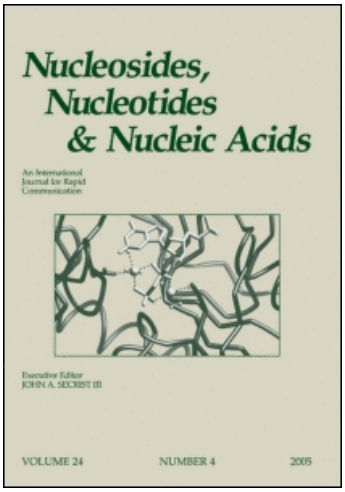

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DO THE 16 MER, 5′-GUGGUCUGAUGAGGCC-3′ AND THE 25 MER, 5′-GGCCGAAACUCGUAAGAGUCACCAC-3′, FORM A HAMMERHEAD RIBOZYME STRUCTURE IN PHYSIOLOGICAL CONDITIONS? AN NMR AND UV THERMODYNAMIC STUDY

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ABSTRACT

In a wide range of salt concentrations, 10–30 mM phosphate buffer containing up to 0.5 M Li_2SO_4 and 300 mM NaCl, 7.5 mM Mg^{2+}, pH 5.5–7.5, a mixture of the 16 mer and the 25 mer RNA strands does not form a hammerhead in any amount detectable by NMR at 600 MHz. The imino-, amino-, aromatic- and anomeric protons in the NMR spectra of both the 16 mer and the 25 mer RNA have been assigned separately. Both the 16 mer and the 25 mer RNA both take up very stable hairpin structures, and when mixed together there is no major change of conformation in neither oligo-RNA.

INTRODUCTION

Ribozymes are small RNA molecules that site-specifically cleave the phosphodiester backbone of target RNA in a non-hydrolytic manner in presence of Mg^{2+}, but in absence of proteins or any external energy source such as ATP. *In vitro* studies have shown that the hammerhead domain can be constructed by two

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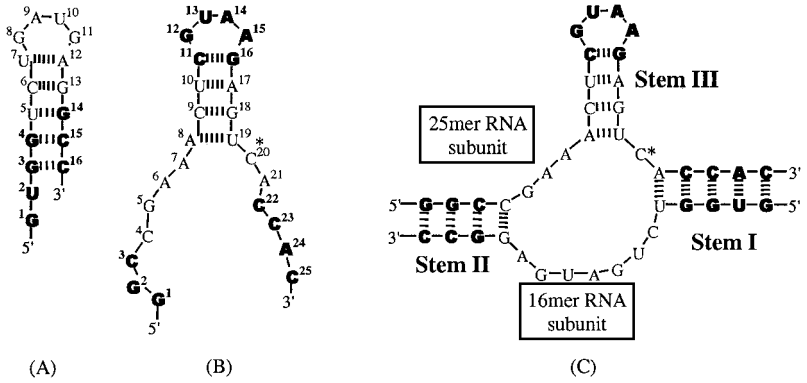

Figure 1. The secondary structures of the 16 mer (A) and 25 mer (B) RNA strands, and the hammerhead ribozyme construct (C) for which X-ray data is available. The residue numbers used is also shown, and the deuterated residues are printed in bold font.

or three RNA strands, forming 3 helices of various lengths surrounding a core of 13 conserved nucleotides, 9 of them situated in single stranded regions (Fig. 1) (1). In the present study we have analysed the possibility of formation of a non-cleavable hammerhead ribozyme (16 mer + 25 mer RNA) in solution for which the X-ray crystal structure (grown in 1.8 M Li_2SO_4) recently has been reported (2) (Fig. 1C). A high resolution NMR structural study of this hammerhead RNA under physiological conditions (low salt concentration) could shed light on the dynamics of RNA folding both in the presence and absence of Mg^{2+}.

RESULTS AND DISCUSSION

Oligo-RNAs, both deuterated (3) (the NMR-window (3c)) and non-deuterated, with 2′-O-methyl cytidine (4) in position ^{20}C (Fig. 1) were used in our NMR study. Only one, solute concentration independent transition is observed in UV for the isolated 16 mer in a 0.1 M NaCl phosphate buffer. $T_m = 65.6 \pm 0.3°C$ (data not shown).

