Synthesis and NMR study of ribooligonucleotides forming a hammerhead-type RNA enzyme system

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ABSTRACT

We designed a hammerhead-type ribozyme system which consists of three RNA fragments and synthesized the component and related ribooligonucleotides by the solid-phase phosphoramidite method using *o*-nitrobenzyl groups for 2'-hydroxyl protection. Improved conditions for photolytic removal of the *o*-nitrobenzyl groups are described. Imino proton NMR spectra of the ribozyme complex were measured. The resonances for the hydrogen-bonded imino protons were assigned by comparison with the spectra of model complexes which contain the base sequences of one or two of the stem regions. The results suggest that the complex indeed forms a hammerhead structure and the loop regions take an ordered conformation in the absence of magnesium ions.

INTRODUCTION

Since the discovery of RNA enzymes in the early 1980's, many types of so-called 'ribozymes' have been found (1). Among them, the 'hammerhead'-type system is an attractive target for chemists because it is shown that an RNA oligomer system containing only about 40 nucleotide residues is fully active in a self-cleavage reaction (2,3). This system apparently contains three double helical regions (SI−SIII), two loop regions (L1 and L2) and one bulged residue (B)(Figure 1). If the RNA strand containing the cleavage site, which is shown by an arrow in Figure 1, is separated from the complex, the remaining part works as an enzyme cleaving catalytically the substrate oligomer in the presence of Mg^{2+}. A 2',3'-cyclic phosphate and a 5'-OH are produced at the cleavage site. Therefore the reaction is transesterification and is quite similar to the first step of ribonuclease action.

In order to elucidate the structure and mechanism of the hammerhead-type ribozymes mainly by NMR, we designed a ribozyme system which consists of three RNA strands of 11, 16 and 12-mers (**1**−**3**, Figure 1). We synthesized these oligomers in sufficient amounts for NMR study by the phosphoramidite method in solid phase using *o*-nitrobenzyl groups for 2'-hydroxyl protection (4,5). Imino proton NMR spectra of the complex were measured and tentative assignments of the resonances were made by comparison with the NMR data for model duplexes.

MATERIALS AND METHODS

Long chain alkylamine derivatized controlled pore glass beads (pore size 500 Å, particle size 125−177 μm) were purchased from Pierce Chemical Co. Nuclease P1 was purchased from Yamasa Shoyu Co. Reverse-phase column chromatography was performed on alkylated silica gel (C_{18}, 55−105 μm, Waters). High performance liquid chromatography (HPLC) was performed on a Gilson MS-3 system using a column (φ6 mm×200 mm) of Senshu-Pack ODS-2202-N (Senshu Kagaku). UV spectra were measured on a Shimadzu UV-2100 spectrophotometer. Proton NMR spectra were recorded with a JEOL GX-500 spectrometer (500 MHz). Proton chemical shifts were determined relative to internal 2-methyl-2-propanol (1.23 ppm). The NMR samples of the complexes were heated at 60−65°C for 5 min prior to measurement. Imino proton spectra of the oligomer complexes in H_2O-D_2O (4:1) containing 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 6.8 or 7.5) were measured with a 1−1 pulse sequence for H_2O signal suppression. The NOE difference spectrum represents the spectrum with an on-resonance preirradiation pulse subtracted by the spectrum with an off-resonance preirradiation pulse.

Synthesis of the ribooligonucleotides

2'-*O*-(*o*-nitrobenzyl)nucleoside derivatives were prepared according to the published procedures (6−10). 5'-*O*-Methoxytrityl-2'-*O*-(*o*-nitrobenzyl)-*N*-acylnucleosides were phosphitylated with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphordiamidite (11) as described previously (5). The resulting phosphoramidites were used as the monomer units. The nucleoside resins were prepared using long chain alkylamine controlled pore glass as a support according to the procedure of Tanaka *et al.* (4). Oligomer synthesis was performed essentially according to the procedures described by Tanaka *et al.* (4) and Sakata *et al.* (5). The reaction cycle is shown in Table 1. Synthesis of the 11-mer (**1**) starting from 5 μmol C-resin was carried out in the following manner. In the condensation step, 8 and 16

