Solution structure of $\psi_{32}$-modified anticodon stem–loop of Escherichia coli tRNAPhe

Javier Cabello-Villegas and Edward P. Nikonowicz*

Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77251-1892, USA

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ABSTRACT

Nucleoside base modifications can alter the structures and dynamics of RNA molecules and are important in tRNAs for maintaining translational fidelity and efficiency. The unmodified anticodon stem–loop from Escherichia coli tRNAPhe forms a trinucleotide loop in solution, but Mg$_2^+$ and dimethylallyl modification of A$_{37}$ N6 destabilize the loop-proximal base pairs and increase the mobility of the loop nucleotides. The anticodon arm has three additional modifications, $\psi_{32}$, $\psi_{39}$, and A$_{37}$ C2-thiomethyl. We have used NMR spectroscopy to investigate the structural and dynamical effects of $\psi_{32}$ on the anticodon stem–loop from E.coli tRNAPhe. The $\psi_{32}$ modification does not significantly alter the structure of the anticodon stem–loop relative to the unmodified parent molecule. The stem of the RNA molecule includes base pairs $\psi_{32}$-A$_{38}$ and U$_{33}$–A$_{37}$ and the base of $\psi_{32}$ stacks between U$_{33}$ and A$_{31}$. The glycosidic bond of $\psi_{32}$ is in the anti configuration and is paired with A$_{38}$ in a Watson–Crick geometry, unlike residue 32 in most crystal structures of tRNA. The $\psi_{32}$ modification increases the melting temperature of the stem by $\sim$3.5°C, although the $\psi_{32}$ and U$_{33}$ imino resonances are exchange broadened. The results suggest that $\psi_{32}$ functions to preserve the stem integrity in the presence of additional loop modifications or after reorganization of the loop into a translationally functional conformation.

INTRODUCTION

Posttranscriptional modification of RNA molecules occurs in all cells (1–3). The modifications, which are primarily localized to the nucleotide bases, can alter the chemical, structural, and thermodynamic properties of RNAs and thereby contribute to RNA function (4). Many nucleotide base modifications have been chemically well characterized but the impact of these modifications on RNA structure and stability has been less thoroughly examined. Pseudouridine is the most common nucleotide base modification and is found in the tRNA and tRNA of all cells (5,6). This modification is formed by an isomerization of uridine resulting in a C5-C1 base–ribose glycosidic bond and a second imino (NH) functionality. Pseudouridine preferentially adopts the syn conformation about the glycosidic bond as the free nucleotide (7,8), but the anti configuration has been most frequently observed for pseudouridine within oligonucleotides and double helices (9). Consequently pseudouridine tends to base pair with adenosine through the N3 imino and the C2 carbonyl groups (Figure 1) (10–13) and imparts thermodynamic stability to helices when located in the interior of an RNA duplex or in a single strand region adjacent to a duplex (8).

Figure 1. Sequences of (A) the $\psi_{32}$-modified and (B) fully modified RNA hairpins corresponding to the anticodon arm of E.coli tRNAPhe. Nucleotide numbering corresponds to the full-length tRNAPhe molecule. $\psi$ designates pseudouridine and ms$_{2}$i6A designates (2-thiomethyl, N6-dimethylallyl)-adenine. $\psi_{32}$-A$_{38}$ base arrangements for (C) the Watson–Crick base pair and (D) the bifurcated hydrogen bond interaction.
In addition to the nearly universally conserved $\psi_{55}$, pseudouridine occurs frequently at several other positions in tRNA including residues 13, 32, 39, and 40 (14), although the frequency of occurrence at these positions varies among species. $\psi_{55}$, located in the T-loop, serves only as an acceptor in its hydrogen bonding with the imino and amino groups of G\textsubscript{18}. The $\psi_{55}$-G\textsubscript{18} pair stacks between the flanking base pairs and contributes to maintenance of the tertiary fold of tRNA (15,16). In yeast tRNA\textsubscript{Phe} and other tRNAs or tRNA complexes, $\psi_{30}$, at the bottom of the anticodon stem, generally aligns with A\textsubscript{31} in a standard A–U Watson–Crick hydrogen bond geometry (15–20), although the hydrogen bonds tend to be somewhat long (2.1–2.3 Å). $\psi_{32}$ also is located in the anticodon arm of six tRNA species in Escherichia coli and adopts a syn configuration about its glycosidic bond in tRNA\textsubscript{Gly} (17). In yeast tRNA\textsubscript{Phe}, $\psi_{32}$ forms a bifurcated base pair with C\textsubscript{38} involving C\textsubscript{32} O4 and the exocyclic amino hydrogens of C\textsubscript{38} (18,21).

Residue 32 in the anticodon arm is important for the translation function of tRNA. A central element of the extended anticodon hypothesis is that nucleotides at the stem–loop junction in the anticodon arm contribute to the translational efficiency of tRNA (22,23). The functional importance of residues 32 and 38 was first demonstrated using amber suppressor tRNAs Su2 and Su7 that incorporate glutamine and tryptophan, respectively, at the stop codon 5′ residues 32 and 38 (23). In addition to the suppressor tRNA studies that correspond to residues G\textsubscript{27}–C\textsubscript{43} of full-length E. coli tRNA\textsubscript{Phe} (24,25), the genome of M. mycoides encodes one tRNA\textsuperscript{Gly} (anticodon 5′-UCC-3′) that is used to decode all four glycine codons and has a C\textsubscript{32}–A\textsubscript{38} mismatch in the anticodon arm. In E. coli, tRNA\textsuperscript{Gly,2} has the anticodon 5′-UCC-3′, but has the base pair U\textsubscript{32}–A\textsubscript{38}. When mutated to a U\textsubscript{32}–A\textsubscript{38} base pair, the ability of the M. mycoides tRNA\textsuperscript{Gly} to decode non-cognate codons dramatically diminishes (25). Similarly, mutation of the U\textsubscript{32}–A\textsubscript{38} base pair of E. coli tRNA\textsuperscript{Gly,2} to C\textsubscript{32}–A\textsubscript{38} leads to decreased fidelity and −1 frameshifting at 5′-GGG-3′ codons (26). These studies demonstrate the ability of the 32–38 interaction to modulate the wobble properties of the anticodon. Thus, the types of interactions formed by residues 32 and 38 may alter the conformation and dynamics of the anticodon loop and/or the interactions within the codon-anticodon complex at the ribosome.

