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Specific labeling approaches to guanine and adenine imino and amino proton assignments in the AMP–RNA aptamer complex

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Summary

The secondary structure of a recently identified ATP-binding RNA aptamer consists of a purine-rich 11-residue internal loop positioned opposite a single guanine bulge flanked on either side by helical stem segments. The ATP ligand targets the internal loop and bulge domains, inducing a structural transition in this RNA segment on complex formation. Specifically, 10 new slowly exchanging proton resonances in the imino, amino and sugar hydroxyl chemical shift range are observed on AMP–RNA aptamer complex formation. This paper outlines site-specific labeling approaches to identify slowly exchanging imino (guanine) and amino (guanine and adenine) protons in internal loop and bulge segments of compact RNA folds such as found in the AMP–RNA aptamer complex. One approach incorporates ^{15}N-labeled guanine (N1 imino and N2 amino positions) and ^{15}N-labeled adenine (N6 amino position), one residue at a time, in the AMP-binding RNA aptamer, with labeling incorporation through chemical synthesis facilitated by generating the aptamer from two separate strands. The unambiguous assignments deduced from the ^{15}N labeling studies have been verified from an independent labeling strategy where individual guanines in the internal loop have been replaced, one at a time, by inosines and assignments were made on the basis of the large 2 ppm downfield shift of the guanine imino protons on inosine substitution. The strengths and limitations of the inosine-for-guanine substitution approach emerge from our studies on the AMP–RNA aptamer complex. The assignment of the internal loop and bulge imino and amino protons was critical in our efforts to define the solution structure of the AMP–RNA aptamer complex since these slowly exchanging protons exhibit a large number of long-range intramolecular NOEs within the RNA, as well as intermolecular NOEs to the AMP in the complex. The current application of specific ^{15}N and inosine labeling approaches for exchangeable imino and amino proton assignments in the nonhelical segments of an RNA aptamer complex in our laboratory complements selective ^{2}H and ^{13}C approaches to assign nonexchangeable base and sugar protons in RNA and ligand–RNA complexes reported in the literature.

Introduction

In vitro selection experiments (reviewed by Chapman and Szostak (1994), Joyce (1994) and Burgstaller and Famulok (1995)) have recently identified an ATP-binding RNA aptamer with a secondary structure as outlined in Fig. 1a (Sassanfar and Szostak, 1993). This aptamer was generated by targeting random RNA libraries to ATP covalently immobilized to a solid support through its C8 purine base position. This RNA aptamer also targets AMP and adenine with micromolar affinity but does not recognize any of the other three nucleotides. The consensus segments required for ATP recognition are restricted to an 11-residue internal loop positioned opposite a guanine bulge, with this segment flanked on either side by nonconsensus duplex stem segments. We have set out to define the tertiary structure of the ATP binding site through a combined application of multinuclear, multidimensional NMR and molecular dynamics calculations (Jiang et al., 1996a). The availability of unambiguous resonance assignments represents a key step in NMR-based structure determination, especially for RNA inter-

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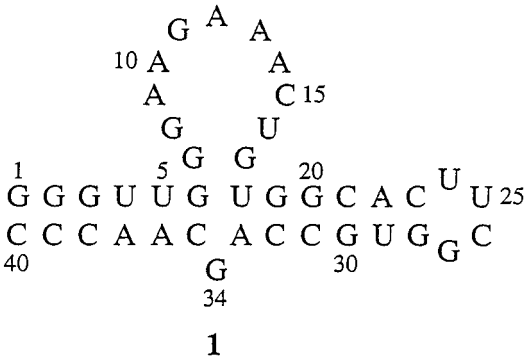

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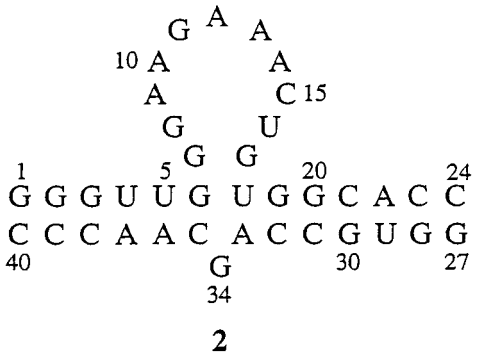

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Fig. 1. (a) The sequence and predicted secondary structure of the ATP-binding 40-mer RNA aptamer. (b) The sequence and numbering system of the ATP-binding RNA aptamer composed of 24-mer and 14-mer strands.

nal loop and bulge segments of unknown secondary and tertiary structure. We have approached this problem by incorporating site-specific labels or analogs at specific positions in the internal loop and bulge positions in the AMP–RNA aptamer complex. In one set of experiments we have incorporated individual guanines isotopically labeled at the ^{15}NH-1 imino and $^{15}NH_2$-2 amino positions and individual adenines isotopically labeled at the $^{15}NH_2$-6 amino position in the internal loop and bulge positions in the complex. This was greatly facilitated by studying the complexes generated with the RNA aptamer outlined in Fig. 1b, which is composed of two separate strands (24 and 14 residues in length) prepared through chemical synthesis and offers the opportunity for labeling one or the other of the two strands in the complex. Such an approach has yielded unambiguous exchangeable imino proton assignments of all guanine residues, as well as amino proton assignments of key guanine and adenine residues in the internal loop and bulge positions in the complex. In an alternate approach, we have sequentially replaced individual guanines by inosines in the 11-mer internal loop segment and have used the distinct spectral characteristics associated with these two purine residues to assign the guanine imino protons within this consensus AMP binding segment in the complex. The guanine imino proton assignments resulting from the ^{15}N labeling and inosine substitution approaches are in complete agreement and establish the robustness of our proposed approach for the assignment of exchangeable purine protons in ligand–RNA aptamer complexes approaching 40 nucleotides in length.