The melting temperature of the main transition of the 16 mer is not RNA concentration dependent, although salt-dependent ($T_m = 65.6 \pm 0.3°C$ in 100 mM NaCl, $71.1 \pm 0.1°C$ in 1M NaCl and $71.8 \pm 0.4°C$ in 2M NaCl), with RNA concentrations in the 0.5 to 8 μM range (Table 1 for thermodynamics). The second, minor component is however both salt and concentration dependent, suggesting a bimolecular process (Fig. 2). The 25 mer RNA shows one major concentration independent transition ($T_m = 62.0 \pm 0.2°C$) in 0.1 M NaCl phosphate buffer (pH 7.5). The UV spectra of this 25 mer is however broad, suggesting the presence of more than one concentration independent conformers (see NMR assignments, Fig. 3c), which prevent reliable thermodynamic calculations. However, the T_m of the 25 mer is comparable to the 16 mer RNA, thereby suggesting that their thermodynamic

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Table 1. The Thermodynamics of the 16 mer Hairpin Melting in 20 mM Phosphate Buffer (100 mM NaCl, pH = 7.5) in kJ/mol

RNA Concentration	ΔH°	$-T\Delta S^\circ$	ΔG°
2 μM	−229	202.6	−26.4
4 μM	−239	211.6	−27.4
8 μM	−234	205.6	−28.4
Average	−234	205.6	−28.4

The error is within 5% in each case.

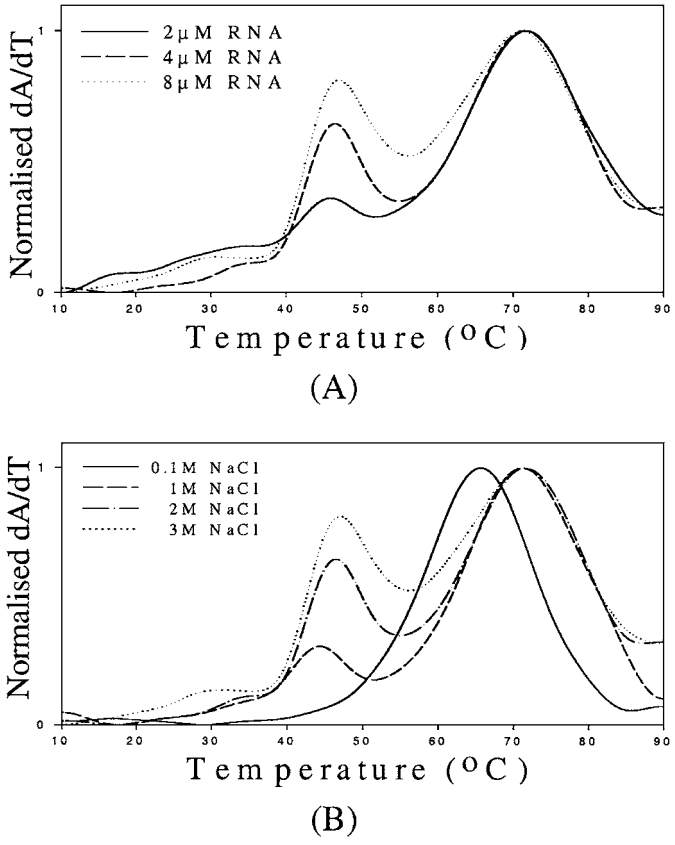

Figure 2. (A) Two transitions are observed in UV for the isolated 16 mer RNA in 2.0 M NaCl phosphate buffer. The main transition is still RNA concentration independent ($T_m = 71.8 \pm 0.4^\circ C$), whereas the second transition appears to be a bimolecular process (see 16 mer NMR assignment below). (B) The relative amount of isolated 16 mer RNA going through the second transition is increasing with increased salt concentration in the phosphate buffer (pH 7.5). The RNA concentration is 8 μM in all samples.

stabilities are quite comparable. The 16 mer RNA has been fully assigned using NOESY/ROESY spectra in combination with DQF-COSY and HSQC (Fig. 3), and was found to be in a hairpin conformation in phosphate buffer alone (10 mM phosphate, pH 6.7). Addition of more than 100 mM NaCl drives the equilibrium towards a duplex structure (Fig. 3B).

