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Base Pairing within the ψ_{32},ψ_{39}-Modified Anticodon Arm of *Escherichia coli* tRNA^{Phe}

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The anticodon arm of tRNA is central to the accuracy and efficiency of protein synthesis. In addition to the three anticodon nucleotides, the extended anticodon hypothesis predicts that the sequence and modification state of nucleotides in the loop-proximal region of the anticodon arm can modulate the decoding function of tRNA.[1] Consistent with this hypothesis is the influence that nucleotides 32 and 38 can have on the accuracy of translation[2] and on the affinity of tRNA for the ribosome.[3] The accuracy conferred by these residues suggests they have a role in fine-tuning the conformational or thermodynamic features of the anticodon arm either in the free tRNA or in the ribosome-bound tRNA. The crystal structures of yeast tRNA^{Phe}, tRNA^{Asp}, and several tRNA-protein complexes reveal the presence of a conserved motif, the bifurcated hydrogen bond, between nucleotides 32 and 38.[4] The bifurcated hydrogen bond allows for an isosteric arrangement of nucleotides 32 and 38 that is preserved among C_{32}−A_{38}, ψ_{32}−A_{38}, and ψ_{32}− C_{38} interactions.[4d] This arrangement leads to partial unwinding of the helix and may help promote formation of the U-turn motif of the anticodon loop.[4d] The unmodified anticodon arm of *E. coli* tRNA^{Phe} also is highly ordered in solution. However, the anticodon adopts a triloop conformation rather than the characteristic U-turn,[5] and U_{32} and A_{38} form a Watson−Crick base pair instead of the bifurcated hydrogen bond. To determine if the native modifications ψ_{32} and ψ_{39} in the anticodon arm lead to formation of the bifurcated hydrogen-bond motif or the U-turn, we have used heteronuclear NMR spectroscopy to investigate their effects on the local hydrogen-bond geometry between nucleotides 32 and 38 and on the global architecture of the anticodon loop.

Pseudouridine is an isomer of uridine in which the N1−C1′ glycosidic bond has been isomerized to C5−C1′ (Figure 1). ψ_{32} and ψ_{39} were introduced into the anticodon arm of *E. coli* tRNA^{Phe} using the pseudouridine synthase RluA.[6] Uniformly ^{13}C/^{15}N-labeled r(GCGGAUUGAAAA*U*CCGAAAUUUUCAAUCCGC) was modified with RluA to produce ψ_{39} precursor anticodon stem-loop. The RNA hairpin was digested with ribonuclease T1 and the 5′ half purified by polyacrylamide gel electrophoresis and modified a second time with RluA to produce the [ψ_{32},ψ_{39}]-modified hairpin (Figure 1). Completeness of the reaction (>99%) was confirmed by loss of the C5−H5 resonances of U_{32} and U_{39}. In addition, an upfield-shifted pseudouridine H1 peak in the NH region of the NMR spectrum often can be observed and is diagnostic of participation of the H1 in a water-mediated hydrogen bond with the phosphate backbone.[7] Both pseudouridine 32 and 39 H1 resonances are present in the imino spectrum, with the ψ_{39} H1 resonance slightly broadened relative to ψ_{32} H1.

The secondary structure in the loop region of the anticodon arm is altered by pseudouridylation. Similar to U_{39}, ψ_{39} forms a Watson−Crick type base pair with A_{31} as evidenced by an intense interbase A_{31} H2−ψ_{39} H3 NOE and an A_{31} H2−ψ_{39} N3 scalar cross-strand correlation. However, the ψ_{32} and U_{33} H3 resonances are broadened beyond detection and no H2−N3 scalar correlations

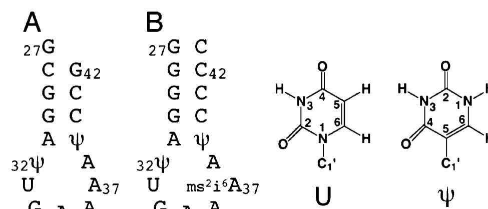

Figure 1. (A) The [ψ_{32}, ψ_{39}]-modified RNA hairpin used in this study and (B) the fully modified anticodon stem-loop of *E. coli* tRNA^{Phe}. The tRNA numbering is used. Base structures and atom numbering of U and ψ are shown.