We have used heteronuclear NMR spectroscopy to examine the solution structure of the $\psi_{32}$-modified form of the anticodon arm of E. coli tRNA\textsuperscript{Phe}. A 17 nt RNA molecule that forms a stem–loop secondary structure in solution and corresponds to the anticodon arm of tRNA\textsuperscript{Phe} was used (Figure 1). Our results demonstrate that the loop of the modified RNA molecule is composed of three nucleotides and lacks the characteristic U-turn motif. The anticodon arm is extended by two base pairs as in the unmodified parent molecule and $\psi_{32}$ increases the stability of the stem. The $\psi_{32}$ base forms a Watson–Crick type base pair with A\textsubscript{38} and not the bifurcated hydrogen bond configuration found in most crystal structures of tRNAs.

### MATERIALS AND METHODS

All enzymes were purchased from Sigma Chemical, except for T7 RNA polymerase and RluA enzymes, which were prepared as described (27,28). Deoxycytidine kinase 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 nuclelease 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% $^{13}$N-ammonium sulfate and 99% $^{13}$C-glucose were purchased from Isotec.

### Preparation of RNA samples

The RNA sequence for E. coli ACSL\textsuperscript{Phe} 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 G\textsubscript{27}–C\textsubscript{43} of full-length E. coli tRNA\textsuperscript{Phe} (17). In yeast tRNA\textsubscript{Gly}, isotonically labeled RNA molecules were prepared from 10 ml transcription reactions using 3 mM uniformly $^{15}$N-enriched and $^{13}$C-enriched 5′-NTPs as described (29). The RNA molecules were purified by passage through 20% (w/v) preparative PAGE, electroeluted (Schleicher and Schuell), and precipitated with ethanol. The purified oligonucleotides were dissolved in 1.0 M NaCl, 20 mM potassium phosphate, pH 6.8, and 2.0 mM EDTA, and dialyzed extensively against 10 mM NaCl, 10 mM potassium phosphate, pH 6.8, and 0.05 mM EDTA, using a Centricon-3 concentrator (Amicon Inc.). The samples were diluted with buffer to a volume of 0.2 ml and lyophilized to a powder. For experiments involving the non-exchangeable protons, the samples were exchanged twice with 99.9% D\textsubscript{2}O and then resuspended in 0.2 ml of 99.96% D\textsubscript{2}O. For experiments involving detection of the exchangeable protons, the samples were resuspended in 0.2 ml of 90% H\textsubscript{2}O/10% D\textsubscript{2}O. The samples contained 80 and 100 A\textsubscript{260} OD units of $^{15}$N-labeled and $^{13}$C-labeled, respectively, RNA oligonucleotides (≈2.5–3.4 mM).

### Preparation of the $\psi_{32}$-ACSL\textsuperscript{Phe}

Pseudouridine was introduced at position U\textsubscript{32} of purified ACSL\textsuperscript{Phe} using the pseudouridine synthase RluA (RluA). Histidine-tagged RluA was expressed in E. coli and purified using Ni\textsuperscript{2+} affinity resin as described (28). The pseudouridylation reaction was carried out using a molar ratio of RluA:RNA of 1:48. The reaction conditions were 50 mM HEPES pH 7.5, 100 mM NH\textsubscript{4}Cl, 0.03 mM ACSL Phe, 0.06 mM ACSL\textsuperscript{Phe}. The reactions were allowed to proceed overnight at 37°C. The RNA was purified from the RluA enzyme by heating the reaction to 90°C for 2 min followed by centrifugation to remove the precipitated protein. The supernatant was dialyzed with NMR buffer. Completion was determined by the disappearance of the C5–H5 resonance of U\textsubscript{32} from 2D 1\textsuperscript{3}C–1\textsuperscript{H} HMBC spectra (Supplementary Figure S1). The reactions could not be monitored using denaturing PAGE because the modified ACSL\textsuperscript{Phe} migrates at the same rate as the unmodified ACSL\textsuperscript{Phe}.

### NMR spectroscopy

All NMR spectra were acquired on a Bruker AMX-500 spectrometer equipped with a 1\textsuperscript{H}–{X} broadband probe, except for...
the $^{31}$P-decoupled $^{13}$C-$^1$H constant time HSQC experiment, which was collected with a $^1$H-$[^{13}$C, $^{31}$P] triple resonance probe. Broadband decoupling of the carbon and nitrogen resonances was achieved using GARP with $\gamma_{B_2} = 3125$ Hz for carbon and $\gamma_{B_2} = 1570$ Hz for nitrogen. H$_2$O spectra were collected at 12°C with solvent suppression using either spin lock pulses or binomial read pulses with maximum excitation at 12.5 p.p.m. D$_2$O spectra were collected at 25°C with presaturation or spin lock pulses to suppress the residual HDO peak. Quadrature detection was achieved using the States-TIPPI method, and acquisition was delayed by a half-dwell in all indirectly detected dimensions. Typically, the data points were extended by 25% using linear prediction for the indirectly detected dimensions and the data were apodized using 1 Hz line broadening and 65° shifted sinebell functions. $^1$H spectra were referenced relative to DSS (0.00 p.p.m.). References for the $^{13}$C and $^{15}$N spectra were calculated using the spectrometer frequencies as reported (30). The $^{31}$P spectra were referenced to an external standard of TMP which was set at 0.00 p.p.m. All spectra were processed and analyzed with Felix 98.0 (Accelrys, Inc.).

