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^{1}H-NMR OF U-G-A AND U-G-A-A IN D_2O: ASSIGNMENT OF NONEXCHANGEABLE PROTONS AND ANALYSIS OF SOLUTION CONFORMATION

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The ribonucleotide oligomers U-G-A and U-G-A-A have been synthesized enzymatically. These oligomers are cognates of the U^{33}-Gm^{34}-A^{35}-A^{36} sequence found in the anticodon loop of t-RNA^{phe}. The ^{1}H-NMR chemical shifts of the base and ribose Hl′ protons as well as the couplings, $J_{1'-2'}$, of the ribose protons have been examined as a function of temperature. Assignments for these resonances have been completed, and used in the analysis of solution conformation for these oligomers. The results are consistent with the A-RNA structure and suggest the absence of alternative ordered solution structures.

1. Introduction

The crystal structure analysis of the t-RNA^{phe} molecule has been completed by Quigley and Rich [1], Stout et al. [2], Hingerty et al. [3] and Holbrook et al. [4]. Their results show that at several locations including the anticodon loop, there exists an oligonucleotide structure designated as a 'U-turn' conformation [1].

The anticodon loop structure contains the sequence U^{33}-Gm^{34}-A^{35}-A^{36}, which forms a U-turn. This name describes the abrupt change in direction incurred by the nucleotide chain following U^{33}, which immediately precedes the anticodon sequence Gm^{34}-A^{35}-A^{36}. This change in direction occurs via a rotation about the guanosine O5′-P bond. As a result, one stacking domain terminates with a uridine, and a new stacking domain, initiated by the anticodon sequence, extends in the opposite direction.

In the light of recent studies suggesting the presence of alternative, non-A-RNA, ordered conformations in solution for several oligonucleotide sequences [5,6], it was of interest to determine whether there is evidence of U-turn structures existing for oligonucleotides in solution. Accordingly, the sequences U-G-A and U-G-A-A were synthesized enzymatically, and the ^{1}H-NMR analysis of these oligomers was completed.

Previous ^{1}H-NMR studies on the anticodon sequence of t-RNA^{phe} have been reported [7,8], as well as CD analysis [9]. These involved isolating the nucleotide sequence mG-A-A-Y-A from intact t-RNA. The long stretch of purines and modified purines has been reported to result in very strong stacking interactions for the anticodon. It was concluded that the presence of mG rather than G further enhances this strong stacking [9].

In this report, incremental analysis is used to arrive at assignments for the base and ribose Hl′ protons of U-G-A and U-G-A-A. The chemical shift of these protons is studied as a function of temperature, and for the ribose Hl′ protons, the temperature dependence of $J_{1'-2'}$ is examined. The data suggest that alternative ordered conformations for U-G-A and U-G-A-A are not present in solution, that the G-A-A sequence is strongly stacked, and that the uracil does not stack strongly. The results are not significantly different from what would be expected for an A′-RNA structure.

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2. Methods

2.1. Synthesis of oligonucleotides

The dimer UpG was obtained as the ammonium salt (Sigma) and used without further purification. Primer-dependent polynucleotide phosphorylase enzyme (PNPase P) [10] (P.L. Biochemicals) was used to achieve chain elongation. In the elongation process, a series of trial reactions was performed to optimize conditions and yield. The 50-μl trial reactions were analyzed using HPLC techniques as developed by McFarland and Borer [11]. The optimal trial was then scaled up to a total volume of 25 ml. This reaction mixture contained 3.0 mM UpG, 9 mM ADP, 10 mM $MgCl_2$, 0.40 M NaCl, 0.2 M Hepes, pH 8.2, and 20% (v/v) PNPase P. This mixture was incubated for 6 days at 37°C. It was then heated to 80°C for 5 min to denature PNPase P and treated with alkaline phosphatase (calf intestine; Boehringer Mannheim) to degrade any remaining nucleotide diphosphate. UGA and UGAA* were separated from the resulting mixture on a DEAE-Sephadex A-25 column, Cl^- form, pH 8.2, using a 3.0 l 0–0.8 M NaCl gradient. Fractions eluting from the column were monitored by the absorbance at 260 nm, and their identity verified by HPLC and paper chromatography (descending mode, 70:30 ethanol/ammonium acetate, v/v). The fractions were desalted by several passes through a Bio-Gel P-2 column, with Millipore-filtered H_2O. Samples were evaporated and quantitated by the absorbance at 260 nm. Extinction coefficients used were 45.7 for UGAA, 33.7 for UGA, and 20.0 for UG (A_{260} units/μmole strand) [12].

