts of the U₃₃ C2 and C4 nuclei (152.2 and 168.2 ppm, respectively) are consistent with participation of the O4 atom in a stable hydrogen bond or in metal ion coordination^{33,34}. The addition of Co(NH₃)₆³⁺ supports metal ion coordination at the U₃₃ O4 by causing additional (~0.2 ppm) upfield and downfield shifts of the C2 and C4 resonances. Coordination of the Mg²⁺ and Co(NH₃)₆³⁺ ions may also involve bridging between phosphate groups across the major groove, however, the ³¹P spectra of both molecules are minimally altered by Co(NH₃)₆³⁺ relative to Mg²⁺.

For the ASL^{Gly,GCC} RNA, intermediate concentrations of Mg²⁺ and Co(NH₃)₆³⁺ ions cause modest exchange broadening of the anticodon nucleotide base resonances. The U₃₃ NH resonance is exchange broadened and not observed and the position of the U₃₉ resonance is shifted upfield ~0.3 ppm. No other resonances in this region exhibit substantial chemical shift changes. The A₃₈ H2 is shifted upfield 0.8 ppm and the A₃₇, U₃₃, and C₃₆ base 6 and 8

resonances are broadened by chemical exchange. Also, no intermolecular NOEs between $\text{Co}(\text{NH}_3)_6^{3+}$ protons and loop nucleotide protons could be identified. Thus, although Mg^{2+} and $\text{Co}(\text{NH}_3)_6^{3+}$ associate with the loop region of $\text{ASL}^{\text{Gly,GCC}}$, the interaction appears weaker than the interaction of these ions with either $\text{ASL}^{\text{Gly,UCC}}$ or $\text{np-ASL}^{\text{Gly,UCC}}$.

Structure Calculations

The structures of the ASL^{Gly} 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 188–211 conformationally restrictive distance constraints and 131–156 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. All converged structures have no constraints violated by more than 0.1 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 0.39 Å², 0.83 Å², and 0.75 Å² for, $\text{ASL}^{\text{Gly,GCC}}$, $\text{ASL}^{\text{Gly,UCC}}$, and $\text{np-ASL}^{\text{Gly,UCC}}$, respectively. The global fold of $\text{ASL}^{\text{Gly,GCC}}$ is a hairpin composed of a seven base pair stem and a three nucleotide loop (Figure 6). The overall fold of $\text{np-ASL}^{\text{Gly,UCC}}$ is a six base pair stem with a five nucleotide loop (Figure 6). The overall fold of $\text{ASL}^{\text{Gly,UCC}}$ (a five base pair stem with a five to seven nucleotide loop) is similar to $\text{np-ASL}^{\text{Gly,UCC}}$, but the loss of some inter-residue NOEs among loop nucleotides and the loss of secondary structure proximal to the loop caused by the absence of the 32–38 base pair results in a somewhat less precisely defined loop conformation (Figure 6). The minimized average structures are shown in Figure 7.

For all of the molecules, the helical base stack along the 5' side of the loop is continuous though residue 34 and is conserved among the converged sets of structures. Base stacking along the 3' side of the loop varies among the structures. For $\text{ASL}^{\text{Gly,GCC}}$, the 3' strand stacking begins with A_{37} . For $\text{ASL}^{\text{Gly,UCC}}$ and $\text{np-ASL}^{\text{Gly,UCC}}$, the 3' strand stacking generally begins with C_{35} , but individual structures show moderate deviations (Figures 6). The C_{35} and C_{36} nucleotides are common to each of the three families of structures and they distribute along the major groove or minor groove sides of the loops depending upon the family. The general positions of these residues within individual structures of each family, though, are uniform and exhibit few excursions to the opposite side.