2D $^{13}$C-$^1$H HMQC and HSQC spectra were collected to identify $^{13}$C-$^1$H chemical shift correlations. 2D HCHC-COSY and 3D HCHC-TOCSY (24 ms DIPSI-3 spin lock) experiments optimized for polarization transfer through the ribose carbons and a 2D $^{13}$C-$^1$H HCHC-TOCSY (52 ms DIPSI-3 spin lock) optimized for polarization transfer through the adenine bases were collected in D$_2$O to identify ribose spin systems and H8-H2 correlations, respectively (31,32). To identify intra-residue base-sugar correlations, a 2D $^{15}$N-$^1$H HSQC experiment was acquired in D$_2$O and optimized for two- and three-bond correlations. A J(N, N)-HNN COSY experiment was acquired in D$_2$O to confirm the presence of A•U base pairs (33) and $^{31}$P assignments were obtained using a $^{31}$P/$^1$H hetero-TOCSY-NOESY spectrum (34).

Distance constraints for the non-exchangeable resonances of $^{32}$-modified ACSL$_{Phe}$ were derived at 25°C from 2D $^1$H-$^1$H NOESY spectra (80, 120, 180, 360, and 480 ms mixing times), $^{13}$C-edited 3D NOESY-HMBC spectra (180 and 360 ms mixing times), and $^{13}$C-edited 3D NOESY-ethHSQC spectra (80, 180, and 360 ms mixing times) optimized for the ribose resonances in $\omega_1$ and $\omega_2$. For the exchangeable resonances, 2D $^{13}$N-$^1$H HSQC spectra were identified to identify $^{15}$N-$^1$H chemical shift correlations. 2D $^1$H-$^1$H NOESY experiments optimized for imino (NH) proton resonances were acquired at 60 and 360 ms mixing time in 90% H$_2$O to obtain distance restraints involving the exchangeable protons.

Backbone torsion angle constraints were derived from $^1$H-$^1$H and $^{31}$P-$^1$H coupling constants obtained from the following experiments. A $^{31}$P-decoupled DQF-COSY experiment and a 2D $^{31}$P-$^1$H HetCor experiment were acquired in D$_2$O with unlabeled RNA samples.

### Interproton distance constraints

Semi-quantitative distance constraints between non-exchangeable protons were estimated from cross peak intensities in 2D NOESY and 3D $^{13}$C-edited NOESY spectra. Using the covalently fixed pyrimidine H5-H6 distance (2.2–4.4 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 1.8 Å. Distance constraints involving exchangeable protons were estimated from 360 ms mixing time NOESY spectra and were classified as either weak, very weak, or extremely weak, except for the intra-base pair distances A•U H2–NH and G•C NH–NH2, which were classified as strong constraints. Only intra-residue sugar-to-sugar constraints involving H5' and H5' were included in the calculations.

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 4.0 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. After the final calculations, virtually all predicted NOEs not in the list could be accounted for by spectral overlap or exchange broadening.

### Hydrogen bonding constraints

Watson–Crick base pairs were identified using two criteria: the 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 A$_{37}$•U$_{33}$ base pair was identified by observation of a cross peak between A$_{37}$H2 and U$_{33}$N3 in the J(N, N)-HNN COSY spectrum. 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. Constraints identified in this way were included in the calculations for base pairs G$_{32}$•C$_{43}$, G$_{28}$•C$_{42}$, G$_{20}$•C$_{41}$, G$_{50}$•C$_{60}$, A$_{31}$•U$_{30}$, and $^{31}$P$_{32}$•A$_{38}$. The U$_{33}$•A$_{37}$ base pair constraint was set to 2.9 ± 1.2 Å and 2.0 ± 1.2 Å between donor and acceptor heavy atoms and acceptor and hydrogen atoms, respectively, to permit conformational freedom of loop residues.

### Dihedral angle constraints

Constraints on the ribose ring and backbone dihedral angles were derived from semi-quantitative measurements of $^3$J$_{HH}$ and $^3$J$_{HH}$ couplings (35,36). Sugar pucker conformations were determined from $^3$J$_{HH}$-$^3$J$_{HH}$ couplings in 31P-decoupled 2D DQF-COSY spectra. Residues with H1′-H2′ couplings $>$7 Hz were constrained to the C2′-endo conformation through two of the torsion angles in the ribose sugar ring (37). Independent confirmation of sugar pucker conformation was provided by the observation of weak ($<$5 Hz) $^3$J$_{HH}$ couplings, C3′ resonances shifted downfield to 76–80 p.p.m. from the main cluster at 70–72 p.p.m., and C4′ resonances shifted downfield to 85–86 p.p.m. from the main cluster at 82–84 p.p.m. Residues with weak ($<$5 Hz) $^3$J$_{HH}$-$^3$J$_{HH}$ couplings were constrained to the C3′-endo conformation. Residues with intermediate $^3$J$_{HH}$-$^3$J$_{HH}$...
couplings were left unconstrained to reflect the possibility of conformational averaging.