2.2. NMR

Each NMR sample contained 1.0 μmole oligomer in 0.5 ml of 0.01 M phosphate buffer, pH 6.6, and 10^{-4} M EDTA. Exchangeable protons were replaced by dissolving samples in 99.7% D_2O (Aldrich) and evaporation, a procedure which was repeated three times. Finally, the samples were dissolved in 99.98% D_2O (Aldrich) and 5 μl of 4.5 M *t*-butanol in D_2O solution was added as an internal reference; shifts were reported with respect to sodium 4,4-dimethyl-4-silapentane-sulfonate (DSS) as described by Borer et al. [13].

Spectra were obtained on various spectrometers, operating in the quadrature FT mode. ^{1}H frequencies of 90, 220, 360 and 500 MHz were used; in all cases with a sweepwidth of 10 ppm. Pulses were accumulated at 3–5 s intervals. At the two lower fields 600–1000 pulses were accumulated, while less than 100 were usually sufficient on the high field instruments. A 16K (8K real) data table was used, except at 500 MHz, where spectra were zero-filled to 32K. A 30–50° flip angle was used in all cases. Temperatures were verified by reference to methanol or ethylene glycol standards [14,15].

* The oligomers U-G-A and U-G-A-A are hereafter referred to as UGA and UGAA for the sake of brevity. None of the oligomers have terminal phosphates.

3. Results and discussion

The ^{1}H-NMR spectra of ribonucleic acid oligomers have two regions of particular interest. The purine H8 and H2 singlets and the pyrimidine H6 doublets appear in the 7.5–8.5 ppm downfield region from DSS. Between 5.9 and 6.1 ppm, one finds pyrimidine H5 doublets and ribose H1′ doublets. The remainder of the ribose protons are extensively coupled and partially obscured by the residual HDO peak, and therefore have not been further examined at this time.

Uracil has a temperature-invariant coupling constant of 8.1 Hz for H5 and H6 resonances. The ribose H1′ protons have coupling constants of magnitude 1–6 Hz and normally decrease with decreasing temperature. The purine H2 and H8 singlets are identified by noting that H8 undergoes deuterium exchange, which results in its intensity being much less than H2. Corresponding nuclei in adjacent residues of the chain are identified by an iterative process, in which oligomer spectra of increasing chain length are examined at elevated temperatures where the resonances are well separated and non-ordered conformations predominate [13].

The two sets of adenosine resonances in UGAA

present the greatest challenge in assignments. These assignments were aided by reference to Kondo et al. [16] and Dhingra and Sarma [17], who utilized selective deuteration of the bases to successfully assign the resonances in the trimer AAA. Both AAA and GAA form strongly stacked species, and the relative position of the A^2 and A^3 resonances of GAA should be the same as for AAA. In addition, comparison to AAA confirms the guanosine assignments of GAA.

3.1. Base protons

The following discussion compares the base protons of UGA with those of UGAA. This allows one to examine the chemical shift effect caused by the addition of an adenosine residue to the trimer UGA. These changes are shown in fig. 1a and b.

The major effects of tetramerization are seen in the chemical shift data of the adenine H2 and H8 protons. The adenine H2 resonances are more shielded than the H8 resonances.

We assign the UGAA A^3H2 as the signal showing the largest temperature dependence of chemical shift; the two curves showing similar behavior in fig. 1a and b are assigned as the terminal adenosine H2 protons of UGA and UGAA, respectively. This assignment is consistent with that achieved by selective deuteration of AAA [16,17]. Adenine has the largest ring current shielding effect, and the shifts observed for A^3H2 in the tetramer as compared to the trimer are indicative of increased shielding resulting from the addition of the terminal adenosine. Some residual shielding effect is seen to remain even at the higher temperatures, where the UGAA protons are still 0.02–0.1 ppm more shielded than the UGA H2.

