Published in final edited form as:

J Mol Biol. 2011 April 22; 408(1): 99–117. doi:10.1016/j.jmb.2011.02.014.

Solution structure of the K-turn and Specifier Loop domains from the *Bacillus subtilis* tyrS T box leader RNA[†]

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Abstract

In Gram-positive bacteria, the RNA transcripts of many amino acid biosynthetic and aminoacyl-tRNA synthetase genes contain 5' untranslated regions, or leader RNAs, that function as riboswitches. These T box riboswitches bind cognate tRNA molecules and regulate gene expression by a transcription attenuation mechanism. The Specifier Loop domain of the leader RNA contains nucleotides that pair with nucleotides in the tRNA anticodon loop and is flanked on one side by a kink-turn, or GA, sequence motif. We have determined the solution NMR structure of the kink-turn (K-turn) sequence element within the context of the Specifier Loop domain. The K-turn sequence motif has several non-canonical base pairs typical of K-turn structures but adopts an extended conformation. The Specifier Loop domain contains a loop E structural motif and the single strand Specifier nucleotides stack with their Watson-Crick edges displaced toward the minor groove. Mg²⁺ leads to significant bending of the helix axis at the base of the Specifier Loop domain, but does not alter the K-turn. ITC indicates that the K-turn sequence causes a small enhancement of the interaction between tRNA anticodon arm with the Specifier Loop domain. One possibility is that the K-turn structure is formed and stabilized when tRNA binds the T box riboswitch and interacts with Stem I and the antiterminator helix. This motif in turn anchors the orientation of Stem I relative to the 3' half of the leader RNA, further stabilizing the tRNA-T box complex.

Keywords

heteronuclear; hairpin; tRNA; transcription regulation; riboswitch; antitermination

Introduction

In Gram-positive bacteria, the transcription of many tRNA synthetase genes and genes involved in amino acid metabolism is regulated in a tRNA-dependent manner by the T box riboswitch 1, ². The transcription of T box regulated genes is prematurely terminated when

[†]This work was supported by Welch Foundation grant C-1277 to E.P.N. and by National Institutes of Health grant GM73969 to E.P.N.

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Accession Numbers

Coordinates have been deposited in the Protein Data Bank under accession number PDB ID: **2KZL**

Chemical shifts have been deposited in the Biomolecular Magnetic Resonance Bank under accession number BMRB ID: **17316**

the relative level of charged to uncharged tRNA is high. The binding of an uncharged tRNA molecule to the T box riboswitch permits read-through transcription of the gene. Each gene regulated by this mechanism responds to a specific species of tRNA (cognate tRNA) that is related to that gene's function. The T box riboswitches are 200–300 nt 5' untranslated regions (or leader RNAs) of the gene transcripts that fold to form a complex set of stem-loop secondary structures including Stems I, II, and III and the mutually exclusive terminator and antiterminator hairpin secondary structures (Figure 1A). Formation of the thermodynamically more favorable terminator hairpin causes transcription to be terminated prior to the translation start codon of the gene³. Read-through of the transcription termination site occurs when uncharged cognate tRNA binds to the leader RNA and stabilizes the antiterminator hairpin by pairing of nucleotides in the 3' end of the tRNA with a seven-nucleotide bulge in the antiterminator helix⁴.

The specificity of the leader RNA-tRNA interaction is achieved through the anticodon bases and the discriminator base of the tRNA molecule^{3, 5}. The Specifier Loop domain is a conserved structural feature of the T box riboswitch Stem I that is variable in size and contains the Specifier Sequence. The Specifier Sequence, three nucleotides complementary to the anticodon nucleotides of the cognate tRNA, is the primary determinant of tRNA specificity. Also conserved in the Specifier Loop domain are a purine residue, 3' to the Specifier Sequence^{6, 7}, that pairs with U₃₃ of tRNA⁸ and a loop E structural motif. Near the 3' end of the leader RNA are the nucleotides that alternately form the terminator and antiterminator helices. In the antiterminator helix, four residues of a seven nt bulge base pair with nucleotides at the 3' end of the tRNA, including the discriminator base. The contributions of these interactions in specifying the leader RNA-tRNA complex have been characterized in *B. subtilis* for the *tyrS* and *glyQS* leader RNAs^{3, 9} and reproduced *in vitro* using a purified transcription assay and the *glyQS* leader RNA^{8, 10}.

In addition to the Specifier Loop domain of Stem I and the bulge in the antiterminator helix, other regions of the T box riboswitch exhibit a high degree of conservation and are functionally important^{6, 7, 11}. The Specifier Loop is flanked on one side by a sequence motif predicted to form a kink-turn (K-turn, or GA) structural motif¹¹. The K-turn motif has been identified in several RNAs¹² including rRNA^{13, 14}, the U4 snRNA^{15, 16}, the archaeal C/D-box snoRNA¹⁷, the *Azoarcus* group I intron¹⁸, and the lysine and S-adenosylmethionine riboswitches^{19, 20}. Most experimentally confirmed K-turns correspond to protein binding sites^{13, 15, 21}, but K-turns also have been observed outside protein-bound contexts^{18, 20}. The net structural effect of the K-turn on RNA is the introduction of a ~120 degree bend in the helix axis towards the minor groove¹³. The reverse K-turn, which has been observed infrequently, leads to a bend toward the major groove^{12, 22}. The direction of the bend created by the K-turn is substantially influenced by elements outside the K-turn²³.

The K-turn sequence motif contains an asymmetric internal loop with flanking stems designated canonical (C-) and non-canonical (NC-). The consensus sequence features a pair of 5'-GA-3' dinucleotide sequences on opposite strands that form tandemly arranged sheared G-A base pairs and terminate the NC-stem¹⁴. The C-stem is separated from the NC-stem by a string of three unpaired nucleotides and is capped by a G-C base pair (Figure 1C). The phosphate backbone turns sharply (kinks) between the second unpaired nucleotide and the third (NC-stem proximal) unpaired nucleotide. The base of the first unpaired nucleotide stacks on the terminal G-C pair of the C-stem and the base of the second unpaired nucleotide stacks against the terminal G-A pair of the NC-stem. A-minor interactions between adenines of the G-A pairs and nucleotides at the terminus of the C-stem also are present, but the relative contributions of these interactions for stabilizing the K-turn conformation are variable²⁴.

The high conservation of the kink-turn motif within Stem I of T box leader RNAs suggests an important role in leader function, including tRNA binding. Indeed, single base mutations predicted to disrupt the K-turn motif significantly reduce the ability of tRNA to promote transcriptional antitermination *in vivo*¹¹. The role of the K-turn sequence motif in this process is not known, but the *glyQS* T box riboswitch functions in a purified *in vitro* system¹⁰, suggesting that it does not serve as a protein recognition site.

We have examined the solution structure, dynamics, and Mg²⁺-binding properties of the K-turn sequence motif and Specifier Loop domain from Stem I of the *B. subtilis* *tyrS* T box riboswitch. The K-turn sequence motif contains three unpaired adenine nucleotides and the C-stem is terminated by an A-U base pair (Figure 1A) in the *tyrS* leader sequence rather than the G-C base pair conserved among K-turns (Figure 1C). The Specifier Loop domain of this leader RNA contains 14 nucleotides and is connected to the K-turn sequence motif by a four base pair helix. Using solution NMR spectroscopy, we find that the K-turn sequence motif and the Specifier Loop domain are conformationally independent. The K-turn sequence motif is well-structured but does not adopt the prototypical K-turn fold in the presence or absence of Mg²⁺. The Specifier Loop domain contains a loop E structural element and the arrangement of the Specifier Sequence nucleotides are minimally altered by the presence of the K-turn. Isothermal titration calorimetry measurements also show that the K-turn sequence motif does not directly facilitate interaction between the Specifier codon and tRNA anticodon nucleotides. The results suggest a model in which tRNA binds to the T box riboswitch and stabilizes the K-turn structural motif with the characteristic bend in the helix axis.

RESULTS

The RNA molecule used for structural analysis in this study, *tyrS*_{SK} (Figure 1B), contains 55 nucleotides and corresponds to the Specifier Loop domain joined to the conserved K-turn motif that together form the lower part of Stem I of the *B. subtilis* *tyrS* leader RNA. The assignments for *tyrS*_{SK} were supplemented by examining spectra for individual RNA hairpins containing the K-turn motif, *tyrS*_{HK}, and the Specifier Loop domain, *tyrS*_{HS}. Cross peaks in the NH ¹⁵N-¹H HSQC spectrum are consistent with the predicted secondary structure including the signature peak at 9.95 ppm from the UUCG tetraloop. The monomeric nature of the RNA is supported by moderate line widths (9–14 Hz ¹H) and the nearly identical cross peak patterns in NH spectra at low (20 μM) and high (1.4 mM) RNA concentrations. In addition, native gel analysis shows that all three hairpins migrate as single bands, supporting the presence of a single form.