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equivalents of the phosphoramidite (40 mM) and *p*-nitrophenyltetrazole (80 mM) in CH_3CN (1 ml) were used. After completion of the chain elongation cycles, the resin was treated with concd. NH_4OH (15 ml)-pyridine (1.5 ml) at 55°C for 16 hr. After filtration and evaporation of the volatile materials, the residue dissolved in 30% CH_3CN in 50 mM triethylammonium acetate (TEAA, pH 7) was chromatographed on a column (ϕ8 mm×150 mm) of C-18 silica gel. The methoxytritylated oligomer (about 400 A_{260} units) was eluted with a linear gradient of CH_3CN (30−60%) in 50 mM TEAA (pH 7, total 200 ml). The pooled fractions were evaporated and the salts were largely removed by repeated evaporation with water. The residue was treated with 80% acetic acid (30 ml) at 30°C for 16 hr with shaking. After removal of the solvent, the residue was dissolved in 50% ethanol (5 ml) and diluted with 0.1 M ammonium formate (pH 3.5) adjusting the oligomer concentration to 5 A_{260} units/ml. The solution was heated at 80°C for 5 min and irradiated with UV light (> 280 nm) at around 55°C for 1hr. After removal of the solvent, the residue dissolved in 0.1 M triethylammonium bicarbonate (TEAB, pH 7.5) was applied on a column (ϕ2.5 cm×60 cm) of Sephadex G-25. Elution was carried out with 0.1 M TEAB (pH 7.5). After desalting by repeated evaporation with water, the oligomer was again purified by chromatography on a column (ϕ8 mm×150 mm) of C-18 silica gel. Elution was carried out with a linear gradient of CH_3CN (4 −15%) in 50 mM TEAA (pH 7) (total 200 ml). 130 A_{260} units (27% overall) of **1** were obtained. The pooled fractions were evaporated and largely desalted by repeated evaporation with water. The residue was desalted by gel filtration on a column of Sephadex G-10 (ϕ2.5 cm×60 cm). The NMR samples were prepared by successive treatment with small columns of Dowex 50 (pyridinium form), Dowex 50 (Na^+ form) and Chelex 100 (Na^+ form) resins. The other oligomers were also synthesized, deprotected and purified in the same manner (Table 2). Oligomer **4** contains 2′-*O*-methylcytidine (Cm) at the position corresponding to B (Figure 1). The UV hypochromicities were determined by complete digestion of the oligomers with nuclease P1 (**1**, 6%; **2**, 14%; **3**, 18%); the ϵ_{260} values were calculated from the data (**1**, 9.7×10^4; **2**, 14.6×10^4; **3**, 12.2×10^4).

Conditions for photolysis

The reaction conditions for photolytic removal of the 2′-*O*-*o*-nitrobenzyl groups were examined using the 16-mer derivative (10 μmol scale) which has only 2′-*O*-protection. After removal of methoxytrityl groups with 80% acetic acid, the solvent was evaporated off. The residue was dissolved in 50% ethanol (5 ml). An aliquot of this solution was taken out and diluted with 0.1 M ammonium formate buffer (pH 3.5) to make the 2-ml solution of a certain concentration (2.5−20 A_{260} units/ml) in a Pyrex tube. In the case of the experiments at 50°C, the solution was heated at 80°C for 5 min prior to irradiation. The irradiation was performed with UV light through a Pyrex filter (2 mm thick) over the Pyrex tube (ϕ1 cm, 1 mm thick) using a photolysis apparatus bearing a 300-W high-pressure mercury lamp (Eikosha, Model PIH 300) and a quarz water-circulating jacket. The ambient temperature was about 25°C. For the experiments at 50°C, the reaction tube was immersed in a water bath and kept at around 50°C. An aliquot of the reaction mixture containing 0.2 A_{260} unit of the starting materials was analyzed by reverse-phase HPLC on a column (ϕ6 mm×200 mm) of C-18 silica gel. Elution was carried out with a linear gradient of CH_3CN (10−17%) in 0.1 M TEAA (total 21 ml) at the flow rate of 0.7 ml/min. The results are shown in Figures 2 and 3.