The adenine H8 of UGA is less shielded than either adenine H8 in UGAA. Extensive overlap in the temperature range between 10 and 30°C makes assignment of the UGAA adenine H8 protons difficult. In fact, these two protons appear to cross over in this temperature range. The results of selective deuteration reported by Dhingra and Sarma [17] indicate that A^3H8 should be less shielded than A^4H8 at lower temperatures. This would agree with theoretical shieldings calculated for stacked A or A'-RNA [18], where in addition,

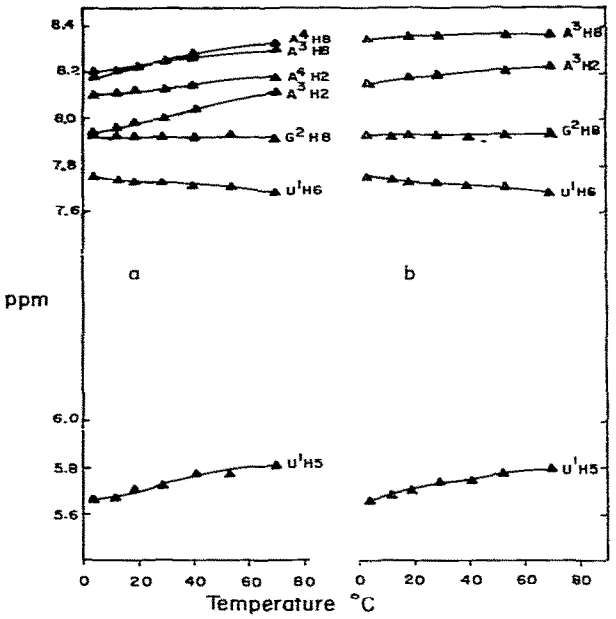

Fig. 1. (a) Base protons of UGAA. (b) Base protons of UGA. δ (ppm from DSS) versus temperature (°C). The UGAA A^3 and A^4 H8 protons cannot be unequivocally assigned using incremental analysis (see text).

A^4H8 should be more shielded at low temperatures due to the fact that it is adjacent to a 5'-neighbor adenosine whereas A^3 is adjacent to a 5'-neighbor guanosine. However, we note that the earlier results of Kondo et al. [16] pertaining to the AAA H8 assignments show the opposite assignment for pAp and pA of AAA. Unequivocal assignment of UGAA A^3 and A^4H8 will require selective deuteration.

The guanine H8 protons appear at approximately 7.92 ppm and are essentially independent of temperature. In addition, the trimer and tetramer curves are virtually superimposed. The lack of dependence on temperature suggests that the guanine H8 proton is in an environment which is not changing as the trimer and tetramer form ordered structures at lower temperatures. The superposition of the two curves suggests that the guanine H8 position is unaffected by the addition of a 3'-adenyl unit to UGA.

The uracil H6 protons become approximately

0.125 ppm less shielded as the temperature is lowered. That no change in behavior is noted in going from the trimer to the tetramer indicates that the environment of the uracil H6 proton also is unaffected by the addition of the 3'-terminal adenosine.

In both UGA and UGAA, the uracil H5 protons become approximately 0.14 ppm more shielded as temperature is decreased. The two curves virtually superimpose upon each other, indicating that the uracil H5 in the tetramer is in an environment not significantly affected by addition of the terminal adenosine. This shift of 0.14 ppm is significantly larger than the theoretically calculated values [18] for A or A'-RNA. Calculated shieldings for a stacked helix indicate that essentially no shielding of a pyrimidine H5 proton should be contributed by a 3'-neighbor guanosine residue. A possible explanation for this shielding effect is that the uridine residue, rather than being rigidly confined to an A-RNA environment, is instead free to assume a 'floppy' state. The 'average' shift observed by NMR will then be shielded due to the presence of some conformations in which shielding due to the guanosine base is occurring. This 'floppy uridine' model is supported by an examination of the $J_{1'-2'}$ couplings of the ribose ring protons, as we discuss in section 3.2.

