Volume 267, number 1, 150–152 FEBS 08576 July 1990

The role of a conserved guanosine residue in the hammerhead-type RNA enzyme

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Received 31 March 1990

We have designed a hammerhead-type RNA system which consists of three RNA fagments for normal and modified complexes which contain a non-cleavable substrate with 2′-*O*-methylcytidine and a guanosine-to-inosine replaced enzyme. Examination of the RNA-cleaving activity and conformational properties of the complexes suggests that the 2-amino group of a conserved guanosine residue in the loop region plays an important role for maintaining both the activity and loop conformation.

Synthetic oligoribonucleotide; RNA enzyme; Hammerhead structure; NMR; Conformation

1. INTRODUCTION

RNA systems, which can form a hammerhead structure, are shown to have a self-cleavage activity at a specific site [1]. The active domain can be as small as about 40 nucleotides in size [2,3]. The domain can be divided into two parts: substrate and enzyme components. The enzyme component catalytically cleaves the substrate RNA chain [2,4–6]. The active complex can be constructed from between one and three pieces of RNA and apparently contains three base-paired stems (SI–SIII), two internal loops (L1 and L2) and one bulged residue (B) whose 3′-side phosphodiester bond is cleaved by the attack of its 2′-OH group (Fig. 1). The active domain contains 13 nucleotides conserved among self-cleaving RNAs from natural sources (6 in L1, 3 in L2 and 4 in SIII) [7]. Some mutagenesis analyses on these conserved residues have been reported [8,9].

In this paper, we describe the preparation and properties of some hammerhead-type systems which consist of three synthetic RNA fragments (**1–3**). We found that a conserved guanosine residue, the third from the 5′-end in L1 (L1-G3), plays an important role in maintaining a special loop conformation which probably involves tertiary interactions.

2. MATERIALS AND METHODS

Oligoribonucleotides were synthesized by the solid-phase phosphoramidite method. Monomer units were prepared from 5′-*O*-monomethoxytrityl-2′-*O*-(*o*-nitrobenzyl)-*N*-acyl nucleosides. The oligomers were purified by gel filtration and reverse-phase chromatography after photolytic deprotection of *o*-nitrobenzyl groups.

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The substrate RNA (**1**) was labeled by kination of the 5′-terminal OH with T4 polynucleotide kinase and [γ-^{32}P]ATP, separated by 20% polyacrylamide/7 M urea gel electrophoresis and desalted with C-18 Sep-Pak (Waters). Cleavage reactions were performed by essentially the same method as described by Uhlenbeck [2]. The reaction mixture (20 μl) contained the labeled substrate (20 pmol), ribozyme fragments (**2** and **3**, 20 pmol each), 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5) and was incubated at 37°C for 1 h. The reaction was stopped by addition of 9 M urea, 20 mM EDTA, 90 mM Tris-borate buffer (20 μl) and heating at 90°C for 5 min. Oligonucleotides were separated on a 20% polyacrylamide/7 M urea gel and subjected to autoradiography.

^{1}H NMR spectra were recorded with a JEOL GX-500 spectrometer (500 MHz). The chemical shifts were determined relative to internal 2-methyl-2-propanol (1.23 ppm). Exchangeable proton spectra were obtained with the complex (1.25 mM) in H_2O/D_2O (4:1) containing 0.1 NaCl, 10 mM sodium phosphate buffer (pH 7.5) or in the same solvent with addition of 6.25 mM $MgCl_2$ using 1-1 pulse sequence for H_2O signal suppression.