RESULTS AND DISCUSSION

Synthesis of the ribooligonucleotides

We designed a hammerhead-type RNA enzyme system, which consists of three ribooligonucleotide strands (**1**−**3**, Figure 1), based on the sequence of the satellite RNA of tobacco ringspot virus (12). We synthesized these oligomers by the solid-phase phosphoramidite method essentially according to the procedure of Tanaka *et al.* (4) where *o*-nitrobenzyl groups were used for 2′-hydroxyl protection and methylphosphoramidite and 5-(*p*-nitrophenyl)tetrazole were used for condensation. We used *N*-acyl-5′-*O*-methoxytritylnucleoside 3′-(2-cyanoethyl)phosphoramidite as a coupling unit. In the condensation step, 8 and 16 equivalents of the nucleoside phosphoramidite and 5-(*p*-nitrophenyl)tetrazole, with respect to the nucleoside resin, were used, respectively, whereas 20 and 40 equivalents of the monomer unit and *p*-nitrophenyltetrazole were used in the previous paper (4). The reaction cycle is shown in Table 1. Thus the condensation step requires 2 min and one chain elongation cycle requires about 25 min by manual operation. The average coupling yield was 95−98%. After treatment of the oligomer bound resin with concentrated ammonia, the oligomer with methoxytrityl and *o*-nitrobenzyl groups was isolated by reverse-phase column

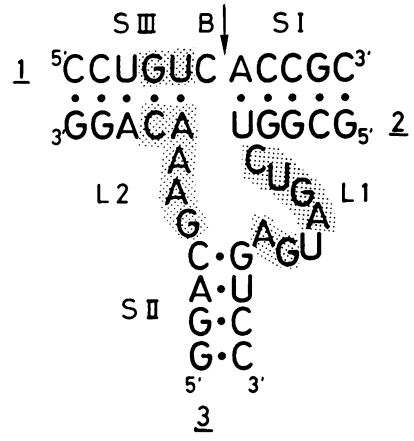

Figure 1. Structure of the designed complex which consists of RNA fragments **1**−**3**. The cleavage site is indicated by an arrow. The three stems, two loops and a bulge are designated as SI−SIII, L1, L2 and B, respectively. The nucleotides conserved among the self-cleaving RNA's from natural sources are stippled.

Table 1. Steps involved in one chain elongation cycle

No.	step	reagent/solvent	time
1	washing	$CH_3CN\times3$, $CH_2Cl_2\times2$	
2	detritylation	5% TCA/CH_2Cl_2	2 min
3	washing	$CH_2Cl_2\times3$, Py, $CH_3CN\times3$	
4	drying N_2 gas	10 min	
5	coupling	phosphoramidite (40 mM) nitrophenyltetrazole (80 mM) CH_3CN (200 μl/μmol-resin	2 min
6	washing	CH_3CN	
7	capping	Ac_2O-0.1 M DMAP/Py (1:9)	30 sec
8	washing	CH_3CN	
9	oxidation	0.1 M I_2/THF-Py-H_2O (8:1:1)	1 min

chromatography. After removal of the methoxytrityl groups with 80% acetic acid, the mixture was subjected to photolysis to remove the *o*-ntrobenzyl groups.