Chemical shift assignments

The sequence-specific resonance assignment of *tyrS*_{SK} was accomplished using ¹H-¹H NOESY and two- and three-dimensional heteronuclear experiments. The NH resonances were assigned using the NOE connectivities between NH proton resonances of neighboring base pairs. These connectivities are continuous from G₂ to G₅, from U₄₇ to G₁₆, and from G₂₄ to G₃₁. The NH resonance of G₄₈ (G₂₆ in *tyrS*_{HK}) is present, but is weak and does not give rise to NOE cross peaks with U₁₂ or U₄₇. The cytidine and adenine NH₂ resonances were assigned for *tyrS*_{HK} and *tyrS*_{HS} molecules using NOESY and HNCCH experiments²⁵.

The non-exchangeable ¹H and ¹³C resonances of the RNA molecules were assigned using standard heteronuclear techniques^{26, 27}. Resonances for *tyrS*_{HK} and *tyrS*_{HS} were assigned first to facilitate assignment of *tyrS*_{SK}. Most of the base and ribose 1' ¹H-¹³C correlations are resolved, with some of the base resonances (G₁₄, A₁₅, A₂₃, G₂₄, and U₄₇ H8-C8/H6-C6 and A₂₃ H2-C2) having spectral characteristics indicative of intermediate exchange (Figure 2A). All residues except U₂₂, A₂₃, and G₂₄ yielded base-ribose correlations in 3D HCN

spectra, and most of the ribose spin systems could be identified using 3D HCCH-TOCSY experiments²⁸.

Assignments for the non-exchangeable resonances were made using 2D NOESY (Figure 2B) and 3D ¹³C-edited NOESY experiments to identify sequential H6/8-H1' NOE connectivities²⁶. The sequential H6/8-H1' NOE connectivities are continuous in the 180 ms NOESY spectrum except at steps A₁₁-U₁₂, A₂₀-G₂₁, G₂₁-U₂₂, and U₄₇-G₄₈. However, G₂₁H8-U₂₂H6, G₂₁H4'-U₂₂H6, and A9H1'-A8H8 cross peaks are present in the spectrum. Interestingly, i to i+2 NOE cross peaks between A²⁰H1'/H2' and U₂₂H6 are observed (Figure 2B) and suggest that the G₂₁ base bulges from the strand. The chemical shifts of U₁₂ H1' (4.57 ppm), G₂₄ H1' (4.17 ppm) and G₂₁H4' (5.86 ppm) are unusual and are discussed below. In general, the chemical shifts of tyr_{S_{SK}} resonances are either similar or are identical with the corresponding resonances of the tyr_{S_{HK}} and tyr_{S_{HS}} hairpin molecules with a few notable exceptions. The C2H2 resonances of A₁₇ and A₄₀ (tyr_{S_{SK}}) have chemical shifts that correspond to A₇ and A₃₀ in the Mg²⁺-bound form of tyr_{S_{HS}} (discussed below). The U₃₉ C6H6 also shows a similar displacement when compared with U₂₉ of tyr_{S_{HS}}.

Several ³¹P resonances could be assigned for tyr_{S_{HK}} and tyr_{S_{HS}} using HCP²⁹ or ³¹P-¹H hetero-TOCSY-NOESY spectra³⁰ and most are dispersed between -3.0 and -5.2 ppm. The G₃₁pG₃₂ resonance in the tetraloop has the expected chemical shift of -2.40 ppm³¹. Two other resonances in tyr_{S_{HK}}, G₁₀pA₁₁ and G²⁶pA²⁷, exhibit even more dramatic down field shifts of -1.47 and -2.24, respectively. As no divalent metal ion was present in this sample, these shifts are indicative of the non-A-form character of the phosphate backbone in these regions³². These unusually downfield-shifted resonances also are present in the tyr_{S_{SK}} ³¹P spectrum (G₁₀pA₁₁ and G₄₈pA₄₉), although complete sequence specific resonance assignment of the spectrum was not possible. The conservation of these unusual ³¹P chemical shifts between tyr_{S_{SK}} and tyr_{S_{HK}} indicates that the conformation the K-turn sequence region is largely preserved in the presence of the Specifier Loop domain.

Effects of Mg²⁺ on the tyr_{S_{SK}} RNA

Mg²⁺ ions are an integral part of RNA structure and function as they aid RNA folding, stabilize tertiary structure, and participate directly in catalysis. The *glyQS* leader RNA requires Mg²⁺ for correct folding⁸ and the conformational equilibria of 23S rRNA helix 7 and 16S rRNA helix 23 K-turns are sensitive to Mg²⁺^{33, 34}. The loop E motif in eukaryotic 5S rRNA is stabilized by Mg²⁺³⁵, but Mg²⁺ has no effect on the same motif sequence located in the contexts of the sNRE and the hairpin ribozyme^{36, 37}. Interestingly, no metal ions are associated with the loop E motif in the crystal structure of the sarcin/ricin loop of 5S rRNA³⁸ nor in nearly all the K-turn motifs for which crystal structures are available^{13, 15, 18, 19, 20, 21, 39}.

The loop E motif and K-turn motif-containing molecules were examined for their response to Mg²⁺. The Mg²⁺-induced chemical shift changes for tyr_{S_{HS}} center on residues adjacent to the loop E motif in the Specifier Loop domain and are generally limited to less than 0.1 ppm for both ¹³C and ¹H. Exceptions are the C₁₈ H6 and C₄₃ H5 resonances that shift upfield 0.8 ppm. The C₁₈ H6 and C₄₃ H5 resonances have shifts of 0.15 ppm and the H2 resonances of A₁₇ and A₄₀ shift 0.2–0.3 ppm downfield. The A₁₉ H2 and A₃₇ H2 resonances have chemical shift changes of ~0.1 ppm. A few resonances also exhibit line width changes indicative of exchange with addition of Mg²⁺. The C₁₈, G₂₁, U₂₂, and C₄₁ C6H6 resonances are broader in the presence of Mg²⁺, but the A₂₃ C2H2 resonance sharpens. Among the 1' resonances, U₃₉ and A₄₀ each shift 1.0 ppm (¹³C) and 0.15 ppm (¹H) downfield, and the A₁₉, A₂₀, U₂₂, and A₃₇ resonances exhibit modest broadening. The NOE cross peak intensity patterns in the presence of Mg²⁺ are qualitatively similar to those in spectra of the metal-free sample. This indicates that the local structural impact of Mg²⁺ is too small to be

reflected in our qualitative classification of cross peak intensities and the corresponding inter-proton distances. However, the additive effects of small changes and effects on the phosphate backbone could lead to long-range helical distortions.

The Mg^{2+} -induced chemical shift changes for tyrS_{HK} (and the K-turn sequence region of tyrS_{SK}) center on residues proximal to the C-stem of the K-turn. The A_{49} and A_{50} H8 resonances each shift upfield ~ 0.15 ppm and the A_7 – A_9 H8, A_{50} H2, and C_{51} H6 resonances each shift downfield ~ 0.1 ppm. The A_7 and A_{49} C2H2, U_{12} and U_{13} C6H6, and A_{50} C8H8 resonances also weaken significantly due to exchange broadening. The G_{10} C1' and C4' resonances shift upfield 0.2 ppm and downfield 0.2 ppm, respectively. The A_{49} C1' and C4' resonances shift downfield 0.7 ppm and upfield 0.4 ppm, respectively. These chemical shift changes indicate the amount of C2'-endo character of residues G_{10} and A_{49} increase and decrease, respectively, in the presence of Mg^{2+} . The U_{12} H1' resonance broadens further with Mg^{2+} but its chemical shift is preserved, indicating that base stacking in much of the NC-stem is not altered by the metal ion association. Mg^{2+} also causes small changes to some of the NH resonances. The U_{13} and G_{48} NH resonances broaden significantly, but most other resonances exhibit only a slight degree of broadening. While accelerated exchange of the G_{48} NH resonance might be expected as Mg^{2+} either stabilizes or enhances the helix kink, the effect on the U_{13} NH resonance may be caused by a moderate increase in dynamics that also is manifest in the C6H6 resonance. The NH resonances of U_{12} and U_{47} shift ~ 0.1 ppm downfield, but the U_{12} – U_{47} base pair is not otherwise altered.