Structure of the loop regions of ASL^{Gly} molecules

The loop of $\text{ASL}^{\text{Gly,GCC}}$ is composed of nucleotides G_{34} – C_{36} and is closed by a distorted U_{33} – A_{37} base pair (Figure 7). The position of the A_{37} base is restricted by NOE cross peaks ($\text{A}_{37} \text{H2-G}_{34} \text{H1}'$ and $\text{A}_{37} \text{H2-A}_{38} \text{H1}'$) that are characteristic of adenine nucleotides in helices. The hydrogen bond functional groups between U_{33} and A_{37} are aligned, but the bases exhibit moderate buckling (25° average among converged structures). The intensity of the U_{33} NH resonance is weak and could result from solvent exchange. However, the U_{33} C2 and C4 chemical shifts (152.8 and 168.6 ppm, respectively) and A_{37} N1 chemical shift (222.1 ppm) are indicative of weak hydrogen bonding and are consistent with the distorted U_{33} – A_{37} pair. The G_{34} base extends across the helix axis with the U_{33} and C_{35} bases stacking above and beneath, respectively, the major groove edge of the G_{34} base. The G_{34} – C_{35} stacking is supported by H8–H6 and H8–H5 NOE cross peaks. The C_{35} base and its NH_2 group point away from the helix axis toward the solvent. The C_{36} base also resides on the major groove side of the loop with its NH_2 group extending toward the solvent, but is not coplanar with the C_{35} base. The ^{15}N and ^1H chemical shifts of the C_{35} and C_{36} NH_2 groups

are shifted upfield (Figure 5) and are consistent with the absence of intra-molecular interactions. The C₃₆ base is vertically displaced from the A₃₇ base and does stack.

The loop of ASL^{Gly,UCC} is composed of seven nucleotides, residues C₃₂-A₃₈, with cobalt hexamine leading to deprotonation of the A₃₈ base. Although the C₃₂ and A₃₈ bases maintain an alignment characteristic of the C₃₂-A⁺₃₈ base pair, the distance between the Watson-Crick faces is increased (Figure 7). The U₃₃ and A₃₇ bases are coplanar and stack beneath C₃₂ and A₃₈, respectively. The A₃₇ base is rotated toward the minor groove causing the N6 amino group to align for hydrogen bonding with the U₃₃ O2 atom. However, the distance between the heavy atoms is long (4.0 Å average) and the U₃₃ C2 and A₃₇ N6 chemical shifts, 153.8 ppm and 80.1 ppm respectively, do not reflect hydrogen bonding involving the O2 and H6 atoms. The base of U₃₄ is on the major groove side of the loop and stacks beneath the U₃₃ base. The bases of C₃₅ and C₃₆ lay on the minor groove side of the helix and stack on each other and beneath A₃₇ with their Watson-Crick edges pointing away from the helix axis toward the solvent. The ¹H and ¹⁵N resonances of the C₃₅ and C₃₆ NH₂ groups are shifted upfield (5.7–6.8 ppm ¹H and 93 ppm ¹⁵N) and are characteristic of solvent exposed cytidine amino groups (Figure 5).

The loop of np-ASL^{Gly,UCC} is composed of residues U₃₃-C₃₇. The bases of U₃₃ and C₃₇ stack beneath C₃₂ and G₃₈, respectively, and neither base is laterally displaced towards the major or minor grooves. However, C₃₇ is rotated so that the Watson-Crick edge of the base points into the minor groove rather than toward the helix axis. The base of C₃₆ is parallel and stacked with C₃₇ in ~80% of the structures, but points down and away from the C₃₇ base in the remaining structures. Both orientations are in agreement with the observed NOE cross peak pattern. Like C₃₇, the Watson-Crick edge of C₃₆ points out toward the minor groove side of the loop in all structures. The base of U₃₄ stacks beneath U₃₃ and is rotated out towards the major groove. C₃₅ is positioned on the minor groove side of the loop at the apex of the phosphate backbone turn. The base of C₃₅ is laterally displaced from and slightly below the base plane of C₃₆. With the exception of U₃₃, the functional groups along the Watson-Crick edges of the loop nucleotide bases are solvent exposed and none form intra-molecular interactions. The C₃₅-C₃₇ NH₂ chemical shifts (Table S1) reflect the solvent exposure of these bases.

The sugar-phosphate backbone conformations of the loop nucleotides are surprisingly uniform (Figure 7). The majority of the nucleotides maintain the C3'-*endo* ring pucker, but the C₃₅ and C₃₆ ribose groups have observable H1'-H2' couplings and ribose ¹³C chemical shifts that indicate a mixture of C2'- or C3'-*endo* ring pucker conformations. In addition, the uniformly small (<5 Hz) P-C2' coupling constants for the loop residues place the ε torsion angles in the *trans* conformation characteristic of RNA. The phosphate backbone torsion angles β, γ, and ε of residues 34–36 were loosely constrained for the calculations, but most have values within the range common to RNA helices, and the majority of deviations from standard values involve residue C₃₄. The ³¹P chemical shifts for all inter-residue phosphorus atoms are tightly clustered between –3.5 and –4.6 ppm, indicating that the α and ζ torsion angles throughout the loop and helix adopt a *gauche* conformation⁴⁶.