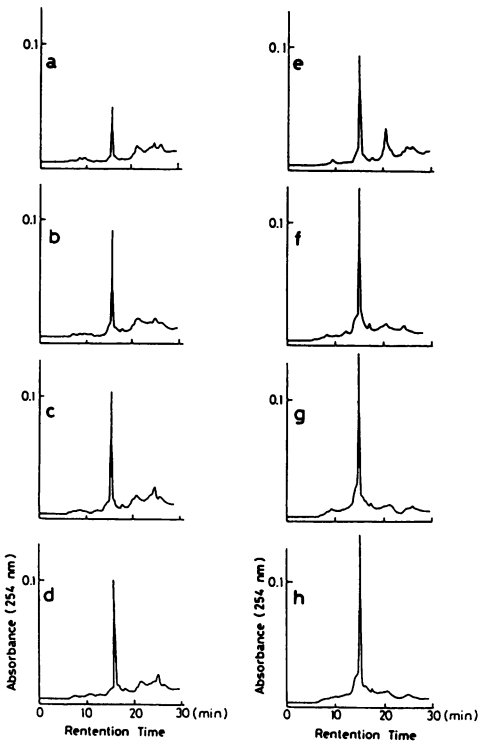

Figure 2. Analysis of the reaction conditions for photolytic removal of the o-nitrobenzyl groups by reverse-phase HPLC. The photolysis was carried out with the oligomer concentration of 20 (a,e), 10 (b,f), 5 (c,g) or 2.5 A_{260} units/ml (d,h) at 25°C (a–d) or 50°C (e–h).

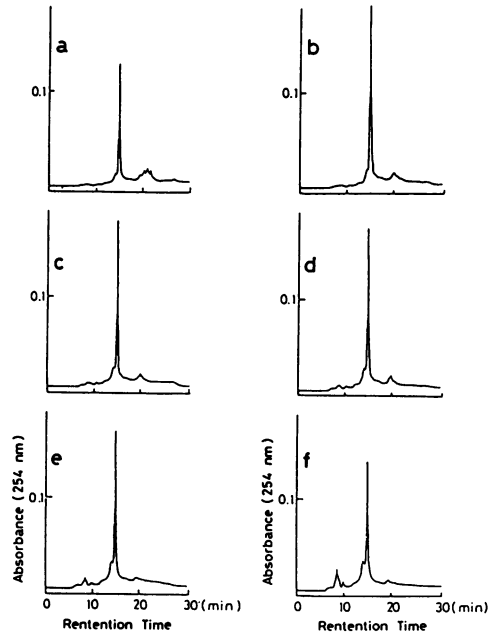

Figure 3. Analysis of the reaction conditions for photolytic removal of the o-nitrobenzyl groups by reverse phase HPLC. The photolysis was carried out at 50°C for the reaction time of 30 min (a), 1 hr (b), 1.5 hr (c), 2 hr (d), 3 hr (e) or 6 hr (f).

In the step of *o*-nitrobenzyl deprotection, UV irradiation was usually carried out with an oligomer concentration below 10 A_{260} units/ml in 0.1 M ammonium formate (pH 3.5) (13) at around 25°C for 1 hr. These conditions are sufficient for oligomers with a chain length shorter than 10. However in the case of longer oligomers, increased amounts of by-products make it difficult to purify the desired product resulting in a further reduction of the isolated yield. We examined the reaction conditions in terms of oligomer concentration, reaction time and temperature using the 16-mer (**2**) with *o*-nitrobenzyl groups. At first, effects of the oligomer concentration (2.5–20 A_{260} units/ml) and the reaction temperature (25°C and 50°C) were analyzed by HPLC (Figure 2). It appears that the reactions with the oligomer concentration below 5 A_{260} units/ml and at 50°C give the best result. Next, effects of the reaction time (0.5–6 hr) were examined running the reactions with a fixed oligomer concentration (2.5 A_{260} units/ml) and at 50°C (Figure 3). The results show that 1–1.5-hr irradiation gives the best result. The

Table 2. Synthetic yields of the ribooligonucleotides

sequence		chain length	reaction scale (μmol)	isolated amount (A_{260} unit)	isolated yield (%)
CCUGUCACCGC	(**1**)	11	5	130	27
GGACGAAACAGG	(**3**)	12	10	230	19
GCGGUCUGAUGAGUCC	(**2**)	16	10	195	13
CCUGUCmACCGC	(**4**)	11	10	280	29
GCGGUCACAGG	(**5**)	11	5	120	22
GGACGA	(**6**)	6	10	83	12
GAGUCC	(**7**)	6	10	146	24