Dihedral angle constraints for the \( \gamma \) torsion angle were derived from \( ^{3}J_{\text{H}_{4^{'}}-\text{H}_{5^{'}}} \) and \( ^{3}J_{\text{H}_{4^{'}}-\text{H}_{5^{'}}} \) couplings in the DQF-COSY spectrum and intra-residue \( \text{H}^{4^{'}}-\text{H}^{5^{'}} \) and \( \text{H}^{4^{'}}-\text{H}^{5^{'}} \) cross peak intensities in the 80 ms mixing time 3D NOESY-ctHSQC spectrum. For residues in which \( \text{H}^{4^{'}}-\text{H}^{5^{'}} \) and \( \text{H}^{4^{'}}-\text{H}^{5^{'}} \) peaks in the DQF-COSY spectra were clearly absent, representing couplings <5 Hz, \( \gamma \) was constrained to the gauche\(^{+}\) conformation (60 \( \pm \) 20) (35,36). For residues with clear \( ^{3}J_{\text{H}_{4^{'}}-\text{H}_{5^{'}}} \) or \( ^{3}J_{\text{H}_{4^{'}}-\text{H}_{5^{'}}} \) couplings >5 Hz and unequal \( \text{H}^{4^{'}}-\text{H}^{5^{'}} \) and \( \text{H}^{4^{'}}-\text{H}^{5^{'}} \) NOE intensities, \( \gamma \) was constrained to include both the trans and gauche\(^{+}\) conformations (\( -120 \pm 120 \)), reflecting the lack of stereospecific assignments for the \( \text{H}^{5^{'}} \) and \( \text{H}^{5^{'}} \) resonances. For residues with only weak or unobservable \( ^{3}J_{\text{H}_{4^{'}}-\text{H}_{5^{'}}} \) or \( ^{3}J_{\text{H}_{4^{'}}-\text{H}_{5^{'}}} \) couplings and unequal \( \text{H}^{4^{'}}-\text{H}^{5^{'}} \) and \( \text{H}^{4^{'}}-\text{H}^{5^{'}} \) NOE intensities, \( \gamma \) was left unconstrained to reflect the possibility of conformational averaging.

Dihedral angle restraints for the \( \beta \) torsion angles were derived from \( ^{3}J_{\text{P}-\text{H}_{5^{'}}} \) and \( ^{3}J_{\text{P}-\text{H}_{5^{'}}} \) couplings measured in 2D \( ^{31}\text{P}-\text{H} \) HetCor spectra. \( \beta \) was constrained to the trans conformation (180 \( \pm \) 40) for residues in which \( \text{P}-\text{H}^{5^{'}} \) and \( \text{P}-\text{H}^{5^{'}} \) peaks in the HetCor spectra were clearly absent, representing couplings <5 Hz (35,36). For residues in which \( \text{P}-\text{H}^{5^{'}} \) and \( \text{P}-\text{H}^{5^{'}} \) peaks could be observed, \( \beta \) was left unconstrained to reflect the lack of stereospecific assignments and the possibility of conformational averaging. All \( \text{P}-\text{H}^{3'} \) couplings that could be clearly identified were >5 Hz which allows for both trans and gauche\(^{-}\) conformations for the \( \epsilon \) torsional angle. A refinement that constrained \( \varepsilon \) angles in the stem (G27-C43 to A31-U39) to \(-125 \pm 80 \) had small improvements (relative to not constraining \( \epsilon \)) on the quality of the stem regions of the structures and were therefore included for the structures reported here.

Dihedral angle restraints for \( \alpha \) and \( \zeta \) were derived from the observation that a trans conformation of either dihedral angle is generally associated with a large downfield shift of the bridging \( ^{31}\text{P} \) resonance (38). Because no such shift is observed for any of the \( ^{31}\text{P} \) resonances in the RNA molecules, \( \alpha \) and \( \zeta \) were loosely constrained to exclude the trans conformation (0 \( \pm \) 120) for all residues except those in the loop regions (nucleotides U33 to A37), which were left unconstrained. No dihedral angle constraints were used for the glycosidic angle \( \chi \). A total of 48 restraints (11 \( \alpha \), 8 \( \beta \), 9 \( \gamma \), 9 \( \epsilon \), and 11 \( \zeta \)) were used constrain the phosphate backbone dihedral angles in the calculations.

**Structure calculations**

All calculations were carried out on Silicon Graphics O2 work stations using X-PLOOR 3.851 (39). The dihedral angles of a linear starting structure (generated using Insight II, Molecular Simulations, Inc.) were randomized to generate 75 structures with randomized coordinates which were used in a simulated annealing/restrained molecular dynamics (rMD) routine (36,37). The calculation protocol was divided into three stages: global fold, refinement, and final minimization. The global fold step consisted of 1000 cycles of unconstrained energy minimization, 10 ps of rMD at 1000 K using only hydrogen bond and NOE constraints, 9 ps of rMD at 1000 K during which repulsive van der Waals forces were introduced, 14 ps of rMD while cooling to 300 K, and 1000 cycles of constrained minimization. The structures were then refined with 500 cycles of constrained minimization, 5 ps of rMD at 1200 K during which the \( \alpha \), \( \beta \), \( \gamma \), \( \epsilon \), \( \zeta \), and sugar ring dihedral constraints were slowly introduced followed by 5 ps of rMD while cooling to 300 K, and 1000 cycles of constrained minimization. The final minimization step consisted of 1000 cycles of conjugate gradient energy minimization using all constraints and repulsive van der Waals potentials. To determine the consistency of the NMR data with the tri-loop conformation of the unmodified anticodon stem–loop, an additional set of calculations was performed using constraints involving overlapped resonances that were derived from spectra of the unmodified RNA molecule. These calculations were performed beginning with the coordinates of converged structures from the global-fold rMD simulation. Structures were viewed using Insight II (Accelrys, Inc.). The structure coordinates have been deposited in the rcsb with accession number 2AWQ.