The current NMR-based structural study absolutely required the site-specific, one at a time, ^{15}N enrichment (guanine and adenine) and inosine substitution (guanine) approach to make definitive imino and amino proton assignments in the AMP–RNA aptamer complex. Our earlier NOE-based efforts to make assignments without specific enrichment or substitution resulted in misassigning the imino protons of G11 and G34 in the complex. This misassignment of key residues involved in the generation of the binding site prevented us from solving the solution structure of the complex for quite some time. We believe that the above outlined enrichment and substitution strategy be given serious consideration for imino and amino proton assignments in the loop and bulge segments of RNA.

Materials and Methods

Labeled RNA sample preparation

The preparation of the specifically ^{15}N-labeled nucleosides (H. Zhao, A. Pagano, A. Shallop, W. Wang and R.A. Jones, manuscript in preparation), and the details of their protection and incorporation into the RNA aptamer by an H-phosphonate method (X. Zhang, B.L. Gaffney, J.L. Abad, Q. Huang, F. Zeng and R.A. Jones, manuscript in preparation), will be reported elsewhere. These chemically synthesized ^{15}N-labeled RNA sequences were purified by preparative 20% acrylamide denaturing gel electrophoresis followed by electroelution. The purified labeled strands were then added to the complementary strand to generate the RNA aptamer.

The inosine-substituted RNA aptamers were synthesized on an ABI DNA/RNA synthesizer using standard RNA synthesis cycles and riboinosine phosphoamidites purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). The synthesized RNA was deprotected with 3:1 ammonium hydroxide:ethanol at 55 °C for 12 h and the silyl protecting group was removed with tetrabutylammonium fluoride at room temperature for 24 h. The RNA was desalted on a Sephadex G25 column followed by gel purification and elution.

NMR spectra

The one- and two-dimensional NMR experiments on the AMP–RNA aptamer complexes labeled with ^{15}N isotopes were acquired on a Varian Unity plus 500 MHz NMR spectrometer, while those on the complexes containing specific inosine substitutions were acquired on a Varian Unity plus 600 MHz NMR spectrometer. Solvent suppression for samples of the complex in H_2O buffer solution was achieved using symmetrically shaped pulses (Smallcombe, 1993) for the 1D spectra and WATER-

GATE suppression schemes (Sklenář and Bax, 1987) for the 2D (^{1}H,^{15}N)-HSQC experiments. Two-dimensional NOESY data sets of the complex in H_2O buffer were recorded at a mixing time of 150 ms, with solvent suppression achieved using a jump-and-return pulse sequence (Plateau and Gueron, 1982).

Results

Proton spectra

We have reported previously on the imino proton spectra of the free 40-mer RNA aptamer and its complex with AMP (Jiang et al., 1996b). The exchangeable proton spectra (8.7–14.8 ppm) of the free RNA aptamer containing 24-mer and 14-mer strands, and its complex with AMP (henceforth designated as the AMP–RNA aptamer complex unless otherwise specified) in H_2O buffer, pH 6.7 at 0 °C, are plotted in Figs. 2a and b, respectively. We can identify 10 new exchangeable proton resonances upon formation of this RNA aptamer complex (Fig. 2b) similar to what was reported previously for the 40-mer RNA aptamer complex (Jiang et al., 1996b). Two of these new exchangeable resonances were identified as uracil imino protons in the 40-mer RNA aptamer complex based on their distinct uracil ^{15}N imino chemical shifts in the (^{1}H, ^{15}N)-HSQC spectrum of the complex (Jiang et al., 1996b). The downfield shifted resonance at 13.75 ppm is assigned to U18 based on its NOE to the imino proton of the adjacent G19 of the right stem, while the other imino proton at 10.20 ppm is assigned by elimination to U16 in the 11-residue internal loop segment in the complex (Fig. 2b).

Five of the remaining eight new exchangeable resonances, designated **1**, **2**, **3**, **4** and **5**, must originate from the imino protons of the four guanines in the internal loop and the single guanine bulge in the complex based on their distinct guanine ^{15}N imino chemical shifts in the (^{1}H,^{15}N)-HSQC spectrum of the complex. We assign exchangeable resonance **6** to the 2'-OH of G34 since it is not covalently linked to a carbon or a nitrogen and based on its strong NOE to the H2' proton of G34 in the 40-mer RNA aptamer complex (Jiang et al., 1996b). The two remaining exchangeable protons in the complex, designated **7** and **8**, are assigned to amino protons based on their through-bond correlation to ^{15}N amino chemical shifts in the (^{1}H,^{15}N)-HSQC spectrum of the complex. We outline below our efforts to assign guanine imino protons **1**, **2**, **3**, **4** and **5** and amino protons **7** and **8** in the AMP–RNA aptamer complex.