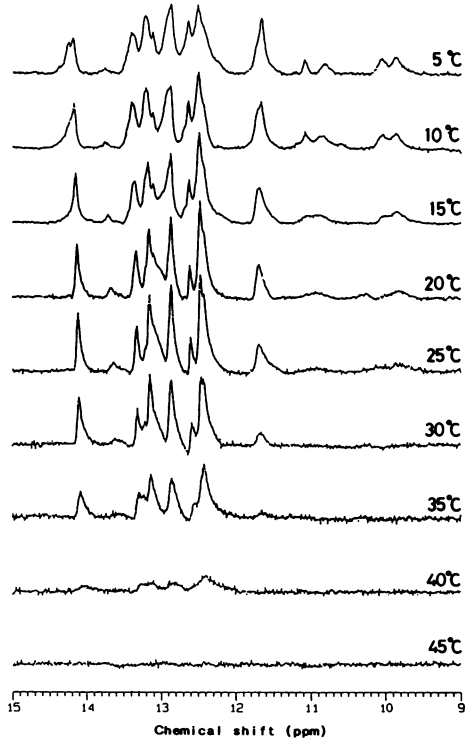

Figure 4. Imino proton NMR spectra of complex A (**1**+**2**+**3**) (1.25 mM) in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 7.5) at temperatures indicated.

by-products eluted after the desired product turn out to be the partially deprotected oligomers since the isolated by-products can be converted to the desired product by second UV irradiation. The by-products, which are most prominent in the 6-hr reaction and eluted before the desired product, may be the degradation products of the oligomer.

Thus deprotection of the *o*-nitrobenzyl groups was performed by photolysis with the oligomer concentration below 5 A_{260} units/ml at 50−55°C for 1 hr. The completely deprotected oligomer was purified by gel filtration and reverse-phase column chromatography. The oligomers **1**−**3** (11-mer to 16-mer) were obtained in good overall, isolated yields (13−27%) and also in sufficient amounts for NMR studies (Table 2). Oligomers **4**−**7**, which can form model duplexes designed for NMR assignments, were also synthesized (see Table 2). Oligomers **4** and **5** form a bulged duplex (complex B) containing the base sequences of the stems III and I (SIII and SI in Figure 1, see also Figure 9d). Oligomers **6** and **7** form a duplex with flanking nucleotides (complex C) containing the base sequences of SII (Figure 9e).

The oligomer **1** was effectively cleaved when mixed with **2** and **3** in the presence of Mg^{2+} (14).

Imino proton NMR spectra of the oligomer complex A (1+2+3)

Proton NMR spectra of an equimolar mixture of the oligomers **1**−**3** (complex A) were measured in H_2O at various temperatures (Figure 4). Hydrogen-bonded base imino proton resonances are usually observed in the 12−15 ppm region and unpaired imino proton resonances for a hairpin are observed in the 9.5−12 ppm region (5,15,16). The complex shows signals in both the regions suggesting that it contains base-paired stems and unpaired loops in which the imino protons are in a

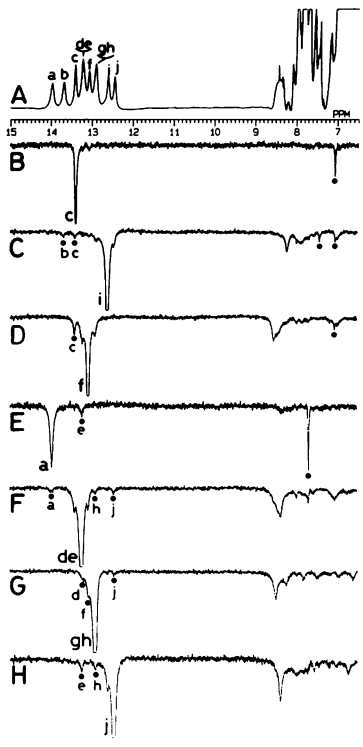

Figure 6. NOE difference spectra for complex B in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 6.8) at 5°C. A: normal spectrum; B − H: NOE difference spectra. The irradiated peak is labeled. The observed NOE's to imino and AH2 protons are indicated by closed circles.