3.2. Ribose Hl' protons

The coupling constants of the ribose Hl' protons, $J_{1'-2'}$, give a measure of the ribose ring pucker. In a right-hand stacked A-RNA structure, the ribose ring exists in the 3'-*endo* conformation (^{3}E), while in the disordered state, a mixture of ^{2}E and ^{3}E conformers is present. Ribose $J_{1'-2'}$ couplings have values ranging between 0 and 10 Hz, increasing with the proportion of ^{2}E conformers. Thus, base stacking can be followed as a function of temperature by examining the observed ribose coupling constant values. These are shown in tables 1 and 2.

At high temperature, less-ordered structures predominate. At 70°C, all of the UGAA ribose Hl' couplings are approximately 5 Hz. As the temperature is lowered and more-ordered structures begin to predominate, the ribose Hl' couplings decrease until at 4°C they are 3.3 Hz or less for the guanosine and adenosine Hl' protons, as is predicted for the A-RNA structure. However, the uridine Hl' decreases only slightly as the temperature is lowered, to approximately 4.5 Hz. This large coupling constant of 4.5 Hz at 4°C indicates that the uridine ribose rings have a significantly larger proportion of ^{2}E conformers than do the purine ribose rings. In the stacked A-RNA conformation, the uridine ribose ring would be expected to be ^{3}E and hence have a small $J_{1'-2'}$ value. The larger proportion of ^{2}E conformers observed supports the concept of a floppy uracil.

The chemical shift transition of the ribose Hl' protons as a function of temperature is shown in fig. 2a and b. The largest difference in the Hl' resonances is noted for the adenosine Hl' protons. As with the adenine base protons, these assignments have been arrived at by comparison to the previously obtained assignments of AAA [16,17]. Both tetramer AHl' resonances are more shielded than the Hl' of the UGA adenosine. This en-

Table 1

UGAA $J_{1'-2'}$ (Hz) versus Temperature (°C)

Temperature (°C)	U^1	G^2	A^3	A^4
4	4.5	≤2.0	2.7	3.2
12	4.5	[a]	3.3	3.2
20	4.7	3.6	3.4	[a]
34	5.0	4.5	4.0	4.0
41	5.0	5.5	4.3	4.3
58	5.0	6.0	4.9	5.0
70	5.0	6.0	5.2	5.0

[a] These values were not determined because of overlap with another resonance. The coupling constants are accurate to approximately ±0.5 Hz.

Table 2

UGA $J_{1'-2'}$ (Hz) versus Temperature (°C)

Temperature (°C)	U^1	G^2	A^3
4	4.5	3.2	3.9
12	4.3	3.8	4.2
20	5.0	4.0	4.5
70	5.5	4.0	5.0

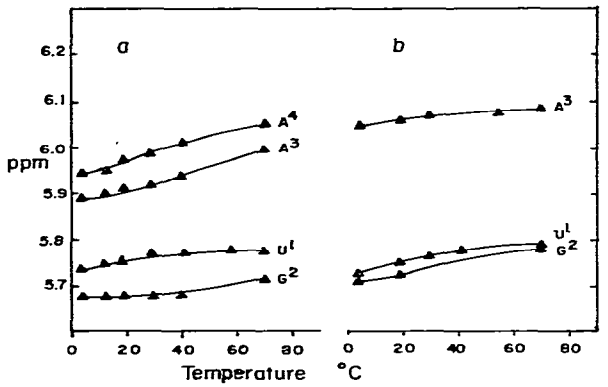

Fig. 2. (a) Ribose Hl′ protons of UGAA. (b) Ribose Hl′ protons of UGA. δ (ppm from DSS) versus temperature (°C).

hanced shielding is more pronounced at lower temperatures. The Hl′ resonances for U^1 and G^2 are more difficult to assign. We assign the least shielded resonance in both UGA and UGAA as U^1Hl′ because it shows the same δ versus T transition in UGA as in UGAA. Addition of A^4 could be expected to affect G^2Hl′ much more than U^1Hl′. The resonance we assign as G^2Hl′ is more shielded in UGAA relative to UGA. This shielding is not nearly as large as for A^3Hl′ in going from UGA to UGAA, consistent with the argument that nearest-neighbor effects should be the greatest. Also, as was discussed in section 3.1., the least shielded of these two resonances in both UGA and UGAA has a larger value of $J_{1'\text{-}2'}$.