Selective incorporation of ^{15}N-labeled guanines and adenines in the complex

The labeling strategy was designed to generate RNA oligomers containing a single ^{15}N-labeled guanine (N1 imino and N2 amino positions) and a single ^{15}N-labeled adenine (N6 amino position) in one or the other of the two strands of the AMP–RNA aptamer complex. The ^{15}N-labeled pairs G11 and A10, G7 and A12, G8 and A9, and G17 and A12 were incorporated in the top 24-mer strand, while the ^{15}N-labeled pair G34 and A33 was incorporated in the bottom 14-mer strand. Sufficient amounts of the selectively labeled AMP–RNA aptamer complexes were available to undertake 2D NMR studies on all the pairs outlined above, except for the labeled G11 and A10 combination where the limited quantities of sample restricted the NMR experiments to 1D difference spectra.

Guanine imino proton assignments from ^{15}N labeling studies

We have recorded the one-dimensional NMR spectrum (8.7–14.8 ppm) of the AMP–RNA aptamer complex containing ^{15}N-labeled A10 and G11 residues on the top 24-mer strand in H_2O solution with and without ^{15}N decoupling. The corresponding difference spectrum in this spectral range exhibits a difference peak at 10.44 ppm (Fig. 3b) which aligns with the chemical shift of the guanine imino proton labeled **5** in the control spectrum (Fig.

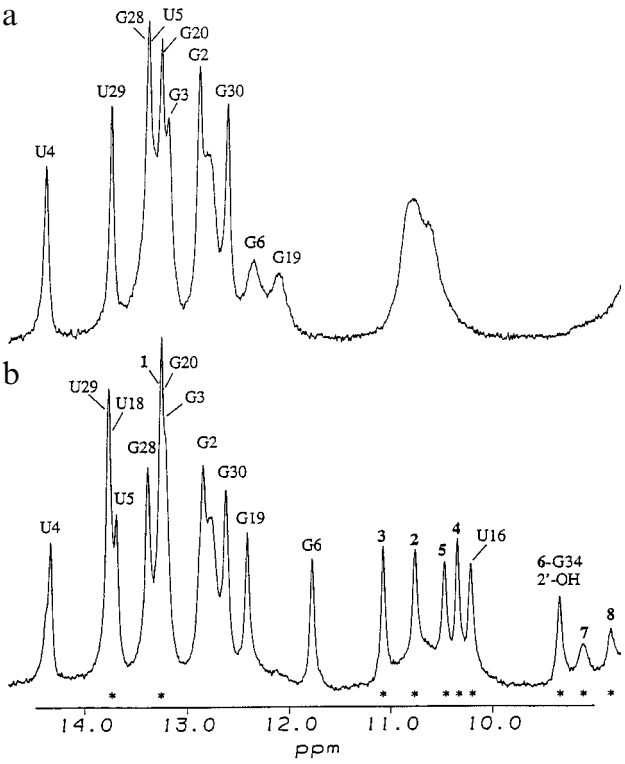

Fig. 2. Exchangeable proton spectra (8.7–14.8 ppm) of (a) the free RNA aptamer composed of 24-mer and 14-mer strands and (b) the AMP–RNA aptamer complex in H_2O buffer (10 mM phosphate, 0.2 mM EDTA), pH 6.7 at 10 °C. The assignments of Watson–Crick imino protons are based on the observed sequential NOEs between neighboring base pairs within individual stem segments in the complex. The new exchangeable proton resonances which appear following the addition of AMP are designated by asterisks and labeled by numbers in bold in (b).