Cobalt hexamine ($\text{Co}(\text{NH}_3)_6^{3+}$) was added to tyrS_{HK} to more precisely define the site(s) of metal ion association through intermolecular NOEs. It was also expected that the higher charge density of $\text{Co}(\text{NH}_3)_6^{3+}$ relative to Mg^{2+} might more effectively stabilize an RNA conformation that is heterogeneous due to electrostatic repulsion of the phosphate backbone^{40, 41}. However, titration of tyrS_{HK} with $\text{Co}(\text{NH}_3)_6^{3+}$ (up to 5 mM) again resulted in exchange broadening for residues of the K-turn sequence motif proximal to the C-stem and caused chemical shift perturbations similar to Mg^{2+} . No inter-molecular NOEs between tyrS_{HK} and $\text{Co}(\text{NH}_3)_6^{3+}$ could be definitively identified.

The hairpins tyrS_{HS} and tyrS_{HK} also were titrated with Mn^{2+} to identify the general location of divalent metal ion association around the Specifier Loop domain and K-turn sequence motif. The paramagnetic property of Mn^{2+} causes a distance dependent line broadening of resonances whose nuclei are less than about 10 Å away. The base 6 and 8 resonances of U_6 – G_{10} , A_{20} – G_{24} , A_{46} – A_{50} , and G_{36} are broadened beyond detection at 50 μM . The base 8 resonances of A_{11} , A_{20} , and A_{37} and base 2 resonances of A_7 – A_9 (tyrS_{HK}) exhibit a moderate degree of broadening. The significant broadening of the base 6 and 8 resonances and modest effect on the adenine H2 resonances indicate that the Mn^{2+} positions itself along the minor groove of the phosphate backbone. Coordination of the ion to the phosphoryl oxygen atoms is consistent with the results from titration of tyrS_{HK} with $\text{Co}(\text{NH}_3)_6^{3+}$ that indicate moderate affinity of $\text{Co}(\text{NH}_3)_6^{3+}$ for RNA and a position distal to RNA protons.

Although the spectral effects of Mg^{2+} for tyrS_{SK} mirror those observed for the individual motif-containing RNA hairpins, two resonances of the Specifier Loop domain are noteworthy. The H2 resonances of A_{17} and A_{40} of tyrS_{SK} in the absence of Mg^{2+} have chemical shifts that approximate those of the corresponding two resonances in tyrS_{HS} in the presence of Mg^{2+} . All of the other resonances of tyrS_{SK} corresponding to nucleotides in tyrS_{HK} have chemical shifts observed in the Mg^{2+} -free form of tyrS_{HK} . Notably, the addition of Mg^{2+} to tyrS_{SK} shifts the A_{17} H2 resonance further downfield. Thus, replacement of the six base pair helix of tyrS_{HS} with a nominal four base pair helix and K-turn motif may alter the base stacking at the proximal end of the Specifier Loop in a fashion similar to that of Mg^{2+} .

Structure of tyrS_{SK}

The structure of tyrS_{SK} was calculated using a restrained molecular dynamics routine. The calculations used a total of 703 conformationally restrictive distance constraints and 222 dihedral angle constraints. RDC constraints for tyrS_{SK} were obtained for Mg²⁺-free (71) and 7 mM Mg²⁺ (19) samples (Table 1). The average structure and a superposition of the Specifier Loop and K-turn sequence domains of the converged structures are shown in Figure 3. NOE-derived distance restraints were obtained from spectra of tyrS_{SK} and from spectra of the individual tyrS_{HK} and tyrS_{HS} molecules where resonance overlap (mostly involving H2' and H3' resonances) was severe. The converged structures had an average of 7 distance constraint violations between 0.5 and 0.9 Å that were randomly distributed throughout the RNA molecule. None of the converged structures had NOE constraint violations >0.8 Å. The heavy atoms of the converged structures superimpose on the average structure with an average RMSD of 1.26 Å. The local RMSDs for the Specifier Loop (A₁₇–A₂₃ and G₃₆–A₄₂) and K-turn sequence region (A₇–U₁₂ and U₄₇–A₄₉) are 0.49 Å and 0.68 Å, respectively.

The conformations of the K-turn sequence regions and flanking stem nucleotides (residues U₆–G₁₄ and C₄₅–G₅₀) are defined with good precision (0.82 Å rmsd) (Figure 3C and Table 1). The conformation of the Specifier Loop domain (residues G₁₆–G₂₄ and C₃₅–C₄₃) is similarly well-defined (0.63 Å rmsd) (Figure 3B and Table 1). The ribose ring puckers of several residues within the Specifier Loop domain and K-turn motif do not adopt A-form, C3'-*endo*, conformations. Residues A₁₉, A₂₀, U₂₉, A₄₂, and A₄₉ adopt C2'-*endo* ring puckers and residues A₈, G₁₀, G₂₁, A₂₃, G₂₆, A₃₈, G₄₈, and A₅₀ exhibit conformations intermediate between C2'-*endo* and C3'-*endo* and are consistent with those identified in the isolated tyrS Specifier Loop domain⁴². Within the prototypical K-turn structure, the C2'-*endo* pucker is conserved among the adenine nucleotides of the sheared G-A base pairs and the guanine nucleotide of the terminal G-A pair of the NC-stem. The puckers of several of the corresponding K-turn sequence residues of tyrS_{SK} differ as the pucker of A₁₁ is C3'-*endo* and G₁₀ and G₄₈ have ribose puckers that are C2'-*endo*/C3'-*endo* mixed.

The loop E and K-turn motifs of tyrS_{SK}—The internal loop containing the Specifier Sequence within tyrS_{SK} was shown to form a loop E structural motif when the loop is flanked by two regular helices⁴². The loop E motif is characterized by three non-standard base pairs, a sheared A-G pair, a U-A trans-Hoogsteen interaction, and a parallel A-A base pair, that stack on one another^{43, 44}. In some cases, a bulged G or C nucleotide is present between the non-canonical U-A and A-A interactions. The nucleotide sequence of this motif in tyrS_{SK} is similar to that found in several prokaryotic and eukaryotic rRNAs⁴⁴ and the hairpin ribozyme⁴⁵. The spectral data support the presence of the loop E motif in tyrS_{SK} (Figure 4), but the arrangement of nucleotides is not as well-ordered as observed in other studies^{38, 43, 46}. The H1' resonance of G₂₄ has a chemical shift of 4.17 ppm and is consistent with a sheared configuration between A₂₃ and G₃₆. The trans-Hoogsteen U-A arrangement inverts the orientation the uridine ribose ring relative to flanking residues and leads to an unusual feature in the NOESY spectrum, an NOE between the uridine H1' and the 5'(_{i-2}) base proton. This characteristic interaction is observed as A₂₀H8–U₂₂H1'. Spectral signatures for the hydrogen bonds that compose the trans-Hoogsteen U-A pair, between UO2 and AN6H₂ and between UN3H and AN7⁴⁴, could not be identified in either tyrS_{SK} or tyrS_{SH}. Adjacent to the U-A interaction is a parallel A-A base pair in which both adenine residues maintain the *anti* configuration about the glycosidic bond. This interaction is made possible by a reversal of the phosphate backbone between A₂₀ and G₂₁³⁸ that positions A₂₀ in an antiparallel orientation relative to the rest of the strand. The reversal leads to a characteristic NOE pattern^{43, 46} involving A₂₀ that includes weak intensity A₂₀ H4'–U₂₂ H6 and A₂₀ H4'–U₂₂ H5 cross peaks and moderate intensity A₂₀ H4'–U₂₂ H1' and A₂₀ H8–U₂₂

H1' cross peaks. The parallel A-A base pair is stabilized by two symmetric N6H₂-N7 inter-base hydrogen bonds^{38, 44}. The presence of the A₃₈N6H₂-A₂₀N7 hydrogen bond is reflected in the significant downfield shifts of the A₃₈N6 and A₂₀N7 ¹⁵N resonances, 84.2 and 237.0 ppm respectively, (Table S1) (Figure 4). Several i, i+2 NOE cross peaks between A₂₀ and U₂₂ indicate stacking of these residues and an extra-helical position of the G₂₁ base.