Structure of the ASL^{Gly} stems

The conformations of the stems of the three ASL^{Gly} molecules are very similar. The geometry of the hairpins from the terminal G₂₇-C₄₃ base pair to 32–38 interaction is primarily A-form (Figure 6). The C₃₂-A⁺₃₈ of ASL^{Gly,UCC} is deprotonated with addition of cobalt hexamine, but the relative arrangement of the two bases is largely unchanged as evidenced by intra- and inter-strand NOEs involving C₃₂ and A₃₈. The base-base and base-ribose NOE cross peaks among the stem nucleotides are continuous and their intensities are consistent with the A-form helical geometry. The torsion angles of the sugar-phosphate

backbones involving the stem residues of all molecules also are within the limits of A-form geometry and are supported by chemical shift and J-coupling data.

Discussion

Although the bulk of tRNA in the cell is destined for aminoacylation and delivery to the ribosome for protein synthesis, a variety of alternative functional roles exist for tRNA^{Gly}. Besides their central role in translation, tRNA molecules participate in the addition of amino acids to membrane lipids and the N-termini of peptides^{9, 10}, the biosynthesis of antibiotics and of the crosslinks in peptidoglycan cell walls^{3, 4, 11, 12}, the regulation of transcription and translation², and viral replication^{5, 8}. Not all tRNA molecules are multifunctional, but in some bacteria the glycyl-tRNA family has extra-ribosomal roles in transcriptional regulation and cell wall biosynthesis. Here we have examined the structural features of three glycyl-tRNA molecules that serve these three functions.

Comparison of the ASL^{Gly} structures

The structures of the tRNA^{Gly} anticodon arm stems (residues 27–32 and 38–43) are nearly the same even though ASL^{Gly,UCC} has a C-A mismatch rather than the U-A and G-C pairs present in ASL^{Gly,GCC} and np-ASL^{Gly,UCC}, respectively. The A₃₈ of ASL^{Gly,UCC} is deprotonated in the presence of Co(NH₃)₆³⁺ but the basic structural features of the C₃₂-A₃₈⁺ base pair, coplanarity of the two bases and minimal lateral displacement from the helix axis, are conserved. In all of the molecules, the base of residue 34 stacks with U₃₃, and is moderately displaced toward the major groove edge of U₃₃. Also common to the three structures is the minor groove displacement of the base of residue 37. The most notable structural differences among the molecules are the orientations of nucleotides C₃₅ and C₃₆. In ASL^{Gly,GCC}, these nucleotides are positioned on the major groove side of the loop whereas in ASL^{Gly,UCC} and np-ASL^{Gly,UCC}, these residues are located on the minor groove side of the loop (Figure 7). The anticodon nucleotides of the three sequences also exhibit similar sugar pucker behavior. The riboses of nucleotides C₃₅ and C₃₆ appear to oscillate between C2'-*endo* and C3'-*endo* conformations as they give rise to cross peaks with modest (≈ 5 Hz) coupling in the DQF-COSY spectrum and intermediate ¹³C chemical shifts. To determine if the base positions are equally accommodated by the canonical ribose conformations we performed a series of simulations where the ribose pucker conformations of C₃₅ and C₃₆ were fixed to C2'-*endo* or C3'-*endo*. In about 50% of the ASL^{Gly,GCC} structures, the C2'-*endo* pucker leads to a small downward rotation of the C₃₆ base toward the helix axis and away from the major groove side of the loop. For ASL^{Gly,UCC} and np-ASL^{Gly,UCC}, the enforcement of C2'-*endo* pucker widens the curvature of the phosphate backbone through the loop with minimal effect on the positions of the bases in the loop. Neither the number of NOE violations nor the overall energies for the three molecules are changed significantly by applying the C2'-*endo* constraints. Thus, the average conformational states of the ribose puckers are consistent with the calculated sets of structures introduce a conformational malleability that may be needed to optimize anticodon loop nucleotide interactions in different contexts.