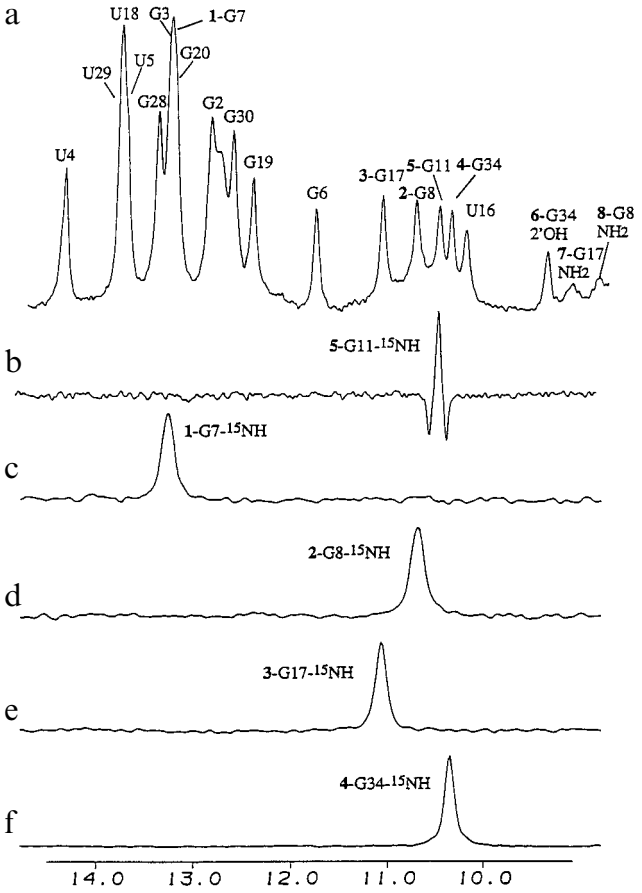

Fig. 3. (a) Exchangeable proton spectrum (8.7–14.8 ppm) of the AMP–RNA aptamer complex composed of 24-mer and 14-mer strands in H_2O buffer (10 mM phosphate, 0.2 mM EDTA), pH 6.7 at 0 °C. (b) One-dimensional difference spectrum corresponding to on and off ^{15}N imino decoupling for the complex containing ^{15}NH (guanine) and $^{15}NH_2$ (guanine and adenine) labels at the G11 and A10 residues in the RNA aptamer sequence. (c–f) One-dimensional traces through the relevant ^{15}N guanine imino frequencies of the two-dimensional (^1H,^{15}N)-HSQC contour plots of complexes specifically ^{15}NH (guanine) and $^{15}NH_2$ (guanine and adenine) labeled at the (c) G7 and A12, (d) G8 and A9, (e) G17 and A12, and (f) G34 and A33 residues in the RNA aptamer sequence, respectively. The assignments of Watson–Crick imino protons are based on the observed sequential NOEs between neighboring base pairs within individual stem segments in the complex. The new exchangeable proton resonances which appear following the addition of AMP are labeled by numbers in bold.

3a). This permits the assignment of the guanine imino proton labeled **5** to the G11 residue in the internal loop sequence in the complex.

One-dimensional traces through the relevant ^{15}N imino frequency in the two-dimensional (^1H,^{15}N)-HSQC spectra of the AMP–RNA aptamer complexes specifically ^{15}N-labeled at G7 and A12 (Fig. 3c), G8 and A9 (Fig. 3d), and G17 and A12 (Fig. 3e) on the top strand and at G34 and A33 (Fig. 3f) on the bottom strand readily yield the assignments of the guanine imino protons labeled **1**, **2**, **3** and **4** in the spectrum of the complex (Fig. 3a). These results conclusively allow us to assign the guanine imino proton labeled **1** at 13.24 ppm to G7 (Fig. 3c), that labeled **2** at 10.72 ppm to G8 (Fig. 3d), that labeled **3** at 11.07 ppm to G17 (Fig. 3e) and that labeled **4** at 10.35 ppm to G34 (Fig. 3f). The imino proton chemical shifts for these internal loop and bulge guanines in the AMP–RNA aptamer complex are listed in Table 1 along with their corresponding ^{15}N imino chemical shifts in the complex. It should be noted that these internal loop and bulge guanine imino proton chemical shifts are virtually identical for the AMP complexes with the 40-mer RNA aptamer and its counterpart generated from 24-mer and 14-mer strands.

Guanine and adenine amino proton assignments from ^{15}N labeling studies

We detect three pairs of amino protons originating from purine residues (linked by horizontal lines) in the (^1H,^{15}N)-HSQC spectrum of the 40-mer RNA aptamer complex in H_2O buffer solution (Fig. 4a). One-dimensional traces through the relevant ^{15}N amino frequency in the two-dimensional (^1H,^{15}N)-HSQC spectra of the AMP–RNA aptamer complexes specifically ^{15}N-labeled at G8 and A9 (Fig. 4b), G17 and A12 (Fig. 4c), and G7 and A12 (Fig. 4d) on the top strand readily yield the assignment of the amino protons labeled **7** and **8** in the complex. These results conclusively allow assignment of the amino proton labeled **7** at 9.11 ppm (and its 7.42 ppm partner) to G17 (Fig. 3c) and the amino proton labeled **8** at 8.84 ppm (and its approximately 5.0 ppm partner) to G8 (Fig. 3b), with the downfield shifts within each amino proton pair indicative of these guanine amino protons participating in hydrogen bonds. These conclusions are consistent with the strong NOEs observed between the 10.72 imino and 8.84 amino protons which have been assigned above to G8 and between the 11.07 imino and 9.11 amino protons which have been assigned above to

TABLE 1
IMINO AND AMINO PROTON AND NITROGEN CHEMICAL SHIFTS (ppm) IN THE AMP–RNA APTAMER COMPLEX

Resonance	Imino proton	Imino nitrogen	Amino proton	Amino nitrogen
1 G7	13.24	150.5		
2 G8	10.72	147.7		
5 G11	10.44	–[a]		
3 G17	11.07	147.0		
4 G34	10.35	143.5		
7 G17			9.11[b]	82.3
8 G8			8.84[b]	76.9

The complex is composed of 24-mer and 14-mer strands. The spectrum was recorded in H_2O buffer (10 mM phosphate, 0.2 mM EDTA), pH 6.7 at 0 °C.