The bases of the Specifier Sequence (U₃₉-A₄₂) form an intra-strand stack that is continuous with A₃₈, but not C₄₃. The Watson-Crick edges of U₃₉ and A₄₀ are solvent exposed and point toward the minor groove whereas C₄₁ and A₄₂ are sequestered and point toward the helix axis (Figure 4). The remaining three nucleotides of the Specifier Loop domain (A₂₇-A₂₉) also show intra-strand stacking that is continuous with G₂₆. No cross-strand interactions are observed between the partner strand nucleotides A₂₇-A₂₉ and U₃₉-A₄₂. The discontinuity of orientation between Specifier bases A₄₀ and C₄₁ necessitates a conformational rearrangement to align the Watson-Crick edges of these bases and allow pairing with the nucleotides of the tRNA anticodon loop.

The nucleotide sequence within tyrS_{SK} that composes the second internal loop is similar to the sequences of RNA molecules that adopt the K-turn structural motif and include the U4 snRNA⁴⁷, ribosomal protein-RNA complexes^{12, 13, 39}, the S box riboswitch regulatory element²⁰, and the reverse K-turn element identified in *Azoarcus* group I intron¹⁸. The C-stems and NC-stems of these K-turns are closed by C-G and sheared tandem G-A base pairs, respectively, and are separated by three- or four-nucleotide bulges (Figure 1C). This nucleotide sequence combination enables the formation of a significant bend (~120°) in the helix axis that is stabilized by protein-RNA or RNA-RNA contacts often distal to the turn itself²³. In isolation, the K-turn sequence motif has been observed to exist as a dynamic population of conformations³³ or to preferentially adopt an extended conformation⁴⁷. In the predicted secondary structure of tyrS_{SK}, the C-stem is closed by a U-A base pair (Figure 1B). The formation of this base pair was inferred from peaks in the NOESY and HSQC spectra and is accommodated in the calculated structure of tyrS_{SK}. Data supporting the presence of the sheared G-A base pairs is primarily in the form of chemical shifts. A spectral signature diagnostic of the sheared G-A pair that is stacked in a helix is an upfield shift of the H1' resonance of the residue 3' to the A^{43, 46, 48}, as seen for the G₂₄ H1' that follows the A₂₃-G₃₆ pair in the Specifier Loop domain. The U₁₂ H1' resonance exhibits this unusual shift (4.57 ppm) and is consistent with stacking against a sheared A₁₁-G₄₈ arrangement. However, the H1' chemical shift signature cannot be used to support the G₁₀-A₄₉ base pair. This base pair stacks against the A₁₁-G₄₈ but does not stack on the U₆-A₅₀ base pair that terminates the C-stem. Therefore, the relative orientation of the G₁₀-A₄₉ and U₆-A₅₀ base pairs would not produce the characteristic upfield shift of the A₅₀ H1' resonance and indeed the chemical shift of this resonance in tyrS_{SK} is 5.87 ppm. The sheared G-A arrangement has two hydrogen bonds, AN6H₂-GN3 and AN7-GN2H₂ (Figure 5). These hydrogen bonds shift the adenine N6 and N7 resonances downfield. The A₄₉ N7 ¹⁵N resonance has a significant downfield chemical shift of 233.9 ppm (comparable to the A₂₀ and A₃₈ N7 resonances that resonate at 234.3 and 236.9 ppm, respectively), but the remaining A₁₁ and A₄₉ N6 and N7 ¹⁵N resonances exhibit shifts that reflect modest hydrogen bonding (Table S1). HNN experiments were used to probe the sheared G-A arrangements but failed to yield correlations that confirm the predicted hydrogen bonds. These results are consistent with sheared G-A base arrangements with weak hydrogen bonding.

The remaining nucleotides of the GA loop region overlay well (0.68 Å r.m.s.d.) on one another among the converged structures (Figure 3C). The U₁₂ and U₄₇ residues that flank the A₁₁-G₄₈ sheared base pair in the NC-stem form an asymmetric U-U base pair. The U₁₂-U₄₇ base pair is arranged with the hydrogen bond pattern U₁₂N3H-U₄₇C2O and U₄₇N3H-U₁₂C4O (Figure S1). On the other side of the loop are three single strand adenine

nucleotides, A₇–A₉. The base of A₇ is displaced toward the helix axis and stacks on the loop-closing U₆–A₅₀ base pair. The base of A₈ stacks on the A₇ base (Figure 5). The base of A₉ flips out of the helix and occupies a range positions among the converged structures. Between A₈ and the G₁₀–A₄₉ base pair, the direction of the helix axis is broken. The positions of A₇ and A₈ appear to be stabilized by base stacking as none of the bulged adenine bases exhibit a consistent pattern of intra-molecular interactions.

The internal loop containing the K-turn sequence motif does not have spectral features compatible with the K-turn structural motif. Inter-strand base H2 to ribose H1' and H4' NOE cross peaks were identified between residues A₁₁ and A₄₉. The A₄₉ H2–A₁₁ H1' cross peak is the most intense and corresponds to a distance less than 3.5 Å. These data are consistent with stacking of the A₁₁–G₄₈ and G₁₀–A₄₉ similar to the two sheared G–A base pairs found in most K-turn structures^{13, 15, 20, 21, 39}. However, for a K-turn conformation, the base-base proton distances between sequential residues A₇–G₁₀ are predicted to be >9 Å and would result in the discontinuity of sequential base-base NOE cross peaks around the junction of the C- and NC-stems. This loss of base-base or base-1' NOE continuity is not the case for either tyrS_{HK} or tyrS_{SK}. The K-turn structural motif also has conserved A-minor interactions involving the adenines of the sheared G–A base pairs, A₁₁ and A₄₉ in tyrS_{SK}. For a K-turn structural motif, hydrogen bonds would be predicted between the 2'-OH of A₇ and A₅₀ and the N1 of A₄₉ and A₁₁, respectively²⁴. The N1 resonance chemical shifts of A₁₁ and A₄₉, 225.3 and 224.6, are comparable to those of A₇–A₉ and are consistent with N1 atoms not involved in intra-molecular hydrogen bonds (Table S1). Thus, evidence of the A-minor interactions conserved in K-turn conformations is absent. Table 2 compares ¹H–¹H NOE intensities observed for tyrS_{SK}/tyrS_{HK} and predicted intensities when the K-turn sequence motif is modeled using conserved K-turn fold features. Finally, the position of the G₄₈ base in the tyrS_{SK} structure extends across the helix (Figure 5). This position of the G₄₈ base is supported by the G₄₈ NH resonance that has an upfield chemical shift (10.05 ppm) and gives rise to several cross-strand NOE peaks. These spectral features are consistent with sequestration of the G₄₈ imino proton and protection from rapid exchange with the solvent. The imino proton is not within hydrogen bond distance of phosphoryl oxygen atoms or ribose 2' OH groups of the partner strand that could similarly cause an upfield chemical shift and reduced solvent exchange.

¹³C Relaxation measurements

In addition to potential structural effects, the K-turn sequence motif may alter the dynamic properties of the Specifier Loop domain and this can be manifest in the rapid time-scale motions of interatomic bonds. The reorientation of a ¹³C–¹H bond vector on the picosecond time scale can be assessed through its carbon T_{1ρ} relaxation: the longer the relaxation time, the more mobile the ¹³C–¹H pair. Therefore, to test the effect of the K-turn sequence on residues in the Specifier Loop domain, T_{1ρ} relaxation times for the base C2, C6, and C8 positions of the three hairpins were compared. Cross peak overlap and chemical exchange prevented accurate measurement for some of the nuclei, especially for tyrS_{SK}. The majority of resonances from internal loop and stem residues have relaxation times between 40 and 60 ms, with those from tyrS_{SK} approximately 10% shorter on average than the corresponding resonances of tyrS_{SH} or tyrS_{KH}. In general, no significant differences in the relaxation rates were identified for residues in the Specifier Loop domain or the K-turn sequence regions between the individual hairpins, tyrS_{SH} and tyrS_{KH}, and the full-length molecule, tyrS_{SK}. One exception is the C8 resonance of the G₁₆–C₄₃ base pair that closes the Specifier Loop domain. The G₁₆ C8H8 cross peak exhibits a modest degree of exchange broadening and so does not allow for direct comparison of relaxation with the C8 resonance of G₆ in tyrS_{SH}. The addition of Mg²⁺ increased chemical exchange among nucleotides in the K-turn

sequence region, but did not significantly alter the relaxation profiles of resonances of tyrS_{SK} (data not shown).