Comparison with structures of other anticodon stem-loop sequences

The crystal structure of fully modified yeast tRNA^{Phe} revealed what is now known to be a common RNA structural motif designated the U-turn^{48, 49}. This functionally important motif has since been observed in the structures of a host of other RNA molecules including the signal recognition particle⁵⁰ and RNA tetraloops^{51, 52} (Figure 8). In the context of the tRNA anticodon arm, the U-turn has been observed in crystal forms of unmodified *E. coli* tRNA^{Phe} (anticodon 5'-GAA-3')⁵³, an *E. coli* tRNA^{Cys}-EF-Tu complex (anticodon 5'-GCA-3')²³, the ribosome-bound *E. coli* tRNA^{Lys,3} anticodon 5'-UUU-3')⁵⁴, and the

solution forms of partly modified *E. coli* tRNA^{Phe} and fully modified tRNA^{Lys,3 43, 55} (Figure 8). The U-turn motif of the tRNA anticodon arm is characterized by a 120° turn of the phosphate backbone between residues 33 and 34 and stacking of the three anticodon nucleotide bases on the 3' side of the loop. The U-turn of tRNA has been observed to contain hydrogen bonds between the U₃₃ 2'-OH and the N7 or N4H₂ of purine or cytidine residues at position 35 and between the U₃₃ N3H and the non-bridging phosphoryl oxygen 5' to residue 36.

The U-turn structural motif that is ubiquitous among anticodon loops of tRNA molecules in the crystal state, is not adopted by any of the ASL^{Gly} molecules in this study. In a classic U-turn, the nonsequential U₃₃ H1' to N₃₅ H6/8 distance is ≈3.8 Å and should give rise to a moderately intense NOE cross peak. Although spectral overlap would occlude the non-sequential U₃₃-C₃₅ 1'-base NOE cross peak in np-ASL^{Gly,UCC}, this peak is not present in the NOE spectra of ASL^{Gly,GCC} or ASL^{Gly,UCC}. In addition, NOE cross peaks in the spectra of the ASL^{Gly,GCC} and ASL^{Gly,UCC} (including U₃₃ H1' to A₃₈ H2 and G₃₄ or U₃₄ H1' to A₃₇ H2) are not compatible with the U-turn motif. The reversal of the phosphate backbone occurs smoothly between residues 34 and 36 (Figure 7) and does not turn abruptly between U₃₃ and G₃₄/U₃₄ as observed for the classic U-turn^{44, 48, 49, 53}. In the U-turn, the *trans* conformation of the G₃₄/U₃₄ backbone angle α facilitates the sharp turn and is expected to cause the corresponding ³¹P resonance to shift downfield 2–3 ppm^{46, 56}. None of the phosphorus spectra of the ASL^{Gly} molecules display this unusual feature and are consistent with the *gauche* angles adopted by the converged structures.

The absence of the U-turn fold from the tRNA anticodon loop in solution is not unique to the ASL^{Gly} molecules. The anticodon arms of unmodified and N6-dimethylallyl-A₃₇ (i⁶A₃₇)-modified tRNA^{Tyr}, ψ₃₉-modified and N6-threonylcarbamoyl-A₃₇ (t⁶A₃₇)-modified human tRNA^{Lys,3}, unmodified *E. coli* tRNA^{Phe}, and unmodified *E. coli* tRNA^{Val} also do not have the U-turn structure^{57–59}. Interestingly, the addition of Mg²⁺ to unmodified *E. coli* tRNA^{Val}, i⁶A₃₇-modified *E. coli* ASL^{Phe}, and [5-methylcarboxymethyl, 2-thiouridine-34 (mcm⁵s²U₃₄), t⁶A₃₇]-modified *E. coli* ASL^{Lys} molecules leads to formation of the U-turn^{43, 56, 59}. These data highlight crucial roles for base modification and Mg²⁺ in the formation of the U-turn fold in some sequences. In the glycyl-tRNAs, though, modification of the anticodon loop is sparse or non-existent. There are no modifications in the anticodon arm of bacterial tRNA^{Gly,GCC}. Although the presence of Mg²⁺ disrupts the U₃₃-A₃₇ base pair and increases the mobility of the loop nucleotides, it does not cause reorganization of the loop nucleotides into the U-turn motif. In some bacterial species, the anticodon arm of tRNA^{Gly,UCC} contains the 5-carboxymethylaminomethyl (cmnm⁵) functionality on U₃₄ (Figure 1). This modification is chemically similar to the mcm⁵s²U₃₄ of *E. coli* tRNA^{Lys,3}. Like tRNA^{Gly,UCC}, the anticodon sequence of tRNA^{Lys,3} is composed only of pyrimidine bases (5'-UUU-3') with a uridine nucleotide at position 34. In its modified form, a salt bridge exists between the U₃₃pU₃₄ phosphoryl oxygen and the cmnm⁵ modification. However, the adoption of the U-turn in tRNA^{Lys,3} also requires thiolation at the base C2 position which strongly promotes the C3'-endo ribose pucker, and the t⁶A₃₇ modification⁵⁵. The cmnm⁵ modification also promotes the C3'-endo ribose pucker, but much more weakly than C2 thiolation^{55, 56}. Thus, it is possible that introduction of the cmnm⁵ modification into ASL^{Gly,UCC} could predispose the loop to adopt a U-turn fold, however, this modification alone is unlikely to substantially change the structure of the pyrimidine rich ASL^{Gly,UCC} loop⁵⁵. Our results do not exclude the possibility that a very small fraction of the population of molecules dynamically samples the U-turn conformation, but evidence for alternative conformations is not visible in HSQC, the most sensitive spectra. It is also possible that although the anticodon loop is physically distal to the body of the tRNA, the conformation of some loop sequences could be altered by the presence of the remainder of