with cross-strand adenines could be observed, indicating loss or weakening of the 32−38 and 33−37 base pairs. Relative to the unmodified molecule, ψ_{32} does not alter the loop structure,[6] but ψ_{39} can disrupt the loop secondary structure.[8] To determine if ψ_{32} participates in a bifurcated hydrogen bond with A_{38}, the ^{15}N and ^{13}C chemical shifts of possible hydrogen-bond donor and acceptor groups were examined. The carbonyl C2 and C4 resonances are shifted downfield by hydrogen bonding of the O2 and O4 atoms, respectively, and can be used to deduce base−base interactions involving these functional groups.[9] In the Watson−Crick ψ−A base pair, the ψ O2 forms a hydrogen bond with the A N6H_2 and the ψ O4 does not participate in an intramolecular hydrogen bond. In the bifurcated hydrogen-bond motif, ψ O4 would serve as an acceptor of the A_{38} N6H_2 group, and the ψ O2 and H3 would be solvent exposed (Figure 2). Since ψ_{39} and A_{31} form a Watson−Crick pair, the C2 and C4 chemical shifts of ψ_{39} serve as an internal standard. The CCH-relay spectrum (Figure 2A) identifies the C4 resonances via the C4−H6 correlation. The chemical shifts of ψ_{32} and ψ_{39} C4 resonances differ by <0.1 ppm, indicating the ψ_{32} O4, like the ψ_{39} O4, does not participate in a base−base hydrogen bond. The H(N)CO spectrum can be used to identify both the C4 and C2 resonances via the H3 resonance, but the ψ_{32} H3 is not present in the NH spectrum owing to exchange, and only the ψ_{39} H3 resonance is observed. However, the H1 resonances of both ψ_{32} and ψ_{39} are observed, permitting identification of the C2 resonances via the H1−C2 correlation (Figure 2B). Loss of the interbase hydrogen bond when switching from the Watson−Crick to the bifurcated hydrogen-bond configuration is expected to produce a ∼2 ppm upfield shift of the C2 resonance.[9] The ψ_{32} C2 resonance is 0.4 ppm upfield of ψ_{39} C2. This upfield shift suggests that the ψ_{32} C2 forms a hydrogen bond but that the bond is longer or geometrically suboptimal relative to the A_{31}−ψ_{39} interaction.

The chemical shifts of the adenine amino nitrogen resonances also are sensitive to the base hydrogen-bond pattern. The hydrogen bond formed by H6 through base pairing leads to a downfield shift of the N6 resonance.[10a] In the A−ψ bifurcated hydrogen bond, both of the adenine H6 atoms form hydrogen bonds with the pseudouridine C4 oxygen atom (Figure 1) and are predicted to shift the N6 resonance further downfield.[10b,c] The chemical shift of the A_{38} N6 resonance is 82.6 ppm and is nearly degenerate with the N6 of

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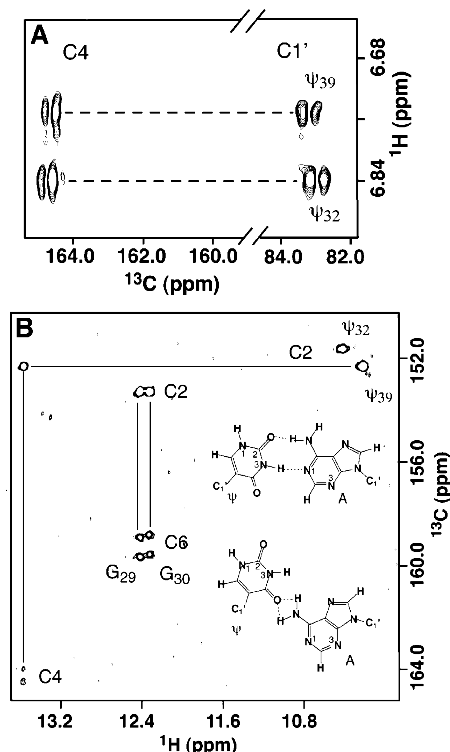