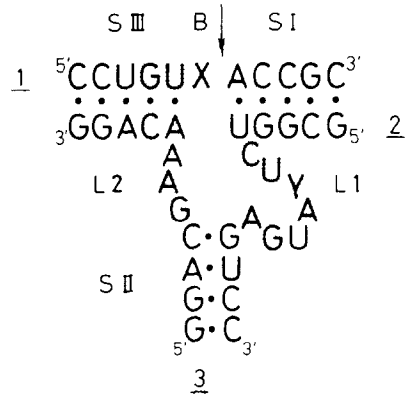

Fig. 1. Structure of the designed complex which consists of RNA fragments **1–3** and where X = C or Cm and Y = G or I. The cleavage site is indicated by an arrow. Three stems, two loops and a bulge are designated as SI–SIII, L1–L2 and B, respectively. The nucleotides conserved among self-cleaving RNAs from natural sources are stippled.

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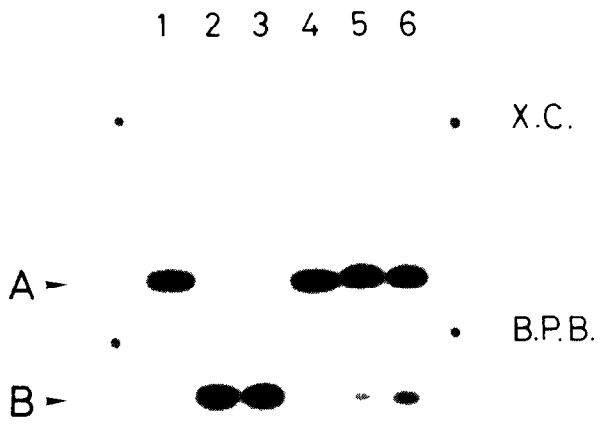

Fig. 2. 20% polyacrylamide/7 M urea gel electrophoresis for the cleavage reactions. (Lanes 1–3) For the complex containing **1**(X = C), **2**(Y = G) and **3**, without Mg^{2+} (lane 1), with 10 mM $MgCl_2$ at 37°C for 20 min (lane 2) and 60 min (lane 3). (Lanes 4–6) For the complex containing **1**(X = C), **2**(Y = I) and **3**, without Mg^{2+} (lane 4), with 10 mM $MgCl_2$ at 37°C for 20 min (lane 5) and 60 min (lane 6). A and B show the 5′-labeled substrate **1** and the cleaved product, respectively.

3. RESULTS AND DISCUSSION

We have designed a hammerhead-type RNA system which consists of three RNA fragments (**1–3**) and contains base sequences similar to those of satellite tobacco ringspot virus RNA [10] (Fig. 1). We chemically synthesized ribooligonucleotides for the normal complex and some modified fragments; a substrate fragment (**1**, X = Cm) which contains 2′-*O*-methylcytidine (Cm) instead of cytidine in B, and an enzyme fragment (**2**, Y = I) which contains inosine instead of L1-G3. **1**(X = Cm) is a non-cleavable substrate which is necessary for structural studies in the presence of Mg^{2+}. **2**(Y = I) was designed in order to examine the effect of the 2-NH_2 group of L1-G3.

Cleavage reactions for a mixture of **1**, **2** and **3** (1 μM each) were performed in 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5) at 37°C (Fig. 2). In the case of the normal complex containing **1**(X = C), **2**(Y = G) and **3**, substrate **1** was cleaved to 89% within 20 min. 91% cleavage was observed at 60 min. When **1**(X = Cm) was used as a substrate, no cleavage was observed as expected (data not shown). A similar result was obtained for a different system which contains a Cm at B position [6]. In the case of the mutant complex containing **1**(X = C), **2**(Y = I) and **3**, **1** was cleaved only to 4% and 9% in 20 and 60 min, respectively. The greatly reduced cleavage rate suggests the importance of the 2-amino group of L1-G3 for catalytic activity. In the absence of Mg^{2+} ions, no cleavage was observed for both the complexes.