the tRNA molecule⁵³. Modification and/or the presence of metal ions, though, are sufficient to promote the U-turn fold in various sequence-diverse isolated anticodon arms^{43, 56, 59}.

The tRNA^{Gly} molecules are functionally diverse and have sequence variation in the anticodon loops, but all are charged by a single glycyl aminoacyl tRNA synthetase (GlyRS). The orientations of the anticodon nucleotides imposed by the U-turn motif are important for the functional roles of tRNA^{Gly,GCC} and tRNA^{Gly,UCC}, but a similar presentation of anticodon bases is not required for np-tRNA^{Gly,UCC} function. Additionally, C₃₇ of np-tRNA^{Gly,UCC} does not provide the same base stacking contribution for the U-turn that is offered by the purine base at position 37 of ribosomal tRNAs. Thus, the different cellular roles of these tRNA^{Gly} molecules and their nucleotide sequences do not require or direct the anticodon loops to adopt a common fold. In contrast, the open arrangement of the anticodon loops may aid formation of the GlyRS-tRNA^{Gly} complex. Although anticodon residues C₃₅ and C₃₆ are important for synthetase recognition of tRNA^{Gly}⁶⁰, the details of the interaction are not known⁶¹. Further, the structures of other tRNAs bound to their cognate synthetases offer little insight into the structural details of the GlyRS-tRNA^{Gly} interaction. The anticodon loops in synthetase-tRNA complexes exhibit a range of conformations, including the U-turn motif, and only weakly correlate with synthetase class or nucleotide identity. But, the loop region structures of the synthetase-bound tRNA molecules depend on the approach (major groove or minor groove side) of the synthetase to the anticodon loop^{23, 62–67}. Thus, the open configurations adopted by the ASL^{Gly} anticodon loops may facilitate readout of the anticodon bases by GlyRS if the loop is accessed from the minor groove side⁶¹.