[a] This nitrogen chemical shift could not be obtained because of the low concentration of the RNA aptamer sample that was specifically ^{15}N-labeled at the imino and amino protons of G11 and A10 in the complex.

[b] Hydrogen-bonded guanine amino proton.

G17 in the complex. In addition, these experiments also identify the amino protons of A12 (7.40 and 6.21 ppm) in the complex (Figs. 4c and d). It should be noted that the ^{15}N amino chemical shifts of G8 (76.9 ppm) and A12 (86.2 ppm) (Fig. 4a) fall within the expected range of ^{15}N amino chemical shifts for guanine and adenine residues, respectively. The amino proton chemical shifts for these internal loop guanine and adenine residues in the AMP–RNA aptamer complex are listed in Table 1 along with their corresponding ^{15}N amino chemical shift counterparts.

It is our view that it would not have been possible to definitively distinguish among guanine amino protons and differentiate them from adenine amino protons without specific ^{15}N labeling of the guanine and adenine residues in the AMP–RNA aptamer complex. Our experience tells us that ^{15}N amino chemical shifts cannot always be used to distinguish between guanine and adenine residues since the ^{15}N amino chemical shift of G17 (82.3 ppm) resonates at the boundary of the ^{15}N amino chemical shift range for guanine and adenine residues (Fig. 4a).

Guanine imino proton assignments based on selective inosine-for-guanine substitution studies

We have also attempted to assign the internal loop and bulge guanine imino protons through the replacement of individual guanines by inosines in the AMP–RNA aptamer complex. This substitution can be achieved through solid-phase chemical synthesis due to the availability of inosine phosphoamidites. The assignment procedure is based on the approximately 2 ppm downfield shift of the imino proton of guanine on inosine substitution, provided such a substitution does not alter the global fold of the RNA or the ligand–RNA alignments in the complex. Such an approach also requires that the amino protons of guanine not be an indispensable component of the folding or recognition processes, since their replacement by hydrogen on substitution by inosine has the potential of disrupting the RNA fold or weakening the recognition event. We have therefore monitored the global fold of the AMP–RNA binding site for each inosine-for-guanine substitution analog by following the NOE fingerprint patterns (listed in Table 2) that are characteristic of the guanine imino protons labeled **1**, **2**, **3**, **4** and **5**, the hydroxyl proton **6** and the amino protons labeled **7** and **8** in the exchangeable proton spectrum of the complex. Indeed, inosine-for-guanine substitution was found to weaken the affinity of AMP for its RNA aptamer target since excess ligand (up to a threefold excess) was necessary to drive complex formation towards completion for several of the inosine analogs.

The exchangeable proton spectrum (8.7–15.5 ppm) of the AMP–RNA aptamer complex in H_2O buffer at 0 °C is plotted in Fig. 5a with the internal loop and bulge guanine imino protons labeled **1**, **2**, **3**, **4** and **5** above the