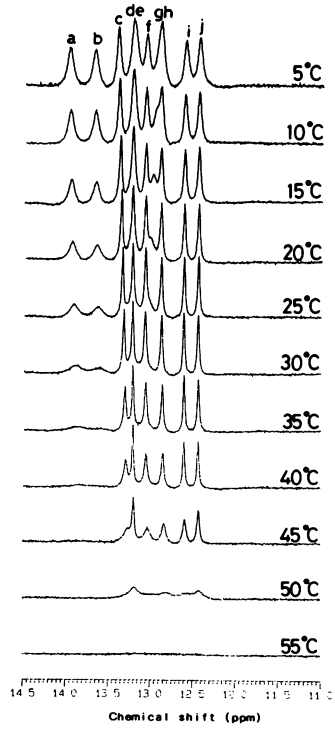

Figure 5. Imino proton NMR spectra of complex B (4+5) (4.9 mM) in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 6.8) at temperatures indicated.

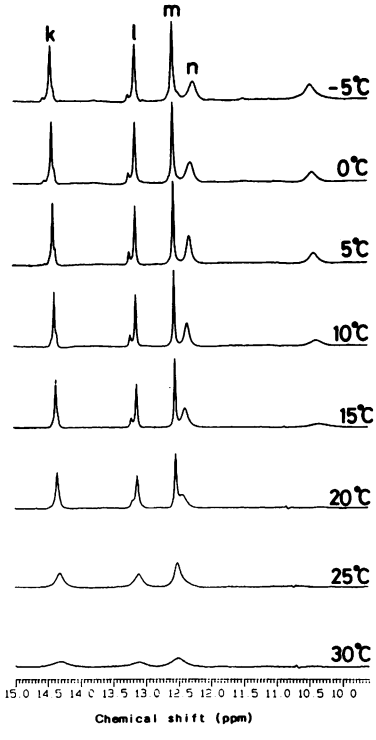

Figure 7. Imino proton NMR spectra of complex C (6+7) in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 6.8) at temperatures indicated.

hydrophobic environment. Upon increasing temperature, the resonances in the 9.5−12 ppm region rapidly broaden and completely disappear at 35°C where the resonances in the 12−15 ppm region are still observed. This result is consistent with the notion that the resonances in the 9.5−12 ppm region originate from the loop imino protons. It appears that most of the resonances for the stems show an upfield shift by about 0.1 ppm over the range of 5°C to 35°C and completely disappear above 45°C because of the fast proton exchange with water. In order to assign the resonances for the stems, imino proton NMR spectra of two model complexes B and C (**4**+**5** and **6**+**7**) were measured.

Imino proton NMR spectra of the oligomer complex B (4+5)

The spectra of the bulged duplex (**4**+**5**) (complex B), which contains the base sequences of SI and SIII, at various temperatures are shown in Figure 5. The duplex should contain 10 hydrogen-bonded imino protons. The spectrum at 5°C indeed shows resonances corresponding to 10 protons in the 12−15 ppm region. The peaks designated as de and gh contain two resonances, respectively. In each case, one of the two resonances (d and g) seems to rapidly broaden and disappear upon raising temperature. The resonance g shows a downfield shift and can be clearly observed at 15°C. This result suggests that these signals (d and g) are those of the G:C base pairs at the outer termini of the two base-paired regions. The two peaks designated as a and b ,which appear in the lowest field region, also broaden rapidly and completely disappear at 40°C where relatively sharp, 6 resonances still remain. This result suggests that these signals (a and b) are those of the U:A base pairs (U5:A18 and U16:A7) which are located at the inner terminals of the two base-paired regions (see Figure 9d for the numbering).

These resonances were assigned by nuclear Overhauser effect (NOE) experiments at 5°C (Figure 6). Irradiation of peak c gives an NOE to the sharp signal at 7.1 ppm, which is assumed to be that of adenine H2 (A20H2), suggesting that peak c originates from the uracil imino proton (UH3) of U3:A20. Peak c also shows a small NOE with peak f. Irradiation of peak i gives small NOE's to peaks b and c as well as to the sharp signal at 7.4 ppm probably of A18H2. This result suggests that the proton for peak i is guanine imino proton (GH1) of G4:C19 which is sandwiched with the U3:A20 and U5:A18. Peak b shows NOE's to peak i as well as to A18H2 (7.4 ppm) (data not shown). Peak f gives an NOE to peak c (U3:A20) suggesting that it is of the GH1 of C2:G21. The results described above suggest that peak a is