Functions of the tRNA^{Gly} molecules

The physical properties of the glycyl anticodon stem-loops will have an impact on their various functions. In bacteria that utilize glycine for synthesis of the peptidoglycan cell wall, it appears important that the pool of charged np-tRNA^{Gly,UCC} not be diverted into the translation machinery. Exclusion of the np-tRNA^{Gly,UCC} from the protein synthesis can be accomplished by two mechanisms. First, the absence of guanine nucleotides at the base of the T Ψ C stem of np-tRNA^{Gly,UCC} is predicted to weaken the complex with EF-Tu:GTP^{20, 24}, thereby limiting ribosome binding of aminoacyl np-tRNA^{Gly,UCC}. A second feature of np-ASL^{Gly,UCC} that may protect against participation in ribosome-catalyzed translation is the cytidine nucleotide at position 37. In proteinogenic tRNA molecules, residue 37 is a purine nucleotide that is modified in most cases. The purine-37 residue confers additional stacking energy for the codon-anticodon helix. tRNA^{Phe} molecules containing unmodified G or A nucleotides at position 37 bind the ribosome A-site more rapidly and are released more slowly than tRNA^{Phe} molecules containing C or U⁶⁸. Thus, the C₃₇ of np-tRNA^{Gly,UCC} may act to further limit its contribution to protein biosynthesis, a possible stop-gap since the sole glycyl-tRNA synthetase enzyme relies in part on a common fold of the tRNA^{Gly,UCC} and np-tRNA^{Gly,UCC} anticodon loops for activity. Interestingly, although the structures of the proteinogenic and non-proteinogenic tRNA^{Gly} anticodon arms are remarkably similar and exhibit similar responses to metal ions, the T_m of the np-ASL^{Gly,UCC} stem is 10 °C higher than the T_m of the ASL^{Gly,UCC} stem which can be attributed in part to the different 32–38 pairings in the two molecules (Figure 2). If np-tRNA^{Gly} lacks the ability to interact with the codon GGC to any significant degree, then regulation of the *glyQS* riboswitch would be responsive only to the pool of uncharged proteinogenic tRNA^{Gly}.

Glycine is a member of the four-codon box family in which the codon nucleotide sequences differ only at the third, or wobble, position and the four possible codon combinations designate a single amino acid. In *E. coli*, there are three glycine isoacceptor tRNAs. tRNA^{Gly,CCC} is the most discriminating and translates only the codon GGG to any significant degree²⁷. *E. coli* tRNA^{Gly,UCC}, in which the U₃₄ modification is not identified

but could be mnm⁵ or cmnm⁵, translates all four glycine codons but translates codons GGU and GGC with ~25% the efficiency of tRNA^{Gly,GCC} 27. In *B. subtilis*, which contains only two isoacceptors, tRNA^{Gly,GCC} and tRNA^{Gly,UCC}, U₃₄ of tRNA^{Gly,UCC} has the modification cmnm⁵. The cmnm⁵ modification can facilitate stacking of residues 34 and 35 and increase codon discrimination. tRNA^{Gly,UCC} is the sole glycyl-tRNA present in *Mycoplasma mycoides* and the only anticodon arm modification is N6-methyl A₃₇ (Figure 1). Like the *E. coli* tRNA^{Gly,UCC}, the *M. mycoides* glycyl-tRNA translates the four glycine codons without discrimination 27, 69, but the translational efficiency of codons GGU and GGC is ~50% the efficiency of *E. coli* tRNA^{Gly,GCC} 27. A key to the ability of the *M. mycoides* tRNA^{Gly,UCC} to read codons efficiently without discrimination was determined to be the C₃₂-A₃₈ base pair 69. Although *E. coli* tRNA^{Gly,UCC} also contains the C₃₂-A₃₈ base pair, the modification of U₃₄ and/or lack of modification of A₃₇ may curtail the ability of tRNA^{Gly,UCC} to read the four glycine codons with equal efficiency.

The ability of tRNAs to discriminate codon triplets based on the third codon nucleotide is a balance of contributions from modification of tRNA residues 34 and 37, the identity of residues 32 and 38, and the purine/pyrimidine composition of residues 35 and 36 25, 69. The property of codon discrimination exhibits modest correlation with the propensity of the U₃₄ ribose pucker to adopt the C3'-endo conformation, which leads to a more conformationally ordered anticodon loop. The C2 thiolation present in some split codon boxes strongly reinforces the C3'-endo conformation 56. The mcm⁵ and cmnm⁵ also tend to favor the C3'-endo conformation, as does the 5-oxyacetic acid (cmo⁵) modification, but to an even lesser extent 56, 70. The ribose conformations of residue 34 in ASL^{Gly,GCC} and ASL^{Gly,UCC} are a mixture of C3'- and C2'-endo conformations. While cmnm⁵ modification of U₃₄ could change this equilibrium in *B. subtilis* (or *E. coli*) tRNA^{Gly,UCC}, the tRNA^{Gly,GCC} is unmodified and would be unchanged *in vivo*.