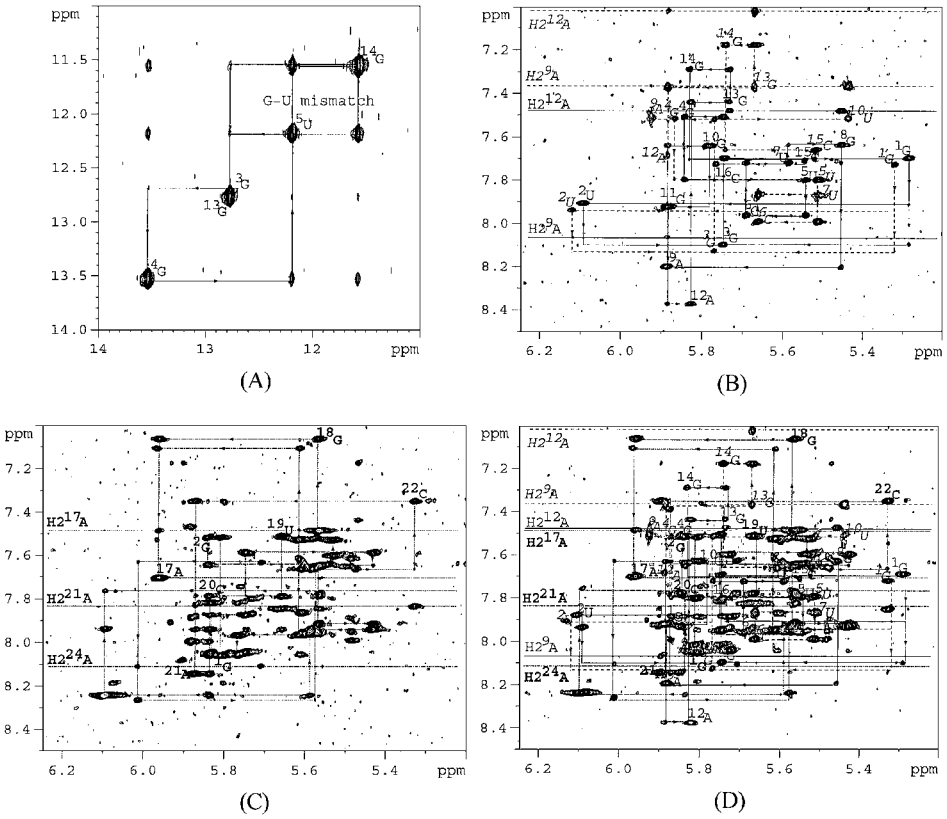

Figure 3. *Expandable pdf figures in colour are available at our website [http://bioorgchem.uu.se/].* (A) The imino region of the 16 mer (0°C), at 600 MHz, shows the internal basepairings, including the characteristic G-U mismatch, giving rise to misleading basepaired imino resonances in 1D titration experiments (data not shown) in H_2O:D_2O (90%:10%). (B) 16 mer assignment (20°C). The solid lines trace the aromatic-H1′ connections in the 16 mer hairpin structure. The dashed lines trace a secondary duplex structure that only exists to any large extent in salt concentrations above 100 mM NaCl and temperatures below 40°C. The breaks in inter-residue connectivity in the duplex trace between residues 7–12 suggest a flexible bulge at the 4 central residues, between the two ^{8}G-^{11}G mismatches. (C) The sequential H6/H8-H1′ assignment of the 25 mer RNA (20°C). A secondary uni-molecular structure, possibly a different fold, also exists in approximately 4:6 ratio. (D) The H6/H8-H1′ region of the mixture of 16 mer and 25 mer resembles the superimposition of the spectra of the two components isolated. Thus, there is no significant change of conformation taking place, meaning that no hammerhead-like structure is formed when the two oligo RNAs are mixed in a 100 mM NaCl phosphate buffer (20°C).

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The imino, H6/H8 and the H1′ protons of the 25 mer could be assigned for the loop region (residues ^{8}A to ^{16}U), but flexibility of the ends and the existence of a secondary structure complicated full assignment of the 25 mer (Fig. 3C). When the 16 mer RNA and the 25 mer RNA are mixed together in the above buffer, the hairpin formed by the 16 mer remains intact (Fig. 3D), preventing the formation of any hammerhead-like structure to any significant extent to NMR. Thus, we conclude that the 16 mer catalytic part and the 25 mer substrate part of the ribozyme alone do not form the active hammerhead complex spontaneously in 10–30 mM phosphate buffer containing as much as 300 mM NaCl and 7.5 mM Mg^{2+}. This means that the hairpin formation under physiological condition is thermodynamically favoured, and therefore the free energy of hammerhead formation in this media must be significantly higher than the −27.4 kJ/mol found for the hairpin. Further increment of the salt concentration (300 mM NaCl and 7.5 mM Mg^{2+}) and the addition of 500 mM $Li_2SO_4^{2-}$ give rise to significant intractable changes for most of the resonances in the imino- and H6/H8-H1′ regions (most importantly the disappearance of the G-U mismatch), except for the crosspeaks belonging to stem III and the GUAA tetraloop (Fig. 1C), which are less affected.

This, together with the appearance of new imino resonances, allow us to suggest that the observed line-broadening (even the cancellation of some crosspeaks) is not only caused by the added salt, but could also be attributed to slow exchange between the hairpin/duplex structure of the 16 mer and a higher ordered structure.

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