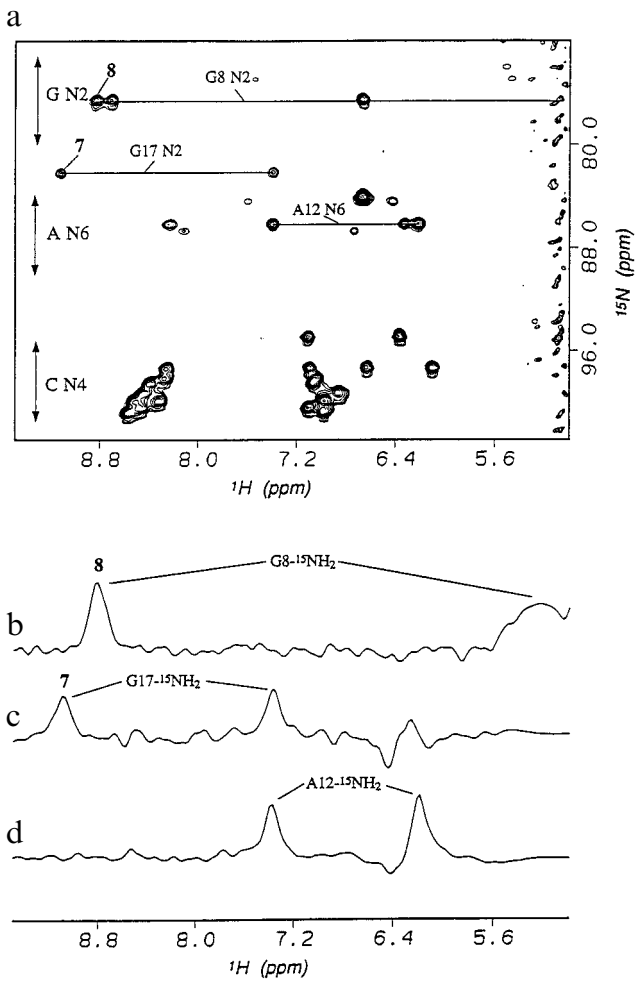

Fig. 4. (a) An expanded contour plot of the amino proton region of the (^1H,^{15}N)-HSQC experiment on the AMP–uniformly (^{13}C,^{15}N)-labeled RNA 40-mer aptamer complex in H_2O buffer (10 mM phosphate, 0.2 mM EDTA), pH 6.7 at 0 °C. Three pairs of amino protons from internal loop guanine and adenine residues in the complex are indicated by solid horizontal lines. (b–d) One-dimensional traces through the relevant ^{15}N amino frequencies of the two-dimensional (^1H,^{15}N)-HSQC contour plots of the AMP–RNA aptamer complexes containing 24-mer and 14-mer strands specifically ^{15}NH imino (guanine) and $^{15}NH_2$ amino (guanine and adenine) labeled at the (b) G8 and A9, (c) G17 and A12, and (d) G7 and A12 residues in the RNA aptamer sequence, respectively.

spectrum. The corresponding spectrum of the complex containing I7 substituted for G7 is plotted in Fig. 5b. A new imino proton resonance is detected at 15.32 ppm and can be assigned to **1** based on the signature of NOEs to other exchangeable and nonexchangeable protons in NOESY spectra of the complex. This corresponds to a 2.08 ppm downfield shift of the imino proton on proceeding from G7 to I7 in the AMP–RNA aptamer complex. The other exchangeable protons labeled **2**–**8** in Fig. 5b have been assigned based on their NOE pattern distribution and exhibit slightly shifted chemical shifts in the I7-for-G7 analog (Fig. 5b) relative to its control counterpart (Fig. 5a).

TABLE 2
A LISTING OF NOEs ASSOCIATED WITH EXCHANGEABLE PROTONS **1**–**8** OF THE AMP–RNA APTAMER (COMPOSED OF 24-MER AND 14-MER STRANDS) COMPLEX

Resonance	NOEs
1 G7 NH-1	G6 (NH-1), **2** G8 (NH-1), G11 (H8, H2')
2 G8 NH-1	A10 (H8, H1', H2', H3', H4', H5',5''), G11 (H8, H2', H3', H4', H5',5''), AMP (H2)
5 G11 NH-1	G34 (H8, H1'), C35 (H6, H1', H4', H5',5''), AMP (H8, H1', H4')
3 G17 NH-1	A10 (H2), A12 (NH_2-6, H2), U16 (NH-3), U18 (NH-3), A33 (H2), **4** G34 (NH-1, H8), AMP (H1')
4 G34 NH-1	A12 (H8, H1', H2'), **3** G17 (NH-1), A33 (H8, H2', H3')
6 G34 2'-OH	G11 (H4'), A12 (NH_2-6, H8, H1', H2'), A33 (H1'), G34 (H8, H1', H2', H3', H4', H5',5''), AMP (H1')
7 G17 NH_2-2	A10 (H2), A12 (NH_2-6), U18 (NH-3), A33 (H2), G34 (H8), AMP (H1', H2', H3')
8 G8 NH_2-2	A10 (H8, H1', H2', H3'), G11 (H8, H2', H3', H5',5''), AMP (H2)

The exchangeable proton spectrum of the complex for the RNA aptamer analog containing an I8-for-G8 substitution is plotted in Fig. 5c. We note that the imino proton labeled **2** no longer resonates around 10.7 ppm (Fig. 5a) but has shifted downfield to 13.03 ppm (Fig. 5c). This large 2.31 ppm downfield shift of the imino proton labeled **2** on I8-for-G8 substitution permits the assignment of this exchangeable resonance to the guanine imino proton of G8 in the control complex. It should be noted that we no longer detect the exchangeable amino proton designated **8** in the I8-for-G8 substituted RNA aptamer complex (Fig. 5c), consistent with peak **8** originating in the amino proton of G8 in the control spectrum of the complex.

The exchangeable proton spectrum of the complex for the RNA aptamer analog containing an I11-for-G11 substitution is plotted in Fig. 5d. We note that the imino proton labeled **5** is missing from the 10.4–10.5 ppm region but we are unable to detect its expected downfield shifted counterpart as a result of inosine substitution in the complex (Fig. 5d). The imino proton labeled **5** is the first to broaden with increasing temperature among the internal loop and bulge guanine imino protons in the AMP–RNA control spectrum. It is possible that the imino proton labeled **5** exchanges rapidly in the I11-for-G11 substituted RNA aptamer complex and hence cannot be detected in the proton spectrum in Fig. 5d. The exchangeable proton labeled **5** is assigned to G11 in the control spectrum of the complex. We do not detect the exchangeable proton labeled **6** in Fig. 5d, suggesting that the 2'-OH group of G34 also exchanges rapidly in the I11-for-G11 substituted RNA aptamer complex.

The exchangeable proton spectrum of the complex for the RNA aptamer analog containing I17-for-G17 substitution is plotted in Fig. 5e. The imino proton labeled **3** no longer resonates in the 11.0–11.1 ppm region but has instead shifted downfield to 12.88 ppm in the I17-substituted complex (Fig. 5e). The observed 1.80 ppm downfield shift of the imino proton labeled **3** on I17-for-G17 substitution permits the assignment of this exchangeable resonance to the guanine imino proton of G17 in the control complex. It should be noted that we no longer detect the exchangeable amino proton designated **7** in the I17-for-G17 substituted RNA aptamer complex (Fig. 5e), establishing that peak **7** must originate in the amino proton of G17 in the control spectrum of the complex.