**Thermal stability**

UV melting studies were performed using 2.2 \( \mu \)M RNA hairpin dissolved in NMR buffer (10 mM NaCl, 10 mM potassium phosphate, pH 6.8, and 0.05 mM EDTA). The samples were heated to 90\(^{\circ}\)C for two minutes and snap cooled on ice before each melt experiment. A260 absorbance spectra from 20–95\(^{\circ}\)C and from 95–20\(^{\circ}\)C were acquired (1.0\(^{\circ}\)C per minute) on a Pharmacia Ultrospec 2000 UV-Visible spectrophotometer equipped with a peltier melting heating apparatus. The melting curves were acquired in triplicate but could not be fit to a two-state model.

**RESULTS**

**Effect of \( \psi_{32} \) modification on RNA stability**

The thermal stability of the ACSL\(^{\text{Phe}} \) and \( \psi_{32} \) ACSL\(^{\text{Phe}} \) RNA hairpins (Figure 1) was investigated using UV melting experiments to determine overall molecular stability (\( T_{m} \)). The UV thermal denaturation curves indicate that the hairpins melt in two stages (Figure 2). The lower temperature (\(<50^{\circ}\)C) transitions presumably correspond to the destacking of the loop

![Figure 2](image-url)
nucleotides. The pseudouridine modification shifts the transition midpoint for the hairpin 3.5°C ± 0.5°C higher. This indicates that ψ32 increases the stability of the stem. The anneal spectra from 95–20°C also were acquired and showed 1.2°C ± 0.5°C of hysteresis at the Tm. Slow cooling of the RNA can lead to duplex formation and to hysteresis. The increased thermal stability of the modified RNA hairpin is consistent with other studies that predict pseudouridine enhances stability when located in a stem or at a loop–stem junction (8, 9). Increased stability of the upper stem and slightly lower stability of the loop is supported by the NH spectrum (Figure 3). The guanine NH protons sharpen with the introduction of ψ32 and the U33 resonance is broadened.

**Resonance assignments of ψ32-ACSL**

The non-exchangeable 1H and 13C resonances of ψ32-ACSL were assigned using standard heteronuclear methods (31, 40). Most of the base and ribose 1H-13C correlations are resolved, and none of the resonances have spectral characteristics indicative of intermediate exchange. All 17 ribose spin systems, except for the incompletely labeled 5' terminal nucleotide, were identified using 2D HCCH-COSY and 3D HCCH-TOCSY experiments. The five adenine intra-base H8-H2 correlations also were identified using the HCCH-TOCSY experiment. Intra-residue base-to-sugar correlations were identified using 2D HCCH-COSY and 3D HCCH-TOCSY experiments (33). A weak G NH resonance, which has a chemical shift that corresponds to a non-base paired guanine, was assigned to G34. The cytidine NH2 resonances were assigned using the strong intra-base pair C NH2 to G NH NOE cross peaks. The NH2 resonances of A37 and of all guanine nucleotides except G34 were not observed. The A31 NH2 resonances were assigned based on their NOE cross peaks with U39 H3. The NH2 proton sequential walk is traced with intra-residue peaks labeled. The ψ32 H1' resonance has a chemical shift of 4.56 p.p.m. and is data not shown. The arrows (a) points to the inter-residue sequential NOE between U33 H1' and G34 H8 and (b) non-sequential NOE between U33 H1' and A35 H8. The presence of the sequential NOE is not compatible with a U-turn motif for the loop whereas the non-sequential NOE can be produced by non-U-turn loop conformations.

The exchangeable NH and NH2 resonances were assigned using 2D NOESY experiments. The NH proton spectrum is shown in Figure 3. The three strong G NH resonances corresponding to G=C base pairs and one U NH resonance corresponding to an A=U base pair are connected by NOE cross peaks between NH proton resonances of adjacent base pairs. These connectivities are continuous in the helix from G28 to U30. The weak NH resonance of the terminal G=C base pair does not yield cross peaks in the NOESY spectrum. The U33 NH resonance is weak (Figure 3) and could only be assigned through a U33 N3–A37 H2 cross peak in the J(N, N)-HNN COSY spectrum (Figure 5) (33). A weak G NH resonance, which has a chemical shift that corresponds to a non-base paired guanine, was assigned to G34. The cytidine NH2 resonances were assigned using the strong intra-base pair C NH2 to G NH NOE cross peaks. The NH2 resonances of A37 and of all guanine nucleotides except G34 were not observed. The A31 NH2 resonances were assigned based on their NOE cross peaks with U39 H3. The NH2 15N resonances of A35, A36 and A38 were assigned based on intrabase H2 to N6 correlations in the J(N, N)-HNN COSY spectrum. The NH2 proton resonances of A35 and A36 are broad and have chemical shifts indicative of solvent-exposed protons.

Resonance assignments for ψ32 were accomplished using conventional heteronuclear methods. The ψ32 C1' resonance is shifted upfield into the region of the C4' resonances at consistent with the non-C3'-endo ribose ring conformations of these residues (described below).
pair would produce a y produced by the Watson–Crick base pair configurations. The shows cross-strand H2-N3 crosspeaks for residues 31–39, 38–32, and 37–33 ing intra-residue U H5 to N3 and y about the 31P assignments. The 5 nitrogen–carbon bond of uridine. The carbon glycosidic bond that replaces the more electronegative 82.78 p.p.m. This position is consistent with the carbon– carbon bond of uridine. The 32 glycosidic bond and participation of 31N in the 32-A38 base pair would produce a 31N-A38 H2 crosspeak (dashed circle). The U33-A37 crosspeak is weak and is indicative of a weak hydrogen bond.

Figure 5. (A) HNN-COSY and (B) multiple-bond 15N-1H HSQC spectra showing intra-residue U H5 to N3 and y H6 to N1 correlations. The HNN-COSY shows cross-strand H2-N3 crosspeaks for residues 31–39, 38–32, and 37–33 produced by the Watson–Crick base pair configurations. The syn configuration about the 31 glycylidic bond and participation of 31N in the 32-A38 base pair would produce a 31N-A38 H2 crosspeak (dashed circle). The U33-A37 crosspeak is weak and is indicative of a weak hydrogen bond.