In six of the eight four-codon boxes, the cognate tRNA molecules with U₃₄ contain the modification cmo⁵ that suppresses wobble base discrimination 70 (Figure 1) and expands codon reading. One question that arises is why U₃₄ of glycyl and arginyl tRNAs carry the mnm⁵ or cmnm⁵ modifications rather than cmo⁵ when restricted codon reading appears unnecessary. In tRNA^{Gly} and tRNA^{Arg}, U₃₄ is followed by a cytidine whereas in the six other four-codon boxes, U₃₄ is followed by a purine nucleotide. It is possible that A or G at position 35 facilitates a stacking of the loop bases, as evidenced by Mg²⁺-induced U-turn formation in unmodified tRNA^{Val} 59, that is less easily accomplished by the cytidine base and modification of U₃₄ with cmo⁵ indirectly compensates for this positive contribution to loop ordering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

tRNA^{Gly,GCC}

tRNA with anticodon sequence 5'-GCC-3'

tRNA^{Gly,UCC}	tRNA with anticodon sequence 5'-UCC-3'
ASL^{Gly,GCC}	anticodon stem-loop of <i>Staphylococcus aureus</i> tRNA ^{Gly, GCC}
ASL^{Gly,UCC}	anticodon stem-loop of <i>Staphylococcus aureus</i> tRNA ^{Gly, UCC}
np-ASL^{Gly,UCC}	anticodon stem-loop of <i>Staphylococcus aureus</i> non-proteinogenic tRNA ^{Gly, UCC}
NTP	nucleoside triphosphate
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	NOE spectroscopy
2D	two dimensional
3D	three dimensional
HetCor	heteronuclear correlation
HSQC	heteronuclear single quantum coherence
MD	molecular dynamics
RMSD	root mean square deviation
NH	imino
NH₂	amino

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Figure 1.

Sequences corresponding to the anticodon arms of *S. aureus* (A) tRNA^{Gly,GCC}, (B) tRNA^{Gly,UCC}, and (C) np-tRNA^{Gly,UCC}. Nucleotide numbering corresponds to the full-length tRNA molecule. Residues 27–30 and 40–43 were changed to permit transcription using T7 RNA polymerase and to facilitate comparison of structural and thermodynamic effects of the loop sequences. Also shown are the chemical structures of modifications cmnm⁵-U, 5-carboxymethylaminomethyl uridine, cmo⁵-U, 5-oxyacetic acid uridine, and m⁶A, (N6-methylallyl)-adenine.

Figure 2.

The UV melting curves of ASL^{Gly,GCC} (red), np-ASL^{Gly,UCC} (blue), and np-ASL^{Gly,UCC} (green). The ASL^{Gly} molecules exhibit a single melting transition that occurs above 55 °C—suggesting minimal stacking of the unpaired loop nucleotide bases. The melting transitions generally agree with the predicted secondary structures of the molecules with ASL^{Gly,UCC} displaying the lowest melting transition and having the fewest Watson-Crick base pairs. The addition of Mg²⁺ to ASL^{Gly,UCC} (light blue) raises the T_m by ~10 °C but causes an ~1.5 °C decrease when added to np-ASL^{Gly,UCC} (light green). In the absence of Mg²⁺, the apparent T_m 's of ASL^{Gly,GCC}, ASL^{Gly,UCC}, and np-ASL^{Gly,UCC} are 72.0 °C, 60.4 °C, and 73.1 °C, respectively.

Figure 3.

Comparison of two-dimensional ^{13}C - ^1H HSQC spectra of the base C6/8 regions of ASL^{Gly,UCC} in the (A) absence and (B) presence of $\text{Co}(\text{NH}_3)_6^{3+}$ and of np-ASL^{Gly,UCC} in the (C) absence and (D) presence of Mg^{2+} . Addition of $\text{Co}(\text{NH}_3)_6^{3+}$ leads to substantial sharpening and upfield movement of the A₃₈ crosspeak of ASL^{Gly,UCC}. The effect of $\text{Co}(\text{NH}_3)_6^{3+}$ addition on the protonation state of ASL^{Gly,UCC} A₃₈ is shown in the inset (B). The adenine C2 resonances in the absence (*gray*) and presence (*black*) of $\text{Co}(\text{NH}_3)_6^{3+}$.

Figure 4. Sequential connectivities in the 400 ms mixing time NOE spectrum of the ASL^{Gly,GCC} molecule. The correlations between N₃₄ and U₃₅ are weak in the spectra of all ASL^{Gly} molecules. Also, the sequential connectivity between C₃₅ and C₃₆ is weak in this spectrum.