TABLE 3
EXCHANGEABLE PROTON CHEMICAL SHIFTS IN THE CONTROL AMP–RNA APTAMER (COMPOSED OF 24-MER AND 14-MER STRANDS) COMPLEX AND ITS ANALOGS CONTAINING SPECIFIC INOSINE-FOR-GUANINE SUBSTITUTION IN THE TOP 24-MER STRAND

Resonance	Control	I7	I8	I11	I17
G6 NH-1	11.75	11.65	11.67	11.77	11.76
1 G7 NH-1	13.24	15.32	12.85	12.96	13.08
2 G8 NH-1	10.72	10.56	13.03	10.68	10.62
5 G11 NH-1	10.44	10.12	10.30	–[a]	10.48
U16 NH-3	10.19	10.18	10.04	10.14	10.11
3 G17 NH-1	11.07	11.08	10.95	11.02	12.88
U18 NH-3	13.77	13.77	13.76	13.82	13.83
4 G34 NH-1	10.35	10.31	10.30	10.14	10.28
6 G34 2'-OH	9.34	9.16	9.52	–[a]	9.41
7 G17 NH_2-2	9.11	9.22	9.16	9.18	–[b]
8 G8 NH_2-2	8.84	8.80	–[b]	8.73	8.89

The spectra were recorded in H_2O buffer at pH 6.7 and 0 °C

[a] These exchangeable protons could not be detected in the spectra.

[b] Inosine-for-guanine substitution replaces these amino protons by hydrogens, accounting for their disappearance in the spectra.

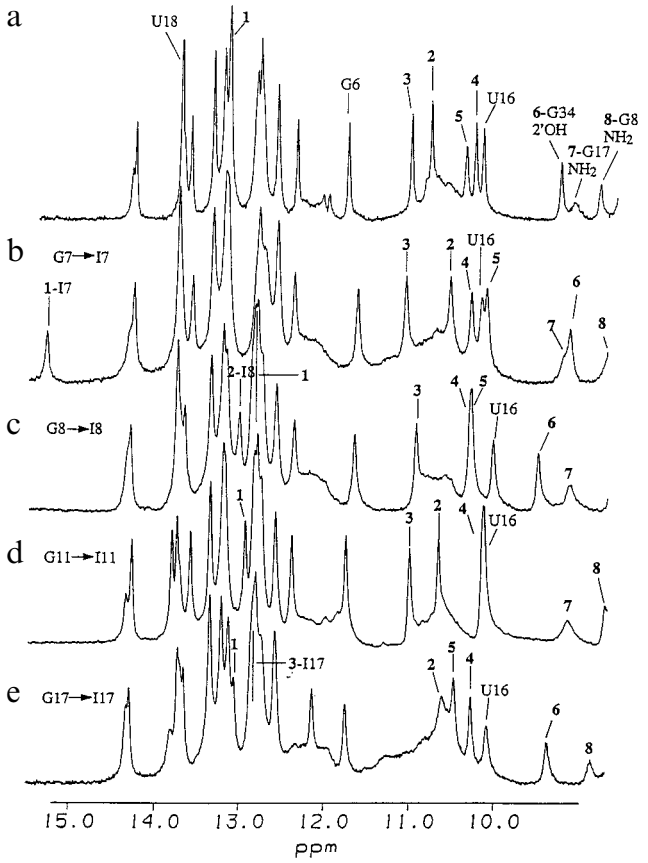

Fig. 5. Exchangeable proton spectra (8.7–15.5 ppm) of (a) the control sequence and (b–e) specific inosine-for-guanine substitution analogs of the AMP–RNA aptamer complex composed of 24-mer and 14-mer strands in H_2O buffer (10 mM phosphate, 0.2 mM EDTA), pH 6.7 at 0 °C. The inosine-for-guanine substitutions are at positions (b) G7, (c) G8, (d) G11 and (e) G17 in the 24-mer top strand in the complex. The ratio of RNA aptamer to AMP was 1:1.1 for the control sample in (a), 1:2 for the I7 analog in (b), 1:3 for the I8 analog in (c), 1:2.5 for the I11 analog in (d) and 1:3 for the I17 analog in (e). The new exchangeable protons observed on complex formation are labeled **1**–**8**. The imino protons of U16 in the internal loop and G6 and U18 of the flanking base pairs are also labeled in (a).

Our attempts to anneal the 24-mer RNA top strand with the 14-mer RNA bottom strand containing the I34-for-G34 substitution were not successful in the absence and/or presence of AMP. This implies that the exocyclic amino group of G34 is critical for both RNA folding and AMP recognition and that replacement by a proton on inosine substitution is not tolerated at this position. Nevertheless, the exchangeable proton labeled **4** is the only internal loop and bulge guanine imino proton that remains to be assigned using the inosine-for-guanine substitution approach and is assigned by elimination to the imino proton of G34 in the complex.