Structure calculations

The structure of 32-ACSL Phe was calculated using a rMD routine starting from 75 sets of coordinates with randomized backbone dihedral angles. The calculations used a total of 238 NOE derived distance constraints, 36 bp constraints, and 74 dihedral angle constraints (Table 1) resulting in 10 converged structures (Figure 6). Structures were classified as converged if they had low energy, few constraint violations, and predicted only NOEs that could be experimentally verified or explained. The converged structures have an average of 5 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. The average root mean square deviations (RMSDs) of the heavy atoms between the individual structures and the minimized mean structure is 0.67 Å for the loop region (residues 33–37) and 0.92 Å for the stem region (residues 27–32 and 38–43) (Figure 6).

Structure of the loop region of 32-ACSL Phe

The 32-ACSL Phe loop is made up of the anticodon nucleotides G34–A36 and is closed by the U33•A37 base pair (Figure 7). The base of G34 against the base of U33, consistent with the observed H6-H8 and H5-H8 NOEs. The bases of G34 and A35 are approximately coplanar and are parallel. This configuration satisfies the observed G34 H8–A35 H8 NOE cross-peak. The A36 base stacks beneath A37 base but is displaced toward the minor groove edge of A37 (Figure 7). Several NOEs involving the A36 H2 resonance help position this base and include interactions with the H2’ of U33, the H8 of A35, and the G34 and/or A35 H1’ (these ‘ resonances are degenerate). These cross-peaks indicate that the Watson–Crick base pair functional groups are oriented to the interior of the molecule and point toward the residues

Table 1. Summary of experimental constraints and structure calculation statistics for 32-ACSL Phe

| Constraint | Count |
|------------|-------|
| NOE distance constraints | 88 |
| Interresidue | 150 |
| Mean number per residue | 14.0 |
| NOE constraints by category | |
| Very strong (1.8–3.0 Å) | 7 |
| Strong (1.8–4.0 Å) | 24 |
| Medium (1.8–5.0 Å) | 70 |
| Weak (1.8–6.0 Å) | 88 |
| Very weak (1.8–7.0 Å) | 49 |
| Base pair constraints | 36 |
| Dihedral angle constraints | |
| Ribose ring | 26 |
| Backbone | 48 |
| Mean number per residue | 4.3 |
| Violations | |
| Average distance constraints > 0.3Å | 0 |
| RMSDs for distance constraints (Å) | 0.027 |
| Average dihedral constraints > 0.5° | 3.7 |
| RMSDs for dihedral constraints (°) | 0.26 |
| RMSD from ideal geometry | 0.006 |
| Bonds (Å) | 1.4 |

0. Only conformationally restrictive constraints are included.
1. The number of base pair constraints includes U33•A37 base pair constraints.
2. Three torsion angles within each ribose ring were used to constrain the ring to either the C2’-endo or C3’-endo conformation. The ring pucker of residues U33 and A37 were not constrained.
3. A dihedral angle violation of 0.3 Å corresponds to 5.0 kcal energy penalty.
4. A dihedral angle violation of 0.5° corresponds to 0.05 kcal energy penalty.
5. Calculated for the minimized average structure.
The loop nucleotides also have unusual sugar–phosphate backbone conformations. The large H1′–H2′ couplings of residues G34–A36 indicate that the ribose sugar rings have the C2′-endo conformation. The strong intra-residue and weak sequential H2′ to H8 NOE cross-peaks as well as the substantial downfield shift of the C3′ and C4′ resonances involving residues G34–A36 also support the C2′-endo conformation. Residues U33 and A37 at the junction of the loop and stem have a mixture of C2′- and C3′-endo conformations as evidenced by their intermediate H1′–H2′ couplings and the modest downfield chemical shifts of their C3′ and C4′ resonances. The ε torsion angles of A35 and A36 have the -gauche conformation not typical of A-form geometry.

A superposition of the loop regions from the ten converged structures is shown in Figure 6B and the minimized average structure is shown in Figure 7. The helical base stack is continuous along the 3′ side of the loop, with G34 stacking against the U33 base. On the 3′ side of the loop, the A37 base straddles the bases of A35 and A36. The A36 N6 is 3 Å from the U33 O2′ in half of the converged structures, suggesting the possibility of a cross strand base–sugar hydrogen bond. However, this interaction could not be confirmed since the A36 NH2 proton resonances could not be assigned and no 2′-OH proton resonances were identified.

The loop of the ψ32-ACSLPhe does not contain the classical ‘U-turn’ motif. Although weak, the inter-residue U33 H1′-G34 H8 and U33 H6–G34 H8 NOE cross-peaks (Figure 4) are consistent with the U33 and G34 positions shown in Figure 7 but not with their positions in the U-turn (Figures 8B and C). A U33 H1′ to A35 H8 NOE is observed, but only at long mixing time and is even less intense than the sequential U33 H1′–G34 NOE (Figure 4). The ribose puckers of the anticodon nucleotides of the U-turn also tend toward the C2′-endo conformation rather than the C2′-endo conformation observed in this study. Another feature characteristic of 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 (Supplementary Table S1). The chemical shift of this resonance is not consistent with the 31P chemical shift predicted for a phosphate having the trans conformation of the α torsional angle (38).