Table 3. Chemical shifts at 5°C and assignments for the imino proton resonances for complexes B and C arranged in the order of the chemical shifts

chemical shift (ppm)	assignment	corresponding position in complex A
14.3	U*4*	SII-2(U2:A*2*)
13.95	U16	SI-1(A1:U*1*)
13.65	U5	SIII-1(A1:U*1*)
13.4	U3	SIII-3(A3:U*3*)
13.2	G12	SI-5(C5:G*5*)
13.2	G15	SI-2(C2:G*2*)
13.1	G*3*	SII-1(G1:C*1*)
13.0	G21	SIII-4(G4:C*4*)
12.85	G10	SI-4(G4:C*4*)
12.85	G22	SIII-5(G5:C*5*)
12.6	G4	SIII-2(C2:G*2*)
12.55	G*8*	SII-3(C3:G*3*)
12.35	G14	SI-3(C3:G*3*)
12.3	G*7*	SII-4(C4:G*4*)

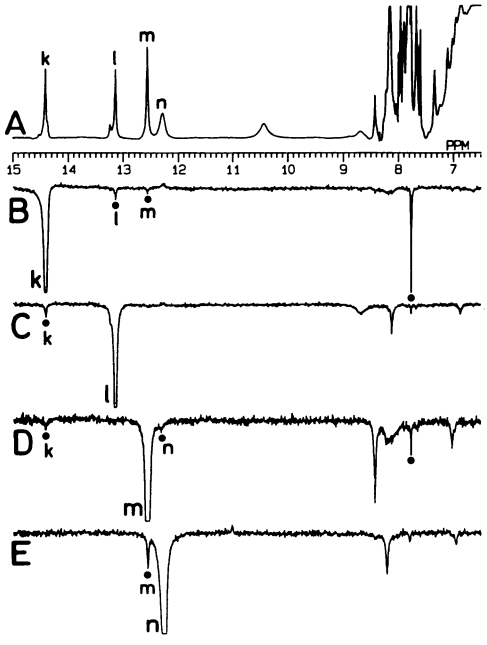

Figure 8. NOE difference spectra for complex C in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 6.8) at 0°C. A: normal spectrum; B − E: NOE difference spectra. The irradiated peak is labeled. The observed NOE's to imino and AH2 protons are indicated by closed circles.

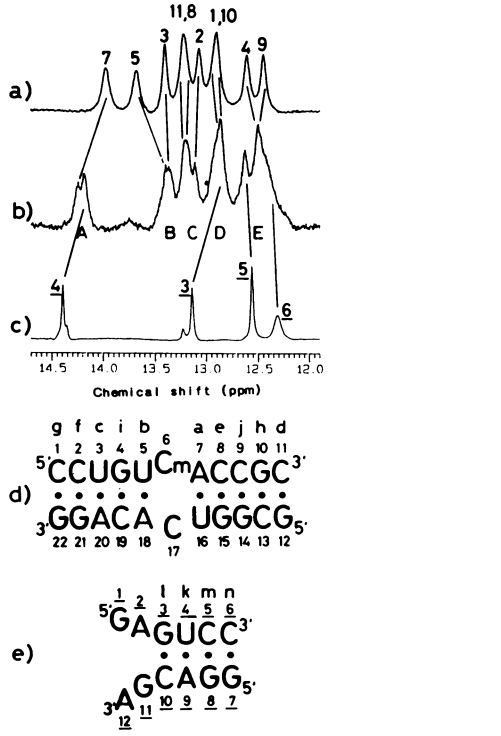

Figure 9. Tentative assignment for the hydrogen-bonded imino proton resonances of complex A by comparison with those of complexes B and C. a: spectrum of complex B at 5°C; b: spectrum of complex A at 5°C; c: spectrum of complex C at 5°C; d: sequence and numbering for complex B; e: sequence and numbering of complex C. The peaks of complexes B and C are labeled with the base-pair numbers.