Isothermal titration calorimetry

The sequence elements corresponding to the K-turn sequence motif are crucial for read-through transcription *in vivo*¹¹ and the proximity of this sequence to the Specifier domain suggests that it contributes positively to the tRNA anticodon-Specifier nucleotide interaction. However, the structural and dynamical effects introduced by the K-turn sequence motif at the Specifier domain are small. Therefore, isothermal titration calorimetry (ITC) was used to determine the affinity of the tRNA anticodon for the Specifier domain in the presence and absence of the K-turn sequence motif to assess possible thermodynamic contributions to the Specifier nucleotide-tRNA anticodon interaction.

The ITC measurements were performed using the anticodon stem-loop of tRNA^{Gly, GCC} (ASL^{Gly, GCC}) and hairpins corresponding to the *glyQS* Specifier Loop domain and *glyQS* Specifier Loop domain/K-turn sequence motif (Figure 6). This system was selected because and because the ASL^{Gly, GCC} anticodon bases do not contain natural base modifications⁴⁹ and the K-turn sequence motif for *glyQS* matches the consensus sequence of the K-turn that includes the terminal C-G base pair. The anticodon loop of tRNA^{Tyr} contains multiple hypermodified bases⁴⁹ that may play a significant role in anticodon-Specifier Loop domain binding and introduce an additional layer of complexity. The affinity of ASL^{Gly, GCC} for the Specifier nucleotide sequence in the context of the K-turn sequence motif was determined to have $K_d \sim 2.5 \mu\text{M}$, the same as that determined using fluorescence quenching⁵⁰. This affinity is only slightly stronger than the affinity of ASL^{Gly, GCC} for the Specifier nucleotide sequence in the absence of the K-turn motif ($K_d \sim 8 \mu\text{M}$). As a negative control, the Specifier Sequence was mutated from GGC to GCC so that a C-C mismatch between the Specifier Sequence and anticodon would be introduced. This modification resulted in the loss of the anticodon-Specifier Loop domain interaction (Figure 6). The limited effect of the K-turn sequence on the affinity of the Specifier Loop domain-anticodon arm interaction is consistent with the marginal effects on the structure and dynamics of the Specifier Loop domain by the K-turn sequence and suggests a functional role for the K-turn sequence within the global architecture of the T box-tRNA complex.

DISCUSSION

Gram-positive bacteria employ the T box riboswitch mechanism to regulate expression of aminoacyl tRNA synthetase genes and genes involved in amino acid metabolism^{1, 2, 3}. Two common RNA structural motifs are highly conserved in Stem I of the T box riboswitch and both are crucial for proper transcriptional regulation of the *tyrS* operon^{11, 51}. A loop E motif, located in the Specifier Loop domain, provides a stable platform that appears to help position the Specifier nucleotides to accept the anticodon of the cognate tRNA⁴². A K-turn, or GA, sequence motif is joined to the Specifier Loop domain by a 3–5 base pair helix. Although transcription read-through *in vivo* is dependent upon the K-turn sequence element¹¹, its role in the T box regulatory mechanism has not been established.

Structure of conserved residues in the *tyrS* leader RNA Specifier Loop domain

The proximity of the K-turn sequence motif to the Specifier Loop domain suggests a possible role in facilitating tRNA binding by altering the conformations or dynamics of the Specifier Sequence nucleotides. The lower portion of stem I of the *tyrS* leader RNA that includes the Specifier Loop domain and a K-turn sequence motif overall is well-ordered with only a few nucleotides exhibiting a moderate degree of mobility. The nucleotides in the upper portion of the Specifier Loop domain of *tyrS*_{SK} form a loop E structural motif. The

Specifier Loop domain structure is similar to the structure of the isolated *tyrS* Specifier Loop domain. As previously observed⁴², the structure of this domain is well-ordered, but the motif adopts a more open conformation than found in other loop E structural motifs^{38, 43, 46}. The hydrogen bonding pattern characteristic of the loop E fold is supported through ¹⁵N chemical shifts, but could not be mapped directly using cross-strand base-base correlation experiments that rely on strong and stable hydrogen bonds. These results are consistent with a less compact arrangement of nucleotides that would lead to longer and somewhat weaker hydrogen bonds.

The Specifier nucleotide bases are stacked, but their Watson-Crick edges are not uniformly displayed Figure 4. The bases of C₄₁ and A₄₂ are rotated toward the minor groove and readily accessible to the tRNA anticodon whereas U₃₉ and A₄₀ are rotated toward the major groove with their base pairing edges pointing toward the helix axis. This base arrangement also is adopted in *tyrS_{HS}*, but differs somewhat from the arrangement adopted by a sequence variant (C₁₆-G₄₃) of the *tyrS* Specifier Loop domain⁴². When the Specifier loop is closed by the C₁₆-G₄₃ base pair, the U₃₉-to-C₄₁ bases stack and rotate toward the minor groove. In addition, A₄₂ does not stack with C₄₁ but instead stacks on G₄₃ with the Watson-Crick edge pointing toward the helix axis⁴². The G₁₆-C₄₃ closing base pair of *tyrS_{SK}* is representative of the highly conserved purine-pyrimidine (R₁₆-Y₄₃) arrangement at this position among T box riboswitches⁷. Thus, the identity of the flanking base pair may serve to establish the placement of the Watson-Crick edge of the A₄₂ base to readily pair with U₃₃ of the incoming tRNA^{Tyr} molecule.

The structure formed by the K-turn sequence motif in *tyrS_{SK}* contains a three-nucleotide bulge and two sheared G-A base pairs, but the conformation is not reflective of the prototypical K-turn motif (Figure 7, Table 2). The sheared G-A base pairs are well-stacked in *tyrS_{SK}* whereas the terminal sheared G-A pair of the canonical K-turn structure exhibits a significant roll angle relative to the penultimate G-A (Figure 7A). The K-turn kinks the phosphate backbone and creates a 120°-A angle between the helix axes of the C-stem and NC-stem, but the helix axis of *tyrS_{SK}* is bent ~25°. Globally, the structure of this region of *tyrS_{SK}* is similar to the structures of Watson-Crick RNA duplexes interrupted by three-nucleotide bulges^{52, 53}. Although the unpaired nucleotide bases tend stack on one another, the bend in the helix axis introduced by the asymmetric bulge is modest. The isolated K-turn sequence motifs from the spliceosomal U4 RNA and the mRNA binding site for yeast ribosomal protein L30 are the only other K-turn motifs to have been examined using solution NMR^{47, 54}. Alone, these motifs exhibit different dynamic properties and neither forms the canonical K-turn fold, although in the protein-bound state they adopt the prototypical fold. The spliceosomal U4 RNA K-turn has a well-ordered internal loop with an overall extended conformation similar to *tyrS_{SK}*⁴⁷. Nucleotides within the internal loop of the K-turn element from the mRNA binding site for yeast ribosomal protein L30 are dynamic and do not adopt a unique and stable conformation⁵⁴. Interestingly, the strings of unpaired residues from the U4 and L30 kink-turn systems are adenine rich with nucleotide sequences 5'-AAU-3' and 5'-AGA-3', respectively. Unlike these sequences, though, the C-stem of *tyrS_{SK}* is terminated by a U-A base pair rather than a C-G base pair. All experimentally established K-turns have a C-G base pair at the terminus of the C-stem¹². This G residue forms a type-1 A-minor interaction with the A of the penultimate G-A base pair, an interaction not achievable in this context by a U-A base pair. Although the type-1 A-minor interaction is not required to form the spliceosomal U4 RNA-15.5K protein complex²⁴, the functional importance of this interaction in other sequence contexts is unknown.

Effect of Mg²⁺ on the *tyrS_{SK}* structure

The consensus K-turn sequence motif has the intrinsic capacity to adopt the familiar K-turn fold, but this conformation appears to be stabilized by intra- or inter-molecular interactions

involving the flanking RNA helices distal to the kink or interactions with proteins that bind K-turns²³. Indeed, FRET measurements have shown that the K-turn motif by itself can be dynamic and converts between linear and bent conformations and that these conformational states appear to be equally populated^{33, 34}. Those experiments also show that addition of Mg^{2+} displaces the population equilibrium towards the bent conformation^{33, 55}. The NMR results indicate that Mg^{2+} associates with the K-turn sequence motif with moderate affinity as some resonances begin to exhibit the effects of intermediate exchange with addition of Mg^{2+} . The Mn^{2+} broadening pattern indicates that divalent metal ion(s) can bind proximal to the three unpaired adenines and that coordination involves the phosphate backbone rather than the base N or O atoms in the major or minor grooves. The distribution of atoms within the calculated structures creates a range of positions that could support the association of Mn^{2+} and would result in a resonance broadening pattern consistent with the experimental data.