**Structure of the stem of ψ32-ACSLPhe**

The geometry of the hairpin stem, base pairs G34–C43 to U33–A37, is primarily A-form (Figure 6A). The sequential base-1′, 2′ and several base–base NOEs are continuous at 180 ms mixing time. The A31–U39 and ψ32–A38 base pairs are sufficiently stable to give rise to a cross-strand NH–H2 NOEs, but the NH proton of the U33–A37 base pair is not. The ψ32 and U33 NH proton resonances are weak, but their participation in Watson–Crick pairing schemes was confirmed using a J(N, N)-HNN-COSY spectrum (Figure 5). Nucleotides A31, A37, and A38 also produce cross-strand H2–H1′ NOEs commonly observed for A/U base pairs within helices. The H2 to N3 through bond correlations clearly confirm that U39 is base paired with A31 and ψ32 is base paired with A38 through its N3 imino group (Figure 7).

The torsion angles of the sugar-phosphate backbone are within the limits of A-form geometry. The small (<5 Hz) H1′–H2′ couplings and the 13C chemical shifts of the 3′ and flanking the 5′ side of the nucleotide. The conserved NOE cross-peaks A36 H2 to A37 H2 and H1′ suggest that A36 is partially stacked with A37. Thus, the observed NOEs support the compact tri-loop conformation.

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Figure 6. Stereoview of the superposition of (A) the stems and (B) the loops of the 10 converged ψ32-ACSLPhe structures. Convergence criteria are given in the text. The views are into the major groove. Only sugar and base heavy atoms are shown and the average r.m.s. deviation for the heavy atoms between the ten structures and the average structure is 1.14 Å. The loop and stem regions are locally well defined, but the propeller twist of the A31–U39 base pair is variable among the structures and slightly increases the r.m.s.d. of the full hairpin.

Figure 7. Stereoview of minimized average structure of ψ32-ACSLPhe (residues G34 to C43). Hydrogen bonding NH and NH2 protons of base pairs ψ32–A38 and A31–U39 are colored purple and the exocyclic amino nitrogens (A31 and A38) are green. The pro-R(p) phosphoryl oxygens of A31 and A38 (residues G30 to C40). Hydrogen bonding NH and NH2 protons of base pairs G34–A38 and A31–U39 are colored purple and the exocyclic amino nitrogens (A31 and U39) are green. The pro-R(p) phosphoryl oxygens of A31 and U39 (residues G30 to C40).
4′ resonances of nucleotides G32–A32 and A38–C42 are indicative of the typical C3′-endo sugar pucker. None of the α or β torsional angles within the converged structures deviated from gauche.

The most remarkable feature of the stem is the minimal structural perturbation caused by ψ32. Although the combination of A31 H8-ψ32 H6 and A33 H2-ψ32 H6 NOEs is unusual, the relative positions of the adjacent A31 and ψ32 bases that give rise to the interactions are accommodated within the A-form helix.

**DISCUSSION**

We are using the anticodon stem–loop of *E. coli* tRNA^Phe^ as a model to probe the cumulative physical effects of sequential modifications in RNA molecules. Pseudouridine is the most common base modification found in RNA and the anticodon stem-loop of *E. coli* tRNA^Phe^ has two of these nucleotides, ψ32 and ψ39. In this study, we have examined the thermodynamic and structural effects of ψ32 on the otherwise unmodified tRNA^Phe^ anticodon stem–loop.

**The structures of ACSL^Phe^ and ψ32-ACSL^Phe^ are similar**

The unmodified anticodon stem–loop of ACSL^Phe^ forms a highly ordered tri-loop conformation (42) and the ψ32-ACSL^Phe^ adopts a similar structure. The stems of the two hairpins are continuous through base pair U33–A37, but this loop-closing base pair is weaker in the ψ32-modified molecule indicating a slightly less compact tri-loop. Also, fewer constraints involving G34 and A35 in ψ32-ACSL^Phe^ lead to greater variability in the positions of these residues compared to the unmodified molecule, including excursions of A33 to the minor groove side of the loop. However, the lower number of experimental constraints in the loop is due to spectral overlap of G34 and A35 H1′ resonances and probably does not reflect conformational heterogeneity inherent in the loop. Nonetheless, the small chemical shift differences between unmodified and ψ32-modified ACSL^Phe^ molecules indicate that the loop structures are not identical. The energy difference between the converged conformations of ψ32-ACSL^Phe^ is small (<15 kcal/mole), and the RMSD between the ψ32-ACSL^Phe^ and ACSL^Phe^ minimized average structures is 1.13 Å.

The pseudouridylation of U32 increases the overall stability of the RNA hairpin. This is consistent with studies that have examined the thermodynamic effects of pseudouridine incorporation (9,13,43,44). Pseudouridine within a helix can increase the melting temperature of the helix 3–5°C depending upon the neighboring base pairs and improves local base stacking (9). A contributing factor to the stabilization effect of ψ32 is its ability to form a water-mediated hydrogen bond with the phosphate backbone (9). This hydrogen bond interaction has been inferred from difference maps of X-ray crystallographic studies of unmodified and fully modified tRNA^Gln^ molecules (19) and is present in molecular dynamics simulations of yeast tRNA^N^ (45). The strong exchange-protected ψ32 N1H resonance (Figure 3) supports this interaction within the ACSL^Phe^ and is modeled in the average structure (Figure 7). Pseudouridine also confers a smaller degree of helix stabilization when present in single strand regions adjacent to a helix (46) or is on the 3′ end of the loop at loop-helix junctions such as ψ30 (46). ψ32 is adjacent to the helix-loop junction (U33–A37), but is at the 5′ end of the loop. Thus, pseudouridine has a comparable stabilizing effect when positioned on either the 3′ or 5′ edge of a helix.