The stability of the complexes containing **1**(X = Cm) and **2**(Y = G) or **2**(Y = I) was examined by UV and CD spectroscopy (Fig. 3). In the absence of $MgCl_2$, both the complexes show a broad UV-temperature profile (Fig. 3a,b). In the presence of 20 mM $MgCl_2$, both the

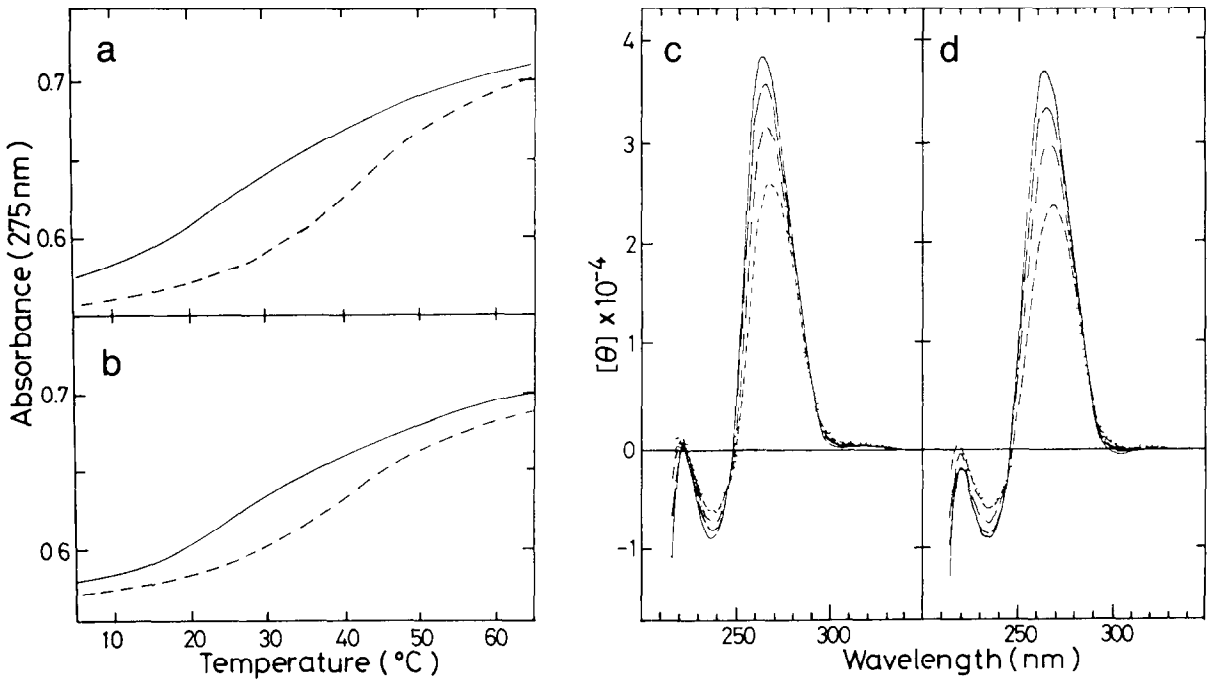

Fig. 3. (a,b) UV-temperature profiles for the complex containing **1**(X = Cm), **2**(Y = G) and **3** (a) and that containing **1**(X = Cm), **2**(Y = I) and **3** (b); without (solid lines) and with 20 mM $MgCl_2$ (broken lines). (c,d) CD spectra of the above complexes containing **2**(Y = G) (c), and **2**(Y = I) (d) with 20 mM $MgCl_2$ at 10°C (——), 20°C (– –), 30°C (– · –), 40°C (-----) and 50°C (· · · · ·).