The guanine and inosine imino proton chemical shifts for the internal loop and bulge guanines in the AMP–RNA aptamer complex along with the chemical shift changes on inosine-for-guanine substitution are listed in Table 3.

Discussion and Conclusions

NMR-based structural studies (reviewed by Varani and Tinoco (1991), Moore (1995) and Pardi (1995)) of RNA and ligand–RNA complexes have been greatly facilitated by current methodology that allows the enzymatic preparation of uniformly ^{13}C- and ^{15}N-labeled RNA in milligram quantities (Batey et al., 1992; Nikonowicz et al., 1992). The application of multinuclear, multidimensional NMR experiments on uniformly labeled RNA has overcome some of the proton assignment problems associated with severe overlap in the nonexchangeable sugar proton spectral region. Further progress towards the assignment of nonexchangeable base and sugar protons has been achieved through the incorporation of ^{13}C (SantaLucia et al., 1995) and ^{2}H (Foldesi et al., 1996) labels at specific base and sugar positions followed by their insertion at defined sites in the RNA sequence. The present paper addresses a related problem, namely the assignment of slowly exchanging imino and amino protons located primarily on purine residues in the internal loop and bulge segments of folded RNA structures. This assignment problem does not arise for imino and amino protons in duplex segments since NOE connectivities can be established between these exchangeable protons and their independently assigned nonexchangeable counterparts across GC and AT base pairs.

^{15}N labeling approaches

The exchangeable imino and amino protons are directly attached to nitrogens and hence can be readily assigned if their attached nitrogens are ^{15}N isotopically labeled site-specifically in the RNA sample. We have achieved this goal by the incorporation of labeled guanine (N1 imino and N2 amino positions) and adenine (N6 amino position), one at a time, at all guanines and some adenines in the internal loop and bulge segments of the AMP–RNA aptamer complex. The resulting spectra have permitted the assignment of the exchangeable protons labeled **1**, **2**, **3**, **4** and **5** to the imino protons of G7, G8, G17, G34 and G11, respectively (Fig. 3), and the assignment of the exchangeable protons labeled **7** and **8** to the hydrogen-bonded amino protons of G17 and G8, respectively (Fig. 4). In addition, it has been possible to assign the amino protons of A12, a residue that plays a key role in the tertiary fold of the AMP–RNA aptamer complex (Jiang et al., 1996a,b). The ^{15}N labeling studies were greatly facilitated by generating the complex using 24-mer and 14-mer RNA segments which permitted the isotopic labeling of specific residues in one or the other of the RNA fragments. The ^{15}N labeling approach gives unambiguous assignments, since there is no structural perturbation associated with the isotope incorporation into the RNA. These imino and amino proton assignments are critical since these exchangeable protons exhibit a large number

of intramolecular and intermolecular NOEs (Table 2) that provide key restraints (Jiang et al., 1996b) for the structure determination of the AMP–RNA aptamer complex (Jiang et al., 1996a).

We decided to combine ^{15}N-labeled guanine and adenine pairs in our labeling strategy in order to optimize the number of samples necessary to get definitive imino and amino proton assignments. The assignment of the amino protons of A12 was critical, and for this case we verified our conclusions by independent studies on the ^{15}N-labeled G17 and A12 pair and the ^{15}N-labeled G7 and A12 pair in the AMP–RNA aptamer complex.

Inosine labeling approaches

The inosine-for-guanine substitution approach for the assignment of internal loop and bulge guanine imino protons in the AMP–RNA aptamer complex is a less direct approach since the exocyclic amino group of guanine is being replaced by a proton on inosine substitution. The concern is that the amino group of guanine may be involved in a critical intramolecular or intermolecular contact and that its disruption on inosine substitution would have an adverse effect on complex formation. Indeed, we used up to a threefold excess of AMP for some of the RNA aptamer analogs containing inosine-for-guanine substitutions and were unable to form a viable RNA aptamer duplex or the AMP–RNA aptamer complex when G34 was substituted by I34. Further, we were unable to monitor the imino proton of I11 when this guanine was substituted by inosine in the AMP–RNA aptamer complex (Fig. 5d), presumably due to the increased lability of this exchangeable proton on inosine substitution.

It is our view that the ^{15}N enrichment approach described above provides unambiguous assignments of the guanine and adenine exchangeable base protons in RNA and ligand–RNA aptamer complexes. This approach is dependent on the availability of ^{15}N-labeled purine nucleosides appropriately protected and modified for solid-phase RNA chemical synthesis. These may not always be readily available, in which case the inosine-for-guanine substitution approach should be used with caution and the deduced guanine exchangeable base proton assignments need to be substantiated by other approaches.

Nevertheless, both the ^{15}N and inosine labeling methods, which use chemical synthesis approaches for the site-specific incorporation/modification of RNA, have yielded the same assignment of internal loop and bulge guanine imino protons in the case of the AMP–RNA aptamer complex. These methods have advantages in that they can be extended to longer RNA sequences, provided the lengths do not exceed the limits achievable with current RNA oligomer synthesis and purification techniques.

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