Although the association of Mg^{2+} with the K-turn sequence motif of $tyrS_{SK}$ is weak and produces only minor effects on the NOE pattern, Mg^{2+} is predicted to increase the bend of the helix axis at the motif³³. Therefore, we examined the possibility that Mg^{2+} induces kinking of the helix axis using RDC data. The structure of $tyrS_{SK}$ was re-calculated using NOE distance restraints and RDCs derived from a Mg^{2+} -containing. (Comparison of HSQC and 3D NOSEY-HSQC spectra reveals the local structures of the molecule in the two conditions appear very similar as manifest by chemical shifts, NOE cross peak identities, and cross peak intensities.) While much of the structure of $tyrS_{SK}$ is maintained in the presence of Mg^{2+} , a significant bend is introduced when the structure is refined using Mg^{2+} -derived RDCs (Figure 8A). Surprisingly, however, the bend occurs at the base of the Specifier Loop domain and not at the K-turn sequence motif. The location of the bend is coincident with the apparent highest affinity Mg^{2+} binding site within $tyrS_{SK}$. It is particularly noteworthy that this bending leads to further rotation of the Specifier codon bases toward the minor groove side of the helix and thus increases the accessibility of the Watson-Crick edges of these residues for pairing with the tRNA anticodon nucleotides (Figure 8B,C). These nucleotides also show some of the biggest chemical shift changes (0.15–0.3 ppm 1H) in the presence of Mg^{2+} . An overlay of the K-turn sequence regions of $tyrS_{SK}$ structures calculated using RDCs derived from Mg^{2+} -containing and Mg^{2+} -free buffer conditions indicates only small differences (1.6 Å r.m.s.d. for heavy atoms) whereas the r.m.s.d. for all residues of the structures in the two conditions is substantial (8.3 Å). The number and magnitude of the constraint violations for each set of structures are small with RDC Q-factors 0.12–0.15 for the two structures⁵⁶. Importantly, the Mg^{2+} -free structures do not satisfy the constraints derived in the presence of Mg^{2+} and likewise the Mg^{2+} -associated structures do not satisfy the constraints derived in the absence of Mg^{2+} . The self-consistency between the structures and constraint sets supports Mg^{2+} as the conformational effector rather than the difference being caused by a limitation of either constraint set to define the overall structure.

Functional significance of the K-turn sequence motif

The loop E and K-turn structural motifs are common features of functional regions in RNA molecules. These elements serve as sites of protein and RNA recognition^{13, 57}. The K-turn sequence motif at the base of stem I of T box leader RNAs is conserved and has been demonstrated to be crucial for the regulatory function of the *tyrS* T box¹¹. In $tyrS_{SK}$, the nucleotide sequence does not form the consensus K-turn sequence motif, a behavior also observed for the isolated K-turn sequences corresponding to the L30-mRNA binding site and the 15.5K protein binding site of the U4 snoRNA^{47, 54}. Unlike these later two molecules though, there is no evidence that links the K-turn element of the T box with a protein binding function. Indeed, the *glyQS* T box riboswitch, which also contains the K-

turn sequence motif, retains its ability to attenuate transcription in an *in vitro* system that utilizes plasmid-derived DNA templates and the purified *B. subtilis* RNA polymerase¹⁰. However, the interaction of the T box K-turn motif with protein(s) *in vivo* cannot be unequivocally excluded.

Our results do not support a role for the K-turn motif in tRNA-binding in which the motif acts directly on the Specifier Loop domain. The K-turn sequence does not alter the structure or dynamics of the Specifier Sequence in the absence or presence of Mg²⁺. The ITC results demonstrate that the K-turn sequence of the *glyQS* T box confers only a three-fold increase in the binding affinity between the *glyQS* Specifier Loop domain and the tRNA^{Gly, GCC} anticodon arm. This effect is in sharp contrast to the decreased level of antitermination activity (100–500 fold) *in vivo* caused by mutations predicted to disrupt the *tyrS* T box K-turn structure¹¹. Further, although the consensus sequence of the K-turn motif is not fully reproduced by *tyrS* K-turn sequence, the *glyQS* T box nucleotide sequence does match the consensus sequence. Although the K-turn sequence of *tyrS* does not form the classic K-turn fold in isolation, it may be stabilized once interactions elsewhere in the tRNA-Tbox complex have been established^{20, 23}. Other RNA molecules with nucleotide sequences that also do not match the consensus sequence have been found to adopt many of the structural features of the K-turn motif and include the *Azoarcus* group I intron that substitutes an A-A pair for the terminal G-A pair¹⁸ and the K-turns in helices 11 and 23 of the 16S rRNA from *T. thermophilus* that substitute noncanonical A-A and non-Watson-Crick A-U pairs, respectively, for the penultimate G-A pairs 34[·] 39[·]. We propose that formation of the structural motif occurs with the binding of tRNA to the T box riboswitch. The K-turn could then function to anchor the position of Stem I (and the Specifier Loop domain) relative to the antiterminator helix and result in greater T box-tRNA affinity to ensure transcription read-through.

MATERIALS AND METHODS

Materials

All enzymes were purchased from Sigma Chemical (St. Louis, MO) except for T7 RNA polymerase which was prepared as described⁵⁸. Deoxyribonuclease I type II, pyruvate kinase, adenylate kinase, and nucleotide monophosphate kinase were obtained as powders, dissolved in 15% glycerol, 1 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.4, and stored at –20 °C. Guanylate kinase and nuclease P1 were obtained as solutions and stored at –20 °C. Unlabeled 5' nucleoside triphosphates (5'-NTPs) were purchased from Sigma, phosphoenolpyruvate (potassium salt) was purchased from Bachem, and 99% [¹⁵N]-ammonium sulfate and 99% [¹³C]-glucose were purchased from Spectra Stable Isotopes (Branchburg, NJ).

Preparation of RNA Samples

The RNA sequences shown in Figure 1B were prepared by *in vitro* transcription with T7 RNA polymerase using synthetic DNA templates⁵⁹ and either unlabeled or ¹³C/¹⁵N -labeled 5'-NTPs⁶⁰. The RNA molecules were purified using 20% (w/v) preparative polyacrylamide gels, electroeluted (Schleicher & Schuell), and precipitated with ethanol. The purified RNA molecules were resuspended in 1.0 M KCl, 20 mM KPi, pH 6.8 and 2.0 mM EDTA and extensively dialyzed against 10 mM KCl, 5 mM KPi, pH 6.7 and 0.02 mM EDTA using a Centricon-3 concentrator (Millipore, Bedford, MA). All RNA samples were concentrated to a volume of 310 µL, lyophilized to powders, and resuspended in either 90% H₂O/ 10% D₂O or 99.96% D₂O. The samples were then heated to 90 °C for 60 s and snap cooled on ice. The sample concentrations varied between 100 and 200 A₂₆₀ O.D. in 310 µL (~0.9–2.2 mM). Partial alignment of ¹³C/¹⁵N-labeled RNA for residual dipolar coupling

measurements was achieved by adding RNA to concentrated Pf1 filamentous phage in 99.96% D₂O NMR buffer, yielding final concentrations of 14 mg/ml Pf1 and 0.4 mM RNA. The degree of alignment was quantified using the quadrupole splitting of the ²H₂O resonance ⁶¹.

NMR Spectroscopy

All spectra were acquired on a Varian Inova 500 MHz spectrometer equipped with a ¹H – [¹³C, ¹⁵N, ³¹P] probe and Inova 600 and 800 MHz spectrometers equipped with cryogenically cooled ¹H – [¹³C, ¹⁵N] probes. Solvent suppression for ¹H homonuclear spectra collected in 90% H₂O was achieved using WATERGATE. Typically, the data points were extended by 25% using linear prediction for the indirectly detected dimensions. NMR spectra were processed and analyzed using Felix 2007 (Felix NMR Inc., San Diego, CA).

Two-dimensional (2D) ¹³C-¹H HSQC spectra were collected to identify ¹³C-¹H chemical shift correlations. Sugar spin systems were assigned using 3D HCCH-TOCSY (16 ms and 24 ms DIPSI-3 spin lock) experiments collected in D₂O. A 3D HCCH-TOCSY (56 ms DIPSI-3 spin lock) was collected to establish the intra-base H2-C2-C8-H8 correlations in adenine residues ⁶². A 3D HCN experiment ⁶³ was used to identify intra-residue base-ribose correlations.