responsible for the remaining UH3 of A7:U16. The U16H3 shows an NOE with the proton of peak e as well as with A7H2 (7.7 ppm) suggesting that peak e is from the GH1 of C8:G15. The remaining resonances (h and j) for the internal base pairs to be assigned are those of C9:G14 and G10:C13. Since peak h is much broader than peak j at 45°C (Figure 5), peak h can be assigned to that of G10:C13 which is the second one from the outer terminus of the paired region and assumed to be more labile than the third and central one, C9:G14, against thermal perturbation; peak j can be assigned to the resonance of C9:G14. Irradiation of peak de gives NOE's to peak a, hg and j (Figure 6F). Since resonance e should show NOE's to peaks a and j and resonance g is that of the outer terminal G:C pair, resonance d is responsible for the NOE with resonance h and therefore is that of C11:G12; the remaining resonance g is of the C1:G22. The results in Figures 6G and 6H are consistent with these assignments.

Imino proton NMR spectra of the oligomer complex C (6+7)

The spectra of the complex C (**6**+**7**), which contains the sequences of SII, at various temperatures are shown in Figure 7. The spectrum at −5°C shows 4 resonances, which are designated as k, l, m and n, in the 12−15 ppm region and a resonance in the 9.5−12 ppm region. The former should be those of the base-paired imino protons and the latter may be that of an unpaired GH1. Resonance n, which is the broadest one among the four resonances in the 12−15 ppm region, can be assigned to the terminal base pair, *C6*:G*7* (see Figure 9e for the numbering). It should be noted that this resonance shows a downfield shift upon raising temperature as observed for resonance g (G22H1) of the complex B, while the other imino proton resonances generally show an upfield shift. G*7* is at the 5′-end with a 5′GGA3′ sequence while G22 is at the 3′-end with a 5′AGG3′ sequence. The resonances were assigned by NOE experiments at 0°C (Figure 8). Irradiation of resonance k gives small NOE's to resonances l and m and a large NOE to a sharp signal at 7.7 ppm probably of A*9*H2. This result suggests that resonance k is for U*4*H3 of U*4*:A*9*. Irradiation of resonance n (C*6*:G*7*) gives an NOE to resonance m suggesting that resonance m is of C*5*:G*8* (Figure 8E). Irradiation of resonance m (C*5*:G*8*) gives very small NOE's to resonances k and n as well as to A*9*H2 (Figure 8D). The remaining resonance l is of G*3*:C*10* (the inner terminal base pair of the stem region); the assignment is confirmed by the fact that resonance l shows an NOE with resonance k (U*4*:A*9*) (Figure 6 C).

CONCLUSION

The chemical shifts and assignments for the imino proton resonances of complexes B and C are summarized in Table 3. In order to describe the hammerhead-type ribozyme system, we adopt the following numbering system. At first, the system is divided into 6 parts (SI−SIII, L1, L2 and B) as described earlier (Figure 1). In the case of loops, the residues are numbered in the 5′ to 3′ direction. In the case of stems, the base pairs are numbered starting from the inner terminus next to the loop or bulge as shown in Table 3. The residues of a strand running 5′ to 3′ in the same order are numbered such as A1−C5 for SI and those of the complementary strand are numbered such as U*1*−G*5* for SI (from 3′ to 5′ in this case). The tentative assignment for the hydrogen-bonded imino proton resonances of complex A is shown in Figure 9. It is assumed that difference in chemical shifts between the model complexes and complex A is most profound for the imino protons of the base-pairs at the inner termini of the stems (SI-A1:U*1*, SII-G1:C*1* and SIII-A1:U*1*). It is very interesting to note that SII-U2 also shows considerable difference. It appears that correspondence between the chemical shifts for the other imino protons is fairly good. The blocks of the signals designated as D and E in Figure 9 seem to contain resonances which show a downfield shift upon raising temperature (Figure 4) in accordance with the present assignment.

The present results suggest that complex A indeed forms a hammerhead structure in terms of the base-paired stems. The loops may take an ordered conformation since the resonances in the 9.5 - 12 ppm region show considerable stability against thermal perturbation (Figure 4). Our study on a non-cleavable complex (**4**+**2**+**3**) shows that addition of Mg^{2+} causes no big chemical shift changes in the 12−15 ppm region but causes specific broadening of the loop imino proton resonances (14). The detailed analysis of the non-cleavable systems will be published elsewhere.

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