Figure 5.

Comparison of the cytidine NH₂ regions from ¹⁵N-¹H HSQC spectra of (A) ASL^{Gly,GCC}, (B) ASL^{Gly,UCC} with Co(NH₃)₆³⁺, and (C) np-ASL^{Gly,UCC} with Mg²⁺ RNA hairpins. Hydrogen bonding leads to a downfield shift of the N4 and participating H4 resonances. The C₃₅ and C₃₆ (and C₃₇ of np-ASL^{Gly,UCC}) NH₂ resonances of all three molecules cluster upfield, indicating the absence of hydrogen bonding. In the U-turn formed by the anticodon loop of tRNA^{Cys}, the C₃₅ NH₂ group hydrogen bonds with the 2'-OH of U₃₃²³. An analogous interaction, and corresponding downfield shift of the C₃₅ NH₂ resonances, would be predicted if the glycyl-tRNA anticodon loops adopted U-turn motifs.

Figure 6. Superposition of 8 converged structures of (A) ASL^{Gly,GCC}, (B) ASL^{Gly,UCC}, and (C) np-ASL^{Gly,UCC} 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.

Figure 7.

Stereoviews of the loop regions of structure of (A) ASL^{Gly,GCC}, (B) ASL^{Gly,UCC}, and (C) np-ASL^{Gly,UCC} RNA hairpins. The coloring scheme is as follows: the anticodon bases N₃₄, C₃₅, and C₃₆ (*Red*), N₃₁ and N₃₉, (*blue*), N₃₂ and N₃₈ (*green*), U₃₃ (*pink*), and N₃₇ (*pink/brown*). The base of C₃₅ 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 the three molecules. The spectral data show that the protonated A₃₈-C₃₂ base pair of ASL^{Gly,UCC} is lost with addition of divalent metal ion.

Figure 8.

Comparison of the U-turn motifs from (A) the solution NMR structure of fully modified human tRNA^{Lys,3} (PDB 1FL8), (B) the X-ray crystal structure of ribosome bound *E. coli* tRNA^{Lys,3} (PDB 1XMO), (C) the X-ray crystal structure of modified *E. coli* aa-tRNA^{Cys} in complex with EF-Tu/GDPNP (PDB 1B23), and (D) the X-ray crystal structure of the hammerhead ribozyme (PDB 299D). The U-turn folds present in A–C are characterized by stacking of the anticodon bases (*purple*) along the minor groove side of the helix and the NH-P hydrogen bond from U₃₃ (*green*). In D, the sequence GUAA shows the U-turn fold characteristic of the GNRA tetraloops where the G (*green*) forms the NH-P hydrogen bond⁵¹. The first (n_i) and third (n_{i+2}) nucleotides of the turns are labeled. The 2'-OH-base hydrogen bond between n_i and n_{i+2} involves the base N7 when n_{i+2} is a purine and the exocyclic amino N4H₂ when n_{i+2} is cytidine. The n_i - n_{i+2} interaction has not been observed in ASLs when uridine occupies the n_{i+2} position (A and B), although the O4 in principle could perform this function. Modifications on residues 34 and 37 (A and B) provide additional hydrogen bonds in the turn.

Table 1

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

Constraint	ASL ^{Gly,GCC}	ASL ^{Gly,UCC}	np-ASL ^{Gly,UCC}
NOE distance constraints			
intraresidue ^a	116	106	110
interresidue	94	81	95
mean number per residue	12.4	11	12.1
NOE constraints by category			
strong (1.8 – 3.0 Å)	32	18	6
medium (1.8 – 4.5 Å)	108	80	89
weak (1.8 – 6.0 Å)	56	82	84
very weak (1.8 – 7.0 Å)	14	7	26
base pair constraints			
total	32	26	32
dihedral angle constraints			
ribose ring ^b	48	51	51
backbone	71	91	93
H bonds	12	12	12
mean number per residue	7.7	9.1	9.2
Violations			
average distance constraints > 0.3 Å	0	0	0
average dihedral constraints > 0.5°	0	0	0
RMSD from ideal geometry^c			
Heavy Atoms (Å)	0.39 ± 0.17	0.83 ± 0.27	0.75 ± 0.9

^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.

^c Calculated against the minimized average structure.