Pseudouridine tends to form a Watson–Crick base pair with adenine in helical contexts and the ψ32 is no exception (43). The HNN-COSY spectrum confirms the hydrogen bond conformations of the ψ32-A38 and U33–A37 base pairs (Figure 5). However, the H3 resonances of U33 and ψ32 are weaker than the corresponding H3 resonances of unmodified ACSL^Phe^ (Figure 3). This weakening may reflect altered alignment of the hydrogen bonds of these base pairs as the base of ψ32 is stabilized toward the major groove by the water-mediated N1H-phosphate hydrogen bond (Figure 7). A ψ32 H6-A31 H2 NOE is consistent with the major groove displacement of the ψ32 base. This NOE is not observed in the unmodified molecule (42) and is not typical of 5′-AU-3′ base stacking in helices with regular A-form geometry.

**The 32–38 bp in tRNA**

The nucleotide composition of residues 32 and 38 is not an equal distribution of the pyrimidine and purine bases. tRNA sequence data show that position 38 is often adenine (67%),
but a significant fraction (30%) of position 38 nt are pyrimidines (14). Residue 32 is almost exclusively occupied by a pyrimidine base (>97%) and is modified in approximately a third of sequenced tRNA molecules on either the base or ribose group. The most common base modification at position 32 is pseudouridine and although this base does not alter the overall structure of the ACSL₃ loop compared to uridine, it partially destabilizes the tri-loop conformation and increases the stem melting temperature by about 3.5°C. Other modifications at position 32 are frequently those that favor the C₃’ endo conformation of the ribose, such as pyrimidine 2’-O-methyl and the pyrimidine C2-thiol (47), which may help to maintain the helical integrity of the stem of the anticodon arm (48). Whether these modifications can alter or stabilize anticodon loop conformations or function as pseudouridine primarily to stabilize the stem has not been investigated.

The geometry of the ψ₃₂ base in solution is different from that observed for position 32 residues in crystal structures of tRNAs. The crystal structures of several tRNAs reveal a highly conserved structural motif, the bifurcated hydrogen bond, between residues 32 and 38 and 49 (Figure 8). The bifurcated hydrogen bond frequently involves proton donation by an exocyclic NH₂ group and acceptance by the O2 of C₃₂ or U₃₂. This hydrogen bond configuration imposes an underwinding of the helix geometry at the 32–38 base step. For *E.coli* tRNA²Ph, the configuration would correspond to hydrogen bonding between A₃₈ N₆H₂ and ψ₃₂ O₄ (adjacent to the C₅-C’ glycosidic bond). This base arrangement of ψ₃₂ and A₃₈ is isosteric with other 32–38 pairs, such as C₃₂–A₃₈ and U₃₂–C₃₈, that adopt the bifurcated hydrogen bond (49) (Figure 8). Auffinger and Westhof (49) have proposed the geometry of nt 32 and 38 resulting from the bifurcated hydrogen bond creates a transition between the stem and loop in the anticodon arm and facilitates formation of the U-turn. Thus, the 32–38 interaction may allow an open conformation of the loop without distortion of the canonical A-form stem. The results of this study indicate that if the bifurcated hydrogen exists in solution and functions in U-turn stabilization, the ψ₃₂ modification alone is not sufficient to organize this motif.

The crystal structure of fully modified Cys-tRNA⁵⁸syn in complex with EF-Tu and GDPNP presents an alternative conformation for the ψ₃₂–A₃₈ base pair. In this structure, the ψ₃₂ base adopts the syn configuration about the glycosidic bond and does not form any interactions with A₃₈ (17). This configuration of the base can accommodate the ψ₃₂ water-mediated hydrogen bond to the phosphate backbone and thus should retain the stabilizing effect conferred by pseudouridine in the anti configuration. Notably though, the anticodon loop in this complex adopts the characteristic U-turn motif with stacking of the anticodon bases G, C, and A. The EF-Tu protein binds at the acceptor stem and has no contacts in the region of the anticodon arm, but the G and C nucleotides of the anticodon form intermolecular base pairs within the unit cell of the crystal. The structure of this molecule is of particular interest since the anticodon arm of tRNA⁵⁸syn contains the same nucleotide base modifications found in fully modified *E.coli* tRNA²Ph, specifically ψ₃₂, ψ₄₉, and ms₃C₆A₃₇. These additional modifications could affect the conformation of the ψ₃₂ base, however, neither ψ₄₉ nor ms₃C₆A₃₇ alone are sufficient to induce the syn conformation of ψ₃₂ in solution (J.Cabello and E.P.Nikonowicz, unpublished data).

Pseudouridylation within the anticodon arm of tRNA is important to the overall fitness of an organism and loss of this modification can lead to a spectrum of growth defects in bacteria and yeast (50–54). Disruption of the truA gene which catalyzes formation of ψ₃₈, ψ₄₉, and ψ₄₉ decreases polypeptide chain elongation rates and reduces cell growth rate ~30% (1,55). A similar effect is observed for the degI gene of yeast that catalyzes ψ₃₈ and ψ₄₉ formation (54,56). *E.coli* that contain a catalytically inactive truB gene which catalyzes formation of ψ₅₅ exhibit no growth defects (52), even though ψ₅₅ is universally conserved (14). Pseudouridylation at position 32 is intermediate in its physiological effects. Cells that lack RluA activity have near normal growth rates, but are strongly selected against when grown in competition with wild type cells (51). However, given that RluA modifies only six tRNA isoacceptors, unlike TruA and TruB that modify their target sites in all tRNA molecules in *E.coli*, the physiological effects of its loss are remarkable and suggest a critical role of ψ₃₂ in proper tRNA function. The specific translational defects associated with loss of ψ₃₂ have not been determined, but studies of position 32 mutations in other tRNAs suggest translational fidelity and wobble base recognition are likely to be impaired (23–25,57). The studies presented here indicate that a primary role of ψ₃₂ is the stabilization of the stem of the anticodon arm. Thus, ψ₃₂ may serve as a buffer to preserve the stability and the architecture of the stem within the context of other loop destabilizing nucleotide modifications (42) or after formation of the U-turn.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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