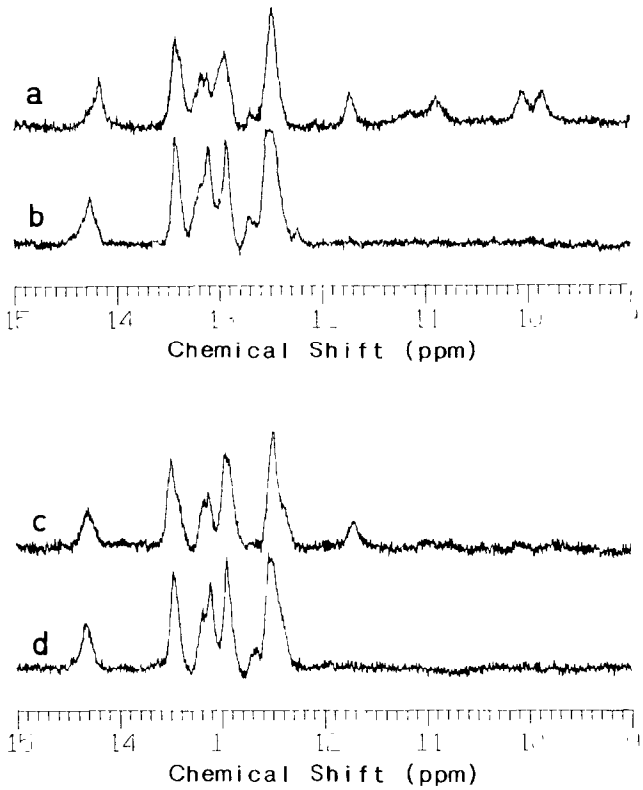

Fig. 4. ^{1}H NMR spectra in the imino proton region of the complex (1.25 mM) containing **1**(X = Cm), **2**(Y = G) and **3** (a,c), and that (1.25 mM) containing **1**(X = Cm), **2**(Y = I) and **3** (b,d) without (a,b) and with 6.25 mM $MgCl_2$ (c,d).

complexes give a sharper UV-transition. The T_ms were determined to be 42°C and 37°C for the L1-G3- and L1-I3-containing complexes, respectively. The difference in the complex stability is also confirmed by CD experiments. The largest change in the positive CD band around 265 nm is observed between 40 and 50°C for the L1-3G-containing complex, while the largest difference is observed between 30 and 40°C for the L1-I3-containing complex (Fig. 3c,d). These results suggest that removal of the 2-amino group of L1-G3 reduces the thermal stability of the complex, although the residue is not included in the base-paired stem region.

In order to examine conformational properties of the complexes, ^{1}H NMR spectra in the imino proton region were measured in H_2O (Fig. 4). In the absence of $MgCl_2$, the spectrum for **1**(X = Cm)-**2**(Y = G)-**3** complex shows signals in the 12–15 ppm region where hydrogen-bonded base imino proton resonances are usually observed, as well as in the 9.5–12 ppm region where unpaired imino proton resonances for a hairpin loop are usually observed (Fig. 4a). These results suggest that the base residues in the loop regions are in a hydrophobic environment not probably forming an ordered structure. Addition of $MgCl_2$ specifically broadens the resonances in the higher field region except for that at around 11.7 ppm (Fig. 4c). Hydrated Mg^{2+} ions may bind directly or closely to the bases in the loops [11] and may enhance the hydrogen-exchange rate with water to give broad resonances. In sharp contrast, the spectra for **1**(X = Cm)-**2**(Y = I)-**3** complex show no signal in the 9.5–12 ppm region either in the absence or presence of $MgCl_2$ (Fig. 4b,d). It is assumed that the ordered structure of the loop regions present in the L1-G3 complex is lost in the L1-I3 complex. This conformational property of the loop regions of the L1-I3 complex may destabilize the thermal stability of the complex as a whole and, on the other hand, reduce the RNA-cleaved activity. Lower stability of the L1-I3 complex was also confirmed by the observation that the hydrogen-bonded imino proton resonances disappeared earlier (40°C vs >45°C) upon the raising temperature.

The present results demonstrate the importance of the 2-NH_2 group of L1-G3 for maintaining both loop conformation and RNA-cleaving activity. Further investigation using these designed hammerhead-type systems is in progress.

Acknowledgement: The authors thank Dr Stuart N. Farrow for reading the manuscript.

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