It has been suggested that the ribose Hl′ versus temperature transitions are due to through-space anisotropies arising from the purine N3 and pyrimidine 2-carbonyl mediated by changes in the glycosidic torsion angles as an oligomer goes from a random to an ordered conformation [19–21]. The observed changes in going from UGA to UGAA may be attributed to changes in the glycosidic torsion angles occurring as a result of adding the terminal adenosine. Only the uridine Hl′ proton shows the same chemical shift versus temperature dependence in UGAA as in UGA. Hl′ protons consistently show increased shielding as A-RNA stacking occurs, so this observation indicates no increase in uridine stacking in the tetramer as compared to the trimer, in agreement with the $J_{1'\text{-}2'}$ coupling constant data for uridine. Examination of the guanosine Hl′ proton shows that it is approximately 0.05 ppm more shielded in the tetramer as compared to the trimer. This may be interpreted as a uniformly increased stacking of the guanosine at all temperatures in UGAA, as compared to UGA. In support of this notion, $J_{1'\text{-}2'} =$ 3 Hz for GHl′ of UGA at 4°C, which indicates less stacking than in UGAA where $J_{1'\text{-}2'} \leq 2$ Hz for the collapsed doublet.

4. Conclusions

The ^{1}H-NMR data observed in this study suggest that the difference between UGA and UGAA is limited to an increased stability in the tetramer caused by a stronger stacking domain comprised of the three purines GAA. An examination of fig. 1a and b shows that only the adenine base protons are affected in going from UGA to UGAA. The uracil and guanine bases appear to be unaffected by the addition of the terminal adenosine, as evidenced by the superposition of the curves representing these resonances in UGA and UGAA, respectively. The adenine has the greatest ring current effect, and the changes in the chemical shift versus temperature curves for the adenine base protons in UGA and UGAA are interpreted as being due to the addition of the terminal adenosine.

This type of behavior is characteristic of an A-RNA helical structure in solution. In such structures, nearest-neighbor effects predominate, and the effect of adding a nucleotide to the sequence is reflected primarily in the chemical shift behavior of what formerly was the 3′-terminal nucleotide.

In the 'U-turn' structure, the uracil base is destacked relative to the GAA sequence, due to a rotation of the P-O5′ torsion angle between U^1 and G^2. This places the phosphate group between G^2 and A^3 (phosphate 2) directly under the uracil ring, and in van der Waal's contact. The plane containing this phosphorus and its two unesterified oxygens should have a deshielding effect. Examination of a Kendrew wire model shows that UH6 and UH5 both lie out of this plane and

therefore should be shielded by the phosphate anisotropy. The experimental data show this to be the case for UH5 but not for UH6. Although UH5 shows a large upfield shift, the H1′ coupling constant for U^1 remains large at low temperature. The U turn structure would predict a small value for $J_{1'-2'}$ as U^1 is ^3E in this structure. Examination of the Kendrew model shows that GH8 is below the plane containing phosphorus 1 and the two unesterified oxygens attached to it, and thus should also be shielded by the phosphate anisotropy. This is not seen in the experimental data; rather the GH8 protons are not temperature dependent, and their chemical shifts are in agreement with the predicted A′-RNA values.

Our results thus appear to indicate that an alternative solution conformation corresponding to the crystalline 'U-turn' structure [1,2] is not present for either UGA or UGAA in solution. The GAA sequence can be interpreted in terms of a standard right helical RNA structure.

The uracil data are not consistent with either the stacked or 'U-turn' predictions. The $J_{1'-2'}$ values for U^1 in particular support a model where U^1 is flopping about adjacent to the strongly stacked GAA sequence. An important experiment will be to construct longer oligomers with bases attached to the 5′-end of UGAA; such longer oligomers should lock the uracil into a particular conformation.

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