Sequential assignments and distance constraints for the non-exchangeable resonances were derived at 28 °C from 2D ¹H-¹H NOESY spectra (τ_m = 120, 160, and 360 ms) and 3D ¹³C-edited NOESY spectra (τ_m = 120 and 320 ms). Pyrimidine C2 and C4 resonances were assigned from H6-C2 and H5-C4 correlations using 2D H(CN)C and 2D CCH-COSY experiments. 2D ¹⁵N-¹H HSQC spectra optimized for 2-bond HN couplings were collected to identify purine N7 and adenine N1 and N3 resonances. H(CN)N experiments ⁶⁴ were used to identify sheared A-A and G-A base pairs. For the exchangeable resonances, 2D ¹⁵N-¹H HSQC spectra were collected to identify ¹⁵N-¹H chemical shift correlations. 2D NOESY spectra (τ_m = 140 and 320 ms) were acquired in H₂O and at 15 °C to obtain distance restraints involving exchangeable protons.

¹H-¹³C residual dipolar coupling constants (RDCs) were determined from the measured frequency difference between corresponding proton doublets in HSQC spectra acquired for isotropic and partially aligned samples. 49 RDC values from base CH bond vectors and 22 from ribose 1' CH vectors were obtained in this way. The axial and rhombic terms were determined within Xplor-NIH using an extensive grid search ⁶⁵, and yielded values of $D_{aH} = -24.93$ and $R_{hH} = 0.14$.

³J_{H-H} coupling constants were estimated for the individual RNA molecules containing the K-turn motif and Specifier Loop domain from DQF-COSY experiments. ³J_{C-P} coupling constants were determined using the spin-echo difference method ⁶⁶. ³J_{P-H} couplings were estimated using ³¹P-¹H HetCor experiments.

¹³C T_{1ρ} relaxation times were measured using 2D ¹³C-¹H ct-HSQC based experiments optimized for C2, for C1' and for C6 and C8 resonances. A 2.1 kHz ¹³C spin lock field was used with delays of 5, 10, 15, 20, 30, 40, 50, 60, 70, 90, 120 ms. The 5 ms experiment was collected twice to provide an estimate of the error of the measured intensities. The ¹³C-¹H cross peak volumes were fitted to a single exponential decay.

Distance and torsion angle constraints

Inter-proton distance estimates were obtained from cross peak intensities in 2D NOESY and 3D ¹³C-edited NOESY spectra. Cross peak intensities were calibrated using the pyrimidine H5-H6 fixed distance of 2.5 Å. NOE cross peak intensities were classified into five

categories assigned upper distance bounds of 3.0, 4.0, 5.0, 6.0, or 7.0 Å and a common lower bound of 1.8 Å. Base pairs were identified by direct detection of hydrogen bonds⁶⁷ or by observation of strong G-C NH–NH₂ or A-U H2–NH NOEs. Hydrogen bonding for the U-U interaction adjacent to the K-turn motif was derived from chemical shift data (Figure S1). Hydrogen bonds distances restraints and planarity constraints were introduced for residues that form base pairs.

Ribose ring pucker and backbone dihedral constraints were derived from ³J_{HH}, ³J_{HP}, and ³J_{CP} couplings⁶⁸. Ribose rings with ³J_{H1'–H2'} > 7 Hz and with C3' and C4' resonances between 76–80 and 85–86 ppm, respectively, were constrained to C2'-endo (A₁₉, U₂₉, C₃₀, and A₄₉). Residues with ³J_{H1'–H2'} < 5 Hz and couplings were constrained to C3'-endo. Residues with either intermediate ³J_{H1'–H2'} couplings or for which no data were available (A₈, G₁₀, G₂₁, A₂₃, G₃₆, A₄₂, G₄₈, and A₅₀) were left unconstrained. For stem residues G₁–C₅₅ to U₆–A₅₀, U₁₂–U₄₇ to G₁₆–C₃₃, and G₂₄–C₃₅ to C₂₇–G₃₂, was constrained to the gauche⁺ conformation (60 ± 30°). For residues A₇, A₉, and G₁₀, γ was loosely constrained to the *trans* conformation (–150 to 150). γ was left unconstrained for all other residues. β was constrained to the *trans* conformation (170 ± 40°) for most duplex and Specifier Loop domain residues except G₂₁ and A₂₇ that were constrained loosely to gauche⁺ (80 ± 50°) as determined from examination of loop E containing crystal structures³⁸. β was loosely constrained to the *trans* conformation (160 ± 50°) for internal loop residues was constrained to exclude the gauche⁺ conformation (–150 ± 50°) for residues with ³J_{P–H3'} > 5 Hz or ³J_{P–C2'} > 5 Hz⁶⁶. α and ζ were constrained to –70 ± 60° for the stem residues (G₁–C₅₅ to U₆–A₅₀, U₁₃–A₄₆ to G₁₆–C₄₃, and G₂₄–C₃₅ to C₂₇–G₃₂). Tetraloop residues were constrained similarly with the exceptions α for U₂₉ and G₃₁ of –150 ± 60 and 60 ± 60, respectively, and ζ for C₃₀ and G₃₁ of 30 ± 60 and 60 ± 60, respectively. For all other residues α and ζ were constrained to exclude the *trans* conformation (0 ± 120°) based on the absence of down-field shifted ³¹P resonances³², with the exceptions of ζ for A₂₀ and G₂₁ where the *trans* conformation was allowed. α and ζ between G₁₀–A₁₁ and G₄₈–A₄₉ were not constrained due to the downfield shifted ³¹P resonance to allow the *trans* conformation.

Structure refinement

Structure refinement was carried out with simulated annealing and restrained molecular dynamics (rMD) calculations were performed using Xplor-NIH v2.19⁶⁵. Starting coordinates for the tyr_{SK} and tyr_{HK} models 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, 100 structures were generated during the first round of structure calculation by 80 ps of rMD at 1200K with hydrogen bond, NOE-derived distance and base-pairing restraints. The system then was cooled to 25 K in 47 cycles of rMD corresponding to a total of 12 ps. During this stage, RDC constraints and repulsive van der Waals forces were introduced into the system and the SANI force constraint used for RDCs was gradually increased from 0.010 kcal mol^{–1} Hz^{–2} to 1.000 kcal mol^{–1} Hz^{–2}. Other 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 1 kcal mol^{–1} rad^{–2} to 100 kcal mol^{–1} rad^{–2} for the dihedral angle constraints. Once the temperature reached the target, each structure was then subjected to another round of constrained minimization⁵⁶. Twenty 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. The eight refined structures were analyzed using Xplor-NIH and Insight II.

Isothermal Titration Calorimetry

A titration calorimeter (MicroCal, Inc.) was used for the ITC experiments. RNA hairpins tyr_S_{HS} and tyr_S_{SK} with the UAC Specifier nucleotide triplet replaced with GGC were synthesized using T7 RNA polymerase as described above. A 17 nt RNA hairpin corresponding to the anticodon arm of tRNA^{Gly, GCC} also was similarly prepared. RNA samples were extensively dialyzed against a buffer of 10 mM potassium phosphate (pH 6.8) and 25 mM NaCl. RNA samples were heated to 90 °C for 60 s, snap cooled on ice and dialyzed against 10 mM potassium phosphate, 25 mM NaCl, and 10 mM MgCl₂. The concentrations of RNA in the injection syringe and sample cell were 270 μM and 22 μM, respectively. For the titration experiments, thirty 10 μL injections into 1.5 mL sample cell volume were performed with 5 minutes between injections. The sample was stirred constantly at 290 rpm and the temperature was set to 12 °C. Control titrations (forward and reverse)⁶⁹ were performed and yielded similar results. No binding was observed in the absence of Mg²⁺. However, the ASL^{Gly, GCC} adopts a relatively compact tri-loop structure that relaxes and becomes dynamic when Mg²⁺ is added (Chang and Nikonowicz, unpublished). Therefore, Mg²⁺ has a role at the level of the ASL anticodon loop, but do not know if Mg²⁺ makes additional contributions.

The ITC data was analyzed using the vendor-supplied software (ORIGIN v7.0) and plots of ΔH versus mole ratio were generated from the raw thermograms. The final 3–5 points from each experiment were extrapolated to obtain a straight line that was subtracted from all the data before determining ΔH , (the overall reaction enthalpy), K_A (association constant), and n (reaction stoichiometry) by fitting the points using a nonlinear least squares model for a single binding site.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

NMR	nuclear magnetic resonance
NTP	nucleoside triphosphate
NOE	nuclear Overhauser effect
NOESY	NOE spectroscopy
2D	two dimensional
3D	three dimensional
HSQC	heteronuclear single quantum coherence
RMSD	root mean square deviation
NH	imino
NH₂	amino
nt	nucleotide

Acknowledgments

We thank Malgorzata Michnicka for preparation of the T7 RNA polymerase and synthesis of the labeled 5'-nucleotide triphosphates and Andrew Chang for assistance with collecting the ITC data. The 800 MHz NMR spectrometer was purchased with funds from the W. M. Keck Foundation and the John S. Dunn Foundation.