Figure 2. Two-dimensional (A) CCH-relay and (B) H(N)CO spectra of [ψ_{32},ψ_{39}]-modified ACSL^{Phe}: geometries of a ψ−A Watson−Crick base pair (upper) and a ψ−A bifurcated hydrogen bond (lower). The C4 carbonyl and C1′ anomeric carbon resonances are correlated with the base H6 resonance (shown in panel A). The ψ_{32} and ψ_{39} C4 differ by ~0.10 ppm. The N1H imino protons of both pseudouridine residues correlate with the intrabase C2 carbonyl carbons (shown in panel B). The N3H imino proton of ψ_{39} correlates to both C2 and C4. The ψ_{32} C2 resonates 0.42 ppm upfield of the ψ_{39} C2 resonance. The small chemical shift differences between the C2 and C4 resonances of ψ_{32} and ψ_{39} support similar hydrogen-bond environments for the ψ_{32} base and the Watson−Crick paired ψ_{39} base.[9] Spectra were acquired at 20 and 12 °C for data A and B, respectively.

A_{32} (82.5 ppm). The N6 of the weakly base paired A_{37} resonates at 80.6 ppm and those of the unpaired A_{35} and A_{36} are 78.9 and 79.8 ppm. The A_{38} N6 chemical shift is consistent with the Watson−Crick configuration of A_{38} and ψ_{32} and hydrogen bonding through a single H6.[10]

Although ψ_{32} and ψ_{39} do not produce the ψ_{32}−A_{38} bifurcated hydrogen bond, these modifications disrupt the U_{33}−A_{37} base pair and may alter the conformation of the anticodon loop. The U-turn motif in tRNA^{Phe} is characterized, in part, by two hydrogen bonds, one between U_{33} H3 and the A_{35}−A_{36} bridging phosphate and the other between U_{33} HO2′ and A_{35} N7.[4a] However, the spectra of the modified molecule do not contain features diagnostic of this hydrogen-bond network,[11,12] and NOE data confirm that the anticodon loop maintains the triloop conformation (Figure 3).

These results demonstrate that the pseudouridine modifications alone are not sufficient to generate the bifurcated hydrogen bond or the U-turn motifs in the anticodon loop of *E. coli* tRNA^{Phe}. A similar result was observed for the N6-isopentenyl modification of A_{37} also present in *E. coli* tRNA^{Phe}. This modification relaxes the loop structure, but does not change the fold of the loop.[5] Also within the context of the ψ_{32} and ψ_{39} modifications, the presence of Mg^{2+} leads only to minor broadening of loop nucleotide resonances and does not change the loop fold. ψ_{32} partly compensates the secondary structure disruption in the loop region caused by ψ_{39}[8] and may stabilize the helix of the anticodon arm when the A_{37} modification is introduced. That the structural motifs present in the crystal structures are not observed in the partially modified molecules in solution suggests a possible synergy among the anticodon loop modifications. Alternatively, these motifs may represent only one of multiple conformational states sampled by an anticodon loop made flexible in solution through base-modification and divalent metal−ion interactions.

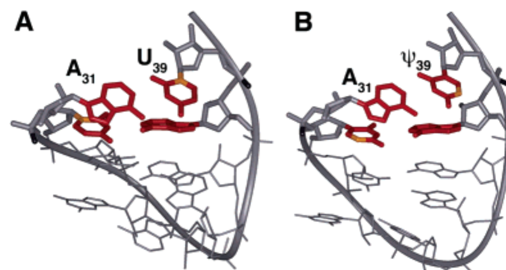

Figure 3. Comparison of residues 31−39 of the solution structures of *E. coli* tRNA^{Phe} anticodon arms (A) unmodified[5] and (B) [ψ_{32},ψ_{39}]-modified. The N1 atoms are highlighted in yellow. In structure B, ψ_{32} is in the anti configuration about the glycosidic bond and forms a Watson−Crick type hydrogen bond with A_{38}. Neither A or B structures contain the U-turn motif. The [ψ_{32},ψ_{39}]-modified ACSL^{Phe} was modeled using base−base and base−ribose NOEs and hydrogen-bond data.

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Supporting Information Available: A table listing sequence specific ^{1}H, ^{13}C, ^{15}N, and ^{31}P chemical shifts, a figure showing the N6−H2, H8 correlations from the HNCCH−TOCSY spectrum, and a summary of the modeling of the [ψ_{32},ψ_{39}]-modified ACSL^{Phe}. This material is available free of charge via the Internet at http://pubs.acs.org.

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