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Figure 1.

Sequence and proposed secondary structures of (A) the T box riboswitch of the *Bacillus subtilis* *tyrS* gene, (B) the 38 nt (*tyrS*_{HS}), 33 nt (*tyrS*_{HK}), and 55 nt (*tyrS*_{SK}) RNA molecules, corresponding to the Specifier Loop domain and K-turn sequence motif of the *tyrS* leader RNA, and (C) the K-turn consensus sequence motif. The Specifier Loop domain and K-turn sequence motif of the *tyrS* leader RNA are boxed. The Specifier Sequence also is highlighted.

Figure 2.

(A) Two-dimensional ^{13}C - ^1H HSQC spectrum of the base C6/8 and C2 regions of tyr_{SK} RNA molecule. The sequence-specific resonance assignments are shown. The A₂₃ C2H2 cross peak is significantly weakened by chemical exchange. The U₄₇ C6H6 and A₁₅ and A₄₆ C2H2 resonances also display moderate exchange broadening. The *in vitro* transcription reaction was primed with unlabeled 5'-GMP, therefore the G₁ C8H8 does not appear in this spectrum. (B) Sequential connectivities through the base-1' region of the 180 ms mixing time two-dimensional NOE spectrum. The dotted lines trace the connectivities among the Specifier codon nucleotides U₂₉-A₃₁. The sequential connectivity is disrupted between steps A₁₁-U₁₂, A₂₀-G₂₁, G₂₁-U₂₂, and U₄₇-G₄₈ (boxes). The H1' resonances of U₁₂, G₂₄, and G₃₂ are shifted upfield to 4.57, 4.17, and 4.42 ppm, respectively. These chemical shifts are

characteristic of the nucleotides flanking the 3' side of the adenine of a sheared G-A base pair and the guanine of a UNGC tetraloop. See also Table S1.

Figure 3.

(A) Structure of the tyrS_{SK} RNA oriented with the Specifier Loop domain above the K-turn sequence domain. Superposition of (B) the Specifier Loop domains and (C) the K-turn sequence domains from the 8 converged structures of tyrS_{SK} . Views are into the minor groove at the center of the Specifier Loop domain and the major groove at the center of the K-turn sequence domain. The RMSDs between the individual structures and the average structure are listed in Table 1. The Specifier Loop and the K-turn sequence regions are generally well defined locally. The disorder of the bulged G_{21} base reflects the lower number of constraints for this residue.

Figure 4.

Stereoview of the structure of the Specifier Loop domain. The Specifier Sequence bases are colored green and the loop E motif nucleotide bases are A₂₀–A₃₈ (*pink*), G₂₁ (*orange*), U₂₂–A₃₇ (*blue*), and A₂₃–G₃₆ (*brown*). Hydrogen bonds are indicated by dotted red lines. The functional groups on the Watson-Crick edges of the specifier nucleotides are colored orange. The S turn of the sugar-phosphate backbone can be seen between residues A₁₈–G₂₁.

Figure 5.

Stereoview of the K-turn sequence region of the tyrS_{SK} RNA molecule showing the arrangement of base-base interactions. Hydrogen bonds, supported by ¹⁵N chemical shifts, are indicated by dotted red lines. The U-U (pink) and G-A (brown) base pairs of the NC-stem stack against each other. The first and second unpaired adenine residues (cyan) stack on the closing A-U base pair (blue) of the C-stem. The third unpaired adenine (cyan) is shifted out from the helix center.

Figure 6.

(A) Representative ITC experiment for the binding of the tRNA^{Gly, GCC} anticodon arm with the *glyQS* T box Specifier Loop domain. A 270 mM solution of and RNA hairpin corresponding to the *glyQS* T box Specifier loop and K-turn sequence motif (i) was titrated into 1.4 ml of a 22 mM solution of the anticodon arm of tRNA^{Gly, GCC} (iii). Both RNA molecules were in a buffer of 10 mM potassium phosphate (pH 6.8), 25 mM NaCl, and 10 mM MgCl₂. For (A), the upper and lower panels correspond to the raw thermogram data and the integrated heat data for each injection (10 mL), respectively. Fitting of the data using a single-site binding model gave $K_A = 6.8 \times 10^5 \text{ M}^{-1}$, $n = 1.1$, $\Delta H = -16.8 \text{ kcal mol}^{-1}$, and $\Delta S = -32.6 \text{ cal mol}^{-1} \text{ K}^{-1}$. (B) *glyQS* T box RNA constructs used for ITC experiments: (l-r) Specifier loop with the K-turn sequence motif, Specifier loop alone, and anticodon arm of tRNA^{Gly, GCC}.

Figure 7.

Comparison of (A) the K-turn sequence region of tyrS_{SK} and (B) the K-turn from helix 7 (Kt-7) of *H. marimortui* 23S rRNA (PDB=1S72). In both structures, the first unpaired nucleotides (cyan) of the internal loops stack on the closing base pairs (blue) of the C-stems, the penultimate G-A base pair (brown) stacks with the neighboring base pair (pink) of the NC-stem, and the third unpaired nucleotide (green) of the internal loop lies outside the helix. In tyrS_{SK}, the terminal G-A pair (orange) of the NC-stem stacks against the penultimate G-A pair whereas in Kt-7, the plane of the terminal G-A base pair is significantly tilted relative to the plane of the adjacent G-A pair. The angles between the axes of the C- and NC-stems are ~25° and 120° for tyrS_{SK} and Kt-7, respectively.

Figure 8.

(A) Structures of $tyrS_{SK}$ RNA calculated using RDCs measured in the absence (green) and presence (orange) of Mg^{2+} . The K-turn regions are superimposed. The bend in the helix axis created by Mg^{2+} occurs at the base of the Specifier Loop domain. Comparison of the Specifier Loop domains from (B) the Mg^{2+} -free and (C) Mg^{2+} -bound structures. The Watson-Crick edges of the Specifier bases (green) are rotated further towards the minor groove side of the helix in (C), increasing their accessibility to the bases of the tRNA anticodon loop.

Table 1Summary of experimental distance and dihedral angle constraints and refinement statistics for tyrS_{SK}.

Constraint	tyrS _{sk}
NOE distance constraints	
intraresidue ^a	324
interresidue	401
mean number per residue	13
NOE constraints by category	
very strong (1.8 – 2.8 Å)	6
strong (1.8 – 3.8 Å)	194
medium (1.8 – 4.8 Å)	268
weak (1.8 – 6.0 Å)	186
very weak (1.8 – 7.0 Å)	71
base pair constraints	
total	94
dihedral angle constraints	
ribose ring ^b	141
backbone	222
mean number per residue	6.6
Residual dipolar coupling constraints	
base CH	49
ribose 1' CH	22
violations	
average distance constraints > 0.6 Å ^c	7
average dihedral constraints > 0.5° ^d	16.6
RMSD from ideal geometry^e	
Heavy Atoms (Å)	1.26
Backbone Atoms (Å)	1.30

^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. The ring pucker of residues A8, G10, G21, A23, G36, A38, G48, and A50 were not constrained.^c A distance violation of 0.6 Å corresponds to 5.0 kcal energy penalty.^d A dihedral angle violation of 0.5° corresponds to 0.05 kcal energy penalty.^e Calculated against the minimized average structure.

Table 2

Summary of NOE intensities between atom pairs that differ for tyrSSK and those expected for a prototypical kink-turn (distance in Å).

Atom pair	tyrSSK	K-turn-predicted
A ₈ H8-A ₇ H1'	very weak (5.7)	not observed ^a (10.3)
A ₈ H8-A ₇ H8	weak (4.9)	not observed (12.0)
G ₁₀ H8-A ₈ H1'	not observed (9.5)	weak (5.0)
A ₇ H1'-A ₄₉ H2	not observed (8.0)	strong (2.6)
A ₇ H2-A ₈ H1'	medium (3.1)	not observed (9.5)
A ₁₁ H2-A ₅₀ H1'	not observed (7.0)	medium (3.0)

^aPredicted distances have a range ± 0.5 Å and were estimated by examining K-turn structures kt-7 and kt-46 from 1S72 and the K-turn from 1T0K.